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DOCTORAL DISSERTATION

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Title of PhD dissertation (in Polish): Chemiczna i biologiczna ocena aktywności przeciwutleniającej endogennych związków redoks-aktywnych w porównaniu do egzogennych przeciwutleniaczy pochodzenia roślinnego

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Gdańsk, 2022





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Summary of PhD dissertation in English

The research conducted so far has shown that endogenous antioxidants, despite being regarded as the first line of antioxidant defense, may not be sufficient to maintain redox homeostasis in cells exposed to oxidative stress.

The results obtained in the doctoral dissertation show that endogenous redox-active compounds were moderate or weak scavengers of ABTS and DPPH radicals, while in cellular setting, their impact on the reducing capacity of HT29 cells was diversified. The results showed no significant correlation between the cellular antioxidant activity and electrochemical properties of tested compounds. Better compliance with biological activity was found for the kinetic parameter describing the velocity of neutralization of ABTS and DPPH radicals. Most of the tested redox-active compounds did not have significant impact on growth of HT29 cells. The inhibition of growth was observed solely in the case of high concentrations. Endogenous redox-active compounds did not protect DNA from fragmentation induced by oxidants in HT29 cells.

The obtained results showed that endogenous redox-active compounds display weaker antioxidant activity compared to exogenous antioxidants. This research provided the systematic evidence that the maintenance of redox homeostasis, should the oxidative stress challenge occur, requires the support of exogenous dietary antioxidants.

Summary of PhD dissertation in Polish

Dotychczasowe badania wskazywały, że przeciwutleniacze endogenne, mimo pełnienia funkcji pierwszej linii obrony organizmu przed stresem oksydacyjnym, mogą nie być wystarczające do utrzymania homeostazy redoks w warunkach narażenia na stres oksydacyjny.

Uzyskane w rozprawie doktorskiej wyniki wykazały, że endogenne związki redoks-aktywne charakteryzowały się umiarkowaną lub słabą zdolnością neutralizacji rodników ABTS i DPPH, natomiast w warunkach komórkowych wykazywały zróżnicowany wpływ na potencjał redukcyjny komórek linii HT29. W rozprawie wykazano brak znaczącej korelacji między komórkową aktywnością przeciwutleniającą, a własnościami elektrochemicznymi badanych związków. Lepszą zgodność z aktywnościami biologicznymi stwierdzono dla parametrów kinetycznych reakcji neutralizacji rodników ABTS i DPPH. Większość badanych związków nie wpływała znacząco na wzrost komórek linii HT29. Hamowanie wzrostu obserwowano tylko dla wysokich stężeń. Endogenne związki redoks-aktywne nie chroniły DNA komórek HT29 przed fragmentacją indukowaną oksydantami.

Uzyskane wyniki potwierdziły, że endogenne związki redoks-aktywne są znacząco słabszymi przeciwutleniaczami niż przeciwutleniacze egzogenne. Badania te dostarczyły systematycznych dowodów na to, że utrzymanie homeostazy redoks w warunkach narażenia na stres oksydacyjny wymaga wsparcia przeciwutleniaczy egzogennych dostarczanych z żywnością.



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LIST OF ABBREVIATIONS

H₂O₂ , hydrogen peroxide	DPPH , 2,2-diphenyl-1-picrylhydrazyl
HO[•] , hydroxyl radical	E⁰ , standard reduction potential
O₂^{•-} , superoxide radical	E^{0'} , formal reduction potential
ONOO⁻ , peroxyxynitrate	EC , epicatechin
R[•] , alkoxyl radical	ECG , epicatechin gallate
ROO[•] , peroxy radical	EDTA , ethylenediaminetetraacetic acid
¹O₂ , singlet oxygen	EGC , epigallocatechin
3IAN , 3-indolylacetonitrile	EGCG , epigallocatechin gallate
4-HNE , 4-hydroxynonenal	eNOS , endothelial nitric oxide synthase
8-oxo-(d)G , 8-oxo-2'-(deoxy)guanosine	ER , endoplasmic reticulum
α-KG , α-ketoglutarate	EROD , ethoxyresorufin-O-deethylase
AA , L-ascorbic acid	ETC , electron transport chain
ABG , ascorbigen	EtOH , ethanol
ABTS , 2,2-azino-bis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt	FAD , oxidized form of flavin adenine dinucleotide
Ade , adenine	FADH₂ , reduced form of flavin adenine dinucleotide
Ado , adenosine	FBS , foetal bovine serum
AGEs , advanced glycation end-products	FE , ferulic acid
AhR , aryl hydrocarbon receptor	FFA , free fatty acid
ALEs , advanced lipoxidation end products	FRAP , ferric reducing ability of plasma
AMP , adenosine-5'-monophosphate	FTH1 , ferritin, heavy polypeptide 1
AOX , antioxidant	GA , gallic acid
AP-1 , activator protein 1	GE , gentisic acid
APS , Antioxidant Power Series	GCL , γ-glutamylcysteine synthetase
ATCC , American Type Culture Collection	GCLC , glutamate-cysteine ligase, catalytic subunit
ATOX1 , ATX antioxidant protein 1	GCLM , glutamate-cysteine ligase, modifier subunit
ATP , adenosine-5'-triphosphate	GCS , glutamylcysteine synthetase
B2M , beta-2-microglobulin	gDNA , genomic DNA
BNIP3 , BCL2/adenovirus E1B 19kDa interacting protein 3	GFT2I , general transcription factor Iii
BPA , bisphenol A	GI , gastrointestinal
C , catechin	GLO , L-glucono-lactone oxidase
CA , citric acid	Glu , L-glutamic acid
CaA , calcium L-ascorbate	Gly , L-glycine
CAA , cellular antioxidant activity	GMP , guanosine-5'-monophosphate
CCL5 , chemokine (C-C motif) ligand 5	GPx , glutathione peroxidase
CFA , caffeic acid	GR , glutathione reductase
cGMP , cyclic guanosine-5'-monophosphate	GRX , glutaredoxin
CHA , chlorogenic acid	GSH , glutathione
CoQ₁₀ , coenzyme Q10 (ubiquinone)	GSR , glutathione reductase (gene)
CoQ₁₀H₂ , coenzyme Q ₁₀ (ubiquinol)	GSS , glutathione synthetase
COX , cyclooxygenase	GSSG , glutathione disulphide
CVD , cardiovascular disease	GSSR , protein-bound glutathione
CYBA , α-chain of cytochrome b	GSTP1 , glutathione S-transferase pi 1
CYBB , β-chain of cytochrome b	GSTs , glutathione S-transferases
CYP1A1 , cytochrome P450 1A1	GSTZ1 , glutathione transferase zeta 1
CYP2E1 , cytochrome P450 2E1	H₂-DCFDA , 2',7'-dichlorodihydrofluorescein diacetate
Cys , L-cysteine	HBSS , Hanks' balanced salt solution
Cys_P , peroxidatic cysteine	HCy , homocysteine
Cys_R , resolving cysteine	HMA-CoA , 3-hydroxy-3-methyl-glutaryl-coenzyme A
CySS , L-cystine	HOCl , hypochlorous acid
DCF , 2'-7'-dichlorofluorescein	HPRT1 , hypoxanthine phosphoribosyltransferase 1
DHA , dehydroascorbic acid	
DHLA , dihydrolipoic acid	
DKG , 2,3-diketogulonate	
DMSO , dimethyl sulfoxide	

HSPA1A, heat shock 70kDa protein 1A
HT29, human colon adenocarcinoma cell line
I3C, indole-3-carbinol
iAA, D-isoascorbic acid
iCA, isocitrate
iDHA, dehydroisoascorbic acid
iNaA, sodium D-isoascorbate
iNOS, inducible nitric oxide synthase
LA, α -lipoic acid
LDL, low density lipoprotein
LLys, lipoyllysine
LMP, low melting point
LOOH, lipid hydroperoxides
LOX, lipoxygenase
MD, Mediterranean diet
MDA, malondialdehyde
Met, methionine
MGST3, microsomal glutathione S-transferase 3
MPO, myeloperoxidase
MPs, microplastics
MT, melatonin
MT3, metallothionein 3
mtDNA, mitochondrial DNA
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-, naringenin
N+, naringin
NaA, sodium L-ascorbate
NAC, N-acetylcysteine
NAD, nicotinamide adenine dinucleotide
NAD⁺, oxidized form of nicotinamide adenine dinucleotide
NADH, reduced form of nicotinamide adenine dinucleotide
NADP, nicotinamide adenine dinucleotide phosphate
NADP⁺, oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH, reduced form of nicotinamide adenine dinucleotide phosphate
NAs, nucleic acids
NCF1, neutrophil cytosolic factor 1
NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells
NMP, normal melting point
nNOS, neuronal nitric oxide synthase
NO, nitric oxide
NOS, nitric oxide synthase
NOS2, nitric oxide synthase 2, inducible
NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
NQO1, NAD(P)H:quinone dehydrogenase 1
Nrf2, nuclear factor-erythroid factor 2-related factor 2
OPs, organophosphates
OXSRI, oxidative-stress responsive kinase 1
PBS, phosphate buffered saline
P-CA, p-coumaric acid
PCR, polymerase chain reaction
PDLIM1, PDZ and LIM domain 1
PR, protocatechuic acid
PRDXs, peroxiredoxins
PRNP, prion protein
PTGS, prostaglandin-endoperoxide synthase
Q, quercetin
qPCR, quantitative polymerase chain reaction
RCS, reactive chloride species
RNF7, ring finger protein 7
RNS, reactive nitrogen species
ROS, reactive oxygen species
RPLP0, Ribosomal protein, large, P0
RSS, reactive sulphur species
RT-PCR, real-time polymerase chain reaction
Rut, rutin
SA, sinapic acid
SAH, S-adenosylhomocysteine
SAM, S-adenosylmethionine
SCS, Seven Countries Study
SD, standard deviation
SEPP1, selenoprotein P, plasma, 1
SIRT, sirtuin
SOD, superoxide dismutase
SRXN1, sulfiredoxin 1
STATs, signal transducers and activators of transcription
STK25, serine/threonine kinase 25
SVCT1, sodium-dependent vitamin C transporter 1
T2D, type 2 diabetes
TE, Tris-EDTA
TEAC, Trolox equivalent antioxidant capacity
TNF- α , tumour necrosis factor alpha
TXN, thioredoxin
TXNR, thioredoxin reductase
UA, uric acid
VA, vanillic acid
VIMP, selenoprotein S
WD, western diet
XO, xanthine oxidase
XOR, xanthine oxidoreductase



1. INTRODUCTION

1.1. *Oxidative stress – definition and evolution of the concept*

Stress, in a biological sense, was first recognized by Hans Selye, an endocrinologist working on hormonal changes in rats, who described it in a letter published in *Nature* in 1936 as a *syndrome* that appears in response to damage [1]. In biological or medical sense, the term oxidative stress began to appear in scientific literature no sooner than in the 1970's, however, it was not defined until 1985 when Helmut Sies published his book entitled simply "Oxidative Stress" [2,3]. The term *oxidative stress* was initially understood as "a disturbance in the prooxidant-antioxidant balance in favour of the former" [3] and at that time, researchers focused mainly on oxidative damage and its role in disease development. As new discoveries were demonstrating the inefficacy of antioxidant-based therapies on one hand [4–7] and physiological roles of reactive oxygen species (ROS) on the other [8], the limitations and inadequacy of the above definition of the oxidative stress became evident [9]. With time, antioxidants and reactive oxygen species were no longer considered solely as respectively "the good" and "the bad guys". The research interests shifted from ROS-induced damage of biomolecules to redox signalling and regulation. The understanding of oxidative stress was evolving in parallel and currently it is defined as "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to the disruption of redox signalling and control and/or molecular damage" (Figure 1.1) [10].

Nowadays, oxidative stress is further classified based on its intensity as *eustress*, corresponding to the low level of ROS necessary to maintain physiological cellular functions, and *distress*, referring to an excessive oxidative burden that results in cellular damage and dysfunction [11]. With regards to dose and frequency of oxidative challenge, stress can be also divided into acute, chronic or repetitive, all of which can play a role in oxidative stress adaptation [12]. Furthermore, depending on the trigger, oxidative stress can take a form of dietary postprandial, photooxidative, radiation-induced, nitrosative, glyco-oxidative, endoplasmic reticulum-derived or environmental stress [10]. The opposite processes, *i.e.* depletion of ROS and surplus of reducing agents pushes the redox equilibrium towards *reductive stress* [13].

After several decades of research, the evidence documenting the importance of oxidative stress in disease development seemed well established. Consequently, the idea of antioxidants as cure-all remedies was easily translated into clinical applications. However, the diversification of forms of oxidative stress and its physiological role makes it hard to define sharply the border between *eustress* and *distress* [14], and thus preventive role of antioxidants requires also redefinition.

1.2. *Reactive oxygen species*

Even though the presence of free radicals in biological systems was observed for the first time in 1950s, it was not until the mid-1970's when it became evident, that they are not the only molecules participating in free radical chain reactions [15,16]. Therefore, the expression "reactive



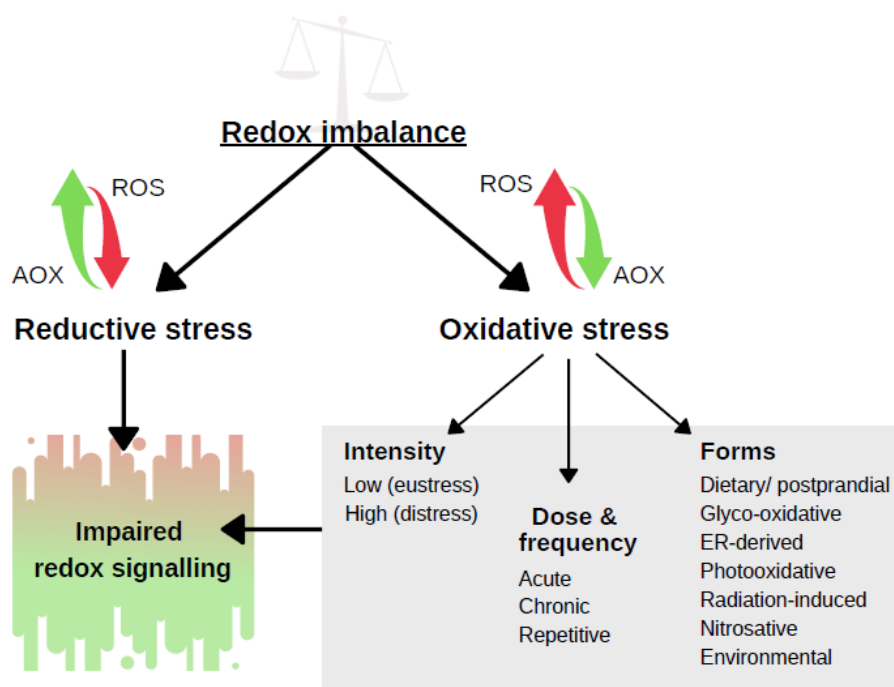


Figure 1.1. The concept of oxidative stress and related terms. Abbreviations used: AOX – antioxidant; ROS – reactive oxygen species; ER – endoplasmic reticulum.

oxygen species” (ROS) was introduced to name collectively all of the oxygen-derived molecules, of free-radical or non-radical nature, formed upon reduction of molecular oxygen (Figure 1.2). Most abundant ROS include free radicals such as superoxide anion ($O_2^{\cdot-}$) or hydroxyl radical (HO^{\cdot}) and hydrogen peroxide (H_2O_2). Due to the presence of an unpaired electron, ROS of radical origin are more reactive than relatively stable H_2O_2 [17]. There are also other reactive species present in cellular redox network, which include nitrogen, sulphur and chloride-centred entities and by analogy are called RNS, RSS and RCS, respectively. Among RNS, which contain also oxygen atom thus may be treated as ROS, nitric oxide (NO) and peroxynitrate ($ONOO^-$) are of uttermost importance. The subject of RSS and RCS is beyond the scope of this chapter and will not be covered in this review.

1.2.1. Formation and physiological roles of ROS

Initially, ROS were recognized solely as deleterious agents responsible for the development of various pathologies and aging, however, between 1970’s and 1990’s the evidence regarding their more widespread physiological roles began to emerge. Currently, it is well recognized that ROS are generated as by-products of aerobic metabolism (e.g. in mitochondria and peroxisomes) and at appropriate physiological levels, they act as signalling messengers and regulators of numerous cellular processes, such as cell proliferation, adhesion, differentiation, survival and death [18]. ROS can be also produced as a result of catalytic activity of some enzymes (e.g. NADPH oxidases in phagocytic and non-phagocytic cells), metal ion-catalysed reactions (e.g. Fenton reactions) or in response to various environmental stressors

embraced under the term exposome (e.g. ionizing radiation, ultraviolet radiation, smoke, air pollutants, drugs, dietary xenobiotics, psychological stress). The list of endogenous and exogenous sources of ROS is presented in Table 1.1.

Table 1.1. Most important endogenous and exogenous sources of reactive oxygen species (ROS) [19–24].

Triggers of ROS production	
Endogenous	Exogenous
<p>Cytosol</p> <p>Purine catabolism Autooxidation of low-molecular-weight compounds</p> <p>Endoplasmic reticulum</p> <p>Xenobiotic metabolism Unsaturated fatty acid biosynthesis Protein folding Thiol oxidase reactions</p> <p>Mitochondria</p> <p>Electron transport via the respiratory chain</p> <p>Peroxisomes</p> <p>Fatty acid α- and β-oxidation Ether-phospholipid biosynthesis Glyoxylate metabolism Amino acid catabolism Polyamine oxidation Oxidative part of the pentose phosphate pathway</p> <p>Plasma membrane</p> <p>Arachidonic acid oxidation Phagocytic and non-phagocytic oxidative burst</p>	<p>Physical stressors</p> <p>UV radiation Ionizing radiation</p> <p>Chemical stressors</p> <p>Air pollutants Tobacco smoke Heavy metals Drugs (e.g. paracetamol, doxorubicin)</p> <p>Diet-derived</p> <p>Oxidized oils Thermally processed meat Pesticides Alcohol</p> <p>Mental stressors</p> <p>Negative life changes Catastrophic events Daily hassles Chronic emotional stressors</p>

Most of endogenous ROS are generated in mitochondria during oxidative phosphorylation as a result of electron leakage from the electron transport chain (ETC), in particular at the stage where molecular oxygen undergoes one-electron reduction to $O_2^{\bullet -}$. As many as seven sites of $O_2^{\bullet -}$ production have been identified in mammalian mitochondria, however, only two of them (complex I and III of ETC) are recognized to be the major ones [25]. It has been estimated that even 1 up to 4% of molecular oxygen consumed by mitochondrial respiration becomes converted to $O_2^{\bullet -}$, though lower values around 0.15% have also been reported [26], [27]. These values were assessed under *in vitro* conditions in isolated mitochondria, so extrapolation to *in vivo* situation should be done with caution [28]. Still, superoxide anion is considered to be the most abundant by-product of cell metabolism from which other ROS may originate. Dismutation of superoxide anion (spontaneous or enzymatic) gives rise to H_2O_2 that by acquisition of one more electron (e.g. in the course of metal-catalysed Fenton reaction) can form HO^{\bullet} . The latter is the most reactive radical and the least specific ROS. Due to high reduction

potential, it rapidly reacts with any molecule that is present in its closest neighbourhood [29]. Finally, upon addition of one proton and one electron, HO^\bullet gets reduced to water molecule (Figure 1.2) [30]. *In vivo*, this reaction occurs through the abstraction of hydrogen atom from lipid or protein structures, initiating chain radical reactions involving these biomolecules as a result.

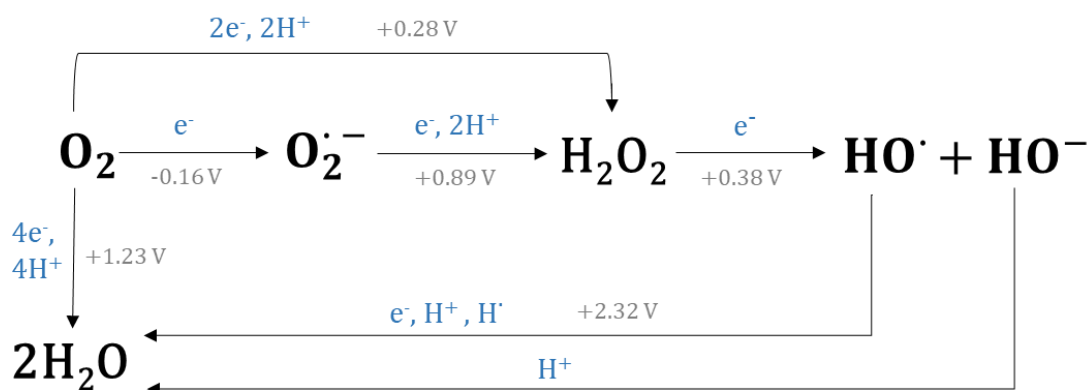


Figure 1.2. The simplified scheme of formation of reactive oxygen species. Formal reduction potential values (at pH 7) are adapted from Li *et al.* (2019) [31].

Peroxisomes constitute another site of endogenous ROS generation due to oxidative metabolism of *e.g.* fatty acids, glyoxylate and amino acids as well as phospholipid biosynthesis, which takes place there. These organelle contain multiple enzymes producing reactive forms of oxygen and nitrogen, however, xanthine oxidoreductase (XOR) is considered as the major one. XOR is a rate-limiting enzyme in purine catabolism as it catalyses the oxidation of hypoxanthine to xanthine, which undergoes further oxidation to uric acid. In the course of these reactions, XOR generates H_2O_2 and $\text{O}_2^{\bullet -}$ as by-products [32]. Additionally, XOR can also produce NO upon reduction of nitrates. Since in peroxisomes both NO and $\text{O}_2^{\bullet -}$ are produced, it is highly probable that they react to generate ONOO^- [21].

In endoplasmic reticulum (ER), a relatively oxidizing environment is maintained in lumen as here H_2O_2 is utilized and produced in the processes of oxidative protein folding [33]. Disturbance of ER redox homeostasis may lead to the accumulation of unfolded proteins and result in so-called ER-stress, in which excessive ROS generation finally causes oxidative stress [34]. Besides, H_2O_2 and $\text{O}_2^{\bullet -}$ can be also generated during oxidative metabolism of xenobiotics, heme and fatty acids by microsomal monooxygenases involving cytochromes P450 and *b5* [35]. Indeed, the microsomal cytochrome P450-dependent monooxygenase system has been suggested as the greatest source of ROS since up to 45% of H_2O_2 was reported to be produced in rat liver where this system is the most active [36].

Membrane-bound NADPH oxidases (NOXs) constitute another major source of ROS, whose generation is established as the primary and probably sole function of these enzymes [19]. Both phagocytic and nonphagocytic isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX1-3) mediate the release of $\text{O}_2^{\bullet -}$ as a side product of electron transfer

from NADPH to molecular oxygen. NOXs present in phagocytic cells release ROS in response to fungal, bacterial and viral infection in order to kill the pathogenic microorganisms [37,38]. These ROS can be excreted either extracellularly at the site of infection or intracellularly following phagocytosis of bacteria. The primary product of oxidative burst is $O_2^{\cdot-}$ that is subsequently dismutated to H_2O_2 [39], which further can either oxidize ferrous (Fe^{2+}) iron with concomitant generation of HO^{\cdot} or serve as a substrate for myeloperoxidase (MPO). MPO present in neutrophils, also generates an array of effective antibacterial substances such as hypochlorous acid (HOCl), singlet oxygen (1O_2), HO^{\cdot} , chloramines and ozone [40]. Nonphagocytic NOXs generate lower levels of $O_2^{\cdot-}$ compared to those present in phagocytic cells, even under pathological conditions [41]. The latter NOXs are expressed in various cell types such as fibroblasts, vascular endothelial cells, neurons and skeletal muscle myocytes, where ROS are involved in intra- and intercellular communication, redox signalling and proper cell function [42–45]. Production of ROS in response to the inflammatory processes occurs also via alternative pathways as a result of lipoxygenase and cyclooxygenase-mediated oxidation of arachidonic acid, which is released from membrane phospholipids to produce prostaglandins, leukotriens and thromboxanes [46]. Arachidonic acid and its metabolites have been also reported to induce ROS generation by NOXs [47–49].

Nitric oxide synthases (NOS) constitute the important source of nitric oxide (NO) formed upon conversion of L-arginine to L-citrulline. Under certain conditions, NOS can also generate $O_2^{\cdot-}$, H_2O_2 and $ONOO^-$. In mammals, there are three different isoforms of NOS: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) [50]. iNOS is stimulated in response to inflammation and infection [51], while eNOS-mediated NO is crucial for cardiovascular health: it regulates blood pressure, vascular tone and platelet function [50,52]. NO maintains normal blood pressure through the activation of soluble guanylate cyclase whose activity leads to elevated levels of cyclic guanosine-5'-monophosphate (cGMP), which results in relaxation of vascular smooth muscle and vasodilation [53]. Furthermore, NO exerts antithrombotic effects through inhibition of activation, adhesion and aggregation of platelets [54]. Even though eNOS is expressed mainly in endothelial cells, it has been detected (together with nNOS) in cardiomyocytes, where NO is also known to control cardiac contractility and heart rate [55,56].

1.2.2. ROS as inducers of oxidative damage of biomolecules

In the organism, excessive production of ROS can be induced in response to impaired homeostasis, for example, as a result of infection and inflammation, acute exercise, mental stress, aging or ongoing development of pathologies (e.g. ischemia, cancer), to name just a few [57]. Overproduction of ROS can lead to oxidative damage of biomolecules such as nucleic acids, lipids or proteins and compromise their function. The below subsections focus on the deleterious effects of ROS on biomolecules, thus on processes that contribute to oxidative stress detrimental effects and may lead to disease development.



Oxidation of nucleic acids

Hydroxyl radical is the major ROS responsible for oxidative damage of DNA, as it can react with all its components (purine/pyrimidine bases and ribose). There are three main types of DNA damage that can initiate mutations: (i) formation of so-called oxygen DNA adducts, (ii) generation of single- and double-strand breaks and (iii) formation of DNA-protein cross-links [58]. Among DNA nucleobases, guanine is the most prone to oxidation due to its low oxidation potential [59]. The attack of HO[•] on 2'-deoxyguanosine results in the formation of 8-oxo-2'-deoxyguanosine (8-oxo-dG) that together with 8-hydroxyguanosine (8-oxoG) are the most studied modifications of nucleic acids. The presence of 8-oxo-dG, if not repaired, may lead to mispairing of G with T and subsequently to mutation, which makes this modification an important risk factor in genome instability and carcinogenesis [60,61]. The presence of 8-oxo-dG in urine has been widely used as a non-invasive biomarker of oxidative stress [62].

Except for the nucleus, around 1% of DNA is also contained in the mitochondria. Due to its location in the close proximity to the electron transport chain, mitochondrial DNA (mtDNA) is exceptionally prone to oxidative damage. In addition, mtDNA is particularly susceptible to damage as it does not contain histones. To make things worse, mitochondrial repair system capacity is very limited. For these reasons, mtDNA is notably more prone to oxidation than genomic DNA (gDNA). Indeed, it has been reported that the level of 8-oxo-dG can be even 10-15-fold higher in mtDNA than in gDNA [63–65]. Similarly to mtDNA, RNA molecules are also more susceptible to oxidation than DNA with 8-oxo-G being the most prevalent modified nucleoside [66].

Lipid peroxidation

Lipid peroxidation is a process of oxidative damage of fatty acids that is initiated by ROS, especially HO[•], and further propagated by chain reactions. This cascade begins with an attack of free radical on methylene group in a structure of unsaturated fatty acid. As a result, alkoxy radical R[•] is formed, which further reacts with molecular oxygen to peroxy radical (ROO[•]). Lipid peroxy radicals abstract hydrogen from other unsaturated lipid molecules and transform them either into lipid hydroperoxides (LOOH) or another alkoxy radical, which enter the next cycle of lipid oxidation. This sequence of reactions, collectively named lipid peroxidation, goes on continuously unless the formed radicals react either with each other or with antioxidant molecules, which terminate the chain reactions by formation of stable nonradical products [67]. The end products of lipid peroxidation can be divided into primary products (lipid hydroperoxides), which can further decompose to secondary products (e.g. lipid hydroxides, ketones, epoxides or furans. In the latter case, as a result of C-C bond cleavage, various lipids and low-molecular aldehydes (e.g., malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) are formed. MDA, 4-HNE and F2-isoprostanes (prostaglandin-like substances that can be formed in the course of nonenzymatic oxidation of arachidonic acid) constitute the most studied end products of lipid peroxidation. All these metabolites are currently used as biomarkers in oxidative stress assessments [68]. Furthermore, electrophilic products of lipid peroxidation can also initiate the formation of various

adducts formed by irreversible conjugation with nucleophilic residues of biomolecules such as proteins, nucleic acids or cell membrane phospholipids. The latter adducts are called advanced lipoxidation end products (ALEs) [69].

The effects of lipid peroxidation on cell function are detrimental since they may cause disruption of cell membranes, alterations of their fluidity and permeability as well as functionality both at lipid and protein level [70]. Structure and function of biological membranes depends on their constituents, that is lipid classes and fatty acid composition. Main components of animal cell membranes include phospholipids, glycolipids and cholesterol, all of which are prone to oxidative modifications [67]. Polyunsaturated fatty acids (PUFAs), which are present in phospholipids and glycolipids, are exceptionally susceptible to non-enzymatic lipid peroxidation due to multiple unsaturated bonds present in their structure [71]. Cholesterol can undergo autooxidation to oxysterols, the process stimulated by free radicals [72]. Besides, lipids can be also subjected to enzymatic oxidation by lipoxygenases (LOXs), cyclooxygenases (COXs) and cytochrome P450 monooxygenases that exacerbate oxidative stress [73]. However, it should be mentioned that lipid oxidation is not only detrimental event. Enzymatic oxidation of arachidonic acid, belonging to n-6 family of fatty acids, produces prostaglandins, leukotriens and thromboxanes, which are involved in *e.g.* inflammatory and pain responses, as well as vasodilation and blood pressure control. Cholesterol hydroxylases present in mitochondria or endoplasmic reticulum catalyse the oxidation of cholesterol to oxysterols. This process is involved in *e.g.* the elimination of excess cholesterol from human organism [74].

Oxidative modifications of proteins

Due to their abundance in the biological systems, proteins may be considered as the major targets for oxidation [75]. These biomolecules can be attacked by almost any type of ROS with HO[•] being again the major oxidant. Similarly to lipid peroxidation process, proteins can also undergo peroxidation; starting from carbon-centred radicals that can be further converted to protein peroxy radicals and then to protein hydroperoxides [76]. Modifications of the residues of lysine, proline, threonine and arginine result in the formation of protein carbonyls. Carbonylated proteins can be also generated upon reaction with the products of lipid peroxidation, which form already mentioned ALEs, or reducing sugars giving rise to advanced glycation end-products (AGEs) [77]. Since the increased quantity of carbonylated proteins has been observed in numerous diseases, their assessment is widely used as a biomarker of oxidative stress [78]. Another type of irreversible protein oxidative modification is tyrosine nitration that arises upon reaction of RNS with tyrosine residues [51]. Oxidative modifications of proteins can force changes in conformation, induce intramolecular crosslinking, aggregation or fragmentation, all of which finally affect function, which can be either reduced or completely abolished.

Cysteine (Cys) and methionine (Met) are the most prone to oxidation, however unlike other amino acids, their modifications are reversible under physiological conditions. This feature is essential for the ability of these amino acids to sense any changes in cellular redox status. Not



surprisingly, Cys and Met govern cellular thiol-redox signalling, which seems to be of the uttermost importance in terms of the maintenance of redox homeostasis [79,80].

1.2.3. *Exposome-derived ROS*

The term exposome embraces all external and internal (but non-genetic) factors that can be divided into 3 groups: (1) *general* being an inseparable part of human our life, such as urban environment, climate factors and stress, (2) *specific*, which refers to lifestyle-based choices such as diet, physical activity, cigarette smoking as well as individual health-related aspects (infections, medical drugs) and exposure to radiation (3) *internal* associated with metabolism, gut microbiota, inflammation and redox status [81], [82]. In this subsection, the impact of selected exogenous factors on ROS formation will be discussed with the special emphasis on diet-related inductors of oxidative stress.

Diet-induced oxidative stress

With the average of 32 m², the gastrointestinal (GI) tract is the biggest surface area in human organism that is being constantly exposed to the external environment. This makes human gut notoriously challenged by numerous pro-oxidants [83], [84]. Most of pro-oxidants in the GI tract are of dietary origin, hence the term *nutritional oxidative stress* defined by Helmut Sies as “an imbalance between the pro-oxidant load and the antioxidant defense as a consequence of excess oxidative load or of inadequate supply of the organisms with nutrients” [85]. The disruption of redox homeostasis in GI tract may be caused either by macronutrients such as carbohydrates, lipids and proteins (known as postprandial oxidative stress), micronutrients (iron, copper, etc.) or by pro-oxidative reactants formed upon food processing, storage and metabolism.

The vivid example of pro-oxidative nutrition is so-called Western diet (WD) that is currently widespread in majority of modern and industrialized countries [86]. WD is characterized by high-caloric meals rich in refined carbohydrates and fat, but poor in fibre, vitamins, minerals and other bioactive compounds. Not surprisingly, in the past decades, westernized lifestyle has led to the dramatic increase in the occurrence of the chronic noncommunicable diseases such as metabolic disorder, cancer, neurodegenerative diseases and depression [87]. All of these diseases are also associated with an impaired redox homeostasis [88,89]. There are several ways by which WD contributes to oxidative stress. Firstly, overconsumption of foods rich in carbohydrates and lipids leads to continuous exposure to elevated levels of glucose and free fatty acids (FFA). Such overnutrition overwhelms mitochondria and further results in overgeneration of O₂^{•-}. Excessive abundance of O₂^{•-} can lead to formation of other free radicals, which if not neutralized, may cause oxidative stress and induction of modifications of biomolecules, e.g. low density lipoprotein (LDL) and cholesterol. Both oxidized LDL and oxysterols are implicated in the development of atherosclerosis [90]. Secondly, overconsumption of vegetable oils rich in omega-6 PUFAs with the simultaneous insufficient intake of omega-3 fatty acids contributes to chronic inflammation, which is interrelated with oxidative stress [87,91,92]. Moreover, individuals who consumed high amounts of omega-6 PUFAs were reported to have high levels of DNA adducts resulting from



reactions with MDA [93]. Thirdly, processed and ultra-processed food products, whose consumption is prevalent in industrialized populations, contain numerous components that can promote ROS generation. On top of high sugar and fat load, whose impact on redox homeostasis has been already mentioned, these products are also rich in food additives such as certain preservatives, artificial sweeteners, sensory enhancers or colourings, which are repeatedly reported to promote oxidative stress [94,95]. Finally, Edalati *et al.* have shown recently that the high intake of ultra-processed food was associated with elevated urinary levels of 8-oxo-dG [96].

Even though according to the current knowledge carbohydrates and lipids are main contributors to postprandial oxidative stress, protein-based meals can also induce generation of ROS at a comparable level to glucose [97]. It has been reported that long-term high-protein intake leads to increased oxidative stress in rats [98,99]. As it comes to individual amino acids, high concentrations of branched-chain amino acids have been shown to induce oxidative stress in peripheral blood mononuclear cells from healthy donors [100]. Similarly, methionine was also reported to increase ROS generation in murine colitis model, which was alleviated when methionine dietary content was restricted [101]. In contrast, the increased intake of dietary cysteine has been demonstrated to boost glutathione (GSH) levels and therefore to prevent postprandial disruption of redox homeostasis in rats fed a high-sucrose diet [102]. Furthermore, moderate amounts of proteins were shown to lower hyperglycemia and hyperlipidemia after consumption of high-fat and high-carbohydrate meals [103]. In line with these observations are some clinical trials involving type 2 diabetes patients, which concluded that high-protein diet (from both animal and plant sources) can be successfully implemented as a strategy towards diabetes prevention and treatment [104].

In terms of the impact of proteins on postprandial oxidative stress response, the most discussed protein-rich food is meat. Nevertheless, this debate goes beyond the protein content. Food is a complex matrix and besides proteins (including protein carbonyls), meat also contains heme iron that if present in a free form, can initiate lipid peroxidation and formation of lipid peroxidation end-products such as MDA, 4-HNE or oxysterols [105,106]. The impact of repeated consumption of these carcinogenic fat oxidation products is highly discussed in scientific literature as they were shown to be absorbed from GI tract and thus pose a threat to human health [106,107]. Besides, other potential carcinogenic components of processed meat include (i) heterocyclic amines, (ii) nitrosamines, (iii) polycyclic aromatic hydrocarbons and (iv) N-glycolylneuraminic acid [108], some of which are formed in radical mediated reactions.

Alcohol consumption

According to the World Health Organization Global status report on alcohol and health 2018 [109], in 2016 the global alcohol consumption has been estimated at 6.4 litres of pure alcohol per person per year, which equals to the consumption of approximately 1 litre of wine per week. The highest intake of alcohol was observed in Europe with the average of 12-14 litres and 15 litres in Western and Eastern European countries, respectively. In 2019, according to Eurostat, 8.4% of European Union population consumed alcoholic beverages every day [110]. To make things



worse, COVID-19 pandemic increased the frequency of drinking behaviours due to psychological distress [111–113], which will probably lead to greater burden of diseases and/or premature mortality.

Classical pathway of ethanol metabolism involves oxidation reactions catalysed by alcohol dehydrogenase and acetaldehyde dehydrogenase with respectively acetaldehyde and acetate as the reaction products. These processes increase the ratio of reduced to oxidized form of nicotinamide adenine dinucleotide (NADH/NAD⁺) in mitochondria, which may lead to the decreased FFA oxidation and thus, accumulation of intracellular lipids [114,115]. Interestingly, ethanol can be naturally produced and absorbed in the GI tract by host microbiota or in the course of degradation of threonine, deoxyribose phosphate and β-alanine forming acetaldehyde, which is then metabolized in the liver [116]. Ethanol can be also metabolized via cytochrome P450 2E1 (CYP2E1), whose activity is significantly increased in alcohol consumers. Since CYP2E1 possess NADPH oxidase activity, following alcohol consumption CYP2E1 contributes to the elevated generation of O₂^{•-} and H₂O₂ [117]. Both pathways of ethanol metabolism (involving alcohol dehydrogenase and CYP2E1) are implicated in the development of alcohol-induced liver disease [115,118]. Ethanol consumption can also lead to alcohol-induced oxidative disruption of intestinal barrier via upregulation of iNOS and thus, excessive generation of nitric oxide [119]. These processes implicated in alcohol metabolism can affect redox homeostasis either by altering NADH/NAD⁺ ratio in mitochondria or by increasing generation of ROS.

Tobacco smoke

Cigarette smoke is one of the most abundant sources of free radicals, with its gas phase estimated to contain as much as ~10¹⁵ radicals per puff and as much as 10¹⁷ radicals per gram of tar [82]. Gas phase radicals are short-lived and can be either oxygen-, nitrogen- or carbon-centred. The tar contains long-lived semiquinone radical that can further reduce oxygen to give rise to more reactive species such as O₂^{•-}, and then H₂O₂ and HO[•] [120]. Besides, plentiful of carcinogens inhaled during smoking (e.g. benzo[a]pyrene, acrylamide, N-nitrosamines) as well as trace amounts of heavy metals in tobacco smoke can also contribute to intensified ROS production through Fenton-like reactions [82]. Such tremendous doses of free radicals significantly exhaust the endogenous systems of antioxidant protection against oxidative stress. The population-based study revealed that 35% of cigarette smokers were deficient in vitamin C [121]. Another study showed that smokers had decreased activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes compared to non-smokers [122]. Notoriously increased levels of ROS and carcinogens as well as weakened reservoirs of endogenous antioxidants make intracellular damage inevitable. Cigarette smoking is known to induce DNA damage (as shown by increased levels of 8-oxoG in smokers' lungs) [123]. Joehanes *et al.* investigated the relationship between genome-wide DNA methylation and smoking and found that DNA methylation patterns may be affected by smoking in more than 7,000 genes, which are known to participate in cell signalling, metabolism and regulation of gene expression



[124]. Besides, these genes were also found to be associated with smoking-related diseases such as colorectal cancer, cardiovascular disease (CVD) or chronic obstructive pulmonary disease.

Ionizing radiation & chemotherapeutic agents

Ionizing radiation, used for therapeutic purposes, produces ROS such as HO^\bullet , $\text{O}_2^{\bullet-}$ and H_2O_2 upon water radiolysis in the extracellular milieu. Initially, generated ROS propagate free radical reactions, since IR increases the activity of NADPH oxidase and iNOS. Generated by NADPH oxidase $\text{O}_2^{\bullet-}$ can easily react with excess nitric oxide (generated by iNOS) and form ONOO^- and other RNS. It has been shown that irradiation (10 Gy of X-rays) of human lung carcinoma cells increased mitochondrial respiration, membrane potential and therefore, lead to elevated ROS generation [125]. In turn, some studies reported that mitochondria, despite of decreased activity of ETC complexes, still had significantly higher levels of ROS [126]. The abundance of ROS and RNS generated by IR have been shown to contribute to intracellular damage of biomolecules such as DNA [127]. Several reports confirmed that mtDNA is more susceptible to damage than gDNA. After irradiation with 150 Gy gamma radiation in mtDNA and gDNA, isolated from the same rat liver, the occurrence of 8-oxoG was 6-fold higher in mtDNA [63]. In another study, exposure of human colon cancer cells to 560 Gy gamma radiation caused twice more strand breaks in mtDNA than in gDNA [128]. Damage of mtDNA may lead to mutations and therefore, to progressive dysfunction of respiratory processes resulting in exacerbated oxidative stress [129].

Cancer cells, due to enhanced proliferation and metabolism, naturally generate elevated levels of ROS. Constant state of oxidative stress is known to be implicated in the promotion of tumorigenesis, angiogenesis and metastasis. Numerous anticancer drugs are designed to further increase levels of ROS until they reach cytotoxic levels and trigger apoptosis of cancer cells. For example, platinum-based drugs, teniposide as well as 5-fluorouracil were shown to cause cytochrome c release from mitochondria leading to the impairment of ETC, excessive generation of ROS and consequently, cell death [130]. Cancer chemotherapeutic agents can also stimulate formation of ROS that induce toxicity via increased lipid peroxidation [131]. It was shown that doxorubicin competes with coenzyme Q_{10} (CoQ_{10}) in cardiac cells and as such, it redirects electrons to molecular oxygen resulting in $\text{O}_2^{\bullet-}$ generation and cardiotoxicity [132]. Oxidative stress-mediated tissue injuries and worsened nutritional status of cancer patients undergoing pharmacological treatments raised the idea of antioxidant supplementation to help mitigate cancer therapy side effects. Indeed, some researchers reported positive effects of chemotherapy combined with antioxidant supplementation on patients wellbeing, survival and even increased efficacy of the treatment [133]. However, this topic remains controversial since antioxidants have been also reported to diminish the efficacy of chemotherapy, to accelerate tumorigenesis or even to reduce patients' survival [134–136].



Solar UV radiation

Solar UV radiation embraces three wavelengths regions: UVA (320-400 nm), UVB (290-320 nm) and UVC (220-290 nm). Most of the UVB and all UVC is absorbed by ozone layer, while 95% of UVA reaches the Earth [137]. Sun exposure (more specifically, UVB wavelengths) are crucial for humans to synthesize vitamin D. However, UV radiation can also induce ROS formation in the skin and therefore, prolonged exposure to UV radiation can lead to skin damage – erythema in the short term effect, while skin aging as well as induction of carcinogenesis are the long term risks. Among UV radiation spectra, UVA radiation is considered to be the main contributor to oxidative stress, since it induces generation of ROS ($^1\text{O}_2$, H_2O_2). UVA was also shown to induce the release of free iron from ferritin [138], which can further promote lipid peroxidation via iron-catalysed oxidation reactions as shown in human skin fibroblasts [139]. Irradiation of tissue culture model of human skin with high doses of full solar spectrum of UV caused depletion of endogenous antioxidants, with CoQ₁₀ concentrations being the most severely affected [140]. Oxidative stress was also shown to be induced in response to UVB radiation, which increased the activity of NOS and xanthine oxidase (XO) in human keratinocytes. The activated NOS and XO were shown to elevate the generation of NO and $\text{O}_2^{\cdot-}$, respectively, in a dose-dependent manner [141]. Nitric oxide and superoxide anion radical can react with each other and form ONOO⁻ whose concentration was also shown to be increased by UVB radiation in the same study [141]. All these redox-related reactive species are likely to be involved in skin erythema and inflammation caused by exposure to UV light [142].

Environmental pollutants

The industrialization of the modern world has made it virtually impossible to avoid the exposure to various man-made chemicals that are abundantly released into the environment. Humans are surrounded by environmental pollutants in everyday life, which is not indifferent to health [143–146]. Environmental stressors, including endocrine disruptors present in foodstuffs (e.g. bisphenols or pesticides) induce oxidative stress, which is implicated in the development of several health-threatening complications.

Bisphenol A (BPA) is a synthetic compound used in production of packaging materials for foods and beverages (in particular, inner coating of metal cans and plastic bottles). It was reported that BPA starts to migrate into foodstuffs already at the stage of food production, and this release is continued during other stages of food supply chain such as handling, packaging and transportation of food products. The highest concentrations of BPA in foodstuffs were reported in products stored in plastic bottles (e.g. vegetable oils) and canned products (e.g. canned fish) [147]. BPA is classified in the first category of Endocrine Disruptive Chemicals, for which there is sufficient evidence that they can exert detrimental effects on human reproductive system. Besides, it may be also implicated in the development of cancer, diabetes and obesity [148]. One of the mechanisms, by which BPA may contribute to health impairment is the induction of oxidative stress. BPA is metabolized by cytochrome P450 to BPA 3,4-quinone, which was shown

to be a strong oxidant and inducer of ROS-generating enzyme – XO [149]. Exposure to BPA was reported to reduce total antioxidant capacity and activity of antioxidant enzymes in numerous tissues *in vivo* [148]. The systematic review including 20 animal studies showed that BPA could markedly decrease the abundance of GSH and antioxidant enzymes such as catalase (CAT), GPx and SOD, as well as cause significant increase of MDA levels [150]. The markers of oxidative stress were also found to be positively correlated with serum levels of BPA and its analogues in people (n = 353) who were highly exposed to this chemical [151].

As a consequence of the widespread usage of plastics, the presence of microplastics (MPs) in the marine, terrestrial environments and even *in utero*, has become an integral feature of the environment [152,153]. The annual consumption of MPs in the American population was estimated to reach up to 93,000 MPs particles per person, regarding that bottled water was the only source of drinking water [154]. The evidence on the impact of MPs on redox homeostasis *in vitro* and *in vivo* is growing, however, research on this subject is still in its infancy [155]. For example, MPs were shown to increase the level of MDA and to decrease the activity of SOD, CAT and GPx in cardiac tissue, which contributed to the occurrence of cardiotoxicity in rats [156]. Numerous studies have shown that MPs can accumulate in the gut and liver of aquatic organisms. MPs were found to cause inflammation, alter lipid and energy metabolism and increase the activity of CAT and SOD in response to oxidative stress in the liver of zebrafish [157]. Another study on zebrafish showed that MPs induced ROS generation that decrease in the abundance of antioxidant enzymes and apoptosis via p53 signalling [158]. The impact of bioaccumulation of MPs in foods on human health is currently widely debated, however, available information is still limited to draw any hard conclusions [154,159,160].

Pesticides are extensively used in heavily industrialized agriculture and therefore, they contaminate not only foodstuffs, but also water and soil. One study found pesticide residues in almost 40% of tested samples of fruits and vegetables available on the Polish market. The highest amount of pesticide levels were found in gooseberry, apples, grapes, black currant among fruits and celeriac, tomato, sweet pepper and Peking cabbage among vegetables [161]. Exposure to pesticides has been related with the development of human diseases such as cancer, allergies or infertility [162]. Numerous studies showed that agricultural workers chronically exposed to pesticides had significantly elevated levels of oxidative stress biomarkers indicating lipid peroxidation and DNA damage [163–165]. Not surprisingly, oxidative stress is considered to be one of the mechanisms by which pesticides exert detrimental effects on human health [163,166]. For example, organophosphates (OPs), one of the most widely used pesticides worldwide, were shown to affect redox homeostasis in rat tissues such as liver and brain. Exposure of rats to OPs significantly decreased GSH levels in liver and brain tissue with the concomitant increase in GSSG levels [167]. Interestingly, GSH/GSSG ratio was not as much disrupted in the rats, which were fed with a mixture of antioxidative vitamins (A, C and E) for 15 days before exposure to OPs compared to vitamin unfed animals [167]. Another study showed that pesticides may lead to mitochondrial dysfunction as the exposure to OPs markedly decreased CoQ₁₀ levels in human neuroblastoma cells, as well as reduced the activity of ETC and citrate synthase [168]. Exposure



to pesticides *in utero* caused increased levels of MDA in liver and kidney of female Wistar rats as well as the offspring. The observed changes persisted from weaning till adulthood, which implied the possible transgenerational adverse effects of pesticide exposure [169]. OPs were also shown to induce inflammation via increased levels of e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), tumour necrosis factor alpha (TNF- α) and COX-2, as well as increased ROS generation and DNA damage in human epithelial ovary cells *in vitro* [170].

1.3. Antioxidants

ROS and oxidative stress are integral parts of aerobic life, therefore it is not surprising that organisms developed their own antioxidant systems of protection against excessive amounts of oxidants, which could disrupt cell structures and impair function. This endogenous protective systems are supported by exogenous reducing substances derived from dietary sources. In the case of food components, an *antioxidant* is described as a substance that once consumed “delays, prevents or removes oxidative damage to a target molecule” [171,172]. In a chemical sense, antioxidants (or reducing agents) donate electrons to oxidizing agents e.g. free radicals, which gain electrons. As a result, oxidants undergo reduction, while antioxidant molecules are transformed into their oxidized forms [173]. The ability of a compound to accept electrons under standard conditions is defined by the *standard reduction potential* (E^0). The lower the value of E^0 , the stronger the antioxidant activity, as the compound is more likely to donate rather than to accept electrons. Most of the data found in scientific literature refers to the values of the *formal reduction potentials* E^0 , i.e., determined at pH 7.0 or 7.4, as they are recognized to be more relevant in terms of biological systems [174,175]. Besides thermodynamics, the reactivity of redox-active compounds is also determined by kinetics of redox reaction in which they are involved [176].

As a matter of fact, the systematic classification of antioxidants does not exist [177]. They can be assigned to various groups according to, e.g. their origin (natural, synthetic), source (exogenous, endogenous), solubility (hydrophilic, lipophilic), size (low or high molecular weight) or activity (enzymatic, nonenzymatic). The proposed mechanisms of antioxidant activity include: sequestration of free radicals, chelation of transition metals, termination of chain reactions, repair of the damaged biomolecules and enhancement of the activity of endogenous antioxidant enzymes. Furthermore, antioxidants can be considered as the first (antioxidant enzymes), the second (endogenous antioxidants) or even the third (antioxidant enzymes involved in the repair of oxidative damage) line of antioxidant defense [178,179]. These protective networks correspond to the *strategies of antioxidant defense* named (i) prevention, (ii) interception, (iii) repair and (iv) adaptation [180]. The aim of preventive measures is, just as this term implies, to avert the generation of ROS primarily at source, while interception is about the neutralization of ROS, which managed to evade the protective mechanisms [10,181]. Repair refers to the ability of certain enzymes, such as methionine sulfoxide and cystine reductases, to contribute to a reversal of oxidative damage of biomolecules by reduction of the oxidized residues of amino acids in proteins [182,183]. Such reversible redox modifications are also fundamental for redox signalling



pathways [79]. Finally, adaptation processes sustain the expression of antioxidant proteins to improve resistance to oxidative stress [11].

1.3.1. Enzymatic antioxidants

Antioxidant enzymes constitute the most effective antioxidative defense, since their cellular abundance (estimated at around 3% of the total cellular protein content) as well as high reaction rates ensure effective ROS neutralization [184]. Numerous enzymes contribute to the maintenance of redox homeostasis, however, SOD, CAT and GPx are considered as the major ones. Other enzymes indirectly involved in redox homeostasis control include thioredoxin (TXN) system, peroxiredoxins (PRDXs) and sulfiredoxin (SRXN1). The interplay between antioxidant enzymes is presented in Figure 1.3.

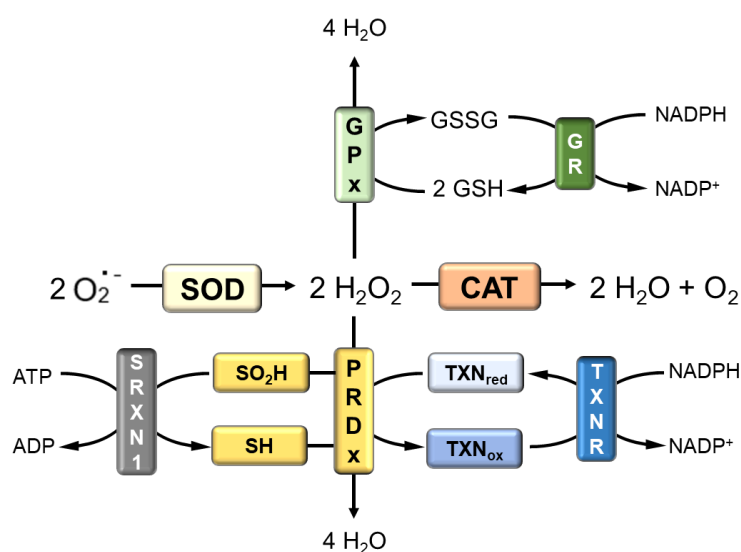


Figure 1.3. Concerted action of major enzymatic and non-enzymatic factors constituting endogenous antioxidant system: CAT, catalase; GSH, glutathione; GSSG, glutathione disulfide; GPx, glutathione peroxidase; GR, glutathione reductase; PRDX, peroxiredoxins; SOD, superoxide dismutase; SRXN1, sulfiredoxin 1; TXN, thioredoxin; TXNR, thioredoxin reductase.

Superoxide dismutase

Superoxide dismutase (SOD) is an ubiquitous enzyme that in mammalian cells occurs in 3 isoforms: located in the cytosol SOD1 that is associated with copper and zinc ions, mitochondrial SOD2 associated with manganese and/or iron ions, and extracellular SOD3 that requires copper and zinc ions to perform catalytic function. SOD catalyses the reaction of superoxide anion radical dismutation to hydrogen peroxide and oxygen at a rate of $\sim 2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is $\sim 10^4$ times more efficient than spontaneous dismutation. Moreover, under homeostatic conditions the abundance of SOD in cells is at least 5 orders of magnitude higher than that of superoxide radical [185,186].

Catalase

Catalase (CAT) is the first discovered enzymatic antioxidant and the major enzyme responsible for removal of hydrogen peroxide from cellular milieu. It is expressed in all major tissues in the human body with the highest activity in liver, kidney and red blood cells [187]. Human CAT possesses one heme prosthetic group and it utilizes NADPH as a cofactor. CAT is localized mainly in peroxisomes, where multiple oxidative reactions take place as already discussed in section 1.2.1. With a catalytic rate of $\sim 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, it has been estimated that every minute this enzyme can convert around 6×10^6 molecules of hydrogen peroxide into water molecule and oxygen [188].

Glutathione peroxidases

Glutathione peroxidases (GPxs) catalyse reduction of hydrogen peroxide and hydroperoxides with the release of water molecule or corresponding alcohols at the expense of GSH that becomes oxidized to GSSG. The rate constant of these reactions is estimated to be $\sim 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [189]. Reduction of GSSG back to two molecules of GSH is catalysed by glutathione reductase (GR) that utilizes FADH_2 or NADPH as cofactors.

There are 8 mammalian GPxs, 5 of which require selenocysteine in their catalytic centre to perform their functions (GPx1-4 and 6), while the other 3 (GPx5, 7 and 8) are cysteine-dependent and do not possess GSH-binding domain. Different isoforms are present in different locations; GPx1 can be found in cytosol and mitochondria, GPx2 in epithelial cells of the intestine, GPx3 in the plasma, GPx6 in the olfactory epithelium lining the nasal cavity, whereas GPx4 provides protection to cellular membranes. The non-selenium dependent GPxs are found in testes (GPx5), while GPx7 and 8 were shown to exert low GPx activity and act rather as protein disulphide isomerase peroxidases in ER [190], [191].

Peroxiredoxins

Peroxiredoxins are another group of thiol-dependent enzymes that catalyse reduction of hydrogen peroxide, hydroperoxides and peroxy nitrates. They catalyse reduction reactions at a rate of $\sim 1-4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ but unlike CAT and GPx, they do not require cofactors to perform their functions. There are 6 different isoforms of human PRDXs: PRDX1-2 and 6 are located in the cytosol, PRDX3 in the mitochondrial matrix, PRDX4 in ER and PRDX5 in the mitochondria, cytosol and peroxisomes [192]. Based on the structure of their catalytic centre, PRDXs can be further subdivided into typical 2-Cys PRDXs1-4, atypical 2-Cys PRDX5, and 1-Cys PRDX6. Typical 2-Cys PRDXs are most ubiquitous in cellular milieu. These enzymes perform catalytic function with the aid of two conserved cysteines: one of them is called catalytic or peroxidatic Cys (Cys_P), and the other one is resolving Cys (Cys_R). Upon reduction of hydroperoxides, Cys_P undergoes oxidation to sulfenic acid, which can be subsequently further oxidized to sulfinic acid. Acquirement of this form results in enzyme inactivation. SRXN1 is indispensable for typical 2-Cys PRDXs reactivation, because as shown in Figure 1.3, it reduces sulfinic to sulfenic acid of Cys_P



[193,194]. This process is slow and requires adenosine-5'-triphosphate (ATP), which suggests that besides providing protection against oxidative damage, SRXN1 contributes to the regulation of hydrogen peroxide-mediated cellular signalling [195]. Sulfenic acid group restored by SRXN1 can be further reduced to a thiol group with the aid of TXN system that involves also thioredoxin reductase (TXNR) and NADPH as a cofactor [196].

1.3.2. *Non-enzymatic antioxidants*

Foods of plant origin have been demonstrated in a large number of studies to play a pivotal role in chemoprevention and maintenance of good health. Health benefits of plant-based diet are frequently reported to stem from the high content of antioxidant compounds, which act as ROS scavengers or enhance the activity of endogenous antioxidants. Thus, plant-derived antioxidants may support endogenous antioxidant systems in the maintenance of redox homeostasis [197]. The chemopreventive efficacy of dietary antioxidants depends on their dose, bioavailability, metabolism, delivery method (food vs. supplements) or even interindividual variability (at e.g. metabolic level) [198].

Dietary antioxidants include thousands of phytochemicals (mainly polyphenols and carotenoids), antioxidant vitamins (vitamin A, C and E) as well as other low-molecular-weight compounds, which besides being present in foods, are produced endogenously in human organism, e.g. GSH, CoQ₁₀, α -lipoic acid (LA) or melatonin. In this thesis, GSH and quercetin (Q) were used as reference antioxidants, therefore these two compounds are described in detail below. Both of them can be found in foods of plant origin. GSH is recognized as a major intracellular endogenous antioxidant, while Q is a strong antioxidant widespread in foods of solely plant origin..

Glutathione

Glutathione (GSH) is the most abundant endogenous antioxidant keeping the cellular redox state in balance. The formal reduction potential (E^0) of GSH/GSSG couple is estimated to range from -260 mV to -150 mV depending on the cellular compartment [199]. GSH directly scavenges ROS/RNS, serves as a substrate for antioxidant enzymes (described in 1.3.1. section), as well as regenerates some other antioxidants, vitamin C in particular. Besides being a major cellular redox buffer, glutathione also (i) constitutes a reserve form of cysteine, (ii) participates in the reduction of ribonucleotides to deoxyribonucleotides, (iii) indirectly affects transcription factors involved in redox signalling (e.g. nuclear factor-erythroid factor 2-related factor 2 – Nrf2, activator protein 1 – AP-1, NF- κ B) and (iv) participates in detoxification processes of both endogenous metabolites and xenobiotics, where it serves as an electrophile-conjugating reactant for glutathione S-transferases (GSTs) [200].

GSH synthesis is maintained by the integration of three pathways, which embrace GSH biosynthesis cycle, methionine cycle and transsulfuration pathway. The formation of GSH in its biosynthesis cycle is catalysed in two enzymatic steps: in the first step, the rate-limiting γ -glutamylcysteine synthetase (GCS) binds glutamate with cysteine forming γ -glutamylcysteine,

to which subsequently glutathione synthetase (GSS) attaches glycine [200]. Therefore, the decreased uptake of cysteine and any alteration in GCS or GSS activities may result in decreased levels of cellular GSH. For example, the activity of GCS has been reported to be inhibited by such factors as α,β -unsaturated compounds formed during processing or storage of food lipids. These products, as mentioned earlier, are regarded as possible casual factors of numerous diseases [201]. Other pathways linked to GSH synthesis are methionine cycle and transsulfuration pathway which share first three reactions that convert methionine to homocysteine. In the first step, methionine adenosyl-transferases generate S-adenosylmethionine (SAM) by addition of methyl group to methionine. Afterwards, SAM is demethylated to S-adenosylhomocysteine (SAH), which is finally hydrolysed to homocysteine (HCy) and adenosine. From this point, HCy can be incorporated into the transsulfuration pathway, where it is converted to cysteine, which may further enter the GSH biosynthesis cycle [200]. Thus, the decrease in methionine uptake may lead to reduced SAM formation and corresponding decrease of SAM/SAH ratio, which subsequently may affect GSH biosynthesis [202]. For that reason, the insufficient supply of dietary methyl-donors, such as methionine, folate, betaine or choline may perturb GSH levels.

GSH reserves may be also replenished from exogenous sources, because it is ubiquitous in foods, e.g. in meat (50-200 mg/ kg wet weight), *Brassica* vegetables, asparagus, potatoes, peppers, carrots, avocados, squash or spinach (40-150 mg/ kg wet weight) [203]. Dietary intake of GSH was estimated to range from 13 to 110 mg per day [204], however, its direct tissue distribution is not effective since it undergoes rapid hydrolysis, except for the small intestine [205]. Therefore, in contrast to intracellular milieu where GSH reaches millimolar concentrations (up to 10 mM), GSH in plasma is present at low micromolar concentration range (below 10 μ M) [206].

Total cellular GSH content embraces free forms of reduced (GSH) and oxidized (GSSG) molecules as well as protein-bound forms (GSSR). Even up to 70% of the total cellular glutathione occurs in the cytosolic part, while the other 30% is found in nucleus, endoplasmic reticulum and mitochondria. Cellular compartments are characterized by different GSH/GSSG ratios and disturbances in redox homeostasis in one compartment does not necessarily affect other compartments and as such, their redox states are independent. The occurrence of chronic oxidative stress may increase the requirement for reduced form of GSH, lowering its reserve with simultaneous increase of levels of its oxidized form. For that reason, GSH/GSSG ratio is considered to reflect the redox balance in cells. Under normal conditions, GSH/GSSG ratio in the cytoplasm equals up to 100:1 and when oxidative stress occurs, this ratio may decrease down to the value of 10:1 or even 1:1. Such observations of decreased GSH/GSSG ratio were made during ageing and in the pathogenesis of neurodegenerative diseases (Alzheimer, Parkinson, multiple sclerosis), brain tumours, cardiovascular and respiratory diseases, diabetes, rheumatoid arthritis as well as cancer [207]. In contrast, lower levels of GSH may be desirable during chemotherapy to prevent detoxification of cytostatics via mercapturic acid pathway. Thus GSH levels must be monitored in oncological patients due to its potential to support chemoresistance [208].



Quercetin

Quercetin (Q) is an ubiquitous dietary polyphenolic compound belonging to the group of flavonoids. Besides its omnipresence in plant-derived foods, it is also recognized as one of the most powerful antioxidants. Q represents around 75% of flavonol intake, the class of polyphenols that accounts for 4-19.3% of the total flavonoid intake [209]. In foodstuffs, it is present mainly in the form of glycosides, which is in contrast to the dietary supplements where Q occurs in the form of aglycone [210,211]. After consumption, a majority of food-derived Q is absorbed in small intestine after removal of sugar moiety by enzymes present in a brush border of the enterocyte [212]. In the form of aglycone, Q is more lipophilic, which facilitates its absorption [213]. The concentration of Q in human plasma was shown not to exceed low nanomolar range in terms of dietary sources or low micromolar range upon supplementation with 1 g of Q per day [214,215]. Beneficial effects of Q on human health are strongly associated with antioxidant activities of this polyphenol. Due to its chemical structure, Q turned out to be potent ROS scavenger *in vitro* as well as efficient chelator of iron [209,216]. Q was also shown to inhibit XO activity, which further strengthens protection against oxidative injuries [217]. The antioxidant activity of Q as an aglycone was proven to be far stronger than that of its most common glycoside – rutin, however, only in terms of its thermodynamic properties [176]. Except for direct ability of Q to scavenge ROS, this compound was also reported to boost GSH levels and to enhance the expression and activity of antioxidative enzymes [218], [219,220].

1.4. *Understanding the redox homeostasis – beyond the concept of oxidative stress*

Increased consumption of fruits and vegetables, rich in antioxidants, has been recently highly recommended in order to lower the risk of cancer and other non-infectious chronic diseases. The scientific reports documenting health benefits provided by antioxidant-rich diet popularized the term “antioxidant” in mass media and brought up its global acceptance among the general public. The dietary supplements industry took advantage of this situation and ensured the still growing range of products for widespread supplementation of antioxidants backed up by ads promising extended lifespan and good health [221]. This created the general belief that antioxidants serve as the universal antidote to a variety of diseases and age-related failing health. These claims however were proved to be overstated. Even though antioxidant-rich diet may be helpful in maintaining good condition for healthy individuals, recent investigations have shown that some redox-active compounds may not be so promising in the case of already initiated pathologies [222]. The reason why some antioxidants may act in the way that contradicts expectations is that ROS are not only harmful oxidizing agents, but they also act as signalling molecules necessary for proper function of the body. Thus, over-scavenging of ROS by antioxidants may lead to disturbances in ROS-mediated signalling pathways, which subsequently may promote initiation and then progression of diseases [223]. For example, it has been reported that the decrease in ROS levels caused by vitamin E and N-acetylcysteine (NAC) supplementation reduced p53 expression levels and induced tumour progression in mice since

apoptosis being under control of this protein and subsequent tumour cell death cannot take place [224]. In humans, this issue may be of concern in smokers and patients with chronic obstructive pulmonary disease, who use NAC as a mucolytic drug [224].

These findings reveal that it is essential to better understand how redox regulation drives cellular functions and what are the possibilities to improve human health with the aid of learned dietary choices in everyday life. The overview of the components that constitute the core of redox homeostasis and therefore may affect both physiological functions and the development of pathophysiological processes are presented in Figure 1.4. Accordingly, Meng *et al.* [225] have proposed the Precision Redox strategy for successful antioxidant therapies. Precision Redox takes into account human variability in the redox status and should be applied taking into consideration the right chemical species (either oxidants or antioxidants), right time (regarding dynamic redox fluctuations), right place (organelle-, cell- or tissue-specific), right level (twofold effects of ROS/antioxidants depending on their doses) and target (delay of oxidative modifications of macromolecules through personalized antioxidant strategies). There are thus numerous variables that decide whether an antioxidant-based therapy will be successful or not. Current state of knowledge seems not sufficient to precisely predict the outcome of antioxidant treatment.

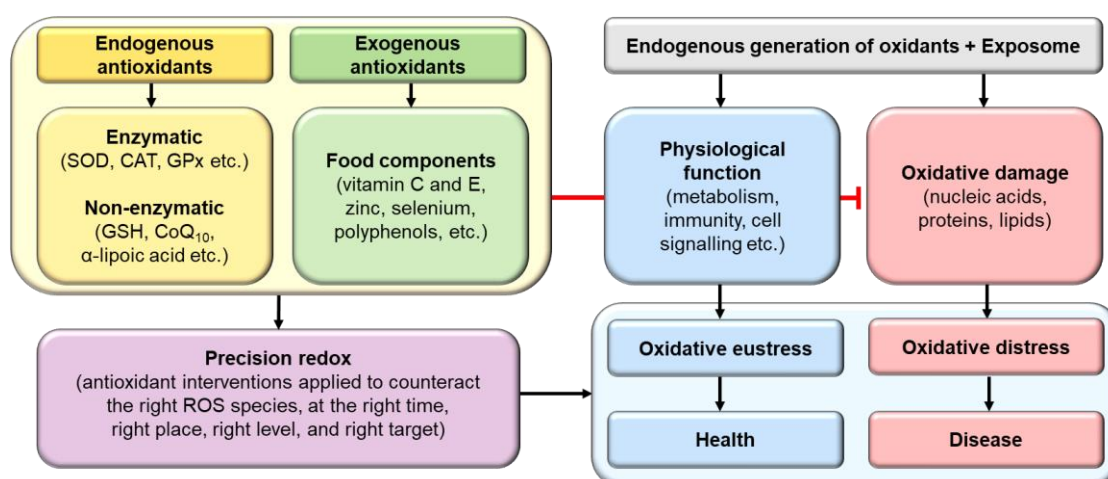


Figure 1.4. Overview of the components that constitute the core of redox homeostasis and therefore may affect both physiological functions and the development of pathophysiological processes. Precision redox strategy points to crucial factors to design successful redox-based therapies. Illustration based on work of Sies *et al.* [10]. Abbreviations used refer to: CAT, catalase; CoQ₁₀, coenzyme Q₁₀; GPx, glutathione peroxidase; GSH, glutathione.

The understanding of redox networks is crucial to design effective antioxidant interventions

Cellular redox status is regulated in a spatiotemporal manner. The formal reduction potential of certain redox couples, such as GSSG/GSH, depends on their intracellular localization: in mitochondria it is around -300 mV, -220 to -260 mV in cytoplasm, -130 to -153 mV in cellular membranes, -150 mV in ER and -240 mV in lysosomes [225]. This indicates that basal cellular redox state is compartment-specific and may be either more reducing or more oxidizing, depending on this compartment role in the maintenance of cellular function. For example, more

oxidizing milieu is necessary for proper protein folding in ER. Slightly oxidizing environment in the cytoplasm triggers redox signalling events through changes in the hydrogen peroxide levels. Moreover, redox state can also change periodically following different stages of the cell cycle [226]. So much feared cellular damage resulting from the elevated levels of oxidizing agents, such as ROS/RNS, occurs only when the homeostasis is disrupted. In the case of tumour-targeted redox therapies, it was shown that low levels of ROS stimulate tumour growth, whereas ROS overload leads to cell cycle arrest and apoptosis. In the case of antioxidants, they can either prevent the initiation of carcinogenesis by curbing oxidative stress or, in contrast, support different stages of tumorigenesis, as it was observed in the case of β -carotene supplementation, which increased the incidence of lung cancer in smokers and workers exposed to asbestos [227], [228]. Currently, there is neither clear threshold set to define proper redox homeostasis nor indications how much ROS is too much. Beyond redox orchestration for proper cellular function, redox-regulated intracellular instructions can be also altered upon circadian rhythms, seasons, external stimuli and disease development processes, not to mention individual human variability. For instance, in the case of carcinogenesis, the levels of ROS and antioxidants change as the disease progresses. At initial stages, the antioxidant defense declines with concomitant increase of ROS production, which results in oxidative DNA damage and elevated risk of mutations. In more advanced stages of cancer, the levels of antioxidants are elevated to mitigate the toxicity of ROS overgeneration [229]. For example, even though NAC is generally considered a safe drug, recent studies in genetically engineered mouse models of cancer have suggested that its use can promote tumorigenesis by preventing apoptotic tumour cell death (the phenomena described in more detail in the previous paragraph) [224]. Therefore, antioxidant supplementation in conjunction with cancer treatment therapies remains a controversial and highly arguable issue [230,231]. The double-sword nature of both ROS and antioxidants is now referred to as *antioxidant paradox* [232,233].

Efficacy of antioxidants

The first indications indirectly suggesting beneficial effects of antioxidants on human health emerged from epidemiological observations, in which the increased intake of foods rich in flavonoids or vitamin E was inversely associated with the incidence and mortality from cardiovascular diseases (CVD) [234–237]. Subsequently, in numerous specifically designed preclinical *in vitro* and *in vivo* studies dietary antioxidants showed the ability to delay or inhibit lipid peroxidation or LDL oxidation, both of which are known to contribute to the development of atherosclerosis [238]. In 2018, the systematic review and meta-analysis summarizing 69 prospective observational studies concluded that the increased intake of vitamin C, carotenoids and α -tocopherol was inversely correlated with the incidence of CVD, cancer and all-cause mortality [239]. Consequently, since oxidative stress was frequently accompanying numerous human diseases, such as CVD, cancer, neurodegenerative and metabolic disorders, great expectations were set for chemopreventive strategies and antioxidant-based therapies [2,240,241]. Disappointingly, these promises failed to be fulfilled in human clinical trials [242].



Neither studies verifying the efficacy of antioxidative vitamins (A, C and E), nor those applying vitamins from B group, minerals (such as zinc, selenium) or beta-carotene were successful [232]. For example, the meta-analyses of randomized controlled trials published in 2009 and 2013 by Myung *et al.* concluded that there is no clinical evidence for the efficacy of antioxidants such as vitamin A, E and β -carotene in cancer prevention nor in prevention of cardiovascular diseases [243,244]. Since different issues concerning efficacy of antioxidants in clinical trials have been thoroughly discussed elsewhere [177,188,225], here only a few points worth reminding will be raised.

There are three issues, which could have contributed to the observed inefficacy of antioxidants in clinical trials. Firstly, most of clinical trials investigated the efficacy of indispensable nutrients (such as vitamins), whose functions go far beyond being just antioxidants. For example, vitamin C is a necessary cofactor of enzymes involved in tissue regeneration, collagen formation or biosynthesis of neurotransmitters [245]. Supplementation of such antioxidant nutrients may be expected to be effective when deficiency is diagnosed, however, not necessarily in well-nourished populations. Secondly, antioxidative nutrients were expected to delay the occurrence of diseases if taken in high doses, which also proved to be ineffective. Already mentioned β -carotene taken at doses of 20 or 30 mg/day, which exceeded the recommended daily intake (6 mg in Poland) by several-fold, increased the risk of lung cancer by 17% and 28%, respectively, in male smokers and workers exposed to asbestos [227,246]. This outcome questioned the use of excessive doses resulting in the endogenous concentrations of nutrients falling beyond the optimal range, which may lead to adverse effects rather than improved health. Last but not least, it was expected that providing the antioxidative nutrients in a form of cocktail would exert additive or synergistic effects. However, such an approach also failed to reduce the risk of e.g. colorectal cancer (mix of 1 g vitamin C, 25 mg β -carotene, 400 mg vitamin E) or lung cancer (mix of 30 mg β -carotene and 25,000 IU vitamin A) [5,6]. Currently, it seems evident that the activity of compounds in a mixture may not necessarily exhibit enhanced function, let alone the unlikely resemblance of such mixtures to the complex food matrix [176,247,248].

The disappointing outcome denying health promoting efficacy of antioxidants stems mainly from studies focused on antioxidative vitamins and β -carotene, whose levels in human organism must be strictly controlled. Nevertheless, this resulted in labelling all antioxidants as ineffective or of questionable protective potency in human organism. However, besides the discussed antioxidative nutrients, there is a plethora of foodborne antioxidants, whose efficacy has not been thoroughly investigated in controlled human studies. With the current expectations, much focus has been set on Mediterranean diet (MD). The health-promoting effects of MD were first discovered in the Seven Countries Study (SCS), which was first epidemiological study that investigated the relationship between diet, lifestyle and health. SCS showed that populations living in Southern Italy and Greece had significantly lower risk of CVD compared to Western countries, such as United States [249]. These differences were not fully understood at that time, however, it was hypothesized that Mediterranean nutrition pattern could provide some answers. Indeed, MD is rich in olive oil, herbs, garlic, onion and other plant-borne foods, which constitute



rich sources of plant-derived antioxidants from a group of polyphenols. So far, the evidence on health-promoting potential of MD has been overwhelming [250,251]. In addition, more and more human intervention trials evaluating supplementation with polyphenols such as resveratrol or curcumin confirmed their potency in the treatment of diet-related diseases. For example, meta-analysis of 6 human trials showed that resveratrol supplementation improved cardiometabolic biomarkers in patients with type 2 diabetes (T2D), thus could be considered as an adjuvant during pharmacological treatment of this disease [252]. Another meta-analysis of 16 controlled trials concluded that resveratrol supplementation improved metabolic parameters (e.g. levels of glucose and triacylglycerols) in metabolic syndrome patients [253]. A randomized controlled trial involving 240 prediabetic patients showed that 9-month curcumin supplementation successfully prevented the development of T2D [254]. Meta-analysis of 7 human trials showed that curcumin and turmeric consumption improved blood lipid profile among patients at risk of CVD, suggesting potential benefits of their combination with pharmacological treatment [255]. Importantly, polyphenols are not the only antioxidants, which may bring benefit. The results of trials, in which supplementation with such endogenous antioxidants as LA or CoQ₁₀ was tested also brought promising results. Meta-analysis of 15 human trials showed that LA significantly reduced levels of MDA, a biomarker of lipid peroxidation, though it did not improve other markers of oxidative stress [256]. Another meta-analyses showed that supplementation with LA improved glycemic and inflammatory biomarkers (fasting glucose, hemoglobin A1c, C-reactive protein, interleukin 6 and TNF- α) among patients at risk of cardio-metabolic disorders [257,258]. In meta-analysis of 16 human trials, supplementation with CoQ₁₀ was shown to reduce levels of MDA and to increase the activity of SOD as well as total antioxidant capacity [259]. The potential of CoQ₁₀ to alleviate oxidative stress could also contribute to the reduced risk of CVD in diabetic patients, which was reflected by lowered total cholesterol and LDL levels according to the meta-analysis of 12 human trials [260].

1.5. *Conclusions*

The oxidative stress has been known in biological sciences for decades, however, the understanding of this concept has highly evolved since its foundation in the mid 1980's. Currently, the extended definition covers not only the subject of the oxidation-reduction balance, but also the consequences of altered redox signalling. One of the most significant changes in the discussion on oxidative stress is that it is now understood as the intrinsic component of life; ROS are no longer perceived exclusively as unwanted and deleterious molecules. Nevertheless, the exposure to various factors in everyday life that contribute to the intensified ROS generation is inevitable and thus, the recommendations of antioxidant-rich diets are justified [2,9,177]. Though initially, it seemed that the more antioxidants, the better, currently, the other side of the coin – the risk associated with reductive stress – has emerged. Nowadays, the question about antioxidants seems to be “how much is enough?”. Furthermore, similarly to ROS, the term “antioxidants” also works as an “umbrella” term. Redox-active compounds differ in chemical structure, electrochemical properties, mechanisms of antioxidant activity, bioavailability and therefore,



the efficacy in providing protection against excessive load of oxidants that vary in redox properties as well. These factors make it difficult to expect that so diversified group of molecules would affect human organism in the same way, just because all of them possess antioxidant activity. In the light of our current understanding, the past assumptions that any antioxidant would act in the similar way turn out rather naive. Fortunately, the failure of clinical trials to prove the efficacy of dietary antioxidants (mainly nutritive) in disease prevention have not ceased the research focused on the health-promoting properties of dietary antioxidants. Therefore, new strategies are being designed and it is now well understood that their effectiveness may depend on the type of antioxidant (endogenous or exogenous), whose activity could either maintain redox balance (under challenging conditions) or restore it when disrupted (state of oxidative or reductive stress). Still, more research on antioxidants of either endogenous or exogenous origin is required for Precision Redox strategy to be successfully implemented.

2. OBJECTIVES AND AIMS OF DOCTORAL THESIS

The doctoral research presented herein was carried out in the framework of the large-scale project, whose aim was to organize dietary antioxidants into Antioxidant Power Series (APS) according to scientifically confirmed strength of their antioxidant activity evaluated by electrochemical, chemical and biological methods. The objective of this project was to verify the hypothesis assuming that APS built on the basis of electrochemical properties of model redox-active compounds can be applied to predict the efficacy of food-derived antioxidant substances in protecting cells against cellular damage under conditions of oxidative stress. Should this hypothesis be verified positively, APS could be applied as a tool for rational design and assessment of health promoting properties of functional foods based on antioxidant phytochemicals.

Research conducted previously within the scope of this project revealed the striking differences between polyphenols (exogenous antioxidants) and glutathione (GSH, endogenous antioxidant) in terms of their impact on cellular redox homeostasis in human colon cell line used as a model of alimentary tract. The antioxidant activity of GSH was demonstrated to be notably weaker compared to polyphenols according to electrochemical, spectrophotometric and biochemical measurements. Moreover, the clear distinction between biological impact of polyphenols and GSH was also observed at the genomic level. The treatment of colon cells with GSH was shown to affect the expression of numerous redox-related genes, which could be implicated in the maintenance of redox homeostasis. In contrast, polyphenols seemed to provide antioxidant protection rather by direct ROS scavenging than engaging intracellular antioxidant systems. These observations led to the formulation of another working hypothesis that **weaker endogenous antioxidants, such as GSH, must rely more on cellular antioxidant mechanisms than on their reducing properties**. GSH, a major intracellular antioxidant, in cooperation with other endogenous redox-active low-molecular-weight compounds, constitutes a chemical part of such cellular antioxidant system and their concerted actions involving also biological mechanisms (*e.g.* antioxidant enzymes) are sufficient to maintain proper redox homeostasis under eustress. It is further presumed that strong antioxidants such as some dietary polyphenols are better or even indispensable when redox homeostasis is pushed towards oxidative stress. The aim of this thesis was to verify the above hypothesis by finding out the efficacy of endogenous low molecular weight redox-active compounds in maintaining redox balance in cells remaining in eustress versus when exposed to exogenous ROS. This goal was addressed by finding out how strong is the impact, if at all, of endogenous antioxidants, however applied exogenously as if they were derived from dietary sources, on the cellular antioxidant capacity and other markers of protection against oxidative stress.

The experiments included 20 redox-active compounds that can be either produced endogenously as well as be provided with food or be only derived from dietary sources, yet by definition support endogenous antioxidant barrier, such as vitamins. Investigated compounds were divided into 5 groups:

- (i) endogenous thiol antioxidants: α -lipoic acid, L-cysteine, N-acetyl-L-cysteine,
- (ii) adenine and its derivatives: adenosine and adenosine-5'-monophosphate,
- (iii) mitochondrial redox-active agents: coenzyme Q₁₀, melatonin, nicotinamide adenine dinucleotide in reduced and oxidized form, citric acid,
- (iv) endogenous pro-oxidants: glutathione disulphide, hydrogen peroxide,
- (v) ascorbic acid and its derivatives: sodium L-ascorbate, calcium L-ascorbate, D-isoascorbic acid, sodium D-isoascorbate, ascorbigen.

The results obtained for each investigated group were compared to, and discussed in the light of data available for two selected reference antioxidants: GSH and quercetin, serving as representatives of endogenous and exogenous antioxidants, respectively.

Methods used to assess the antioxidant activity of redox-active compounds involved both chemical and biological approaches. Chemical methods included two spectrophotometric tests applying ABTS and DPPH radicals, which are commonly used to determine the antioxidant activity of foods and beverages. Biological tests were carried out in human colon adenocarcinoma HT29 cells, which served as a model of gastrointestinal tract, where intestinal cells are in direct contact with ingested food. Measurements of antioxidant activity of tested compounds in cells were conducted with Cellular Antioxidant Activity (CAA) test, which is recognized to be superior to commonly used chemical colorimetric antioxidant activity assays, as it takes into account the uptake of compounds by cells and their cellular metabolism. The impact of tested redox-active compounds on cellular antioxidant activity was studied either under normal (10% FBS) or serum-deprived (0% FBS) conditions. Verification of genotoxicity and the ability of investigated antioxidants to provide protection against oxidative DNA damage was assessed by comet assay. Such a set of methods was applied in previous investigations of our group focused on polyphenolic antioxidants, which made it possible to directly compare the activities of exogenous antioxidants with the activity of antioxidants produced also endogenously in human cells.

Doctoral thesis presented herein was realized at the Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry at Gdańsk University of Technology under supervision of Prof. Agnieszka Bartoszek. Presented research was carried out in the framework of the project MAESTRO 6 (application number 2014/14/A/ST4/00640 lead by the late Prof. Jacek Namieśnik) financed by National Science Centre, Poland.



3. MATERIALS AND METHODS

3.1. Chemical reagents, preparation of buffers and research instruments

3.1.1. Chemical reagents

Reagent	Manufacturer	Purity/ comments
Adenine	Sigma Aldrich (USA)	≥ 99%
Adenosine	Sigma Aldrich (USA)	≥ 99%
Adenosine-5'-monophosphate	Acros Organics (USA)	99%
Agarose, low melting point (LMP)	Sigma Aldrich (USA)	For molecular biology
Agarose, normal melting point (NMP)	Sigma Aldrich (USA)	For molecular biology
α-Lipoic acid	Sigma Aldrich (USA)	≥ 99%
2,2-Azinobis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS)	Sigma Aldrich (USA)	≥ 98%
β-Mercaptoethanol	Sigma Aldrich (USA)	For electrophoresis, ≥ 98%
β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH)	Sigma Aldrich (USA)	≥ 97%
β-Nicotinamide adenine dinucleotide hydrate (NAD ⁺)	Sigma Aldrich (USA)	≥ 96.5%
Calcium L-(+)-ascorbate	Sigma Aldrich (USA)	Pharmaceutical standard; certified reference material
Citric acid	Sigma Aldrich (USA)	≥ 97%
Coenzyme Q ₁₀	Sigma Aldrich (USA)	≥ 98%, ubiquinone
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma Aldrich (USA)	-
Dimethyl sulfoxide (DMSO)	Sigma Aldrich (USA)	For molecular biology, ≥ 99.5 %
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich (USA)	98%
D-(-)-Isoascorbic acid	Sigma Aldrich (USA)	98%
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich (USA)	≥ 99%
Ethanol	Avantor Performance Materials (Poland)	96%, analytical grade
Foetal bovine serum	Sigma Aldrich (USA)	-
Glutathione	Sigma Aldrich (USA)	Pharmaceutical standard; certified reference material
Glutathione disulphide	Sigma Aldrich (USA)	≥ 98%
Hanks' balanced salt solution (HBSS)	Sigma Aldrich (USA)	Modified, with sodium bicarbonate, without phenol red, calcium chloride and magnesium sulfate

Reagent	Manufacturer	Purity/ comments
Hydrogen peroxide	Sigma Aldrich (USA)	30% (w/w) in water, contains stabilizer
L-(+)-Ascorbic acid	Sigma Aldrich (USA)	Pharmaceutical secondary standard; certified reference material
L-Cysteine	Sigma Aldrich (USA)	≥ 97%
Melatonin	Sigma Aldrich (USA)	≥ 98%
Methanol	Avantor Performance Materials (Poland)	Analytical grade
McCoy's 5A Medium	Sigma Aldrich (USA)	Modified with L-glutamine and sodium bicarbonate
N-acetyl-L-cysteine	Sigma Aldrich (USA)	≥ 99%
OxiSelect Cellular Antioxidant Activity Assay Kit	Cell Biolabs (USA)	-
Penicillin/ streptomycin	Sigma Aldrich (USA)	Activity of penicillin 10 000 U/mL, streptomycin concentration 10 mg/ mL
Phosphate buffered saline (PBS)	Sigma Aldrich (USA)	In tablets
Quercetin	Sigma Aldrich (USA)	≥ 95%
RNase-free DNase set	Qiagen (Germany)	-
RNeasy mini kit	Qiagen (Germany)	-
RT ² First strand kit	Qiagen (Germany)	-
RT ² Sybr green kit	Qiagen (Germany)	-
Sodium chloride	Sigma Aldrich (USA)	≥ 99%
Sodium D-(-)-isoascorbate	Sigma Aldrich (USA)	97%
Sodium hydroxide	Sigma Aldrich (USA)	≥ 98%
Sodium L-(+)-ascorbate	Sigma Aldrich (USA)	Pharmaceutical secondary standard; certified reference material
Sodium persulfate	Sigma Aldrich (USA)	≥ 98%
Sybr Green I nucleic acid gel stain	Sigma Aldrich (USA)	10,000 x in DMSO
4-(1,1,3,3-Tetramethylbutyl)phenylpolyethylene glycol solution (Triton™ X-100)	Sigma Aldrich (USA)	-
Tris-base (Trizma™ base)	Sigma Aldrich (USA)	≥ 99.9%
Trypsin-EDTA solution	Sigma Aldrich (USA)	10 x concentrated, 5 g porcine trypsin and 2 g EDTA · 4 Na/L of 0.9% sodium chloride
Uric acid	Sigma Aldrich (USA)	≥ 99%



3.1.2. Preparation of buffers and other solutions

Buffer/ solution	Preparation
ABTS solution	First, 5.831 mg of sodium persulfate was dissolved in 10 mL of ultra-pure water. Then, 10 mL of prepared sodium persulfate solution was added to 38.36 mg of ABTS and incubated in the dark at room temperature overnight. Just before use, a stock solution of ABTS radical was diluted in methanol before measurement until absorbance reached 0.8 ± 0.05 at $\lambda = 734$ nm.
0.5% agarose LMP	10 mL of PBS was added to 50 mg of LMP agarose and heated in a microwave oven until a clear solution was obtained.
1% agarose NMP	100 mL of ultra-pure water was added to 1 g of NMP agarose and heated in a microwave oven until a clear solution was obtained.
Buffer RLT	Buffer RLT for cell lysis was prepared just before use by mixing 10 mL of buffer RLT (provided with RNeasy mini kit, Qiagen) with 100 μ L of β -mercaptoethanol.
DPPH solution	Solution of DPPH radical was prepared in methanol just before measurements until a value of absorbance reached 0.9 ± 0.05 at $\lambda = 515$ nm.
Electrophoresis buffer (10x conc.)	0.372 g of EDTA was dissolved in 800 mL of ultra-pure water and then 12 g of NaOH was added gradually while stirring. The final pH of a buffer should be higher than 13. The total volume of a buffer was adjusted to 1 L. Just before use, buffer was diluted 1:10 (v/v) with cold ultra-pure water. The concentrated buffer (10x) was stored at 4°C.
Lysis buffer A	146.1 g NaCl, 37.2 g EDTA and 1.2 g Tris-base were dissolved in 800 mL of ultra-pure water. Subsequently, 7 g of NaOH was added gradually while stirring. 1 M HCl and 1 M NaOH were used to adjust pH to 10. The buffer was stored at 4°C.
Lysis buffer B	10 mL of Triton X-100 was added to 90 mL of ultra-pure water. The suspension was mixed and kept in water bath at 37°C until a homogenous solution was obtained. Buffer was stored at 4°C.
Lysis buffer (A + B)	Just before use, 90 mL of lysis buffer A was mixed with 10 mL of lysis buffer B. The obtained solution must be kept at 4°C.
MTT solution	MTT powder was mixed with ultra-pure water (4 g/L) and subsequently the obtained suspension was sonicated at room temperature for 1 min. MTT solution was stored in a dark-glass bottle in 4°C.
Neutralizing buffer	48.5 g Tris-base was dissolved in 800 mL of ultra-pure water. 1 M HCl and 1 M NaOH were used to adjust pH to 7.5. The total volume of a buffer was adjusted to 1 L using ultra-pure water. The buffer was stored at 4°C.
PBS solution	One tablet of PBS was dissolved in 200 mL of ultra-pure water as suggested by the manufacturer.
Sybr Green solution	10 μ L of concentrated Sybr Green solution was dissolved in 990 μ L DMSO. Subsequently, the obtained solution was added to 49 mL of 1xTE buffer. The final Sybr green solution was stored in a dark-glass bottle in 4°C.

Buffer/ solution	Preparation
TE buffer (10x conc.)	1.576 g Tris-HCl and 0.372 g EDTA were dissolved in 800 mL of ultra-pure water. 1 M HCl and 1 M NaOH were used to adjust pH to 7.5. The total volume of a buffer was adjusted to 1 L. Just before use, buffer was diluted 1:10 (v/v) with cold ultra-pure water. The 10x concentrated buffer was stored at 4°C.

3.1.3. *Materials*

Material	Manufacturer	Comments
24-well culture plates	VWR International (USA)	Surface treated
48-well cell culture plates	VWR International (USA)	Surface treated
96-well cell culture plates	VWR International (USA)	Surface treated
96-well cell imaging plates	Eppendorf (Germany)	Black with clear 25 µm film bottom
96-well RT ² Profiler™ PCR array	Qiagen (Germany)	Human oxidative stress, GeneGlobe ID: PAHS-065Z
Microscope slides	VWR International (USA)	Ground edges frosted
Microscope cover slips	Menzel Gläser (Germany)	22 x 22 mm, 22 x 60 mm
Microscope slide staining jars	Ted Pella, Inc. (USA)	-
Millex-GV sterile syringe-driven filters	Millipore (USA)	33 mm diameter with a 0.22 µm pore size hydrophilic PVDF membrane
Nunc® EasYFlasks™, cell culture flasks	Thermo Scientific (USA)	Culture area 25 cm ² and 75 cm ²
QIAshredder	Qiagen (Germany)	-

3.1.4. *Research instruments*

Instrument	Manufacturer
Analytical balance XS204	Mettler Toledo (Poland)
Centrifuge 5415R	Eppendorf (Germany)
Centrifuge Heraeus Megafuge 16R	Thermo Fisher Scientific (USA)
Cytometer Scepter 2.0 cell counter	Merck (Germany)
Fluorescent microscope Axio Imager Z2	Carl Zeiss (Germany)
Horizontal gel electrophoresis apparatus, model A2	Owl Separation Systems, Inc. (USA)
Juli™ Smart fluorescent cell analyser	Digital Bio (Korea)
Laminar flow hood Airstream ESCO Class II BSC	ESCO (Poland)
Magnetic stirrer	SunLab Sustainable Lab Instruments (Germany)
Metafer4 scanning and imaging platform version	MetaSystems (Germany)
Milli-Q Gradient A10 water purifying system	Merck Millipore (Germany)
pH-meter Seven Easy	Mettler Toledo (Poland)

Instrument	Manufacturer
PowerPac™ Basic power supply	BioRad (USA)
Smart Cell CO ₂ incubator HF90	Heal Force (China)
Spectrophotometer Nanodrop 2000c	Thermo Scientific (USA)
Spectrophotometric plate reader Tecan Infinite M200	Tecan Group Ltd. (Switzerland)
Thermoblock QDB4	Grant Instruments (UK)
Thermocycler LightCycler 96 system	Roche (Switzerland)
Ultrasonic cleaner	VWR International (USA)
Vacunsafe Aspiration system	Integra Biosciences™ (Germany)

3.1.5. Softwares

- CometScore 2.0 free software, available online [last access 1/04/2021]: <http://rexhoover.com/index.php?id=cometscore>
- GeneGlobe Data Analysis Center, Qiagen (Germany)
- GraphPad Prism version 8.0 (GraphPad Software, Inc., USA)
- Magellan™ (Tecan Group Ltd., Switzerland)
- Metafer4 software (MetaSystems, Germany)
- Lightcycler 96 SW 1.1 analysis software (Qiagen, Germany)

3.2. Methods

3.2.1. Investigated compounds – preparation of stock solutions

3.2.1.1. Thiol antioxidants

The investigated thiol antioxidants included L-cysteine (Cys), N-acetyl-L-cysteine (NAC) and α -lipoic acid (LA). 100 mM stock solutions of Cys and NAC were prepared in ultra-pure water. 10 mM stock solution of LA was prepared by first dissolving a solute in 96% ethanol and then diluting it 1:10 (v/v) using ultra-pure water. The final concentration of ethanol in cell culture did not exceed 1%.

3.2.1.2. Purine derivatives

The group of purine derivatives included adenine (Ade), adenosine (Ado), adenosine-5'-monophosphate (AMP), and uric acid (UA). Stock solutions of Ade (5 mM), Ado (10 mM), and AMP (10 mM) were prepared in ultrapure water heated to 37 °C with thorough stirring until the compounds were completely dissolved. Uric acid (400 μ M) was dissolved directly in pre-warmed McCoy's 5A culture medium with thorough stirring.

3.2.1.3. Mitochondrial redox-active agents

The group of mitochondrial redox-active agents included coenzyme Q₁₀ (CoQ₁₀) in its oxidized form (ubiquinone), reduced and oxidized forms of nicotinamide adenine dinucleotide

(NADH and NAD⁺, respectively), citric acid (CA) as well as melatonin (MT). Stock solutions of CA, NADH and NAD⁺ (100 mM) were prepared in ultra-pure water. In the case of MT (50 mM) and CoQ₁₀ (2.5 mM), the concentrated solutions were prepared in 100% DMSO. The suspension of CoQ₁₀ was sonicated for 20 min using ultrasound cleaner at 37°C to achieve the complete solubilization of this compound. Occasional stirring was necessary to prevent the deposition of a solute from the top layer of the solution on the wall of a centrifuge tube. Due to the quick precipitation of a compound, a stock solution of CoQ₁₀ was immediately used for the preparation of serial dilutions. The final concentration of DMSO in cell culture did not exceed 0.2%.

3.2.1.4. *Endogenous pro-oxidants*

The investigated pro-oxidants included glutathione disulphide (GSSG) and hydrogen peroxide (H₂O₂). Stock solution of GSSG (10 mM) was prepared using ultra-pure water. Working solutions of H₂O₂ were obtained by diluting the 9.8 M stock solution (provided by the manufacturer) in ultra-pure water.

3.2.1.5. *Ascorbic acid derivatives*

The group of investigated ascorbic acid derivatives included L-ascorbic acid (AA), D-isoascorbic acid (iAA), calcium L-ascorbate (CaA), sodium L-ascorbate (NaA), sodium D-isoascorbate (iNaA) and ascorbigen (ABG). The latter compound was synthesized as a courtesy of Prof. Witold Przychodzeń at the Department of Organic Chemistry, Faculty of Chemistry, Gdańsk University of Technology according to Medjacovic *et al.* [261]. Stock solutions (100 mM) of ascorbic acid, its salts, and isomers were prepared in ultra-pure water. A stock solution of ABG (10 mM) was prepared by first dissolving a solute in 96% ethanol and next diluting it 1:10 (v/v) using ultra-pure water. The final concentration of ethanol in cell culture did not exceed 1%. For the investigation of modulation of gene expression, ABG was dissolved with ultra-pure water (2 mM stock solution).

3.2.1.6. *Reference antioxidants – glutathione and quercetin*

100 mM stock and working solutions of glutathione were prepared in ultra-pure water. A stock solution of quercetin (10 mM) was prepared by first dissolving a solute in 96% ethanol and then diluting it with ultra-pure water, so the final concentration of ethanol was 30%. Working solutions were prepared by diluting a stock solution with ultra-pure water.

3.2.2. *Chemical methods*

3.2.2.1. *Antioxidant activity by ABTS and DPPH tests*

Spectrophotometric tests applying ABTS or DPPH radicals are one of the most popular *in vitro* methods allowing to rapidly assess the ability of pure compounds (or food extracts) to scavenge free radicals. Here, the antioxidant activity was expressed as a stoichiometric value n_{10} defined as a regression coefficient of the slope of the line that represents how many molecules



of a ABTS or DPPH radical were scavenged by one molecule of an antioxidant during a 10 min reaction time.

The colorimetric determination of antioxidant activity of investigated compounds was carried out in 48-well plates. Stock solutions of tested compounds were prepared in ultra-pure water or 96% ethanol and diluted (using the same solvent) to concentrations falling within a linear range of the assay. Just before the experiment, stock solutions of ABTS or DPPH were diluted in analytical grade methanol to obtain the absorbance equal to 0.8 ± 0.05 at 734 nm and 0.9 ± 0.05 at 515 nm, respectively. Next, 1 mL of ABTS or DPPH solution was mixed with 10 μ L or 30 μ L of a tested compound, respectively, and incubated for 10 min at 25, 37 and 41°C. The procedure of DPPH test was slightly modified in the case of ABG, MT and UA. In order to obtain a linear relationship between concentrations of a sequestered DPPH radical and concentrations of a tested compound, 1 mL of DPPH solution was mixed with increasing volumes of a stock solution. The final volume was adjusted with the solvent to maintain the same reaction volume among all the concentrations tested. The ABTS test was modified analogously in the case of UA only. Measurements of absorbance were performed using TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland). The concentrations of tested antioxidants were plotted against the concentrations of the radical remaining in the reaction mixture after 10 min, which were calculated with the use of the Beer-Lambert-Bouguer Law (Beer's Law) according to the Equation 1:

$$(1) S_R = \frac{A_0 - A_f}{\epsilon \times l}$$

where: S_R – the concentration of scavenged radicals [M], A_0 – the initial absorbance of the radical solution; A_f – the absorbance of the radical solution after reaction time; ϵ – the molar extinction coefficient of the radical ($16,000 \frac{1}{M \cdot cm}$ for ABTS radical at 734 nm, $11,240 \frac{1}{M \cdot cm}$ for DPPH radical at 515 nm); l – the optical path length of a cuvette [cm].

3.2.3. *Biological methods*

3.2.3.1. *Cell culture*

All biological tests were carried out using human colon adenocarcinoma HT29 cell line provided by American Type Culture Collection (ATCC, USA). Cells were cultured in McCoy's 5A medium with the addition of 0.22 g/L of L-glutamine, 2.2 g of sodium bicarbonate, 100 mL/L of foetal bovine serum (FBS), 100 U/mL penicillin and 100 g/L streptomycin in a humidified atmosphere (37°C, 5% CO₂) in the Smart cell incubator. Water-soluble compounds were sterilized with syringe-driven Millex sterile R33 mm (0.22 μ m) filters before treatment of cells.

3.2.3.2. *Cell viability by MTT test*

HT29 cells were seeded in a 96-well cell culture plate at the density of 5,000 cells/well in a 0.18 mL of medium. Cells were allowed to settle down for 24 h at 37 °C before the experiment. Then, the cells were treated with 20 μ L of working solutions of tested compounds in four technical



replicates for 6, 24, and 72 h. The final range of concentrations in cell culture fell between 0.01 μM and 10 mM. Control cells were treated with the appropriate solvent only. After the shorter treatment intervals, the medium was removed from wells, and the cells were left to grow until the total incubation time of 72 h. Afterwards, the medium after 72 h was replaced with fresh medium and 50 μL of MTT solution was added to all wells and the incubation was continued for 3.5 h at 37°C. Finally, the medium was carefully aspirated from the wells and formazan crystals produced by metabolically active cells were dissolved in 50 μL of DMSO. The measurement of absorption was performed at 540 nm using a TECAN Infinite M200 plate reader. For each of the investigated compounds, the experiments were performed in 3 independent biological repetitions. Cell viability was expressed as a percentage of control cells, whose growth was regarded as 100%.

3.2.3.3. Cellular antioxidant activity by CAA assay

The determination of the antioxidant activity in the living cells was carried out using the cellular antioxidant activity (CAA) [262]. This method represents more biologically-relevant approach compared to spectrophotometric tests. The principle of CAA assay is to measure the ability of antioxidants to prevent the oxidation of a probe (2',7'-dichlorodihydrofluorescein diacetate, H₂-DCFDA) to its fluorescent form (2'-7'-dichlorofluorescein, DCF), which is reflected by the decrease in cellular fluorescence compared to the control cells. Results are presented as so-called CAA values; the higher the CAA value, the stronger the antioxidant activity of a tested compound.

The CAA assay was performed using commercially available The OxiSelect Cellular Antioxidant Assay Kit (Cell Biolabs Inc.) and black, bottom-transparent 96-well tissue culture plates for fluorescence measurements. All steps of CAA assay were performed according to the manufacturer's protocol available online (https://www.cellbiolabs.com/sites/default/files/STA-349-cellular-antioxidant-activity-assay-kit_0.pdf). Briefly, HT29 cells were seeded at the density of 30,000 cells per well in 0.2 mL of complete medium containing 10% FBS. The next day, the medium was removed and cells were washed with 100 μL of HBSS. Afterwards, 50 μL of 2x concentrated DCFH-DA solution prepared in serum-free medium was added to all wells. Cells were treated with 50 μL of solutions of investigated compounds at final concentrations ranging from 0.1 μM up to 1000 μM and incubated for 1 h at 37 °C. When the treatment was finished, cells were washed again with 100 μL of HBSS, and 100 μL of 2.8% solution of the free radical initiator was added. Measurement of fluorescence was carried out at 37 °C every 5 minutes for 60 min at 485 nm with a TECAN Infinite M200 plate reader. The CAA values for studied antioxidants were calculated according to the equation:

$$(2) \text{ CAA units} = 100 - \frac{\text{SA}}{\text{CA}} \cdot 100$$

where: SA and CA refer to the area under the fluorescence curve plotted against probe excitation time corresponding to tested antioxidants and cells treated with the solvent only, respectively. Experiments were carried out three times with three technical replicates per each biological replicate.



3.2.3.4. Cellular antioxidant activity by CAA assay after serum starvation

HT29 cells were seeded with 30,000 cells per well in black, bottom-transparent 96-well tissue culture plates and left for 24 hours to settle at 37 °C. The next day, the complete medium containing 10% FBS was aspirated off the wells, cells were washed with PBS and then a serum-free medium was added for another 16 to 24 hours to induce cell synchronization. After serum starvation, cells were treated with the studied compounds at doses in the range from 0.1 µM to 200 µM for 1 h, as already described. Further steps were carried out according to section 3.2.3.3. Cell synchronization after serum-starvation was confirmed by flow cytometry as described in Appendix B.

3.2.3.5. Genotoxicity by comet assay

HT29 cells were seeded in 24-well tissue culture plates at a density of 100,000 cells per well and were allowed to grow for 48 h at 37 °C. Then, the cells were treated with tested antioxidants in the concentration range from 1 µM to 1000 µM for 24 h. The cells treated with the appropriate solvent only served as negative controls, whereas cells treated with 200 µM H₂O₂ for 1 h served as a positive control. After treatment, the medium was aspirated off the wells, the cells were washed with 0.5 mL of PBS, which was subsequently replaced with fresh 1 mL of PBS. Cells were detached from the wells using a cell scraper and resuspended in PBS. Finally, the suspension of cells was transferred to 1.5 mL tubes and centrifuged (1,200 rpm, 4 min, 4 °C). The supernatant was removed and the cell pellets were washed with 1 mL of PBS and centrifuged again under the same conditions. Afterwards, the majority of the supernatant was removed and approximately 80-100 µL of PBS was left for a resuspension of cells. The cell suspension (25 µL) was mixed with 150 µL of 0.5% LMP agarose prewarmed and kept at 42 °C in a thermoblock prior to use. The obtained suspension was placed as two spots (25 µL per spot) on a microscope slide pre-coated with 1% NMP agarose, covered with a cover slip and left to solidify on an ice-cold tray. After 5-10 min, cover slips were gently removed and slides were placed in staining jars filled up with lysis buffer (A+B) for at least 2 hours. Three slides with two repetitions on each were prepared for every concentration of the tested compounds. After lysis, slides were placed on an electrophoresis platform filled up with cold electrophoresis buffer. DNA was left to unwind in the electrophoretic buffer for 20 min, unwinding period was followed by 30 min of electrophoresis (26 V, 300 mA) in the dark at 4°C. Eventually, slides were transferred to staining jars filled up with neutralizing buffer for 5 min, followed by 5 min wash in cold ultra-pure water and finally, fixed for 5 min in 70% cold ethanol. DNA was stained with cold SybrGreen solution for 30 min and washed with cold ultra-pure water for another 5 min. The stained nuclei were examined under a fluorescence microscope and scanned using Metafer4 scanning system (Germany). The analysis was performed with the aid of Metafer4 system and involved counting 200 consecutive nuclei per gel. The mean of %DNA in the comet tail was a measure of genotoxicity of the tested compounds.

3.2.3.6. *Protection against oxidative DNA damage by comet assay*

The investigations of protection against oxidative DNA damage were performed by comet assay as described in section 3.2.4.5. with one modification. Right after 24 h treatment of cells with tested compounds, the medium was removed and cells (with exception of negative controls) were exposed to 150 μM H_2O_2 for 1 h. Subsequently, the cells were harvested and submitted to comet assay procedure as already described. The analysis was performed with Comet Score 2.0 software and involved counting 100 consecutive nuclei per gel.

3.2.3.7. *RNA isolation*

HT29 cells were seeded in 24-well tissue culture plates at density of 100,000 cells per well and were allowed to settle for 24 h at 37°C. The next day, the cells were treated with investigated antioxidants (AA, CaA and ABG) at 50 and 200 μM concentrations for 24 h (37°C, 5% CO_2). All tested compounds were dissolved in ultra-pure water and their solutions sterilized using syringe-driven filters (0.22 μm). When the treatment was finished, total RNA was isolated with the use of RNeasy Mini kit (Qiagen) following the protocol "Purification of total RNA from animal cells using spin technology" with the homogenization step carried out using QIAshredders (Qiagen). Briefly, after 24 h treatment, the medium was removed and the cells were lysed directly in cell culture plates by adding 350 μL of the lysis buffer (Buffer RLT mixed with β -mercaptoethanol) per well. The lysates were transferred onto QIAshredder spin columns and centrifuged at maximum speed (13,200 rpm) for 2 min. Homogenization of the lysate occurs as it passes through a QIAshredder spin column. The homogenates were mixed with 350 μL of 70% ethanol, then the total volume (700 μL) of each sample was transferred onto the RNeasy spin columns and centrifuged at 10,000 rpm for 15 s. The flow-throughs were discarded and the additional on-column digestion of gDNA was applied with the aid of RNase-free DNase Set. First, the RNeasy spin columns were washed with 350 μL of the washing buffer (RW1) and centrifuged at 10,000 rpm for 15 s. Again, the flow-throughs were discarded and 80 μL of DNase I incubation mix was added to each of the RNeasy spin columns and incubated for 15 min at room temperature. Afterwards, the washing step with RW1 buffer was repeated as already described. Further, the RNeasy spin columns were washed twice with 500 μL the RPE buffer with the second centrifuging extended to 2 min instead of 15 s. The flow-through was discarded and the additional dry-centrifuging step was carried out at full speed for 1 min. The total RNA was extracted in 30 μL of RNase-free water. The quality and quantity of isolated RNA was measured using Nanodrop 2000c. The isolated RNA was stored at -80°C until analysis.

3.2.3.8. *Reverse transcription*

Reverse transcription was conducted using RT² First Strand kit (Qiagen) according to the manufacturer's protocol. First, the genomic DNA elimination mix was prepared for each sample according to the Table 3.1:



Table 3.1. Genomic DNA elimination mix

Component	Amount
RNA	500 ng
Buffer GE	2 μ L
RNase-free water	Variable
Total volume	10 μ L

The prepared genomic DNA elimination mix was incubated at 42 °C for 5 min and then placed on ice for at least 1 min. The reverse transcription mix was prepared according to the Table 3.2. The 10% surplus of reagents was always included while preparing any of the master mixes:

Table 3.2. Reverse transcription mix

Component	Volume for 1 reaction
5x Buffer BC3	4 μ L
Control P2	1 μ L
RE3 Reverse transcriptase Mix	2 μ L
RNase-free water	3 μ L
Total volume	10 μ L

For each sample, 10 μ L of the genomic DNA elimination mix was added to 10 μ L of the reverse transcription mix and gently mixed. The obtained mixture was incubated at 42 °C for 15 min and right after the incubation was finished, the reaction was stopped by 5 min incubation at 95 °C. Finally, 91 μ L of RNase-free water was added to each sample and gently mixed.

3.2.3.9. Genomic studies by microarray technology

Genomic studies were performed using 96-well RT² profiler PCR arrays (Qiagen) designed for expression analysis of 84 genes related to oxidative stress and antioxidant defense. The array included also 3 controls assessing reverse transcription efficiency, 3 positive PCR controls, a gDNA contamination control as well as 5 reference genes. All instructions are available online at <https://www.qiagen.com/us/resources/resourcedetail?id=f4b13eaa-884f-4357-abe6-1a5f9469bc32&lang=en>. The analysed genes are listed in Appendix C. One 96-well RT² Profiler PCR array was used per one biological repetition for each of the treatments. The obtained cDNA (as described in section 3.2.4.9.) was immediately diluted in qPCR master mix using RT² SYBR Green kit (Qiagen) according to Table 3.3.:

Table 3.3. qPCR master mix

Component	Volume for 1 array
2x RT ² SYBR Green Mastermix	1350 μ L
cDNA synthesis reaction	102 μ L
RNase-free water	1248 μ L
Total volume	2700 μ L

The qPCR master mix containing cDNA was subsequently applied to 96-well RT² Profiler PCR array. The qPCR was performed according to the cycling conditions suggested by the manufacturer for Roche LightCycler 96 (Table 3.4.). Data analysis was performed using software available on manufacturer's website at <https://dataanalysis.qiagen.com/pcr/arrayanalysis.php>. Data were normalized based on the geometric averaging of 3 best-performing reference genes (*B2M*, *HPRT1* and *RPLP0*). The relative changes in gene expression were calculated with the aid of comparative threshold cycle method ($\Delta\Delta C_t$).

Table 3.4. The qPCR cycling conditions

Step	Cycles	Conditions
Preincubation	1	95 °C for 600 s
2 step amplification	45	95 °C for 15 s 60 °C for 60 s
Melting	1	60 °C for 15 s 95 °C for 1 s
Cooling	1	37 °C for 30 s

3.2.3.10. Statistical analysis

All values are expressed as means \pm SD of three independent experiments. All tests were carried out using Prism 8.0 software package (GraphPad Software, Inc., USA). The determination of statistically significant differences in antioxidant activity measured by CAA assay was evaluated by one-way ANOVA with Tukey's or Dunnett's test. Statistical significance between CAA values resulted from treatments of cells grown in serum-free or serum-containing medium was carried out with Student's t-test. Statistical significance in the case of comet assay was performed with one-way ANOVA with Dunnett's test. Relationships between chemical and biological parameters were investigated using linear regression and Pearson's correlation. In each case, the level of statistical significance was set at $p < 0.05$.

4. RESULTS

4.1. Framework and scope of the research

The doctoral research presented herein was carried out in the framework of the project entitled “Antioxidant Power Series as a tool for rational design and assessment of health promoting properties of functional foods based on antioxidant phytochemicals” (application no. 2014/14/A/ST4/00640) financed by National Science Centre, Poland in a programme „MAESTRO 6”. The aim of the project was to organize dietary antioxidants into Antioxidant Power Series (APS) based on their standard reduction potentials by analogy to electrochemical metal series. One of the objectives of this project was to verify the concordance of APS based on physicochemical measurements with antioxidant impact of redox-active substances determined with the aid of biochemical and biological tests. The ultimate goal was to verify whether APS built on the basis of electrochemical properties of the model redox-active compounds can be applied to predict the efficacy of food derived antioxidant substances in protecting cells against cellular damage under oxidative stress conditions.

Research conducted formerly in this project was focused mainly on plant polyphenols, which constitute the most widespread group of dietary redox-active compounds. There are two main observations, which have emerged from these initial experiments carried out for catechins [263], cocoa extract [248] as well as quercetin (Q) and naringenin [176]:

- physiological concentrations of polyphenols seemed not to have any effect on cellular antioxidant status,
- higher concentrations, achievable in the gut, increased cellular antioxidant activity in E^0 -dependent manner in exposed cells representing human alimentary tract.

Moreover, based on the results of genomic studies, the distinct patterns in terms of the pools of genes, whose expression was affected in treated cells, were identified for different antioxidants applied. Polyphenols, which are exogenous antioxidants, modulated the expression of a different and much smaller set of genes than glutathione (GSH), which is a major endogenous antioxidant. For example, the treatment of cells with catechins significantly up-regulated expression of just three genes (*ALB*, *CCL5*, *HSPA1A*) and only when applied at low concentration matching physiological levels (1 μ M). At higher concentration (10 μ M), which could reflect the concentration in the gut, the expression of none of the genes was affected with the exception of *SRXN1*, which was downregulated, thereby protecting cells against reductive stress [263]. In turn, the array of genes affected by GSH, whose antioxidant activity is substantially weaker than that of catechins, was much broader and affected another set of genes. GSH at low concentration (1 μ M) upregulated antioxidant genes (*GSTZ1*, *SEPP1*, *TXNRD2*), while at higher concentration (10 μ M), it induced also the expression of genes involved in ROS generation (*NOX5*, *NOS2*, *NCF1*) probably to prevent reductive stress [263]. These results suggest that, in contrast to GSH, strong exogenous antioxidants provide protection against oxidative stress rather by direct ROS scavenging, without the necessity of involving cellular antioxidant defense systems. It could be hypothesized that weaker endogenous antioxidants, such as GSH, must rely more on cellular



antioxidant mechanisms. Their concerted actions are sufficient to maintain proper redox homeostasis under eustress, whereas strong antioxidants such as some polyphenols are better or even indispensable when redox homeostasis is pushed towards oxidative stress. The aim of current research carried out in the framework of this thesis was to verify the above hypothesis by finding out the efficacy of endogenous low molecular weight antioxidants in maintaining redox balance in cells exposed to exogenous ROS. This goal was addressed by finding out how strong is the impact of endogenous antioxidants, however applied exogenously as if they were derived from dietary sources, on the cellular antioxidant capacity and other markers of protection against oxidative stress in cellular gut model.

The experiments were conducted for 20 redox-active compounds (listed in Table 4.1) that can be either produced endogenously as well as be provided with food or be only derived from dietary sources, yet by definition support endogenous antioxidant barrier, such as some vitamins. The activities and functions of these compounds are described in the introductory sections of the appropriate subchapters of Results: 4.3.1. (thiol antioxidants), 4.3.2. (purine derivatives), 4.3.3. (mitochondrial agents), 4.3.4. (pro-oxidants), 4.4.1. (ascorbic acid derivatives). In this thesis, GSH and Q were chosen as representatives of, respectively, endogenous and exogenous antioxidants. Data illustrating antioxidant properties of reference redox-active compounds are summarized in section 4.2.

Table 4.1. List of investigated redox-active compounds with the corresponding abbreviations as well as their standard (E^0) and/or formal (E^0') reduction potentials.

Investigated redox-active compounds	Ox/Red pairs	E^0 at pH 0 [V]		E^0' at pH 7 [V]		Ref.
		25°C	37°C	25°C	37°C	
N-acetyl-L-cysteine	NAC _{ox} /NAC _{red}	0.308	0.302	-0.130*	-0.154*	[264]
L-cysteine	CySS/Cys	0.145 ^a	0.138 ^a	-0.293 ^{a*}	-0.317 ^{a*} -0.220 ^b	[263] ^a , [264] ^b
α-Lipoic acid	LA/DHLA	n.a.		-0.294 (22°C)	n.a.	[266]
Adenine	Ade [*] /AdO _{red}	1.770	n.a.	1.360	n.a.	[267]
Adenosine	Ado [*] /AdO _{red}	2.030 (temp. n.a.)		1.420 (temp. n.a.)		[268]
Adenosine-5'-monophosphate	AMP [*] /AMP _{red}	1.590	n.a.	1.410	n.a.	[269]
Uric acid	UA [*] /UA _{red}	n.a.		0.590 (20°C)	n.a.	[270]
Coenzyme Q ₁₀ (ubiquinone)	CoQ ₁₀ /CoQ ₁₀ H ₂	n.a.		-0.066	n.a.	[271]
Melatonin	MT [*] /MT _{red}	n.a.		0.950	n.a.	[272]
Nicotinamide adenine dinucleotide	NAD ⁺ /NADH	n.a.		-0.320	n.a.	[273]
Citric acid	α-KG/iCA	n.a.		-0.380	n.a.	[273]
Glutathione disulphide	GSSG/GSH	0.318 ^a	0.310 ^{a,b}	-0.120 ^{a*}	-0.146 ^{a*} -0.240 ^c	[264] ^a [263] ^b

Investigated redox-active compounds	Ox/Red pairs	E^0 at pH 0 [V]		$E^{0'}$ at pH 7 [V]		Ref.
		25°C	37°C	25°C	37°C	
						[265] ^c
Hydrogen peroxide	O ₂ /H ₂ O ₂	0.697 ^a	n.a.	0.281 ^b	n.a.	[274] ^a
	H ₂ O ₂ /HO [•]			0.380 ^b		[275] ^b
	O ₂ ^{•-} /H ₂ O ₂			0.890 ^b		[276] ^c
	H ₂ O ₂ /H ₂ O	1.763 ^c		1.349 ^b		
L-Ascorbic acid	DHA/AA	0.288 ^a	0.287 ^a	-0.150 ^{a*}	-0.168 ^{a*}	[264] ^a ,
				-0.081 ^c		[277] ^b ,
						[278] ^c
Sodium L-ascorbate	Na(DHA)/NaA	0.275	0.272	-0.163 [*]	-0.184 [*]	[264]
Calcium L-ascorbate	Ca(DHA)/CaA	0.021	0.021	-0.092 [*]	-0.097 [*]	
D-Isoascorbic acid	iDHA/iAA	0.265	0.258	-0.173 [*]	-0.197 [*]	
Sodium D-isoascorbate	Na(iDHA)/iNaA	0.256	0.246	-0.182 [*]	-0.210 [*]	
Ascorbigen	ABG _{ox} /ABG _{red}	0.297	0.135	0.184 [*]	0.017 [*]	
Quercetin	Q _{ox} /Q _{red}	0.287 ^a	0.279 ^{a,b}	-0.151 ^{a*}	-0.176 ^{b*}	[264] ^a

n.a. – not available in scientific literature, ^{a, b} superscripts refer to corresponding references

* measurements were carried out at pH 7.4

Endogenously produced redox-active compounds (thiols, mitochondrial agents, purine derivatives and pro-oxidants) are involved in a variety of cellular processes such as energy production, metabolism, mitochondrial function, redox signalling and homeostasis, and as recently discovered, even in epigenetic regulation of gene expression. The investigated compounds included also ascorbic acid (AA), which cannot be synthesized by humans (and a few other mammalian species), yet belongs to physiologically important antioxidants. This nutrient must be provided daily with diet. In this thesis, this exogenous compound is considered as endogenously indispensable in the maintenance of proper redox homeostasis of the organism. Other compounds included in the group of ascorbates are ingested either in the form of food additives or dietary supplements. Most of them possess vitamin C activity and therefore, despite their synthetic origin, can perform a wide range of physiological functions.

Methods used in these investigations to assess the antioxidant activity of redox-active compounds embraced both chemical and biological approaches. Chemical methods included two spectrophotometric tests applying ABTS and DPPH radicals, which are commonly used to determine the antioxidant activity of foods and beverages. The results of these spectrophotometric tests are expressed as the stoichiometric n_{10} value, which is defined as a number of radical molecules scavenged by one molecule of antioxidant within 10 min reaction time. Stoichiometric value n_{10} is calculated as a tangent of a linear relationship between concentrations of reduced radical and studied compounds at three different temperatures: room temperature (25°C), the average physiological temperature of human body (37°C) and elevated temperature (41°C), which in the case of humans may be accompanied by excessive ROS formation and requires medical intervention. Biological tests were carried out in human colon

adenocarcinoma HT29 cells, which served as a model of gastrointestinal tract, where intestinal cells are in direct contact with ingested food. This cell line was chosen according to “3R” concept, *i.e.* human cell lines recommended by National Centre for Replacement, Refinement and Reduction of Animals in Science, UK, for nutritional studies [279]. Measurements of antioxidant activity of tested compounds in cells was conducted with Cellular Antioxidant Activity (CAA) test, which is recognized to be superior to commonly used chemical colorimetric antioxidant activity assays, as it takes into account the uptake of compounds by cells and their cellular metabolism.

In the case of some of the investigated compounds, the results occurred to be non-reproducible (as indicated by high SD values for results obtained for *e.g.* the group of adenine derivatives). This could stem from heterogeneity of cells being at various stages of cell cycle, which affected cellular redox status. Therefore, the modified version of CAA assay involving serum starvation of cells was proposed. The removal of serum from culture medium is regarded as a way to synchronize cells and thereby to create more uniform cell culture for biological experiments [280]. Subculture of cells grown in serum-free medium is more homogenous since under such conditions cells enter and remain in G₀/G₁ phase until experiment. The lack of dividing cells was confirmed by flow cytometry analysis (described in Appendix B). The use of such synchronized cultures ensures more reproducible experimental conditions, therefore such a modification of CAA assay was introduced.

The impact of redox-active compounds on the growth of HT29 cells was studied using MTT test. The ability of investigated antioxidants to provide protection against oxidative damage was assessed by comet assay, which is a simple method for measuring DNA strand breaks at the single-cell level in cells exposed to genotoxic agents such as H₂O₂. In this study, this approach was used to determine whether tested redox-active compounds could both exert genotoxic effects in HT29 cells as well as prevent genotoxicity of H₂O₂ as a representative endogenous oxidant. In both cases, cells were initially incubated with investigated compounds at 1-100 μM concentrations for 24 h. When the treatment was finished, cells were subjected either to the comet assay procedure (in the case of genotoxicity determination) or to 1 h treatment with 150 μM H₂O₂ (to assess protection from genotoxic effects), followed by the comet assay procedure. The equivalent set of methods was applied in previous investigations of our group focused on polyphenolic antioxidants, which makes it possible to directly compare the activities of exogenous antioxidants with the activity of those produced also endogenously in human cells [176,248,263].

Another former approach to study antioxidant activity of dietary redox-active low molecular weight compounds was based on electrochemical methods (potentiometric titration and differential pulse voltammetry) carried out by Klaudia Suliborska, PhD, who was a collaborator in the same research project. Therefore, the results obtained in this thesis will be discussed in relation to electrochemical properties of studied compounds established by Suliborska for GSH, Cys, NAC, AA derivatives and Q, as well as to those data that are available in literature, for other investigated compounds [264]. In this thesis, electrochemical behaviour of investigated redox-active agents is expressed by thermodynamic parameter called reduction potential, which describes the ability of a compound to accept electrons and thus, to undergo reduction. The more



negative is the value of reduction potential, the stronger the reducing properties of a compound. With regard to the former study on catechins [263], whose standard reduction potentials (E^0) were also established by Suliborska, it can be observed that endogenous antioxidants tend to have higher E^0 values, thus are weaker antioxidants. However, most of the values of reduction potentials published in literature for endogenous redox-active compounds were determined at pH 7 or 7.4, and thus are called biological or formal reduction potentials ($E^{0'}$) to distinguish them from standard reduction potentials (E^0) that refer to theoretical pH 0. As shown in Table 4.1, thermodynamic properties strongly depend not only on pH, but also on temperature. Even though the temperature of 37°C is most biologically-relevant in the case of human cells, and for this reason, it was included in measurements of E^0 carried out by Suliborska, majority of published E^0 and $E^{0'}$ values were determined at ambient temperature, which is reasonably similar to standard temperature 25°C. Therefore, in this thesis electrochemical behaviour of endogenous redox-active compounds is discussed based on $E^{0'}$ values determined at 25°C and pH around 7 to enable the broadest comparison between values established by Suliborska and those available in literature. This approach may be criticized by purists, who would be probably in favour of standard reduction potential E^0 , however, the formal reduction potential $E^{0'}$, determined for pH 7.4 and 25°C, seems to better reflect biological situation.

4.2. Reference redox-active compounds

Glutathione (GSH) and quercetin (Q) were chosen as reference compounds to create a frame within which the impact of studied antioxidants on redox cellular homeostasis could be assessed. These two compounds are “gold standard” representatives of, respectively, endogenous and exogenous antioxidants for several reasons. In the case of GSH, it is (i) recognized as a main intracellular antioxidant crucial to maintain thiol redox homeostasis, (ii) it serves as a substrate for antioxidant and detoxification enzymes (GPxs, GSTs), (iii) it plays a role in cellular redox signalling through e.g. protein S-glutathionylation [281]. Q was selected since it is an ubiquitous dietary polyphenolic compound known to be exceptionally biologically effective antioxidant, which is determined by its kinetics-related parameters (*i.e.* anodic current and charge density in cyclic voltammetry measurements). However, thermodynamic parameters, such as E^0 , suggest that Q is a rather moderate reductant [176]. Nevertheless, kinetic-related parameters seem to be more biologically relevant in terms of regulation of redox homeostasis. Moreover, electrochemical properties and impact on redox-related cellular functions of both reference compounds were previously investigated in our group [176,263], which enables convenient comparisons. Data on the electrochemical properties are listed in Table 4.1., while antioxidant activity, genotoxicity, protection against H₂O₂-induced DNA oxidation and the list of genes upregulated by GSH and Q are gathered in Table 4.2. The values highlighted in yellow (Table 4.2.) were either replicated (genotoxicity, CAA) or obtained (ABTS, protection from oxidative DNA damage) in the framework of this thesis; other data are derived from the following references: [176,263].



Table 4.2. Reference data on the antioxidant activity, genotoxicity and protection against H₂O₂-induced DNA oxidation of glutathione (GSH) and quercetin (Q) [176,263].

Test	GSH		Q	
Spectrophotometric tests	n₁₀ value at 37°C			
ABTS	1.522 ± 0.024		4.880 ± 0.050 ^a	
DPPH	0.012 ± 0.000		3.239 ± 0.178	
Cellular antioxidant activity (CAA)	CAA values			
Solvent	Water		Q in 1% EtOH^b	Q in 5% EtOH
1 µM	7 ± 7		10 ± 4	5 ± 5
10 µM	22 ± 6		37 ± 7	32 ± 16
100 µM	23 ± 8		96 ± 0.4	92 ± 2
1000 µM	26 ± 8		-	
Genotoxicity	% DNA in comet tail			
Solvent control	1.4 ± 0.3 ^c	4.7 ± 0.7	1.9 ± 0.4	
0.1 µM	-	4.3 ± 2.7	-	
1 µM	1.4 ± 0.4 ^c	6.7 ± 0.9	2.4 ± 0.8	
10 µM	1.3 ± 0.5 ^c	6.3 ± 2.0	2.8 ± 0.6	
100 µM	1.2 ± 0.3 ^c	6.5 ± 2.4	5.0 ± 1.3	
Protection against H₂O₂-induced DNA oxidation	% DNA in comet tail			
Solvent control	2.1 ± 0.1		1.4 ± 0.3 ^d	
0.1 µM	12.5 ± 4.4		-	
1 µM	9.7 ± 1.5		49.1 ± 5.5 ^d	
10 µM	14.8 ± 5.9		46.1 ± 8.2 ^d	
100 µM	14.2 ± 0.5		35.5 ± 6.7 ^d	
150 µM H ₂ O ₂ control	11.4 ± 2.6		57.5 ± 6.6 ^d	
Genomic analysis	Upregulated genes			
1 µM	<i>GSTZ1, NOX5, SEPP1, TXNRD2</i>		<i>HSPA1A, PTGS1, TXNRD1</i>	
10 µM	<i>BNIP3, NCF1, NOS2, NOX5, SEPP1</i>		<i>HSPA1A, TXNRD1</i>	

^a – data borrowed from [282], ^b – CAA assay for Q was replicated from Baranowska *et al.* [176] using 1% EtOH as a solvent, ^c – comet assay for GSH was replicated from Baranowska *et al.* [263] using modified protocol as described in Materials and methods (section 3.2.3.5.), ^d – original data on protection of DNA against oxidative damage provided in this thesis; data were not available for Q in previously published work.

Based on the former experiments, it can be observed that the antioxidant activity of GSH, the representative endogenous antioxidant, is markedly weaker compared to exogenous plant polyphenols represented by Q. According to electrochemical measurements, GSH has significantly higher standard reduction potential than Q, thus it is much weaker electron donor (Table 4.1). In spectrophotometric tests, GSH was also around 3 times weaker scavenger of ABTS and DPPH radicals than Q (Table 4.2). In the case of cellular antioxidant activity, Q at concentrations not exceeding 10 µM did not differ significantly from GSH, which suggests that

such low concentrations of this polyphenol were not able to override cellular redox buffering capacity of HT29 cells. In the case of GSH, it affected reducing capacity of cells only moderately even at the highest used concentrations (100 and 1000 μM , CAA values 23 and 26, respectively). In contrast, the impact of 100 μM Q on cellular antioxidant activity of HT29 cells reached the highest measurable level (CAA value 96). In line with these results, protection against oxidative DNA damage was provided solely by Q, reaching statistical significance for the highest tested concentration of Q (100 μM). No DNA protection against ROS insult was observed in the case of GSH (Table 4.2 and Figure 4.1C). At the genomic level, in contrast to GSH, Q significantly affected the expression of solely three genes, which is similar to what was observed in the case of catechins as described earlier.

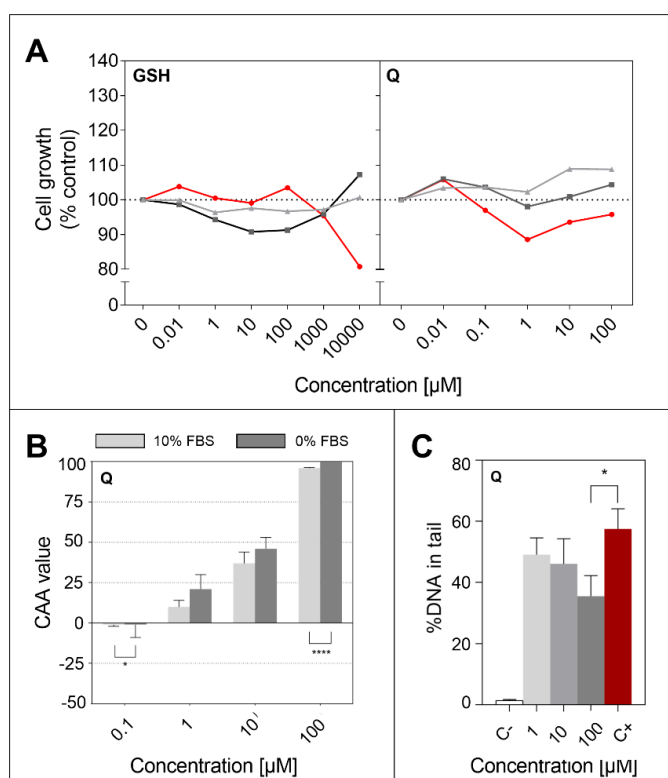


Figure 4.1. The impact of glutathione (GSH) and quercetin (Q) on redox-related cellular effects.

A – Cell growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment expressed relative to control non-treated cells whose growth is regarded as 100%; data borrowed from Baranowska *et al.* [176,263]. **B** – The impact of serum starvation of HT29 cells (10% vs. 0% FBS in medium) treated with Q on cellular antioxidant activity. The results are expressed as means \pm SD of three independent experiments carried out in triplicate. Statistical analysis was carried out with Student's t-test: (*) – ≤ 0.05 and (****) – ≤ 0.0001 . **C** – The antigenotoxic impact of Q in HT29 cells exposed to H_2O_2 used as a model genotoxic oxidant. The cells were treated with Q for 24 h prior to 1 hour exposure to 150 μM H_2O_2 and then submitted to comet assay procedure. The results are expressed as mean percentage of DNA in comet tail \pm SD from three independent experiments carried out in duplicate and compared with C⁻, negative control (cells treated with solvent only) and C⁺, positive control (150 μM H_2O_2). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$ marked as with one asterisk (*).

Figure 4.1 collects the results of experiments assessing the impact of GSH and Q on HT29 cell growth (adapted from Baranowska *et al.* [176,263]), results of determinations of cellular antioxidant activity under normal and serum-deprived conditions, as well as anti-genotoxic effects of Q in HT29 cells exposed to H₂O₂ investigated in the framework of this thesis. Neither GSH, nor Q had notable impact on the growth of HT29 cells (Figure 4.1A). Measurements of cellular antioxidant activity were replicated using lower concentration of the solvent for Q (1% instead of 5% EtOH); the conditions also used for α -lipoic acid (LA) and ascorbigen (ABG). Concentration of ethanol, as shown in Table 4.2, did not have great impact on CAA values of Q. Furthermore, cellular antioxidant activity of endogenous redox-active compounds, included in current research, was investigated under normal and serum-deprived conditions. Since such approach was not applied in the former studies on GSH and Q, these experiments were carried out in the framework of this thesis. As shown in Figure 4.1B, serum starvation of HT29 cells treated with Q did not have significant impact on cellular antioxidant activity. The impact of serum starvation of cells treated with GSH on CAA values is presented and discussed in the following section 4.2.1. concerning all tested endogenous thiol antioxidants. Briefly, CAA values in serum-starved cells treated with GSH were low regardless of concentration used, and in the case of 10 and 100 μ M concentrations, serum-starvation of cells abolished the activity observed under normal conditions.

4.3. *Redox-active compounds under study*

4.3.1. *Endogenous thiol antioxidants*

L-cysteine

L-Cysteine (Cys) is a conditionally essential amino acid, whose levels in the organism are dependent on 3 sources: diet, transsulfuration pathway and catabolism of endogenous proteins. It has been reported that in porcine gastrointestinal tract, 25% of dietary Cys is used for synthesis of mucosal epithelial proteins and, as such, it is indispensable for maintaining intestinal structure and function [283,284]. This amino acid may also serve as a precursor for GSH synthesis. Cys exerts stronger antioxidant properties than either N-acetyl-L-cysteine (NAC) or GSH [285], thus when GSH synthesis is blocked, Cys may efficiently strengthen cellular antioxidative barrier. In certain cases, this may be an undesirable effect. For instance, it has been reported that Cys could compensate for the depletion of GSH in liver cancer HepG2 cell line, thus preventing ferroptosis, which is a target pathway for cancer therapy [286].

The redox properties of Cys depend on its location – in foods, blood and tissue proteins, it is present mainly in its oxidized form – L-cystine (CySS), while the reduced form dominates in the intracellular milieu, due to more reducing environment [287]. Therefore, besides GSH/GSSG ratio, Cys/CySS ratio can serve as an important indicator of cellular redox status as well [288–290]. The oxidative state of Cys plays also a crucial role in redox signalling, since its thiol group can undergo further several steps of oxidation: to sulfenic (-SOH), sulfinic (-SO₂H) and sulfonic (-SO₃H) acids, each of which may affect protein properties. These consecutive oxidation steps of Cys control, for example, peroxiredoxins activity, which has been already described in



section 1.3.1. In response to physiological stimuli, Cys residues in proteins can undergo multiple, though reversible, oxidative post-translational modifications, such as S-nitrosylation, S-glutathionylation, and formation of disulphide bonds. All of them determine protein 3-dimensional structure and thus function [291]. For instance, thiol proteins of mitochondrial complex I in ETC can be reversibly S-glutathionylated by glutaredoxin 2 in response to decreasing GSH/GSSG ratio and increasing concentration of hydrogen peroxide. In this case, S-glutathionylation in complex I proteins prevents irreversible oxidation of Cys to sulfonic acid and thereby, complex I becomes protected against unreparable inactivation [292,293].

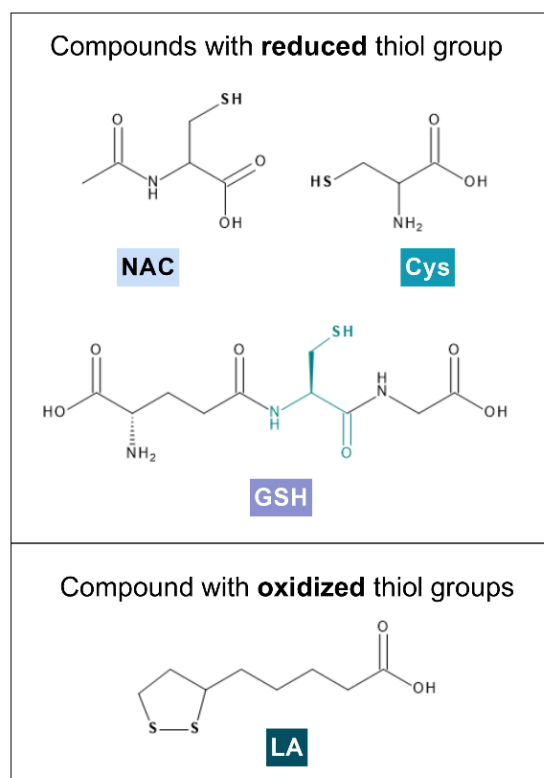


Figure 4.2. Chemical structures of studied thiol compounds. NAC, N-acetyl-L-cysteine; Cys, L-cysteine; GSH, glutathione; LA, α-lipoic acid.

N-acetyl-L-cysteine

N-acetyl-L-cysteine (NAC) is a thiol antioxidant compound approved for clinical use in the treatment of acetaminophen (paracetamol) overdose and in ailments of respiratory tract, in which NAC acts as a mucolytic agent [294]. Therapeutic potential of NAC in the treatment of neurodegenerative and infectious diseases as well as psychiatric disorders, to name just a few, is being tested in numerous clinical trials [295]. Most of clinical applications of NAC are based on its antioxidant activity. For example, NAC reduces disulphide bonds in glycoproteins of mucus, which decreases its viscosity being a problem in pulmonary diseases [285]. In the case of already mentioned paracetamol overdose, NAC replenishes GSH pool in the liver to enhance the detoxification and antioxidant processes [285]. Currently, NAC is also available on the market in

a form of dietary supplements claimed to, for example, improve sport performance by boosting GSH pool, which helps to fight oxidative stress during physical exercise [296,297]. However, NAC is also formed endogenously from S-conjugates of GSH, which are metabolized upon II phase detoxification pathway to the corresponding S-conjugates of NAC, commonly known as mercapturic acids [200,298]. Besides being a drug or dietary supplement, NAC is present in foods of plant origin such as asparagus and red pepper, however, in very small amounts (nM/g of wet weight) [299].

Metabolism of exogenous NAC after oral administration occurs in the small intestine as well as, after absorption, in the liver and kidney, where it undergoes extensive first-pass metabolism. Bioavailability of NAC was assessed to be less than 5-10%, while its plasma concentration was shown not to exceed 20 μM [295,300]. Only 3% of ingested NAC was reported to reach the colon [300], where it may also exert beneficial effects. For example, NAC was found to support the maintenance of remission in patients with ulcerative colitis, whose etiopathology is associated with oxidative stress [301].

The antioxidant properties of NAC are considered to stem predominantly from its ability to increase intracellular pools of GSH, since NAC after deacetylation liberates L-cysteine (Cys), which can be further used for biosynthesis of GSH (chemical structures presented in Figure 4.2) [302]. NAC acts also as a direct ROS scavenger (however weaker than e.g. GSH or Cys) and chelator of transition and heavy metal ions [303]. Other, more specific molecular mechanisms of NAC's antioxidant activity are postulated, but still are not well documented. Recently, it has been reported that strong antioxidative effects of NAC in human cells may result from the activity of hydropersulfides formed upon the catabolism of NAC. Hydropersulfides are considered as "hyperactivated thiols", which appear to be more efficient in scavenging radicals, peroxides and electrophiles compared to thiols [304].

α -Lipoic acid

α -Lipoic acid (LA) is an endogenous thiol short chain fatty acid (systematic name 1,2-dithiolane-3-pentanoic acid). In the form of protein-bound lipoamide, it serves as a cofactor of pyruvate dehydrogenase and α -ketoglutarate dehydrogenase complexes in the citric acid cycle in the mitochondria. LA is also present in foods of plant and animal origin, mostly in the protein-bound form as lipoyllysine (LLys), however, even its richest dietary sources (*i.e.* spinach or meat offal) do not contain more than 400 ng of LLys per 100 g of food product [305]. It has been suggested that dietary LA is absorbed in the form of LLys, since the peptide bond between LA and Lys is not efficiently cleaved by proteolytic enzymes [306].

LA ingested in the form of dietary supplement by healthy volunteers was shown to be readily absorbed from alimentary tract and metabolized in the course of β -oxidation to bisnorlipoic acid, tetranorlipoic acid, 6,8-bismethylthio-octanoic acid, 4,6-bismethylthio-hexanoic acid and 2,4-bismethylthiobutanoic acid [307,308]. Its bioavailability was estimated to be around 30% and after single oral administration of 600 mg, its plasma concentration reached values close to 20 μM in healthy volunteers [309]. In nature, LA occurs in a form of R-enantiomer, while dietary



supplements obtained in the course of chemical synthesis are composed of equal proportions of R and S enantiomers [310]. The bioavailability of R-enantiomer was reported to be 40-50% higher than that of S-enantiomer in 15 healthy men given a racemate of LA [311].

After absorption, LA was shown to be converted mainly to its reduced form – dihydrolipoic acid (DHLA) by cultured cells [312]. Both forms, reduced and oxidized, possess antioxidant activity and owing to their amphiphilic properties, they can scavenge ROS in both lipophilic and hydrophilic environment showing important chemoselectivity [313]. LA and DHLA were shown to scavenge HO^\bullet and HOCl , $^1\text{O}_2$ could be scavenged only by LA, while ROO^\bullet and $\text{O}_2^{\bullet-}$ only by DHLA [314]. Both LA and DHLA were also reported to react with ONOO^- , however, this reaction was considered to be too slow to occur *in vivo* [315]. Besides the ability to scavenge ROS and RNS, DHLA and LA can also chelate transition metal ions (e.g. iron, copper, manganese and zinc) known to promote oxidative stress [306]. Last but not least, LA is called an “universal antioxidant”, as it was shown to regenerate other important intracellular antioxidants such as GSH, vitamin C, vitamin E and coenzyme Q_{10} (CoQ_{10}) [316,317].

4.3.1.1. Antioxidant activity by ABTS and DPPH tests

The results of spectrophotometric tests carried out for all studied thiol antioxidants are expressed as stoichiometric n_{10} values, which are presented in Figures 4.3B,D and listed in Table A1 in Appendix A (Section 7.1.1.). The impact of temperature on the antioxidant activity of NAC and Cys turned out negligible in both ABTS and DPPH tests. Therefore, the description of the results will refer only to those obtained at 37°C as the most biologically relevant. In general, thiol antioxidants occurred to be better scavengers of ABTS than DPPH radical. LA did not react with any of these radicals and therefore linear relationships for this compound are not shown; only n_{10} values (equal 0) are marked in n_{10} value axes in Figures 4.3B and D. In ABTS test, the antioxidant activity of NAC was higher than that of Cys ($n_{10} = 1.118$ vs. $n_{10} = 1.040$, respectively). However, this was in contrast to the results of DPPH test, in which the strongest antioxidant activity was observed for Cys ($n_{10} = 0.742$) that was 2-fold stronger reducing agents than its N-acetylated derivative, NAC ($n_{10} = 0.371$).

The results of spectrophotometric tests obtained for NAC and Cys were compared to the n_{10} values obtained for GSH and Q at 37°C (Figure 4.8), used in this work as a reference antioxidants (data published in [176,263]). In contrast to NAC and Cys, GSH did not show the ability to scavenge DPPH radical. At the same time, GSH turned out to be the best ABTS scavenger among tested thiols. To conclude this stage of studies, the order of antioxidant activity of thiol compounds according to the results of ABTS at 37°C tests was as follows: $\text{LA} < \text{Cys} < \text{NAC} < \text{GSH} \ll \text{Q}$. The antioxidant activity of tested thiols expressed as n_{10} value was similar to that of GSH ($n_{10} = 1.52$), but much weaker than that of Q ($n_{10} = 4.88$).

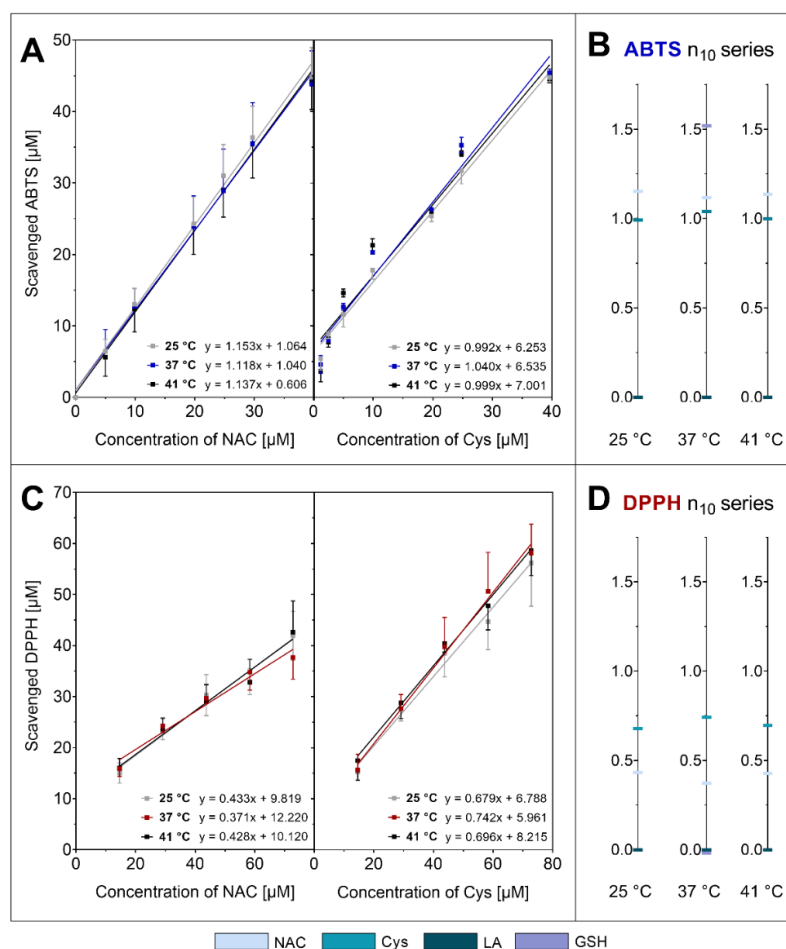


Figure 4.3. The relationship between scavenged ABTS – **A** or DPPH – **C** radicals vs. concentration of N-acetyl-L-cysteine (NAC) or L-cysteine (Cys) at 25, 37 and 41°C and corresponding n_{10} value axes for NAC, Cys, glutathione (GSH) and α -lipoic acid (LA) – **B, D**. The results are expressed as means \pm SD of three independent experiments carried out in triplicates. The ABTS n_{10} value calculated for GSH at 37°C was borrowed from Baranowska *et al.* [263].

4.3.1.2. Antioxidant activity by CAA test

The impact of thiol antioxidants (NAC, Cys, LA) on cellular antioxidant activity was determined by CAA assay. HT29 cells were treated for 1 h with individual compounds at low physiological concentrations, which are achievable in the bloodstream (0.1-10 μM) and higher concentration reachable in the gut (100 μM). Prior to the experiments (20 h before), cells were cultured either in complete (10% FBS) or in serum-free (0% FBS) medium. Cellular antioxidant activity was assessed by the ability of investigated compounds to inhibit oxidation of the probe, which is reflected by proportionally decreased levels of fluorescence. Results are expressed as so-called CAA values, which indicate the percentage of the inhibition of probe oxidation. Thus, the higher the CAA value, the stronger reducing capacity of the compound. Results obtained for investigated thiols were compared to the ones determined for reference compounds, *i.e.* GSH, which is also an endogenous thiol, as well as a polyphenolic compound – Q (Figure 4.5 and 4.6).

As shown in Figure 4.4, in cells cultured under normal conditions (10% FBS) before the experiment, the treatment of cells with NAC and LA resulted in the moderate enhancement of cellular antioxidant activity, which remained at the same level regardless of the applied concentration. In turn, Cys at the lowest 0.1 μM concentration had minor effects on CAA value, which increased at higher concentrations and then maintained at moderate level. In contrast to the rest of thiols, GSH affected cellular antioxidant activity in a concentration-dependent manner. GSH exerted the strongest (but still moderate) reducing capacity at 10 and 100 μM concentrations, while at the lowest concentrations CAA values suggested pro-oxidative effects.

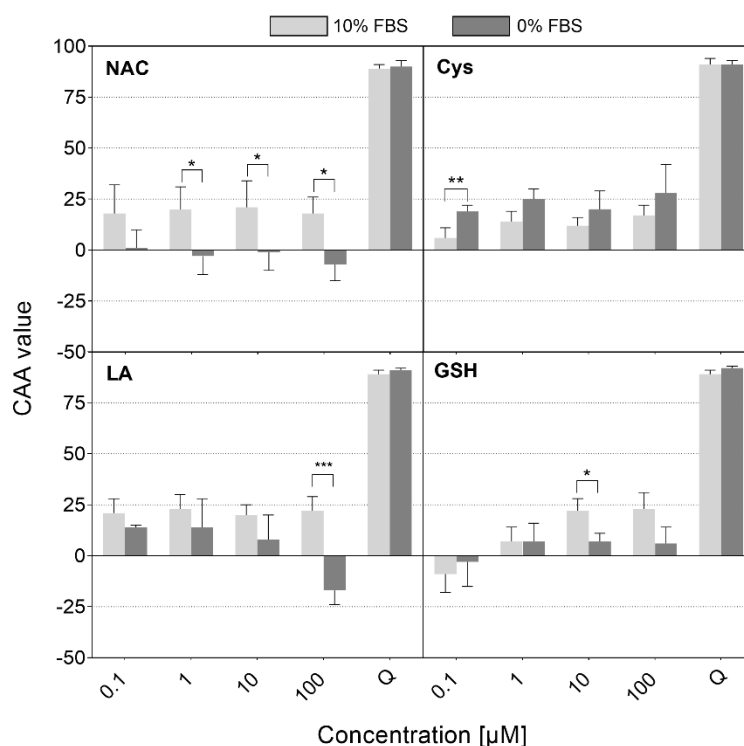


Figure 4.4. The impact of serum starvation of HT29 cells treated with studied thiols or 500 μM quercetin used as a positive control on cellular antioxidant activity. The results are expressed as means \pm SD of three independent experiments carried out in triplicate. Statistical analysis was conducted by Student's t-test and the level of significance was set at $p < 0.05$. The asterisks mark p values as follows: (*) – ≤ 0.05 , (**) – ≤ 0.01 and (***) – ≤ 0.001 . Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before the experiment.

The abbreviations used refer to: Cys, L-cysteine; GSH, glutathione; LA, α -lipoic acid; NAC, N-acetyl-L-cysteine, Q, quercetin.

Serum starvation of cells before the experiment resulted in the complete loss of antioxidant effects of NAC, which showed a concentration-dependent trend towards pro-oxidation. Similarly, reducing capacity of GSH was also abolished in serum-starved cells, regardless of the concentration. In the case of LA, with increasing concentrations, a trend towards decreasing CAA values was observed, which finally turned into significant pro-oxidative effects at the highest 100 μM concentration. Cys was the only compound, whose impact on CAA values



maintained the trend: concentration-dependent increase in cellular antioxidant activity of HT29 cells.

As shown in Figure 4.5A, the reducing capacity of tested thiols is comparable under normal conditions, especially in the case of higher concentrations (10 and 100 μM). In particular, GSH at the lowest 0.1 μM concentration showed activity notably lower than that of NAC or LA (p values 0.014 and 0.007, respectively). In contrast, the antioxidant activity became more diversified within the whole concentration range of studied thiols, when cells were serum-starved. Under serum-deprived conditions, Cys appeared to be the strongest antioxidant at each of tested concentrations except for 10 μM , at which no statistically significant differences between tested compounds were determined. In the range of lower concentrations (0.1 and 1 μM), the second strongest antioxidant appeared to be LA. At 10 μM , CAA value of LA aligned with that of GSH. At the highest 100 μM concentration, strong reducing capacity of Cys was followed by much weaker impact of GSH, NAC and LA.

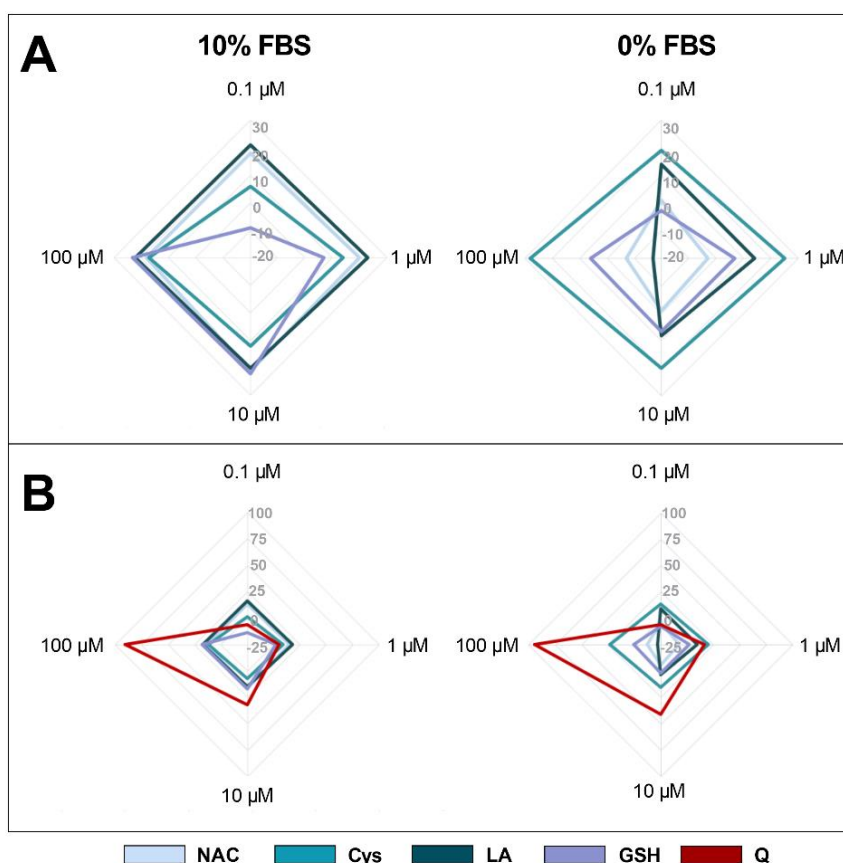


Figure 4.5. Comparison radar charts visualizing differences between CAA values of studied thiol antioxidants – **A**, or studied thiol antioxidants vs. quercetin (Q) – **B**, determined in HT29 cells cultured under either normal (10% FBS) or serum deprived (0% FBS) conditions. The statistical analysis was conducted by one way ANOVA with Tukey's (Panel **A**) or Dunnett's (Panel **B**) tests with the level of significance set at $p < 0.05$. All experimental details are as described in the caption for Figure 4.4. The abbreviations used refer to: Cys, L-cysteine; GSH, glutathione; LA, α -lipoic acid; NAC, N-acetyl-L-cysteine; Q, quercetin.

The impact of quercetin (1-100 μM) on cellular antioxidant activity in HT29 cells was initially determined by Baranowska [176]; data are presented in Table 4.2. Experiments were repeated with additional inclusion of 0.1 μM concentration and serum-deprived conditions as shown in Figure 4.1B in section 4.2 as well as Figure 4.5B. The CAA values obtained herein were in line with those determined by Baranowska [176]. In cells cultured in complete medium, Q at the lowest tested concentration (0.1 μM) was significantly weaker antioxidant than LA or NAC (p values 0.017 and 0.040, respectively). In cells grown in serum-deprived medium, 0.1 μM Q was markedly weaker than Cys (p value 0.015), while 1 μM Q turned out to be notably stronger than NAC (p value 0.023). At 10 μM concentration, the impact of Q on reducing capacity of cells was markedly stronger than Cys and LA (p values 0.006 and 0.040, respectively) under normal culture conditions. Antioxidant effects of 10 μM Q were even more enhanced in serum-starved cells. Under both normal and serum-deprived conditions; 100 μM quercetin had significantly greater impact (p value < 0.0001) on cellular antioxidant capacity of HT29 cells compared to exogenously applied thiol antioxidants (Figure 4.5). According to CAA values at 100 μM , determined in cells cultured under normal conditions, the order of antioxidant activity increases as follows: Cys $<$ NAC $<$ LA $<$ **GSH** $<$ **Q**. However, as it can be seen in Figure 4.5, the differences between individual thiol antioxidants are negligible. In the case of cells cultured in serum-deprived medium before the experiment, the order of antioxidant activity is changed as follows: LA $<$ NAC $<$ **GSH** $<$ Cys $<$ **Q**.

4.3.1.3. Cytotoxicity by MTT test

The impact of antioxidant thiols on the growth of HT29 cells was determined by MTT test. Cells were treated with low, corresponding to physiological concentrations of individual compounds, which are achievable in the bloodstream (0.1-10 μM) as well as higher concentrations that are reachable in the gut (100 μM) after meal consumption. The impact on cell growth after 6, 24 or 72 h treatment is illustrated in Figure 4.6.

Exposure of HT29 cells to NAC for 6 h significantly increased (by approximately 20%) cell growth within 1-1000 μM concentration range. Longer treatment (24 and 72 h) with the highest 5000 μM concentration of NAC was no longer stimulative and cell growth was at the level close to the control. In contrast to NAC, HT29 cells growth was not notably affected after exposure to Cys at 0.1-1000 μM concentrations. The inhibition of growth after treatment with the highest (5000 μM) concentration was negligible after 6 and 24 h exposure, however, after 72 h cell growth was reduced by approximately 15%. Slight increase of HT29 viability (by approximately 10%) was observed after 6 and 24 h exposure to LA at any concentration up to 1000 μM . Growth of cells exposed to 0.1-100 μM LA for 72 h was close to the level of control cells, however, prolonged treatment with higher concentrations (250-1000 μM) of LA significantly suppressed cell growth (up to 80% inhibition).

Compared to reference antioxidants, long treatment with Cys showed the same trend like GSH, especially at the highest tested concentrations (5 mM and 10 mM, respectively), where a 20% drop in cell growth was observed. Due to limited solubility, the impact of Q on cell growth

was tested only at concentrations reaching up to 100 μM , which barely affected cell growth. Within the same range of concentrations, the outcome was similar for Cys. LA and NAC at concentrations up to 100 μM seemed to be more stimulative of cell growth compared to both Q and GSH.

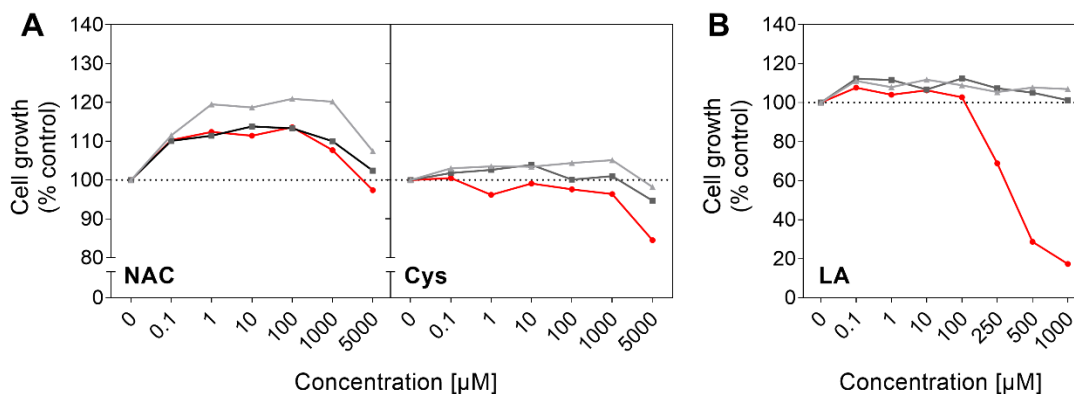


Figure 4.6. The impact of studied thiols on the growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment with **A** – N-acetyl-L-cysteine (NAC), L-cysteine (Cys) or **B** – α -lipoic acid (LA). Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

4.3.1.4. Genotoxicity and protection against oxidative DNA damage by comet assay

To determine whether tested endogenous thiol antioxidants could both exert genotoxic effects in HT29 cells as well as prevent H_2O_2 -induced DNA fragmentation, cells were first incubated with tested thiols at 1-100 μM concentrations for 24 h. When the treatment was finished, the cells were subjected either to the comet assay procedure (in the case of genotoxicity determination) or to 1 h treatment with 150 μM H_2O_2 (to assess protection against genotoxic effects), followed by the comet assay procedure. The measure of genotoxicity was the percentage of DNA in the comet tail.

As shown in Figure 4.7A, the 24 h treatment of cells with tested thiol antioxidants did not lead to DNA fragmentation compared to the control cells treated with the appropriate solvent only. In neither case, DNA damage exceeded 2%. Thus, none of the investigated compounds behaved like a genotoxic agent regardless of the concentration used. In contrast to endogenous thiol antioxidants, treatment of HT29 cells with Q resulted in statistically significant, albeit small (around 3%) increase in percentage of DNA damage (Table 4.2). The results of modified version of comet assay applied to assess if the pre-treatment of HT29 cells with investigated thiols could provide protection against H_2O_2 -induced DNA damage are shown in Figure 4.7B. The only compound among tested thiols that “immunized” cells against H_2O_2 -induced DNA damage was 100 μM LA. In the case of 10 μM LA, the protection was not statistically significant (p value = 0.054). None of the remaining compounds in this group (NAC, Cys, GSH) had a notable impact on prevention of

DNA damage. In contrast, pre-treatment of HT29 cells with 100 μM Q resulted in statistically significant protection against oxidative DNA fragmentation (Figure 4.1C).

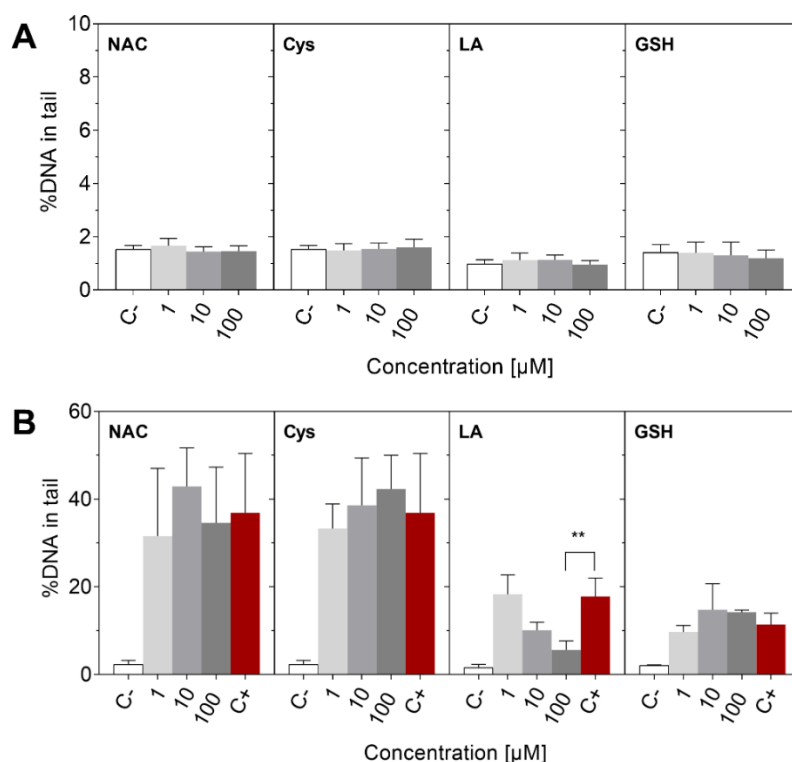


Figure 4.7. The genotoxicity of studied thiol antioxidants towards HT29 cells – **A**, and their antigenotoxic impact in HT29 cells exposed to genotoxic treatment with H_2O_2 (150 μM , 1 h) used as a model genotoxic oxidant – **B**. The cells were treated with studied thiols for 24 h (genotoxicity) or for 24 h prior to 1 hour exposure to 150 μM H_2O_2 (antigenotoxic activity) and then submitted to comet assay procedure. The results are expressed as mean percentage of DNA in comet tail \pm SD from three independent experiments carried out in duplicate and compared with C-, negative control (cells treated with solvent only) and C+, positive control (150 μM H_2O_2). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$, where (**) – p value ≤ 0.01 . The abbreviations used refer to: Cys, L-cysteine; GSH, glutathione; LA, α -lipoic acid; NAC, N-acetyl-L-cysteine.

4.3.1.5. Endogenous thiol antioxidants – recapitulation

The antioxidant properties of studied thiols are summarised in Figure 4.8. The chemical and cellular determinations of antioxidant activity are presented in relation to the compounds' formal reduction potentials measured at 25°C and pH around 7; both conditions can be expected to mirror those in which living organisms must maintain their redox homeostasis. In the case of reduction potentials, both standard and formal, more negative values indicate stronger reducing agents (Table 4.1). However, to simplify graphical presentation of reduction potentials across all groups of compounds investigated in this thesis, formal reduction potentials are presented as absolute values. More oxidizing or more reducing properties are highlighted along axes with red or blue colour, respectively.

Endogenous thiol antioxidants, including glutathione that was used as a representative of intracellular antioxidants, in the light of conducted experiments turned out to exert rather weak to moderate antioxidant activity. Based solely on formal reduction potentials, Cys was twice stronger antioxidant compared to the rest of the group as well as Q, whereas NAC was only slightly better reductor than GSH. LA turned out to be the weakest electron donor due to its oxidized form. These relationships may be altered under cellular conditions, when metabolic processes change the form of compounds applied, e.g. reduction of oxidized form of LA to its reduced form may occur, which markedly increased its antioxidant properties.

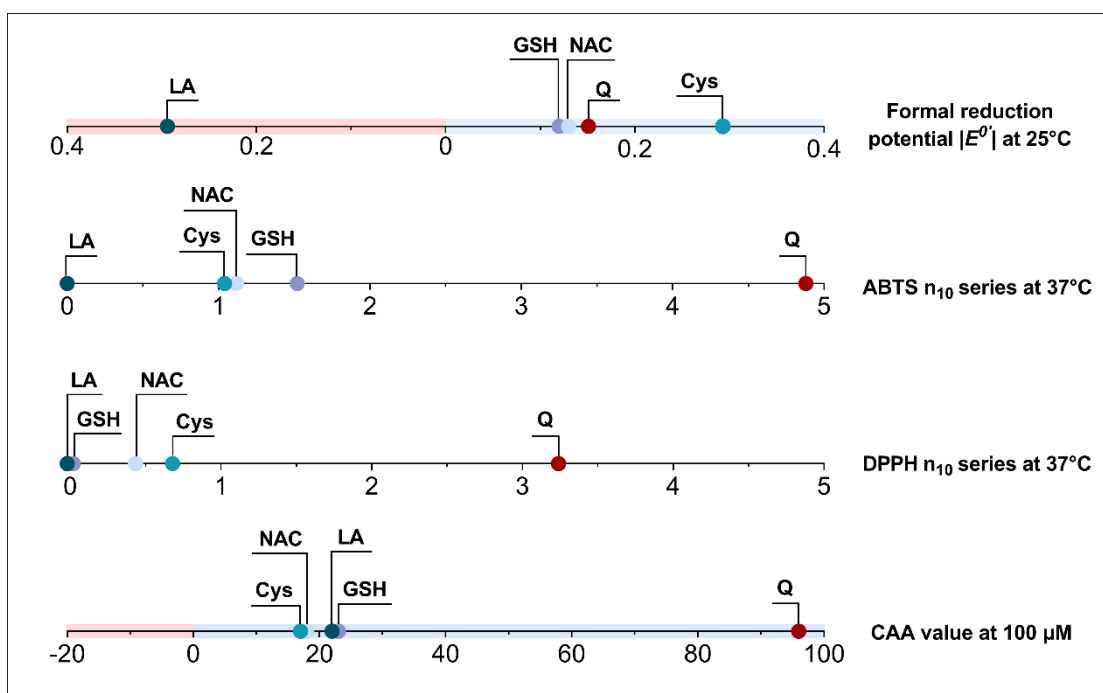


Figure 4.8. Axes comparing redox properties of thiol antioxidants ordered according to: formal reduction potential (E° [V], shown as absolute values); n_{10} values calculated based on ABTS or DPPH tests; the cellular antioxidant activity (CAA) values measured in cells cultured in complete medium and treated with 100 μM concentration of investigated compounds (Cys, cysteine; GSH, glutathione; LA, α -lipoic acid; NAC, N-acetyl-L-cysteine; Q, quercetin). Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity.

Reduction potential describes compound's reducing properties in terms of thermodynamics, however, Baranowska *et al.* showed that biological activity seemed to be rather determined by kinetics-related parameters [176]. This may explain why Q, despite its indistinctive thermodynamic properties, is far stronger antioxidant compared to investigated thiols according to ABTS and DPPH n_{10} series (n_{10} value takes into account also reaction kinetics) as well as CAA values for 100 μM concentration (Figure 4.8). In the case of LA, its thermodynamic behaviour was in line with the results of spectrophotometric tests, since it did not react neither with ABTS nor with DPPH radical. In contrast, in CAA assay, in which intracellular reduction of LA to DHLA may take place, it turned out to be as strong as other investigated thiols and moreover, it was the only

thiol that provided significant protection against H₂O₂-induced DNA fragmentation (Figure 4.7B). Similar effects were observed only in the case of Q, which also significantly decreased the percentage of DNA in a comet tail (Figure 4.1). Finally, LA was the only compound within this group, whose low concentrations led to significant inhibition of cell growth after prolonged treatment. Such an effect may indicate shift towards reductive stress, under which cells are deficient in ROS needed to trigger signalling pathways required to initiate proliferation. In contrast, wide range of NAC concentrations significantly increased cell growth, regardless of incubation time. It is noteworthy that NAC was already reported to support cancer cell growth and prevent p53-mediated apoptosis in mice [224]. Even though NAC undergoes deacetylation to Cys and in this form it can be used as a precursor for GSH synthesis, promotion of cell growth was not observed in the case of the latter two compounds (Figures 4.1 and 4.6).

4.3.2. Purine derivatives

Adenine and its derivatives

Nucleosides and nitrogenous bases are essential for numerous biological processes. They are building blocks of nucleic acids and precursors of S-adenosylmethionine (SAM) required for proper DNA methylation. These nitrogenous compounds are also components of enzyme cofactors (such as NAD, NADP and FAD) involved in redox reactions, thus play a pivotal role in the maintenance of redox status of the organism. For example, NADPH can be used as a reductor of oxidized GSH as well as a substrate for ROS-generating NADPH oxidases [318]. Furthermore, nucleosides and nitrogenous bases are substrates for multiple metabolic enzymes as well as constitute a part of high-energy compounds such as adenosine-5'-triphosphate (ATP) formed in the course of oxidative processes, oxidative phosphorylation in particular [319]. Since cellular bioenergetics is based on redox processes, in which especially adenine and its derivatives are indispensable, they were included in this thesis as vital components of redox-related endogenous compounds.

In humans, the average concentrations of purine bases and nucleosides (such as Ade and Ado) are maintained in the range of 0.4-6 μM, which is several-fold lower than physiological concentration of their actual functional form – nucleotides, e.g. 82 μM or 2 mM in the case of AMP or ATP, respectively [320]. Human organism can produce purine compounds in two ways: by energy-consuming *de novo* synthesis or via the salvage pathway, in which nucleobases recovered during catabolism of nucleic acids are reused for nucleotide and nucleoside synthesis [319]. In both cases, liberated adenine (Ade) is used for synthesis of adenosine-5'-monophosphate (AMP) in reaction catalysed by adenine phosphoribosyltransferase, while hydrolysis of generated AMP releases adenosine (Ado) [319]. A simplified scheme of adenine nucleotide catabolic transformations is presented in Figure 4.9.

Certain amounts of nucleic acids (NAs) can be provided with diet, since they are inherent components of once-living cells. As regards adenine, its average content is highest in organ products (about 50 mg/100 g), while the lowest in dairy and egg products, fruits and sweets

(> 2 mg/100 g) [321]. Nevertheless, metabolism of dietary NAs has attracted relatively little attention compared to other nutrients, so it is not possible to estimate at the moment the share of exogenous NAs in redox homeostasis of human organism. Recently, Liu *et al.* reported that dietary NAs can be digested by pepsin to 3'-phosphorylated fragments, which implies that their metabolism may start already in the stomach [322]. However, despite the fact that pancreatic nucleases are already released into duodenum, NAs digestion is very often claimed to start in the intestine [319,323]. From the pool of ingested NAs, 2-5% can be incorporated into new molecules of NAs, almost half of which becomes incorporated into NAs of intestinal cells [324]. Since capability of enterocytes to synthesize *de novo* NAs is limited, dietary sources of exogenous NAs may be indispensable to meet the demands of gastrointestinal tract [325]. Besides, part of the requirement for NAs in the intestines may be secured by the turnover of intestinal cells, which were reported to yield even 30 mg of NAs per day in rat jejunum *in vitro* [326]. Even though in healthy adults a normal varied diet is rich in exogenous components of NAs, nucleotide supplementation has gained a lot of interest, for example, in personalized sports nutrition to meet energetic requirements during training [327,328].

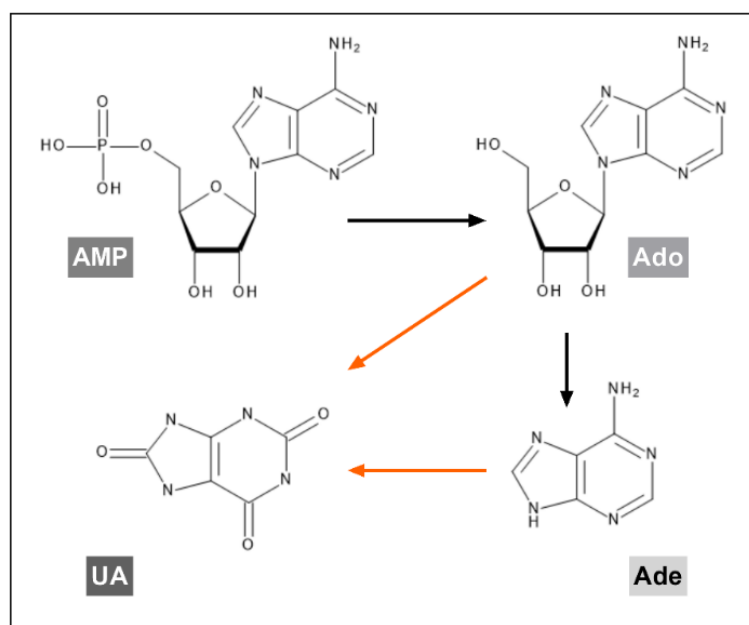


Figure 4.9. Simplified scheme of adenine nucleotide catabolic transformations. Chemical structures represent: **1** – adenine (Ade), **2** – adenosine (Ado), **3** – adenosine-5'-monophosphate (AMP) and **4** – uric acid (UA).

Uric acid

Uric acid (UA) is a final product of catabolism of purines (originating from both endogenous and exogenous sources) formed mainly in the liver, GI tract, muscles, kidneys as well as vascular endothelium [329]. Endogenous synthesis of UA starts with conversion of AMP either to adenosine or inosine monophosphate, which can be further transformed into hypoxanthine and subsequently, to xanthine. The latter compound is oxidized by xanthine oxidase



to UA and H₂O₂. Precursors of UA synthesis include also guanosine-5'-monophosphate (GMP) and xanthosine monophosphate, which can also be enzymatically converted into xanthine [318]. UA levels can be also affected by dietary sources of purines, especially alcoholic beverages (especially beer), meat, seafood as well as by high consumption of fructose, whose metabolism enhances nucleotide turnover and UA formation [330,331]. Absorption of purines by enterocytes occurs via facilitated diffusion and sodium-dependent transporters, mostly in the upper part of the small intestine. Once absorbed in enterocytes, they can be either distributed to other tissues or undergo further catabolism to UA [332,333].

Since humans lack the enzyme urate oxidase (uricase), UA cannot be further oxidized to allantoin as it is the case in other mammals and lower vertebrates. Ames *et al.* proposed a hypothesis that the loss of the enzyme uricase, which led to elevated levels of UA in blood, was a milestone in primates' evolution. Relatively high levels of UA in plasma (140-430 μM) could have recompensated for the loss of ability to synthesize ascorbic acid and have taken over its antioxidant functions. This contributed to life extension and decrease in occurrence of age-specific cancer [334]. Indeed, UA was shown to contribute to 2/3 of antioxidant capacity of human blood. However, in the case of lipids, UA was reported to provide efficient protection against lipid peroxidation in human blood only in the presence of ascorbic acid and thiols [335,336]. In contrast, it was reported that in the intracellular milieu, UA can promote pro-oxidant events through the activation of lipid peroxidation mediated by peroxynitrate, triggering inflammation and reduction of the endothelial levels of NO. The physiological function of this so-called antioxidant-prooxidant paradox is still unclear [337]. UA is thus an abundant endogenous antioxidant that can affect organism's redox homeostasis via different mechanisms.

4.3.2.1. Antioxidant activity by ABTS and DPPH tests

The results of spectrophotometric tests carried out for purine derivatives studied are expressed as stoichiometric n_{10} values, which are presented in Figure 4.10 and listed in Table A1 in Appendix A (Section 7.1.1.). None of the purine derivatives, except for UA, displayed the ability to scavenge either ABTS or DPPH radical. Therefore, linear relationships for Ade, Ado or AMP are not shown in Figures 4.10A and B. Their antioxidant activity in chemical tests was equal zero, which is marked as black blocks in n_{10} value axes (Figure 4.10C). Uric acid turned out to be the only purine derivative in this group that was able to scavenge both radicals. The linear concentration-dependent range of scavenging activity is shown in Figures 4.10A and B. As can be seen, ABTS radical was more efficiently scavenged than DPPH. In most cases, the temperature did not affect the antioxidant activity of UA in DPPH test. The only statistically significant drop was observed between 25 and 41°C in ABTS test (p value = 0.01) according to the one-way ANOVA with Tukey post-hoc test. In ABTS test, the scavenging activity of UA was close to that of Q (n_{10} values 4.43 and 4.88, respectively) and almost 3-fold higher than in the case of GSH. In DPPH test, scavenging capability of Q was 2-fold higher than that of UA, while GSH was incapable of DPPH radical scavenging (Figure 4.3). Thus, UA in chemical tests turned



out to be a stronger antioxidant than the most abundant endogenous reducing agent, but failed to reach antioxidant potential of the reference flavonoid.

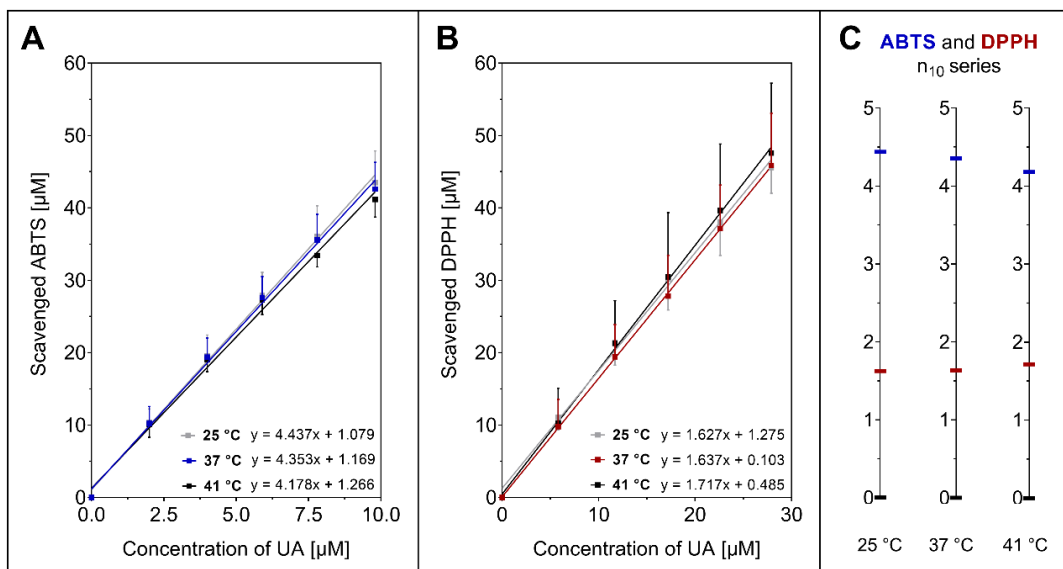


Figure 4.10. The relationship between scavenged ABTS – **A** or DPPH – **B** radicals vs. concentration of uric acid (UA) at 25, 37 and 41°C and corresponding n_{10} value axes – **C**. In panel C, blue coloured blocks represent results of ABTS test, red coloured blocks – results of DPPH test, while black coloured block represents Ade, Ado and AMP that did not react neither with ABTS nor with DPPH. The results are expressed as means \pm SD of three independent experiments carried out in triplicates.

4.3.2.2. Antioxidant activity by CAA test

As shown in Figure 4.11, in cells cultured under normal (10% FBS) conditions prior to the experiments, the treatment of cells with Ade resulted in concentration-dependent decrease in cellular antioxidant activity, which reached CAA values suggesting slightly pro-oxidative effects at higher concentrations. In turn, cellular antioxidant activity of cells treated with Ado, AMP and UA turned out to be moderately enhanced, but no concentration-dependence was observed. In cells cultured in serum-deprived medium, treatment with Ade and Ado significantly enhanced cellular antioxidant capacity. The increase in CAA values was more pronounced in the case of Ade, which switched from weak pro-oxidant activity towards moderate antioxidative effects. In contrast, lower concentrations of AMP and UA decreased reducing capacity of serum-starved cells, which was reversed to antioxidative impact at higher concentrations. Under normal conditions, 200 μ M UA had the strongest impact on cellular antioxidant activity, while under serum-deprived conditions CAA values were the highest in the case of treatment of cells with Ade, Ado and high concentrations of UA. The lack of concentration-dependent effects confirms that the studied adenine derivatives did not act as direct chemical antioxidants, but rather as mediators of redox signalling network that regulate cellular antioxidant potential.

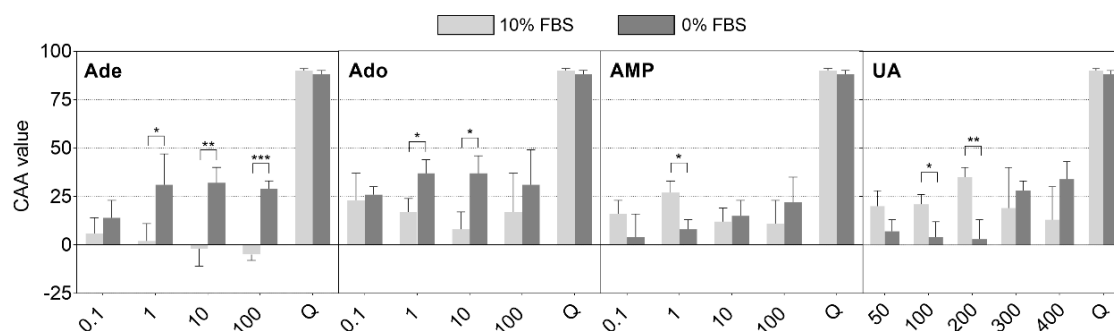


Figure 4.11. The impact of serum starvation of HT29 cells treated with adenine derivatives or 500 μM quercetin used as a positive control on cellular antioxidant activity. The results are expressed as means \pm SD of three independent experiments carried out in triplicate. Statistical analysis was carried out with Student's t-test and the level of significance was set at $p < 0.05$. The asterisks mark p values as follows: (*) – ≤ 0.05 , (**) – ≤ 0.01 and (***) – ≤ 0.001 . Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before the experiment. The abbreviations used refer to: Ade, adenine; Ado, adenosine; AMP, adenine-5'-monophosphate; Q, quercetin; UA, uric acid.

Figure 4.12A suggests that under normal culturing conditions, the reducing capacity of Ade was lower than that of its derivatives, however actually they did not differ significantly between each other. The only significant difference occurred at 1 μM concentration between Ade and AMP (p value 0.009). Ado and AMP showed a similar, more reducing trend in contrast to Ade that seemed to be at the edge of antioxidative-prooxidative balance. In turn, serum starvation of cells changed these trends markedly and Ado appeared to exert noticeably stronger antioxidative effects than AMP at 0.1 μM (p value = 0.046), 1 μM (p value 0.034) and 10 μM (p value 0.020) concentrations. At 10 μM concentration, the activity of Ade was also markedly different from AMP under serum deprived conditions (p value 0.046). UA was not included in this analysis since its activity was investigated in the range of concentrations that was distinct from those applied for other purine derivatives. Therefore, UA was compared to adenine and its derivatives only for one shared concentration (100 μM) as shown in Figure 4.13.

As presented in Figure 4.12B, the impact of Q on cellular antioxidant activity of HT29 cells was stronger than that of Ade derivatives, but only at higher concentrations (10 and 100 μM). Stronger impact of Q on CAA values compared to Ade derivatives was more pronounced in cells cultured in complete medium; all the differences between Q and Ade derivatives were statistically significant. Under serum-deprived conditions, only AMP turned out to be markedly weaker antioxidant compared to 10 μM Q (p value 0.001), whereas at 100 μM , Q again occurred to exert the strongest impact on reducing capacity of cells. In contrast, 0.1 μM Q had significantly weaker impact on cellular antioxidant activity compared to Ado, regardless of culturing conditions (p values 0.021 for 10% FBS and 0.011 for 0% FBS), while 1 μM Q displayed notably poorer antioxidant activity than AMP (p value 0.0391) but only in cells cultured under normal conditions.

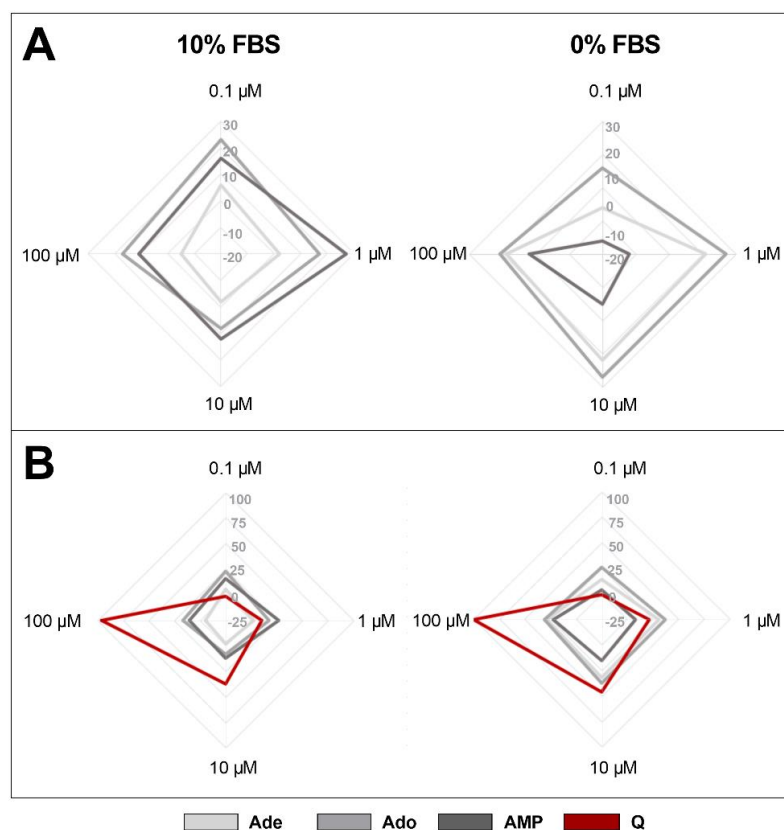


Figure 4.12. Comparison radar charts visualizing differences between CAA values of studied adenine derivatives – **A**, or studied adenine derivatives vs. quercetin – **B**, determined in HT29 cells cultured under either normal (10% FBS) or serum deprived (0% FBS) conditions. The statistical analysis was conducted by one way ANOVA with Tukey's (Panel **A**) and Dunnett's (Panel **B**) tests with the level of significance set at $p < 0.05$. All experimental details are as described in the caption for Figure 4.11. The abbreviations used refer to: Ade, adenine; Ado, adenosine; AMP, adenine-5'-monophosphate; Q, quercetin; UA, uric acid.

As already mentioned, the only concentration of UA that could be compared to Ade, Ado and AMP was 100 μM . As shown in Figure 4.13, UA affected cellular antioxidant activity more profoundly compared to Ade in the case of cells cultured under normal conditions. The opposite could be observed in serum-starved cells, where 100 μM UA was markedly weaker in supporting antioxidant cellular status than Ade and Ado. Thus, surprisingly, the culturing conditions that boosted antioxidant impact of Ade and Ado suppressed the influence of UA as well as AMP. Moreover, 100 μM UA was also significantly weaker in this regard than Q (p value < 0.0001) in cells cultured under both normal and serum-deprived conditions (data not shown). According to CAA values at 100 μM , determined in cells cultured under normal conditions, the order of antioxidant impact increases as follows: Ade $<$ AMP $<$ Ado $<$ UA $<$ GSH $<$ Q. In the case of cells cultured in serum-deprived medium before the experiment, the order of antioxidant impact has changed as follows: UA $<$ GSH $<$ AMP $<$ Ade $<$ Ado $<$ Q.

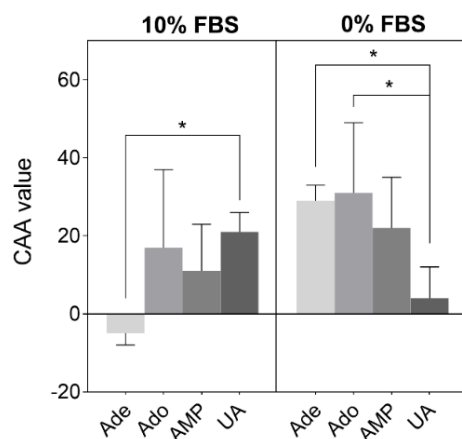


Figure 4.13. Comparison of cellular antioxidant activity determined in HT29 cells cultured under normal (10% FBS) or serum deprived (0% FBS) conditions and treated with 100 μM of adenine (Ade), adenosine (Ado), adenosine-5'-monophosphate (AMP) and uric acid (UA). The results are expressed as means \pm SD of three independent experiments carried out in triplicate. The statistical analysis was conducted by one way ANOVA with Dunnett's test. The level of significance was set at $p < 0.05$.

4.3.2.3. Cytotoxicity by MTT test

The impact of purine derivatives on growth of HT29 cells was determined by MTT test. Cells were treated with physiological concentrations of individual compounds: 0.1-10 μM in the case of Ade and Ado, and 100 μM in the case of AMP, as well as higher concentrations, which may be achievable in the gut (500-1000 μM). The maximum tested concentration of Ade was 500 μM due to its limited solubility. Despite the fact that physiological concentrations of UA exceed these of other adenine derivatives, the range of investigated concentrations of UA was limited to 400 μM , which meets the upper limit of its solubility in water. Results on cell growth inhibition after 6, 24 and 72 h treatment are presented in Figure 4.14.

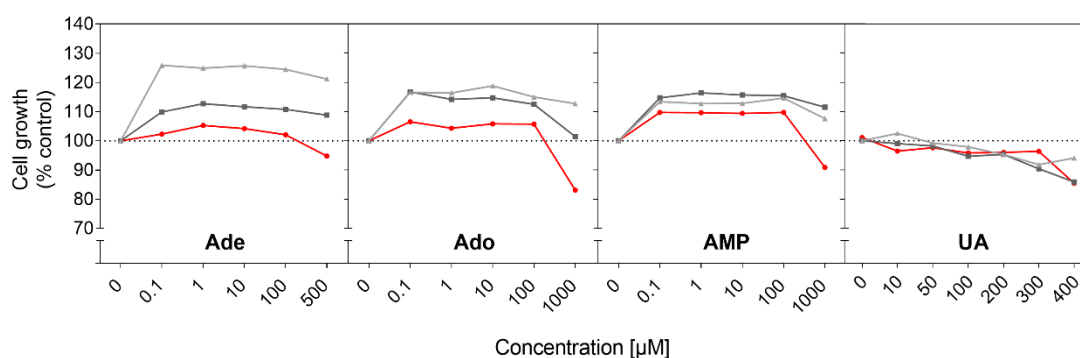


Figure 4.14. The impact of studied thiols on the growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment with adenine (Ade), adenosine (Ado), adenosine-5'-monophosphate (AMP) and uric acid (UA). Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

The treatment of HT29 cells with Ade, Ado and AMP at doses reaching up to 100 μM resulted in cell growth stimulation, in particular after shorter incubation times. Short treatment of cells with Ade induced the strongest cell growth stimulation among this group of compounds regardless of the applied concentration. Exposure of cells to 0.1-100 μM AMP stimulated the growth of HT29 cells regardless of exposure time. Inhibitory effects were observed only after 72 h treatment with the highest concentrations of Ade, Ado and AMP, where the strongest inhibition was caused by 1000 μM Ado. In contrast to adenine derivatives, UA did not stimulate the growth of HT29 cells within the investigated range of concentrations, but rather caused slight growth inhibition at concentrations exceeding 50 μM . More pronounced cytotoxicity occurred after longer treatment timepoints (24 and 72 h) with the highest applied concentration (400 μM). Compared to Q and GSH, purine derivatives had more profound impact on cell growth.

4.3.2.4. Genotoxicity and protection against oxidative DNA damage by comet assay

As shown in Figure 4.15, none of the tested purine derivatives (at concentrations 1-100 μM) exerted genotoxic effects in HT29 cells after 24 h treatment. Similarly, as it was in the

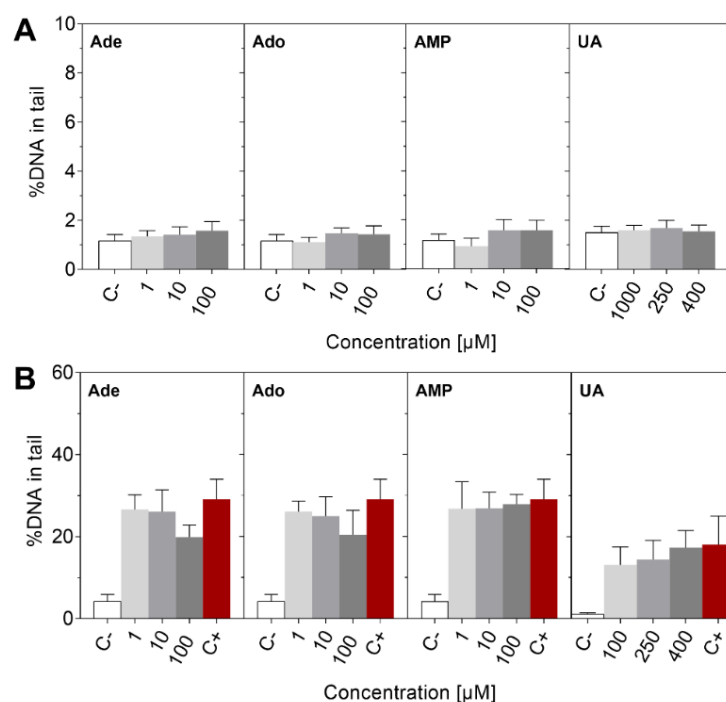


Figure 4.15. The genotoxicity of studied purine derivatives towards HT29 cells – **A**, and their antigenotoxic impact in HT29 cells exposed to genotoxic treatment with H_2O_2 (150 μM , 1 h) used as a model genotoxic oxidant – **B**. The cells were treated with studied purine derivatives for 24 h (genotoxicity) or for 24 h prior to 1 hour exposure to 150 μM H_2O_2 (antigenotoxic activity) and then submitted to comet assay procedure.

The results are expressed as mean percentage of DNA in comet tail \pm SD from three independent experiments carried out in duplicate and compared with C₋, negative control (cells treated with solvent only) and C₊, positive control (150 μM H_2O_2). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$. The abbreviations used refer to: Ade, adenine; Ado, adenosine; AMP, adenine-5'-monophosphate; UA, uric acid.

case of thiol antioxidants (including GSH). The overall percentage of DNA in comet tail did not exceed 2%, both in the case of control (cells treated with the appropriate solvent only) and treated cells. The 24 h pre-treatment of cells with Ade derivatives did not provide significant protection against DNA fragmentation induced by genotoxic treatment with H₂O₂ as in the case of thiol antioxidants. Some trends towards limitation of DNA fragmentation could be observed for cells exposed to pro-oxidant and then 100 μM Ade or Ado, however, no statistically significant differences compared to control were determined (*p* values 0.073 and 0.125, respectively). In relation to Q (Table 4.2), Ade and Ado were less effective in providing protection against DNA fragmentation induced by ROS.

4.3.2.5. Purine derivatives – recapitulation

The redox-related properties of studied purine derivatives are summarised in Figure 4.16. Adenine and its derivatives turned out to support to some extent the antioxidant capacity at cellular level despite their total lack of antioxidant activity in chemical tests. In terms of electrochemical properties, represented by $E^{0'}$ values (Table 4.1) adopted from literature, purine derivatives should act rather as oxidants than antioxidants. Nevertheless, $E^{0'}$ value of UA is around twice lower than that of Ade derivatives, which indicates that UA acts as the strongest reducing agent within this group. The electrochemical behaviour of investigated compounds was reflected by the results of chemical spectrophotometric tests: Ade, Ado and AMP did not show any capability to scavenge ABTS and DPPH radicals, while UA proved to be their efficient scavenger. In ABTS test, UA was slightly weaker than Q and 3 times stronger than GSH. In DPPH test UA was twice less effective than Q, but still better than GSH, which did not scavenge DPPH radical at all (Figure 4.16). At cellular level, the impact of 100 μM UA on reducing capacity of cells was around 5 times weaker than that of Q, but only slightly weaker than that of GSH. According to CAA values at 100 μM under normal culturing conditions, UA was followed by Ado, AMP and Ade, where the latter compound induced slight pro-oxidative effect on cellular redox homeostasis. Interestingly, treatment of cells with Ade caused the most pronounced cell growth stimulation, while UA slightly inhibited cell growth in a concentration-dependent manner. Similarly to thiol antioxidants, Ade derivatives were neither genotoxic towards HT29 cells, nor provided significant protection against H₂O₂-induced DNA fragmentation.

It seems that, at least in the case of Ade, growth stimulation of cells may be reflected in pro-oxidative CAA values, since enhanced proliferation of cells generates excessive ROS. Besides, Ade is one of the components of coenzymes such as NADH, whose utilization may be also increased by bioenergetic processes. On top of it, Ade may be preferentially used as a precursor of a substrate for DNA replication rather than affect cellular redox homeostasis. Interestingly, in serum-starved cells, whose proliferation was stopped, CAA values after treatment of cells with Ade were significantly enhanced, suggesting that if Ade cannot be used for cell bioenergetics purposes or DNA replication, it may indirectly induce cellular antioxidant systems. Similarly, such increase in CAA values after culturing of cells in serum-deprived medium could be observed for Ado, but not in the case of AMP. To conclude, adenine derivatives have rather

indirect impact on cellular redox homeostasis. As reflected by standard reduction potentials as well as the lack of ability to scavenge ABTS and DPPH radicals, they may be presumed to affect cellular reducing capacity rather through endogenous antioxidant systems. In contrast, UA appeared to be efficient ABTS scavenger and its antioxidant activity was reflected by relatively high CAA values in cells cultured under normal nutritional conditions. Interestingly, serum-starvation of cells made them irresponsive to the presence of UA.

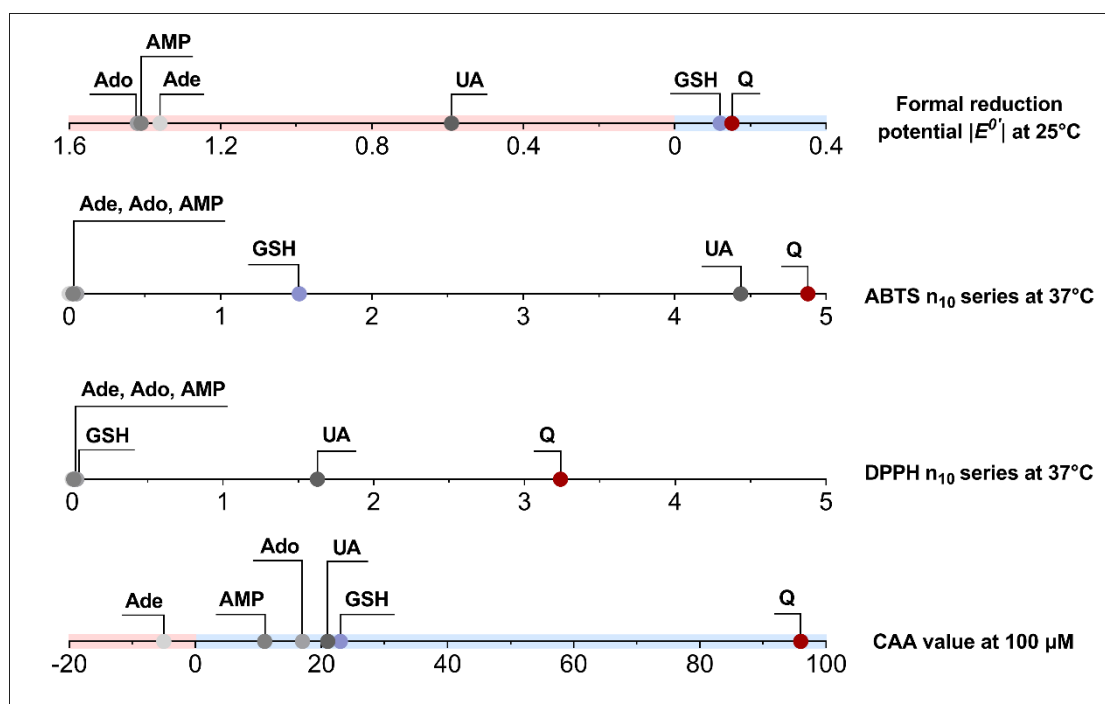


Figure 4.16. Axes comparing redox properties of purine derivatives ordered according to: formal reduction potential (E^0' [V], shown as absolute values); n_{10} values calculated based on ABTS or DPPH tests; the cellular antioxidant activity (CAA) values measured in cells cultured in complete medium and treated with 100 μM concentration of investigated compounds (Ade, adenine; Ado, adenosine; AMP, adenine-5'-monophosphate; GSH, glutathione; Q, quercetin; UA, uric acid). Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity.

4.3.3. Mitochondrial redox-active agents

Coenzyme Q_{10} (ubiquinone)

Coenzyme Q_{10} (CoQ₁₀, 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) is an endogenous redox-active compound involved in mitochondrial bioenergetics. CoQ₁₀ structure includes benzoquinone ring and polyisoprenoid side-chain (Figure 4.17), whose length varies between 6 and 10 isoprenoid subunits among organisms [338]. Benzoquinone ring is a functional group, which is involved in electron and proton transport. Isoprenoid side-chain, in humans built up from 10 subunits, ensures lipophilic character of this molecule necessary to perform functions within biological membranes.

Biological functions of CoQ₁₀ include: electron transport in the ETC, enhancement of integrity of cellular membranes and antioxidant protection. In the inner membrane of mitochondria, CoQ₁₀ is primarily responsible for transport of 2 electrons from the ETC complex I to complex II and the subsequent transfer of one electron at a time to cytochrome c via complex III. Simultaneously, this flow of electrons contributes to the formation of electrochemical proton gradient across the inner mitochondrial membrane, which drives ATP synthesis by ATP synthase [339]. Besides its role in ETC, owing to the ability of isoprenoid side-chain to become anchored in phospholipid bilayer, CoQ₁₀ was shown to increase the density as well as the degree of order of lipid membranes, thereby providing enhanced stabilization of cellular membranes and decreased membrane permeability [340,341], [342].

CoQ₁₀ is the only lipophilic antioxidant produced endogenously in the human body [338,339]. It exists in three redox states: reduced as ubiquinol, partially reduced as ubisemiquinone and oxidized as ubiquinone. Anchored in cellular membranes, CoQ₁₀ in its reduced state can protect phospholipid bilayer from lipid peroxidation. It was reported that ubiquinol was a potent, comparable to α -tocopherol, scavenger of free radicals generated in a model of membranous lipid peroxidation [343]. Besides, CoQ₁₀ has the ability to restore reduced forms of α -tocopherol and ascorbate from α -tocopheroxyl and ascorbyl radicals, respectively [344], [345]. It was shown that without the presence of ubiquinol, lipid peroxidation leads to destruction of the oxidized form of CoQ₁₀ and therefore, inactivation of ETC in beef heart submitochondrial particles [346]. In the same research model, endogenous ubiquinol was shown to concomitantly decrease levels of lipid peroxidation and protein carbonylation, which indicates the importance of ubiquinol in the protection against oxidative stress-induced cellular damage [347]. CoQ₁₀ was also reported to decrease oxidation of DNA *in vitro* in human lymphocytes exposed to H₂O₂ and to support DNA recovery from oxidative damage *in vivo* [348,349]. In rats subjected to exercise, treatment with CoQ₁₀ reduced levels of lipid peroxidation in liver, heart and muscles [350]. The *ex vivo* study performed using low density lipoprotein (LDL) isolated from plasma of healthy human subjects showed that ubiquinol efficiently inhibited LDL oxidation, surpassing the effects of dietary lipophilic antioxidants such as lycopene, β -carotene or α -tocopherol [351]. Human studies confirmed that supplementation of volunteers with CoQ₁₀ led to increased plasma concentration of ubiquinol and enhanced resistance to LDL to oxidation [352].

CoQ₁₀ is present in all cell types and its particularly high concentrations are found in tissues that have high energy demand, such as heart, kidney, liver or muscles [340,353]. In plasma, normal physiological concentrations of CoQ₁₀ range from 0.5 to 1.7 μ g/mL (below 1 μ M) [354]. The declined levels of CoQ₁₀ may occur as a consequence of inherited (primary) deficiency, which leads to e.g. encephalopathy. Decreased levels of CoQ₁₀ are also associated with diseased states such as cardiomyopathy and heart failure or the process of aging [355]. Interestingly, patients undergoing the treatment of cardiovascular diseases with the use of statins had significantly decreased levels of CoQ₁₀ in plasma (even by 50%) [356], since statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMA-CoA) reductase needed in the biosynthesis of



cholesterol as well as other molecules such as CoQ₁₀ [357]. In the case of mentioned conditions, the need arises to increase the intake of CoQ₁₀ either from diet or with dietary supplements.

In foods, CoQ₁₀ is present in its oxidized form, which after ingestion may be reduced at the level of erythrocytes [358]. Indeed, it was reported that most of CoQ₁₀ ingested orally appears in the plasma in reduced form [359]. Foods rich in CoQ₁₀ include mainly spinach, broccoli, soy and different kinds of nuts as well as red meats and fatty fish. Daily dietary intake of CoQ₁₀ was estimated to range from 3 to 5 mg [360]. However, due to its relatively large size, high lipophilicity causing low-aqueous solubility, the bioavailability of exogenous CoQ₁₀ is low (approximately 10%) and it is vastly eliminated with faeces.

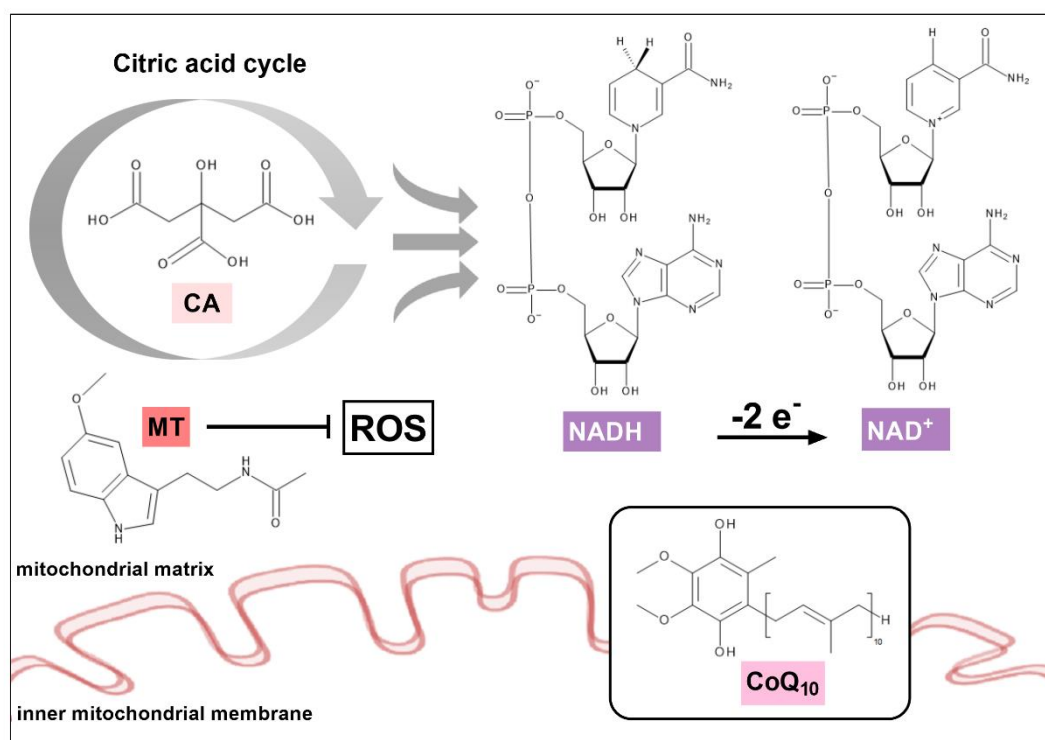


Figure 4.17. Structures and localization of studied redox-active agents found in a mitochondrion. Citric acid (CA) is a first stage component of citric acid cycle, which generates reduced nicotinamide adenine dinucleotides (NADH). In electron transfer chain NADH passes two electrons to coenzyme Q₁₀ (CoQ₁₀), and as a result is converted into its oxidized form – NAD⁺. Melatonin (MT) present in mitochondrial matrix protects mitochondria (components of ETC, inner membrane, mtDNA) from excess ROS and therefore preserves the proper function of this organelle [361].

Melatonin

Melatonin (systematic chemical name N-acetyl-5-methoxytryptamine) is a hormone secreted mainly by the pineal gland in response to darkness. At night, serum concentration of MT reaches 80-150 pg/mL, which decrease during a day to 10-20 pg/mL [362]. Less commonly known is that there are also extrapineal sources of MT that include especially the GI tract, where MT is produced by enterochromaffin cells, but also retina, lymphocytes and skin [362]. MT

concentrations in the GI tract can be even 400 times higher than concentrations found in systemic circulation. The tissue distribution of MT in the GI tract varies: the highest concentrations occur in the rectum and the colon, while the lowest in the jejunum and the ileum [363]. The precursor for MT synthesis is L-tryptophan belonging to the essential amino acids. In the first step of MT biosynthesis, tryptophane is hydroxylated to 5-hydroxytryptophane, which then undergoes decarboxylation to serotonin. Two last steps of MT synthesis include the addition of N-acetyl group to serotonin by arylalkylamine-N-acetyltransferase, and then methylation of N-acetylserotonin to MT by acetylserotonin-O-methyltransferase [362]. In contrast to the pineal MT, whose release into systemic circulation follows a circadian rhythm, the release of MT by the GI tract into the gastrointestinal lumen is not dependent on the exposure to light, but rather follows the periodicity of food (especially tryptophane) intake [363]. Interestingly, in mice exogenous MT was shown to improve the diurnal patterns of gut microbiota known to affect circadian rhythms through the release of microbial metabolites such as short-chain fatty acids and bile [364,365].

However, apart from the regulation of biological clock, MT plays a multitude of physiological functions associated with its redox properties, both as a primary as well as secondary antioxidant. The documented effects embrace stimulation of an immune system, anti-inflammatory and neuroprotective effects and control of the processes involved in the pathogenesis of cancer, diabetes and cardiovascular diseases [366]. In terms of chemical antioxidant activity, MT was shown to efficiently scavenge ROS and RNS, such as HO^\bullet , H_2O_2 , LOO^\bullet , NO , ONOO^- and HOCl , mostly in cell-free systems, nevertheless radical scavenging activity of MT was also proven in *in vitro* and *in vivo* assays [367]. In studies on rats and chicks, exogenously applied MT was also reported to increase the gene expression and the activity of antioxidant enzymes such as GPx and SOD under conditions of oxidative eustress, thus behaving like a secondary antioxidant [368], [366]. These observations were confirmed in animal models of neurodegenerative diseases and aging, representative of elevated oxidative stress conditions, where MT was shown to restore the activity of GPx and SOD in brain tissue, which otherwise would be diminished [368–370]. More recently in human studies, MT was also shown to upregulate SOD, sirtuins 1 (SIRT1) and CAT in peripheral blood mononuclear cells in multiple sclerosis patients [371].

Currently, the role of MT in mitochondria has been investigated in terms of its ability to provide protection against oxidative stress [372]. Compared to other organelles, MT is highly concentrated in mitochondria, where it is involved in regulation of mitochondrial bioenergetics and can positively affect the activity of the respiratory chain [372,373]. Specifically, MT was shown to stimulate the activity of complex I and IV of ETC *in vivo* in Wistar rats and *in vitro* in rat liver and brain [374,375]. Furthermore, MT increased activity of enzymes involved in oxidative phosphorylation, e.g. NADH-coenzyme Q reductase, and enhanced ATP synthesis in mitochondria of rat liver [375]. Numerous studies published so far revealed that MT can exert protective effects in the case of mitochondrial damage caused by ischemia/reperfusion injury or in animal models of neurodegenerative diseases such as multiple sclerosis [376,377]. Beneficial effects exerted by MT included alleviation of oxidative stress in mitochondria, maintenance of the



proper mitochondrial membrane potential and therefore, enhanced ATP generation as well as induction of antiapoptotic pathways [372].

Dietary sources of MT are both of animal and plant origin [378]. Known animal sources of MT include fish (salmon), meats (lamb, beef, pork and chicken), eggs, yoghurt, colostrum as well as both cow and human milk. The latter two sources contain MT at the level no higher than 20 pg/mL, while in the other mentioned animal foods, MT levels are between 0.13 and 6.1 ng/g [378]. In plant foods, MT is ubiquitous: it can be found in cereals (corn, rice, barley, oats, wheat), fruits (grapes, cherries, strawberries), vegetables (tomatoes, peppers), mushrooms, legumes and seeds (germinated soybean and mustard seeds), nuts (pistachio), alcoholic beverages (beer, wine and even coffee), medical herbs, edible oils (e.g. extra virgin olive oil, virgin soybean oil) and yeasts (*Saccharomyces cerevisiae*) [378]. Plant-derived foods contain significantly higher amounts of MT compared to foods of animal origin, with mushrooms being particularly rich source of MT: *Agaricus bisporus* (4.3-6.4 mg/g of dry weight), *Boletus badius* (6.8 mg/g of dry weight) and *Lactarius deliciosus* (12.9 mg/g of dry weight) [379,380].

Nicotinamide adenine dinucleotide (NADH/NAD⁺)

Nicotinamide adenine dinucleotide (reduced and oxidized forms are jointly abbreviated as NAD) is a ubiquitous cellular molecule that plays a role in various, mostly catabolic processes; in anabolism metabolic roles are fulfilled by its equivalent NADPH. NAD is also needed for energy production, epigenetic modifications, DNA repair, inflammatory response, circadian rhythm and longevity [381]. Its reduced form, NADH, acts as an electron donor in the mitochondrial ETC, where it becomes oxidized to NAD⁺ in the course of catabolic processes. Therefore, NAD⁺/NADH ratio informs about the metabolic state of a cell reflected by the balance between NAD⁺ generation and utilization by NAD⁺-consuming enzymes [382]. This ratio may be affected by ageing, physical exercise, caloric restriction as well as diet rich in NAD⁺ precursors, such as tryptophan and different forms of vitamin B3 (namely, nicotinic acid, nicotinamide, nicotinamide riboside) and unrefined foods [383–386]. If not bound to enzymes, in plasma it is present mainly in oxidized state - NAD⁺. Plasma or serum concentrations reported in the literature vary from 0.05 up to 500 μM. The NAD⁺/NADH ratio and total NAD was reported to be higher in women than in men, however, these differences diminished with age. In the same study, NAD⁺ concentration in plasma was determined to fall within 0.1-1.2 μM range [387]. Intracellular concentrations of NAD⁺ were reported to be between 200 and 500 μM with NAD⁺/NADH ratio around 1000:1 in the cytosol, and 10:1 in mitochondria. Exceeded levels of NADH in mitochondria can disturb and alternate metabolic pathways since such situation leads to increased electron flow in ETC, which increases probability of electron leakage and O₂^{•-} generation. This can be associated with the development of metabolic syndrome and diseases such as cancer or neurodegenerative disorders as well as the acceleration of aging processes [388].



Citric acid

Citric acid (CA) occurs in human organism in its ionized form as citrate. It is generated endogenously in the Citric Acid Cycle, known also as the Krebs Cycle, which provides cells with energy in the form of ATP as well as with so-called reducing equivalents – NADH and FADH₂. Plasma concentration of citrate is maintained at around 100-150 μM level and its main endogenous source are bones. It does not need to be provided with food since it can be synthesized endogenously. Nonetheless, citric acid is consumed in high amounts not only from natural sources, such as citrus fruits, but also as one of the most commonly used food additive (E330) [389],[390]. Industrially manufactured CA is applied as an acidulant, regulator of pH, preservative, flavour enhancer and antioxidant. The latter actually is a misnomer as CA cannot directly scavenge ROS, but rather prevents metal-induced oxidation owing to its metal-chelating properties. Recently, CA has been shown to inhibit oxidative stress and inflammatory reactions via its impact on redox signalling [391]. However, mice fed with citrate were shown to develop insulin resistance and fasted hyperinsulinemia, with the increase in the expression of genes involved in hepatic lipogenesis as well as elevated biomarkers of liver inflammation [392]. Such perturbations in lipid metabolism, insulin signalling and inflammation, which may be initiated by CA as suggested in the mentioned study, are known to be implicated in the development of diseases, in which oxidative stress plays a central role [393].

4.3.3.1. Antioxidant activity by ABTS and DPPH tests

The results of chemical determinations of antioxidant activity of redox-active endogenous compounds particularly abundant in mitochondria are expressed as stoichiometric n_{10} values, which are presented in Figures 4.18B and D and listed in Table A1 in Appendix A (Section 7.1.1.). In this group of tested mitochondrial redox-active agents, only MT and NADH exhibited the ability to scavenge ABTS and DPPH radicals. The linear range of their scavenging activity is shown in Figure 4.18A,C. NADH was markedly stronger ABTS and DPPH radical scavenger than MT. The antioxidant activity of both these compounds was significantly higher in the case of ABTS test than in DPPH test, which was also observed for thiol antioxidants and adenine derivatives. In ABTS test, the temperature did not affect the scavenging capability of NADH, however, in the case of MT the significant increase in radical scavenging was observed at 37 and 41°C compared to 25°C (both p values = 0.0015). In DPPH test, the increase of temperature slightly enhanced scavenging activity of NADH, but this change was statistically insignificant. The ability of MT to scavenge DPPH was almost zero, therefore in this case only one temperature (37°C) was tested. Both NADH and MT turned out to be weaker ABTS and DPPH scavengers than GSH and Q, while CoQ₁₀, CA and NAD⁺ did not scavenge neither ABTS nor DPPH radical, thus their antioxidant activity expressed as n_{10} values equalled zero. Therefore, the order of antioxidant activity of mitochondrial redox-active agents compared to reference antioxidants (Table 4.2) according to the results of ABTS tests carried out at 37°C was as follows: CoQ₁₀ = CA = NAD⁺ < MT < NADH < **GSH** << **Q**.

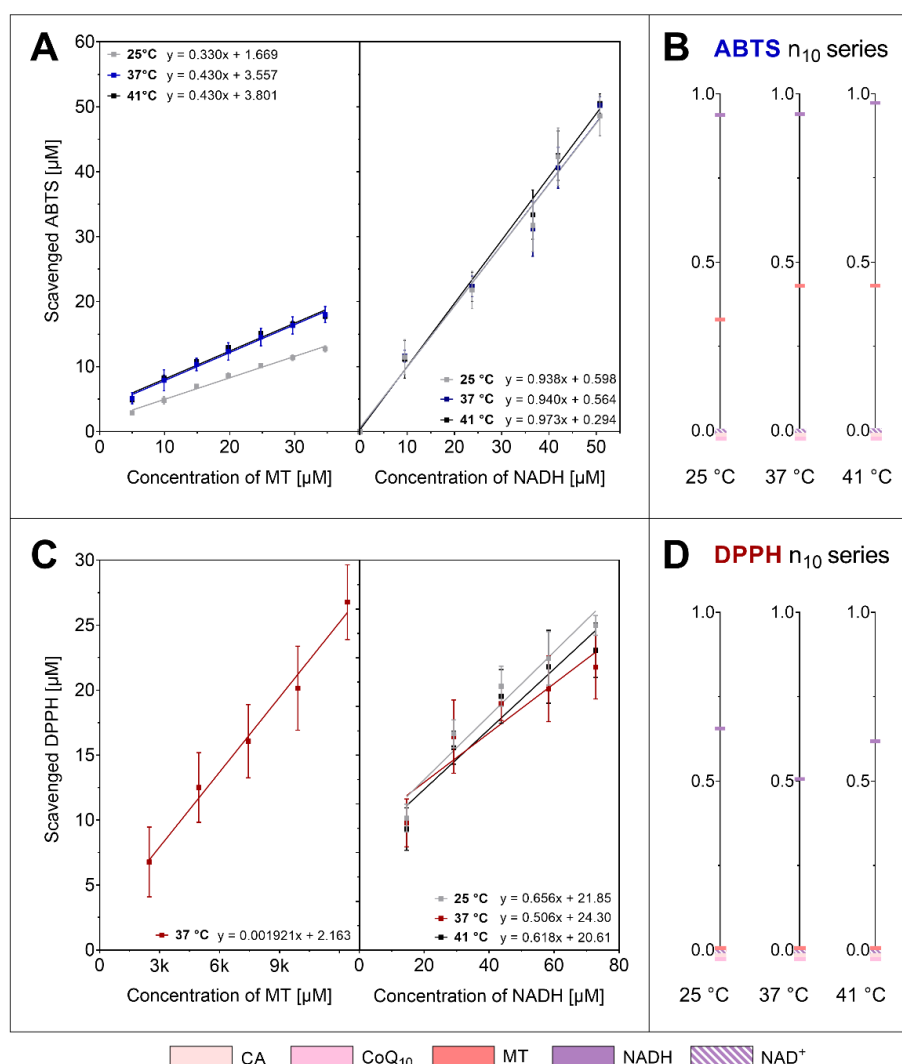


Figure 4.18. The relationship between scavenged ABTS – **A** or DPPH – **C** radicals vs. concentration of melatonin (MT) or reduced nicotinamide adenine dinucleotide (NADH) at 25, 37 and 41°C and corresponding n_{10} value axes for all tested mitochondrial redox-active agents – **B** and **D**. DPPH test for MT was conducted only at 37°C. The results are expressed as means \pm SD of three independent experiments carried out in triplicates.

4.3.3.2. Antioxidant activity by CAA test

As shown in Figure 4.19, under normal culture conditions (10% FBS) most of tested mitochondrial redox-active compounds displayed weak or even slightly pro-oxidant impact on cellular antioxidant activity in HT29 cells. Concentration-dependence occurred mostly in the case of MT and CA, for which CAA values decreased with increasing concentration of these compounds. Treatment of cells with CoQ₁₀ and NADH resulted in pro-oxidative effects, which were more pronounced in the case of CoQ₁₀. In contrast, NAD⁺ had weak not concentration-dependent antioxidant impact on reducing capacity of HT29 cells. Similarly to most of investigated mitochondrial redox-active agents, slight pro-oxidative or weak antioxidative effects could be also observed after treatment of cells with low concentrations (0.1 and 1 μM) of Q or GSH (Figures 4.1

and 4.4, respectively). The impact of tested mitochondrial redox-active agents on cellular antioxidant activity, in most cases, was not significantly affected when cells were cultured in serum-deprived medium prior to the experiments. Similar results were obtained for Q and GSH, however, in the case of the latter compound a concentration-dependent trend towards lower CAA values could be observed (Figure 4.4).

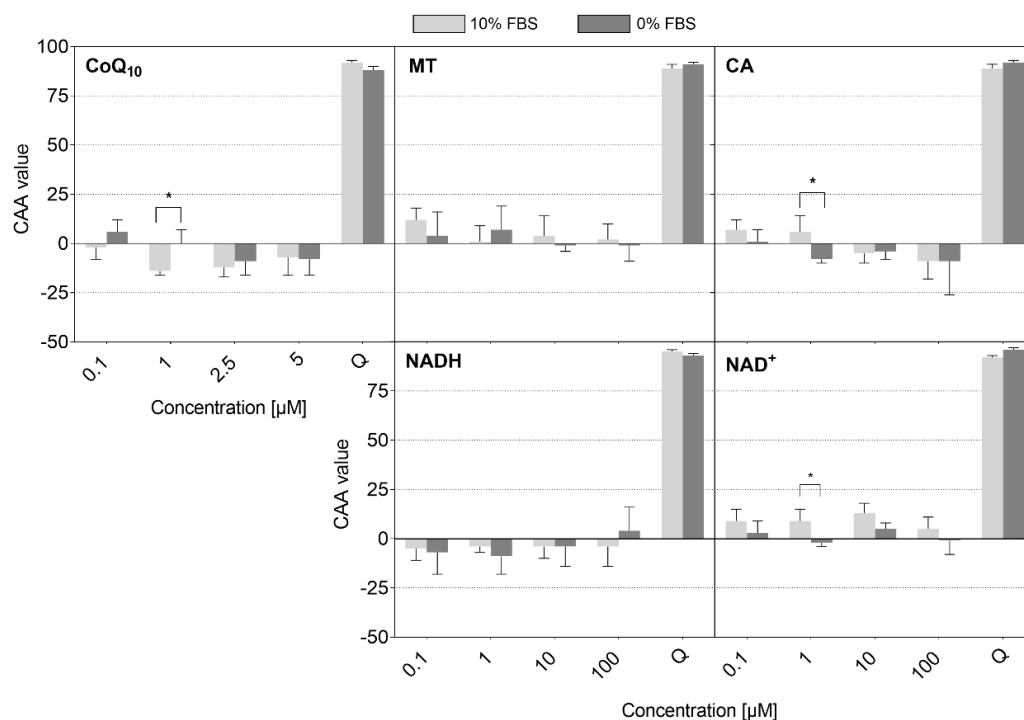


Figure 4.19. The impact of serum starvation of HT29 cells treated with tested mitochondrial redox-active agents or 500 μM quercetin used as a positive control on cellular antioxidant activity. The results are expressed as means ± SD of three independent experiments carried out in triplicate. Statistical analysis was carried out with Student's t-test and the level of significance was set at $p < 0.05$ marked with one asterisk (*). Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before the experiment. The abbreviations used refer to: CA, citric acid; CoQ₁₀, ubiquinone; MT, melatonin; NADH/NAD⁺, nicotinamide adenine dinucleotide reduced and oxidized, respectively; Q, quercetin.

As summarised in Figure 4.20A, most of mitochondrial redox-active agents studied displayed similar, but rather weak antioxidative or even pro-oxidative impact on cellular antioxidant activity. In cells cultured under normal culture conditions before the experiment, the only statistically significant difference between the activity of tested compounds was determined for MT and NADH at 0.1 μM (p value 0.0171). In serum-deprived conditions, the impact of tested compounds was not significantly different (Figure 4.20A). At 10 and 100 μM concentrations, Q showed significantly stronger impact on cellular antioxidant activity than any other tested mitochondrial redox-active compound, regardless of culturing conditions (Figure 4.20B). In cells grown in complete medium, low concentrations of Q occurred to have markedly stronger antioxidant impact on CAA values compared to MT at 0.1 μM (p value 0.029), and NADH at 1 μM

(p value 0.040). In cells cultured in serum-deprived conditions, 1 μM Q exerted also markedly stronger antioxidative effects than CA, NADH and NAD⁺ (p values 0.003, 0.002 and 0.014, respectively).

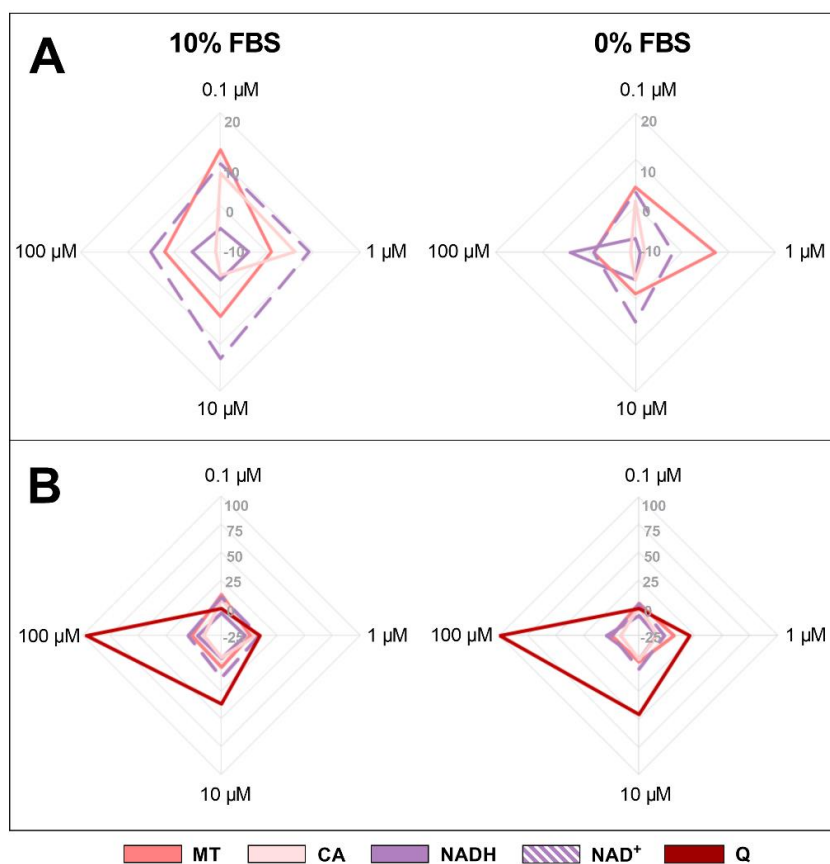


Figure 4.20. Comparison radar charts visualizing differences between CAA values of studied mitochondrial redox-active agents – **A**, or studied mitochondrial redox-active agents vs. quercetin – **B**, determined in HT29 cells cultured under either normal (10% FBS) or serum deprived (0% FBS) conditions.

The statistical analysis was conducted by one way ANOVA with Tukey's (Panel **A**) and Dunnett's (Panel **B**) tests with the level of significance set at $p < 0.05$. All experimental details are as described in the caption for Figure 4.19. The abbreviations used refer to: CA, citric acid; CoQ₁₀, ubiquinone; MT, melatonin; NADH/NAD⁺, nicotinamide adenine dinucleotide reduced and oxidized, respectively; Q, quercetin.

Since the antioxidant activity of CoQ₁₀ was determined for a different range of concentrations, it could be compared to other mitochondrial redox-active compounds only at 0.1 and 1 μM concentrations. Figure 4.21A shows that in cells cultured under normal conditions before the experiment, the impact of 0.1 μM CoQ₁₀ on cellular reducing status of HT29 cells was significantly weaker compared to MT (p value 0.030). In turn as shown in Figure 4.21B, 1 μM CoQ₁₀ was significantly more pro-oxidative compared to MT (p value 0.028), CA and NAD⁺ (p values < 0.01). After serum starvation of cells, any evident distinction between CoQ₁₀ and other mitochondrial redox-active compounds could not be observed regardless of applied

concentration. At 1 μM concentration, CoQ₁₀ displayed also markedly distinct impact on CAA values compared to Q (data not shown), regardless of culture conditions.

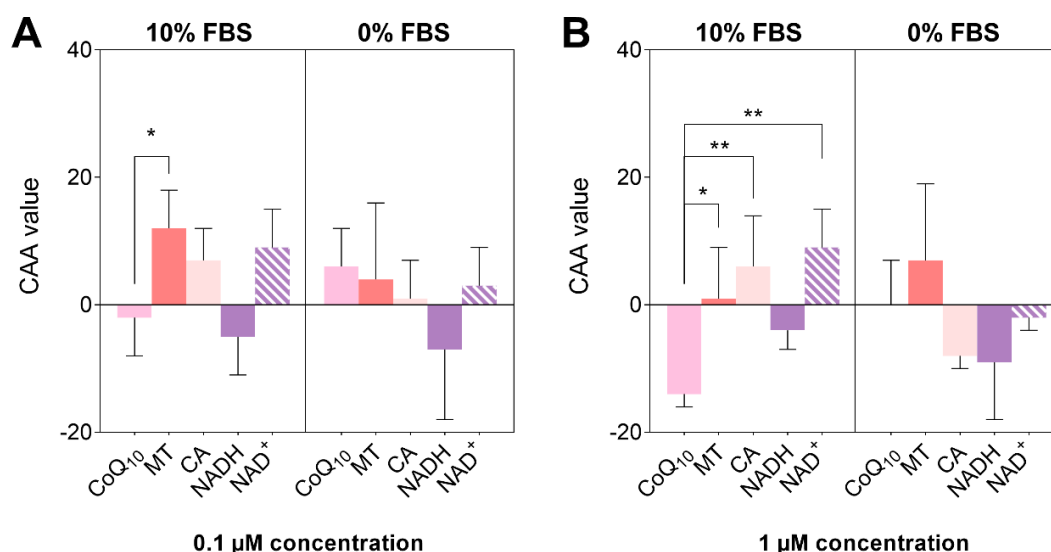


Figure 4.21. Comparison of cellular antioxidant activity determined in HT29 cells cultured under normal (10% FBS) or serum deprived (0% FBS) conditions and treated with 0.1 or 1 μM of coenzyme Q₁₀ (CoQ₁₀), melatonin (MT), citric acid (CA) as well as reduced and oxidized form of nicotinamide adenine dinucleotide (NADH/NAD⁺). The results are expressed as means \pm SD of three independent experiments carried out in triplicate. The statistical analysis was conducted by one way ANOVA with Dunnett's test. The levels of significance are marked with asterisks: (*) for $p < 0.05$ and (**) for $p < 0.01$.

According to CAA values at 100 μM determined in cells cultured under normal conditions, the order of antioxidant activity increases as follows: CA < NADH < MT < NAD⁺ < GSH < Q. Due to solubility limitations, the highest tested concentration of CoQ₁₀ was 5 μM , therefore, as mentioned before, its impact on cellular antioxidant activity could be compared only at 0.1 and 1 μM concentrations, as shown in Figure 4.21. Under normal culture conditions, CoQ₁₀ at 0.1 μM had weak pro-oxidative impact on CAA values similarly to GSH and Q. In contrast, at 1 μM concentration reference antioxidants behaved like weak antioxidants, while CoQ₁₀ turned out to have notable pro-oxidative effects, which diminished after serum-starvation of cells.

4.3.3.3. Cytotoxicity by MTT test

As shown in Figure 4.22, the treatment of HT29 cells with tested mitochondrial redox-active agents did not have significant impact on cell growth. The effects observed after treatment with CoQ₁₀, CA and NAD⁺ were similar to those of GSH and Q (Figure 4.1). In the case of MT and NADH tested at concentrations up to 10 μM , cell growth occurred to be slightly stimulated, but this increase was statistically insignificant. NADH and NAD⁺ at the highest concentration (1000 μM) and after the longest exposure time caused significant drop in cell growth (by around 35%).

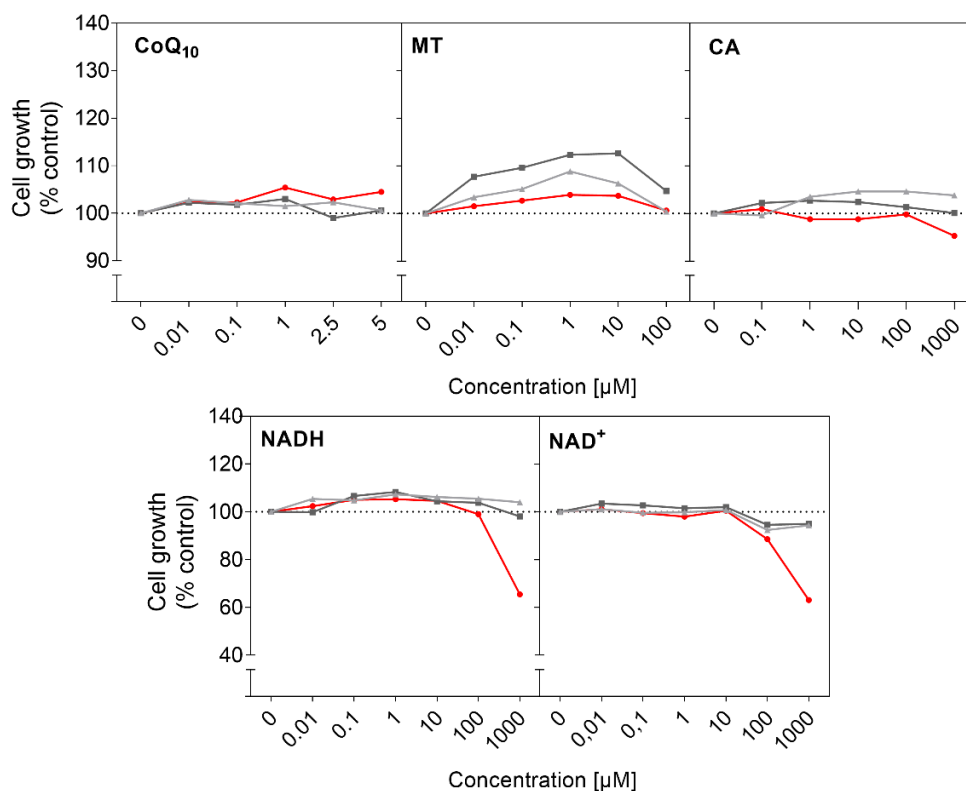


Figure 4.22. The impact of studied mitochondrial redox-active agents on the growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment with coenzyme Q₁₀ (CoQ₁₀), melatonin (MT), citric acid (CA) as well as reduced and oxidized form of nicotinamide adenine dinucleotide (NADH/NAD⁺). Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

4.3.3.4. Genotoxicity and protection against oxidative DNA damage by comet assay

As shown in Figure 4.23, none of the tested mitochondrial redox-active compounds (at concentrations 1-100 µM) turned out to be genotoxic in HT29 cells after 24 h treatment. Similarly, as it was in the case with thiol antioxidants and Ade derivatives, the overall percentage of DNA damage exceeded 2% neither in the control nor in the treated cells. Again, the 24 h pre-treatment of cells with mitochondrial redox-active compounds did not protect DNA against damage induced by genotoxic treatment with H₂O₂. A trend towards decrease in DNA damage could be observed after treatment of cells with 100 µM MT, 10 and 100 µM NAD⁺ as well as 1-100 µM NADH. Nevertheless, these results were not statistically significant (*p* values > 0.3). Therefore, mitochondrial redox-active compounds were less effective in preventing oxidative DNA damage, compared to Q and not distinct from GSH (Figure 4.1 and Table 4.2).

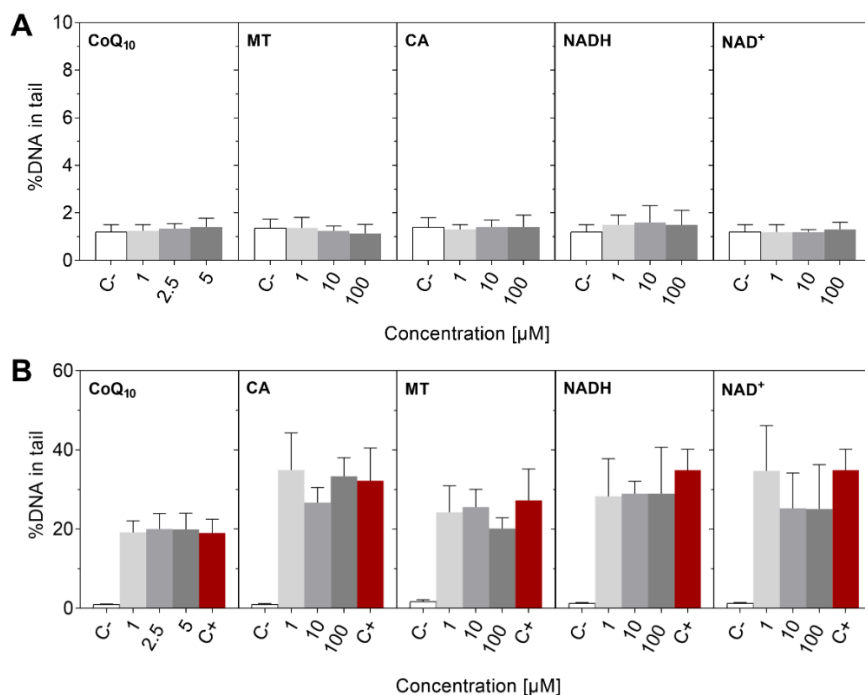


Figure 4.23. The genotoxicity of studied mitochondrial redox-active agents towards HT29 cells – **A**, and their antigenotoxic impact in HT29 cells exposed to genotoxic treatment with H₂O₂ (150 μM, 1 h) used as a model genotoxic oxidant – **B**. The cells were treated with studied mitochondrial redox-active agents for 24 h (genotoxicity) or for 24 h prior to 1 hour exposure to 150 μM H₂O₂ (antigenotoxic activity) and then submitted to comet assay procedure. The results are expressed as mean percentage of DNA in comet tail ± SD from three independent experiments carried out in duplicate and compared with C⁻, negative control (cells treated with solvent only) and C⁺, positive control (150 μM H₂O₂). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$. The abbreviations used refer to: CA, citric acid; CoQ₁₀, ubiquinone; MT, melatonin; NADH/NAD⁺, nicotinamide adenine dinucleotide reduced and oxidized, respectively.

4.3.3.5. Mitochondrial redox-active agents – recapitulation

The redox-related properties of studied mitochondrial redox-active agents are summarised in Figure 4.24. In contrast to other tested compounds, in the case of CA, neither its standard nor formal reduction potentials were available. In the citric acid cycle, CA is transformed to isocitrate by aconitase, and subsequently isocitrate undergoes enzymatic oxidation to α-ketoglutarate with the aid of isocitrate dehydrogenase. Therefore, α-ketoglutarate/isocitrate redox pair was assumed to represent the value of E^0 as the approximate reduction potential of CA.

As shown in Figure 4.24, the antioxidant activity of mitochondrial redox-active agents turned out to be very modest, in all used tests, sometimes inclining towards pro-oxidative effects in the case of biological determinations. Most interestingly, MT was the only compound in the case of which, the lack of ability to scavenge ABTS and DPPH radicals as well as CAA value close to 0 were reflected by its electrochemical behaviour, *i.e.* high E^0 value. Both reference



compounds – GSH and Q – in chemical and biological tests displayed markedly stronger antioxidant activity than mitochondrial redox-active agents, even though they were not the best electron donors according to E° values. Such results indicate that there must be other factors that affect and thus determine the antioxidant activity of mitochondrial redox-active compounds. For example, as it was discussed by Baranowska *et al.* in the case of Q [176], kinetics of electrochemical reaction may be more biologically relevant compared to thermodynamics reflected by reduction potentials.

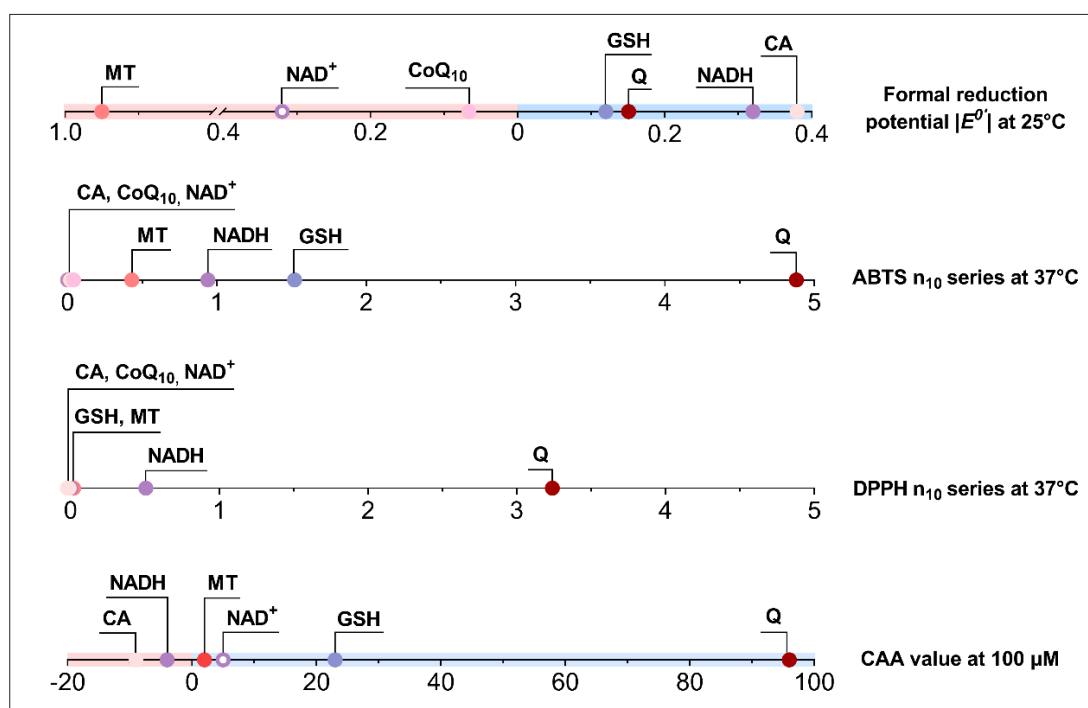


Figure 4.24. Axes comparing redox properties of mitochondrial redox-active agents ordered according to: formal reduction potential (E° [V], shown as absolute values); n_{10} values calculated based on ABTS or DPPH tests; the cellular antioxidant activity (CAA) values measured in cells cultured in complete medium and treated with 100 μM concentration of investigated compounds (CA, citric acid; CoQ₁₀, ubiquinone; GSH, glutathione; MT, melatonin; NADH/NAD⁺, nicotinamide adenine dinucleotide reduced and oxidized, respectively; Q, quercetin). Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity.

Despite strong reducing behaviour in enzymatic reactions, free exogenous NADH applied to HT29 cells displayed slightly pro-oxidative activity in CAA assay (Figure 4.19). Such unintuitive result could be explained by the so-called “NAD ratio redox paradox”. In mitochondria and cytosol, NAD⁺/NADH ratio has been estimated to be 7:1 and 800:1, respectively [394]. Overabundance of NADH in the cytosol leads to the decrease in NAD⁺/NADH ratio, which may deregulate metabolic enzymes, whose activity depends on appropriate levels of NAD⁺ [395]. In the case of mitochondria, the increase in the levels of NADH may disrupt mitochondrial redox homeostasis, since the increased intensity of NADH oxidation leads to elevated generation of O₂^{•-} and H₂O₂ [396]. On the other hand, it has been reported that boosting NAD⁺ levels enhanced the activity of

intracellular antioxidant enzymes as well as increased GSH levels in mice [397]. This may explain why the treatment of HT29 cells with NAD⁺ tended in our experiments to increase CAA values in contrast to NADH that turned out to exert pro-oxidative effects.

In the case of CoQ₁₀, despite reports suggesting that it can be intracellularly reduced to ubiquinol once absorbed, CAA assay conducted in this study failed to confirm its antioxidant impact on cellular redox homeostasis in HT29 cells. This may result from low intracellular bioavailability of the applied form of CoQ₁₀, and therefore insufficient delivery to the mitochondria, where it could perform its antioxidant function. This poor absorption, observed also by other researchers, prompted the development of multiple structural analogues of CoQ₁₀, such as mitoQ, so they could be more efficiently targeted directly to mitochondria. The difference between CoQ₁₀ and mitoQ structures relies on the attachment of a lipophilic triphenylphosphonium cation to the isoprenoid chain, which ensures better accumulation inside mitochondria [398].

Mitochondrial redox-active agents did not have a great impact on HT29 cell growth. Significant cell growth inhibition was observed only in the case of the longest treatment of cells with the highest concentrations of NAD. Similarly to thiol antioxidants and purine derivatives, mitochondrial redox-active agents were also neither genotoxic towards HT29 cells nor provided significant protection against H₂O₂-induced DNA fragmentation. Thus not only cellular antioxidant activity, but also prevention of oxidative DNA damage seem to be markedly more efficiently secured by the exogenous phytochemicals represented by Q than by endogenous reducing agents, even those characterized by lower reduction potentials compared to Q.

4.3.4. *Endogenous pro-oxidants*

Cellular redox homeostasis is affected not only by reducing agents, but also by oxidants, which can be of both exogenous or endogenous origin, as already described in the section 1.2. of the Introduction. Therefore, apart from reducing compounds found in cells, also two endogenous pro-oxidants were investigated in the framework of this thesis: glutathione disulphide (GSSH) and hydrogen peroxide (H₂O₂). GSSG is an oxidized form of a major intracellular antioxidant GSH, that has been chosen as a reference compound in this study, while H₂O₂, an ubiquitous endogenous molecule that depending on its concentration, may perform physiological functions or trigger oxidative damage.

Glutathione disulphide

GSSG is formed in the course of GSH-mediated reduction reactions such as those catalysed by GPx with simultaneous H₂O₂ reduction (Figure 4.25). In cells with maintained redox balance, the regeneration of GSH is ensured by the activity of NADPH-dependent GR and TXN/glutaredoxin (GRX) system and by non-enzymatic antioxidant – dihydrolipoic acid (DHLA), whose formal reduction potential is lower from that of GSH, which makes DHLA a stronger antioxidant capable of reducing GSSG [265,399]. Besides such redox recycling, GSSG can be also removed from cells through specialized transporters [400]. Low GSH/GSSG ratio is

recognized to be indicative of oxidative stress conditions, upon which GSH regeneration may be disrupted. Insufficient levels of GSH (and therefore, the excess of GSSG) has been reported to be prevalent in neurodegenerative diseases or aging. However, since GSSG levels are compartment-dependent, its high concentrations do not always point at cellular perturbations. For example, GSH/GSSG ratio is estimated to be between 1:1 and 3:1 in the ER where more oxidizing milieu is needed to enable disulphide bonds formation to acquire the proper protein folding [33]. In contrast, this ratio is estimated to be 100:1 in the cytoplasm, where 10:1 ratio is already indicative of oxidative stress.

GSSG was found to be present in numerous unprocessed foods, such as meats (beef, chicken breast, pork), fish, green beans, cauliflower as well as yellow squash. In the same study, it was shown that cooking contributes to 25-50% loss of GSH, which could have been converted to GSSG or nonreducible GSH adducts upon reactions with electrophilic compounds contained within food matrix [203]. Interestingly, dietary GSSG was demonstrated to be reduced in the GI tract of rats (it was not specified if reduction occurred lumenally or intracellularly). This suggests that exogenous GSSG, once reduced to GSH, could support, for example, intestinal detoxification of xenobiotics present in foods and thereby enhance protection of intestinal epithelium [401].

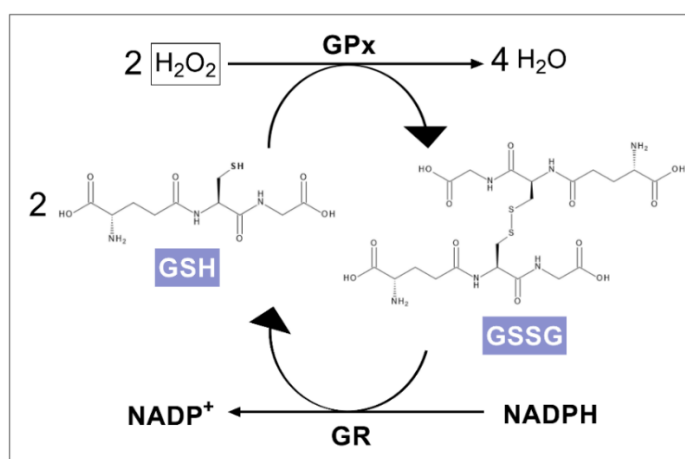


Figure 4.25. Oxidation of GSH and its recycling by enzymatic antioxidant systems. The abbreviations refer to: GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; H_2O_2 , hydrogen peroxide; NADPH/NADP⁺, nicotinamide adenine dinucleotide phosphate reduced and oxidized, respectively.

Hydrogen peroxide

Hydrogen peroxide is a two-electron oxidant, which intracellularly reacts rather poorly with macromolecules and low-molecular-weight antioxidants. However, even though H_2O_2 is not a very reactive oxidant itself, it gives rise to more reactive species such as HO^\bullet , which reacts non-specifically with a whole range of biomolecules and therefore induces cellular damage and genotoxic effects [17]. Hydrogen peroxide can diffuse through cellular membranes, thus if not reduced, it can reach sites that are distant from the site of its generation. Furthermore, H_2O_2 transport into cellular milieu was shown to be additionally facilitated by specialised proteins –



aquaporins, called also peroxiporins [402]. Such a potentially controllable transport caused that besides being one of ROS, in recent years H_2O_2 has also emerged as the major redox signalling molecule [403]. Signalling events modulated by H_2O_2 are triggered mostly through reversible oxidation reactions of thiol groups present in proteins. For example, the activity of PRDXs can be modulated (or even inhibited) by H_2O_2 -driven oxidation of thiol groups localized in the catalytic centres. In yeasts as well as in mammalian cells, PRDXs were shown to further mediate H_2O_2 -dependent redox signalling through redox-sensitive transcription factors such as signal transducers and activators of transcription (STATs), which suggests that antioxidant enzymes are not just so-called “sinks of oxidants”, but they can also actively participate in regulation of redox signalling [404]. Plasma concentrations of H_2O_2 are estimated to be around 1-5 μM , which is relatively high compared to intracellular milieu, where its physiological concentrations are maintained within nanomolar range while micromolar range indicates the occurrence of oxidative stress [403].

Interestingly, H_2O_2 can be also present in foodstuffs, since it is utilized as antimicrobial and sterilizing agent for aseptic packages for fruit juices and milk products and as such, residual amounts of H_2O_2 can be left inside the package upon sealing. Moreover, in several countries (e.g. Canada, New Zealand, USA), H_2O_2 can be applied as a bleaching agent in foods such as wheat flour or edible oils [405]. The maximal amount of residual H_2O_2 allowed by Food and Drug Administration to be present in foods is 0.5 ppm [406].

4.3.4.1. Antioxidant activity

As it was expected for investigated pro-oxidants, neither GSSG nor H_2O_2 were able to scavenge ABTS and DPPH radicals in chemical tests, therefore the n_{10} values, a measure of anti-

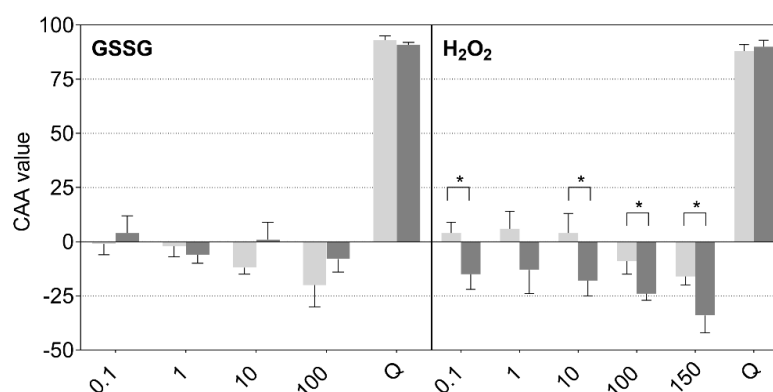


Figure 4.26. The impact of serum starvation of HT29 cells treated with endogenous pro-oxidants or 500 μM quercetin used as a positive control on cellular antioxidant activity. The results are expressed as means \pm SD of three independent experiments carried out in triplicate. Statistical analysis was carried out with Student's t-test and the level of significance was set at $p < 0.05$ marked with one asterisks (*). Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 hour during treatment. The abbreviations used refer to: GSSG, glutathione disulphide; H_2O_2 , hydrogen peroxide; Q, quercetin.

-oxidant activity in these assays, were assumed to equal zero (data not shown). In turn, the impact of tested pro-oxidants on redox homeostasis could be observed at the cellular level as shown in Figure 4.26. The higher the concentration of GSSG, the higher was its pro-oxidative activity in HT29 cells cultured under normal conditions. Prior serum starvation of cells seemed to alleviate these concentration-dependent effects, however, the observed differences did not reach statistical significance. Nevertheless, the most notable difference occurred at 10 μM concentration (p value 0.058). In contrast, culture conditions of HT29 cells had notable impact on the cellular antioxidant activity of H_2O_2 -exposed cells. Under normal conditions, it exerted weak antioxidative effects at concentrations up to 10 μM , which switched towards pro-oxidative activity at higher concentrations. In serum-starved cells, the impact of H_2O_2 on cellular antioxidant activity was exclusively pro-oxidant and increased in a concentration-dependent manner. This may suggest that serum contained substances capable of H_2O_2 neutralization.

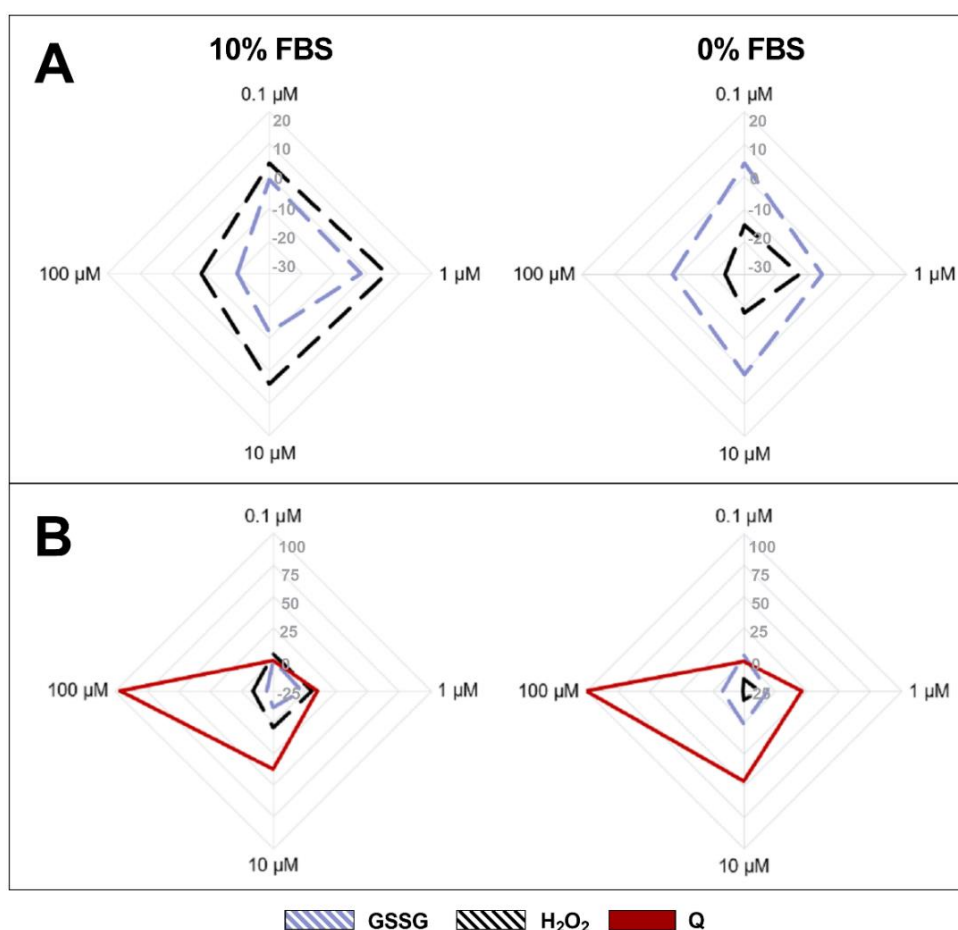


Figure 4.27. Comparison radar charts visualizing differences between CAA values of studied endogenous pro-oxidants – **A**, or studied endogenous pro-oxidants vs. quercetin – **B** determined in HT29 cells cultured under either normal (10% FBS) or serum deprived (0% FBS) conditions. The statistical analysis was conducted by Student's t-test (Panel **A**) and one way ANOVA with Dunnett's test (Panel **B**) with the level of significance set at $p < 0.05$. All experimental details are as described in the caption for Figure 4.26. The abbreviations used refer to: GSSG, glutathione disulphide; H_2O_2 , hydrogen peroxide; Q, quercetin.



Under normal conditions, the impact of tested pro-oxidants on the reducing capacity of HT29 cells was similar (Figure 4.27A). Their activity was significantly different only at 10 μM concentration (p value 0.043). In contrast, under serum starved conditions the impact of H_2O_2 on cellular antioxidant activity was switched towards more pro-oxidative effects and therefore, it was notably distinct from GSSG at 0.1, 10 (both p values 0.036) and 100 μM concentrations (p value 0.015). As shown in Figure 4.27B, in cells cultured under normal conditions, the impact of Q on cellular antioxidant activity was significantly stronger than endogenous pro-oxidants only at higher concentrations (10 and 100 μM). In the case of cells cultured in serum-deprived medium before the experiment, the differences between Q compared to GSSG and H_2O_2 occurred to be statistically significant already at low 1 μM concentration (p values 0.015 and 0.005, respectively). With regard to reference antioxidants, the order of antioxidant activity, according to CAA values at 100 μM , increases in cells cultured under normal conditions as follows: $\text{GSSG} < \text{H}_2\text{O}_2 < \text{GSH} < \text{Q}$.

4.3.4.2. Cytotoxicity by MTT test

As shown Figure 4.28, both GSSG and H_2O_2 did not affect cell growth at low concentrations, regardless of incubation time. At higher concentrations, GSSG significantly reduced cell growth, but only in the case of 72 h treatment (by almost 50%). In the case of H_2O_2 , it caused almost 90% growth inhibition at the highest tested concentration after both short and long exposure times.

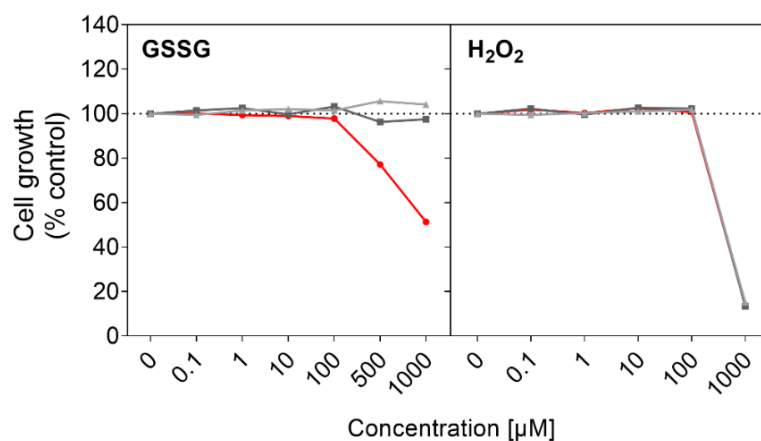


Figure 4.28. The impact of studied endogenous pro-oxidants on the growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment with glutathione disulphide (GSSG) and hydrogen peroxide (H_2O_2). Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

4.3.4.3. Genotoxicity and protection against oxidative DNA damage by comet assay

As shown in Figure 4.29, none of tested endogenous pro-oxidants when applied exogenously in the range of non-cytotoxic concentrations exerted genotoxic effects in HT29 cells



after 24 h treatment compared to the control cells (treated with the solvent only), in the case of which the percentage of DNA in comet tail did not exceed 2%. The pre-treatment of cells with increasing concentrations of GSSG, followed by 1 h exposure to 150 μM H_2O_2 (a model genotoxic treatment used in this thesis), showed a trend towards elevated level of DNA fragmentation, however, the differences compared to control cells were not statistically significant.

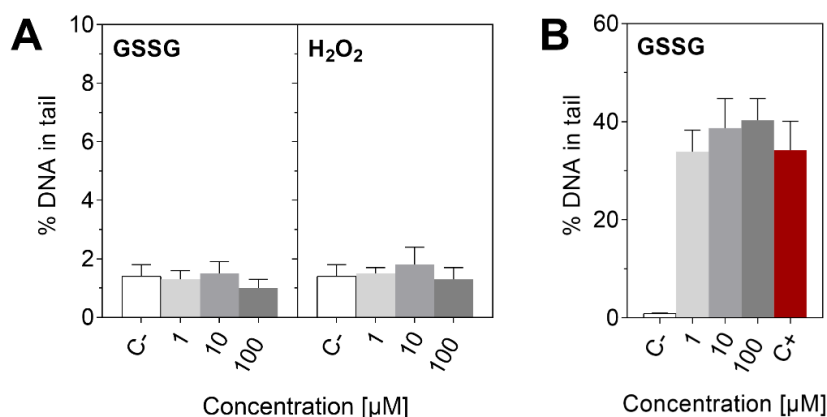


Figure 4.29. The genotoxicity of studied endogenous pro-oxidants towards HT29 cells – **A**, and antigenotoxic impact of glutathione disulphide (GSSG) on HT29 cells exposed to genotoxic treatment with H_2O_2 (150 μM , 1 h) used as a model genotoxic oxidant – **B**. The cells were treated with GSSG and H_2O_2 for 24 h (genotoxicity) or for 24 h prior to 1 hour exposure to 150 μM H_2O_2 (antigenotoxic activity) and then submitted to comet assay procedure. The results are expressed as mean percentage of DNA in comet tail \pm SD from three independent experiments carried out in duplicate and compared with C⁻, negative control (cells treated with solvent only) and C⁺, positive control (150 μM H_2O_2). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$.

The abbreviations used refer to: GSSG, glutathione disulphide; H_2O_2 , hydrogen peroxide.

4.3.4.4. Endogenous pro-oxidants - recapitulation

The antioxidant properties of studied endogenous pro-oxidants are summarised in Figure 4.30. Based on values of standard reduction potentials, H_2O_2 is a better oxidant (*i.e.* acceptor of electrons) than GSSG. However, according to CAA values determined in HT29 cells treated with 100 μM pro-oxidants, GSSG displayed stronger pro-oxidant behaviour than H_2O_2 . The pro-oxidant impact of GSSG on the reducing capacity of HT29 cells was of the same magnitude, though opposite direction, as antioxidant impact of GSH (CAA values -20 vs. +23, respectively). As expected, in chemical tests both GSSG and H_2O_2 did not scavenge neither ABTS nor DPPH radicals, thus their n_{10} values were assumed to equal zero. Both pro-oxidants significantly inhibited cell growth at concentrations exceeding 100 μM , however, in the case of GSSG only after 72 h treatment. In the range of non-cytotoxic concentrations, neither H_2O_2 nor GSSG treatment induced any genotoxic effects in HT29 cells. Pre-treatment of cells with GSSG did not affect the level of DNA fragmentation induced by the exposure to genotoxic treatment (1 h, 150 μM H_2O_2), either.



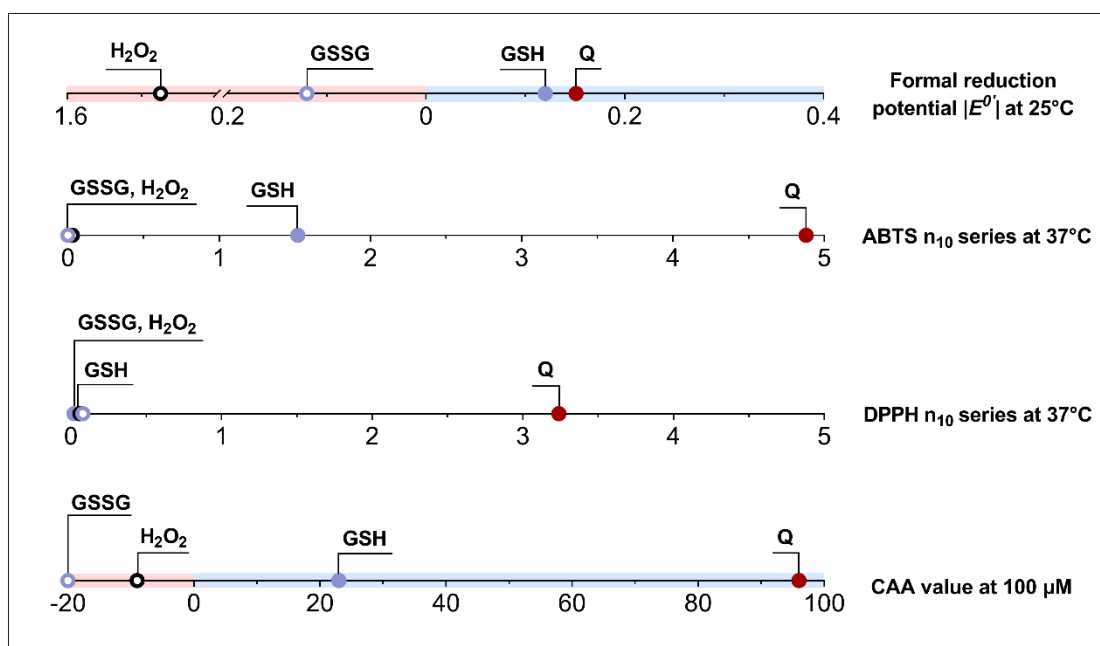


Figure 4.30. Axes comparing redox properties of endogenous pro-oxidants ordered according to: formal reduction potential ($E^{0'}$ [V], shown as absolute values); n_{10} values calculated based on ABTS or DPPH tests; the cellular antioxidant activity (CAA) values measured in cells cultured in complete medium and treated with 100 μ M concentration of investigated compounds (GSH, glutathione; GSSG, glutathione disulphide; H_2O_2 , hydrogen peroxide; Q, quercetin). Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity.

4.4. Human dietary antioxidant nutrients

The term *essential nutrients* refers to substances that are required for normal physiological function and cannot be synthesised endogenously – either at all or in sufficient amounts – and thus, must be provided daily with food [407]. Among these dietary components some display antioxidant activity, e.g. vitamin C. The term “vitamin C” describes a group of derivatives of L-ascorbic acid (AA), which possess the biological activity of vitamin C. Such structurally-related compounds can be also named *vitamers*, or in this particular case – *C vitamers* [407]. In contrast to humans, most of higher organisms – within both plant and animal kingdoms – are able to synthesize AA from glucose [408]. Humans are unable to produce AA endogenously because human variant of GLO gene encoding L-glucono-lactone oxidase needed in the last step of AA biosynthesis is inactive [409]. Other mammals, which are also unable to synthesize AA, include: bat, guinea pig as well as primates such as owl monkey, marmoset, macaque, gibbon, orangutan and gorilla [409]. There are several hypotheses proposed to explain from the evolutionary perspective why humans lost the ability to synthesize AA. One of the explanations refers to the broad accessibility of this vitamin in foods, which could have made it unnecessary to be produced endogenously [181]. Such diet-related explanation appears to be true to all the species that lost the active form of GLO gene, therefore this hypothesis is considered as the most convincing [409]. On top of it, the loss of active variant of *GLO* was found to be positively

correlated with the loss of uricase, the enzyme catalysing the conversion of uric acid to allantoin [334,410]. It has been proposed that UA might have partly replaced AA in terms of its antioxidant role in human plasma. UA is formed upon catabolism of purines, which are released as a result of nucleic acids turnover as well as daily consumed with food. Thus, UA being constantly produced could have been favoured antioxidant compared to AA. The latter can be easily provided with diet in amounts sufficient to fulfill physiological needs, whereas the biosynthesis in mammals requires conversion of L-gulono-1,4,-lactone to AA by GLO with concomitant release of H_2O_2 [411]. This means that the benefits of endogenous production of this antioxidant would be swept away by the release of ROS [412].

AA is thus a unique antioxidant, which can be either endogenous or exogenous, depending on the species. Humans lost the ability to synthesize AA and thus, they must provide AA with daily diet to cover the requirements of an organism for this essential nutrient. In this context, AA can be considered as exogenous antioxidant performing indispensable endogenous functions. This issue differentiates AA from other dietary antioxidants, such as Q used in this thesis as a reference. Although Q exhibits strong reducing properties and was reported to exert other health benefits, it is not required to be consumed every day to maintain proper physiological function of an organism; probably could be replaced by another plant polyphenol without harm. AA was included in this thesis as a representative of exogenous antioxidants, which are simultaneously the indispensable agents enhancing endogenous antioxidant systems and other redox-related processes.

4.4.1. *Ascorbic acid and its derivatives*

Ascorbic acid (AA) belongs to a group of water-soluble vitamins, thus humans must provide it daily with the diet as mentioned earlier. This nutrient is widely distributed in foods of plant origin, but its highest content was reported especially in red peppers, kiwifruits, papayas and oranges. AA bioavailability is determined by the absorption in the alimentary tract and renal re-absorption. Once absorbed, it is distributed to tissues via the circulation. It can be transported into cells with the aid of specific sodium-dependent vitamin C transporters or, if present in the oxidized form that can be further recycled back to the reduced state, by glucose transporters [413]. Plasma concentration of AA is determined by dietary intake. In healthy individuals, who consume several servings of fruits and vegetables daily, its level falls between 40 and 80 μM . In tissues, AA is accumulated in millimolar concentrations, which are not possible to be reached in plasma unless pharmacological doses of AA are provided intravenously. In the case of reported oral supplementation of 3 g administered 6 times a day to healthy volunteers, the peak plasma concentrations of AA reached 200 μM , but did not exceed 220 μM [414].

Vitamin C plays multiple roles in human organism, but most of its physiological and biochemical functions stem from its ability to donate electrons, which makes it physiologically important reducing agent [415]. Under physiological conditions, AA is present in a form of ascorbate monoanion that can undergo two sequential one-electron oxidations. First loss of electron results in the formation of ascorbate radical, a relatively unreactive molecule. The loss



of second electron generates dehydroascorbic acid (DHA), the oxidized form of AA, which maintains biological activity of vitamin C. Ascorbate radical can be reduced back to AA with the aid of NADPH/NADH-dependent mechanisms, while reduction of DHA involves either dehydroascorbic acid reductase or non-enzymatic antioxidant – GSH [416]. DHA is rather unstable under physiological conditions and it may be irreversibly hydrolysed to 2,3-diketogulonate (DKG), the compound that lacks vitamin C activity. Besides, both DHA and DKG can be further oxidized; DHA to L-threonic acid, oxalic acid and their derivatives while DKG to 2-oxo-L-threopentionate. Still, DHA and DKG were shown to act as ROS (H_2O_2 , $\text{O}_2^{\cdot-}$, HO^{\cdot} , $^1\text{O}_2$) scavengers in cell-free experiments, in which studied compounds were incubated with investigated ROS and reaction products were monitored with the aid of electrophoresis [417].

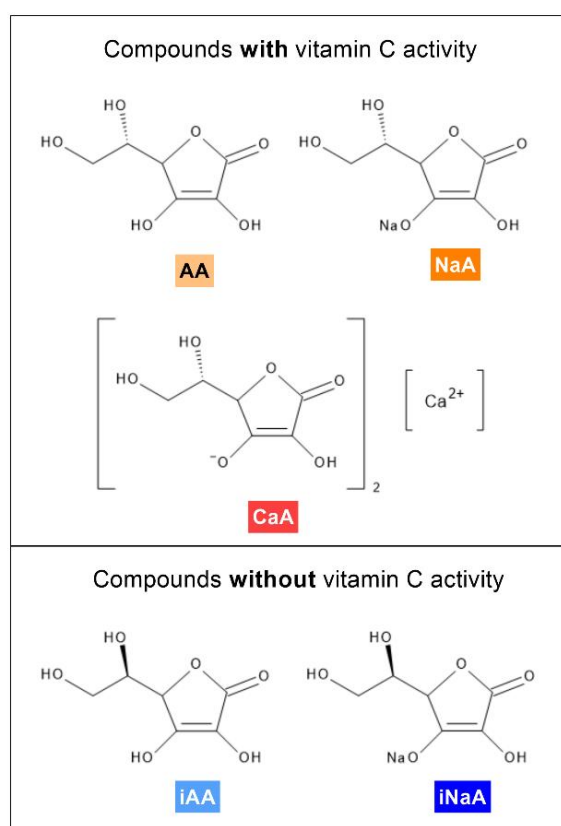


Figure 4.31. Chemical structures of studied compounds. AA – L-ascorbic acid, iAA – D-isoascorbic acid, CaA – calcium L-ascorbate, NaA – sodium L-ascorbate, iNaA – sodium D-isoascorbate.

AA is not only naturally widespread in foods and beverages, but it is also readily applied as a food additive. The group of ascorbates used in food production includes: L-ascorbic acid (E300), L-sodium ascorbate (E301, NaA), L-calcium ascorbate (E302, CaA), fatty acids esters of ascorbic acid (E304), D-isoascorbic acid (E315, iAA, known also as erythorbic acid) and sodium D-isoascorbate (E316, iNaA, known also as sodium erythorbate). Structures of these AA derivatives that were investigated in this thesis are shown in Figure 4.31. The enrichment in AA and its derivatives increases nutritional and sensory value of foods, inhibits browning, stabilizes flavour and colour as well as protects against potential spoilage and oxidation of other food



components [418]. Ascorbates have the Generally Recognized As Safe status and according to European Food Safety Authority, there is no need to define an acceptable daily intake for AA and its salts as their consumption does not pose any health threat [419].

D-Isoascorbic acid is a stereoisomer of AA as these two molecules differ in the orientation of hydrogen and hydroxyl group on the carbon 5. The chemical properties and antioxidant activity of both isoforms are regarded as similar, however, the antiscorbutic activity of iAA in guinea pigs was reported to be negligible compared to AA [420]. The stimulation of collagen synthesis by iAA in primary avian tendon cells was also reported to be less effective compared to AA, probably due to lower intracellular uptake. Nevertheless, it still effectively induced proline hydroxylation once it got inside the cells [421]. Moreover, iAA was reported to be better enhancer of iron absorption from ferrous sulphate comparing to AA [422]. iAA and its salt, iNaA, are not naturally present in foods, but they found application as food additives. Their main function is to prevent oxidation and to protect colour of cured meats, frozen fruits and vegetables, oils and fish as well as to prevent the formation of nitrosamines upon meat curing or cooking [419]. Another ascorbate that found application as food preservative is CaA. This salt is mostly used as an antioxidant in dairy products, the enhancer of the colour intensity of cured and cooked meat, a curing accelerator, inhibitor of browning of freshly-cut fruits and as a synergist to other antioxidants [423,424]. It has also a potential to partially replace sodium chloride in food products, thus may reduce the content of sodium in foodstuffs ensuring simultaneous enrichment in calcium [425].

Ascorbigen

Ascorbigen (ABG) (structure shown in Figure 4.32) belongs to a group of degradation products of indolic glucosinolates found in *Brassica* vegetables such as broccoli, cabbage or cauliflower. Disruption of plant tissues (e.g. during cutting or chewing) triggers myrosinase-mediated hydrolysis of glucobrassicin giving rise to ABG precursors – indole-3-carbinol (I3C) and 3-indolylacetonitrile (3IAN). Then, both I3C and 3IAN can react spontaneously with AA, which is also present in disrupted plant tissues to finally form ABG. For that reason, ABG is sometimes referred to as “a bound version of vitamin C” [426]. *In vivo* experiments carried out in the 1960' showed that synthetic ABG administered to scorbutic guinea pigs displayed some curative effects, even though these animals could utilize only 10-15% of AA bound to ABG [427]. In terms of antioxidant activity, Tai *et al.* reported that ABG was stronger than AA in terms of ABTS scavenging activity, however, ABG could not scavenge DPPH radical [428]. Another study showed that ABG was a weak scavenger of DPPH radical and it could not scavenge $O_2^{\bullet-}$. Compared to AA, it also displayed substantially weaker antioxidant activity assessed with the aid of ferric reducing ability of plasma (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays. In turn, the same study showed that *in vitro* preincubation of human keratinocytes with ABG provided better protection against tert-butylhydroperoxide-induced cytotoxicity and lipid peroxidation than AA, which may result from higher lipophilicity, hence bioavailability of ABG [429].



Modulation of gene expression and activity of the xenobiotics-metabolizing enzymes is known to play a role in chemopreventive properties of *Brassica*-derived phytochemicals, including ABG [426]. Even though health promoting potential of ABG was extensively investigated in the 1990's, recent advances are scarce. So far, most of the research regarding beneficial health effects of ABG has been carried out using cellular and animal models. As an indole-derivative, ABG is known to act as a modulator of the xenobiotics-metabolizing enzymes. For example, the exposure of HepG2 human hepatoma cells to ABG resulted in markedly increased transcription and enzymatic activity of NAD(P)H:quinone dehydrogenase 1 (NQO1). In the same study, these results were confirmed *in vivo* in rats administered with 5 mg/day of ABG for 7 days, where mRNA levels and NQO1 activity were upregulated in the liver of treated rats by 90% and 40%, respectively [430]. In another study, ABG was shown to dramatically increase the activity of ethoxyresorufin-*O*-deethylase (EROD) in the small (45-fold) and large (9-fold) intestines of rats consuming 1600 mg/kg of ABG for 5 days [431]. EROD activity is a biomarker of cytochrome P450 induction (CYP1A1) due to exposure to substances showing affinity to bind the aryl hydrocarbon receptor (AhR) [432]. AhR is a transcription factor activated by various xenobiotics, such as polycyclic aromatic hydrocarbons (e.g. known carcinogen – benzo[*a*]pyrene), but also by so-called “physiological ligands” derived from gut microbiota, host metabolism and diet (e.g. indole-derivatives) [433]. Once induced, AhR is translocated from the cytoplasm into the nucleus, where it controls the expression of numerous genes involved in the regulation of basic cellular functions (e.g. proliferation), immune response, maintenance of intestinal homeostasis and carcinogenesis [434]. Even though ABG was shown to be an efficient inducer of CYP1A, it was reported to poorly bind to AhR; this incompatibility led to the investigation, which resulted in the observation that CYP1A was more efficiently stimulated by indoles formed upon ABG degradation such as indolo[3,2-*b*]carbazole [435]. Recently, the study on brassica vegetables showed that activation of AhR is significantly reduced upon long thermal treatment of broccoli. However, the ligands of this receptor can be still generated under mild heat treatment of broccoli, such as steaming, which preserves intact glucobrassicin and myrosinase activity [436].

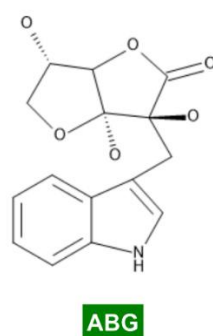


Figure 4.32. Chemical structure of ascorbigen (ABG).

4.4.1.1. Antioxidant activity by ABTS and DPPH tests

As shown in Figure 4.33, C-vitamins and other studied AA derivatives tended to be better scavengers of ABTS than of DPPH radical, regardless of temperature (Figure 4.33B and D). In both tests, the strongest and the weakest radical scavengers at each tested temperature were CaA and ABG, respectively. In contrast to other derivatives of AA, ABG was an extremely weak scavenger of both applied radicals. In ABTS test, the antioxidant activity of AA and its isomer iAA was the same at higher temperatures, while at room temperature iAA turned out to be a better scavenger. In DPPH test, AA exhibited stronger antioxidant activity than iAA, regardless of temperature. NaA was a better radical scavenger than its isomer iNaA in both tests at any tested temperature.

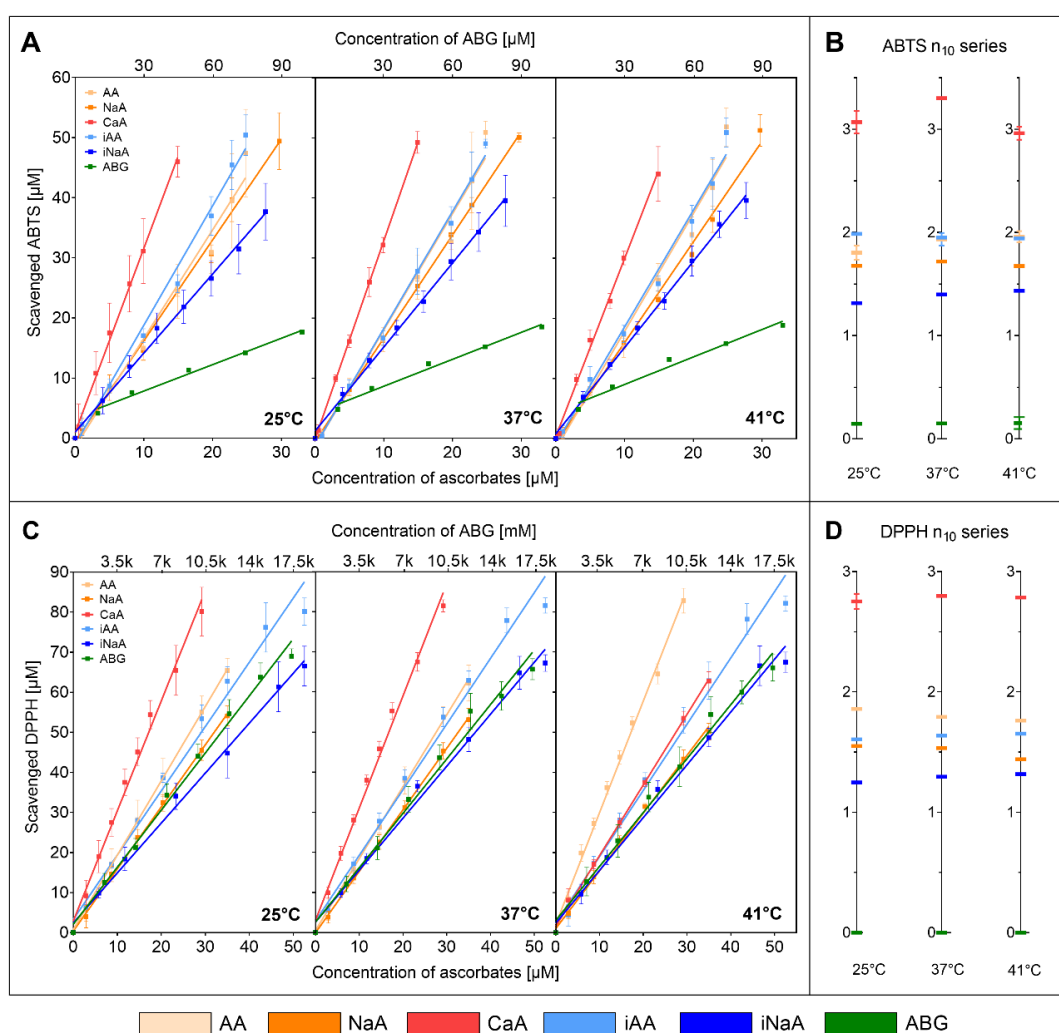


Figure 4.33. The relationship between scavenged ABTS – **A** or DPPH – **C** radicals vs. concentration of ascorbic acid and its derivatives at 25, 37 and 41°C and corresponding n_{10} value axes – **B, D**. The results are expressed as means \pm SD of three independent experiments carried out in triplicates. The abbreviations used refer to: AA, L-ascorbic acid; iAA, D-isoascorbic acid; CaA, calcium L-ascorbate; NaA, sodium L-ascorbate; iNaA, sodium D-isoascorbate; ABG, ascorbigen.

In relation to reference antioxidants, almost complete lack of DPPH scavenging activity observed for ABG resembled that of GSH. In contrast, Q displayed the greatest ability to scavenge both ABTS and DPPH radicals even when compared to the strongest ascorbate antioxidant – CaA. To conclude this stage of studies, the order of antioxidant activity of ascorbic acid derivatives according to the results of ABTS test at 37°C was as follows: ABG < iNaA < **GSH** < NaA < AA < iAA < CaA < **Q**.

4.4.1.2. Antioxidant activity by CAA test

As shown in Figure 4.34, the cellular antioxidant activity of investigated AA derivatives was low to moderate at a wide range of concentrations considered as physiological (reaching up to 200 µM). Within this range, no significant differences between AA derivatives could be observed. Only after exceeding the range of physiological concentrations, though achievable in the alimentary tract (500 µM and higher), the impact of studied compounds on cellular antioxidant activity was noticeably enhanced. At both highest concentrations used, CaA prominently increased cellular antioxidant activity of HT29 cells compared to other ascorbates. At the highest tested concentration CaA caused almost complete inhibition of probe oxidation (83% in the range of maximum 100%). The second strongest ascorbate derivative was NaA. CAA values for other compounds did not however significantly differ from these determined for NaA. In the case of ABG

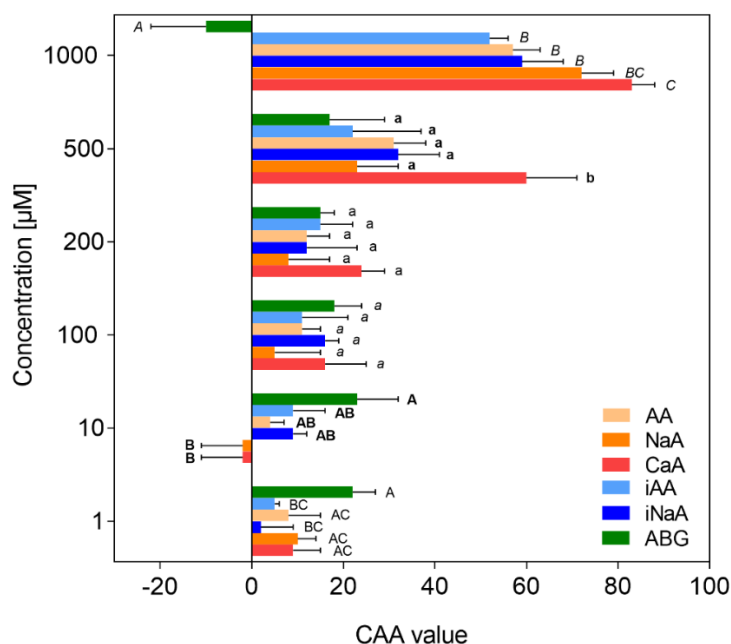


Figure 4.34. The impact of ascorbic acid derivatives on cellular antioxidant activity in HT29 cell line.

The results are expressed as means ± SD of three independent experiments carried out in triplicate. Statistical analysis was carried out with one way ANOVA with Tukey test with the level of significance set at $p < 0.05$. Different letters represent statistically significant differences between specific treatments. The abbreviations used refer to: AA, L-ascorbic acid; iAA, D-isoascorbic acid; CaA, calcium L-ascorbate; NaA, sodium L-ascorbate; iNaA, sodium D-isoascorbate; ABG, ascorbigen.

at low concentrations, its impact on cellular antioxidant activity was similar to that determined for the rest of studied compounds. In contrast, when the concentration was increased to 1000 μM , ABG treatment resulted in CAA values suggesting pro-oxidative effects. Regarding the CAA values of compounds applied to HT29 cells at 1000 μM concentration, the order of antioxidant activity rises as follows: $\text{ABG} < \text{iAA} < \text{AA} < \text{iNaA} < \text{NaA} < \text{CaA}$. Interestingly, the moderate enhancement of cellular antioxidant activity after treatment with physiological range of concentrations of AA derivatives was in line with the effects observed for Q (Figure 4.1B). However, according to the CAA values at 100 μM , both GSH and Q exerted stronger antioxidant activity compared to AA and its derivatives; the order of antioxidant activity increased as follows: $\text{NaA} < \text{iAA} = \text{AA} < \text{ABG} = \text{iNaA} = \text{CaA} < \text{GSH} < \text{Q}$.

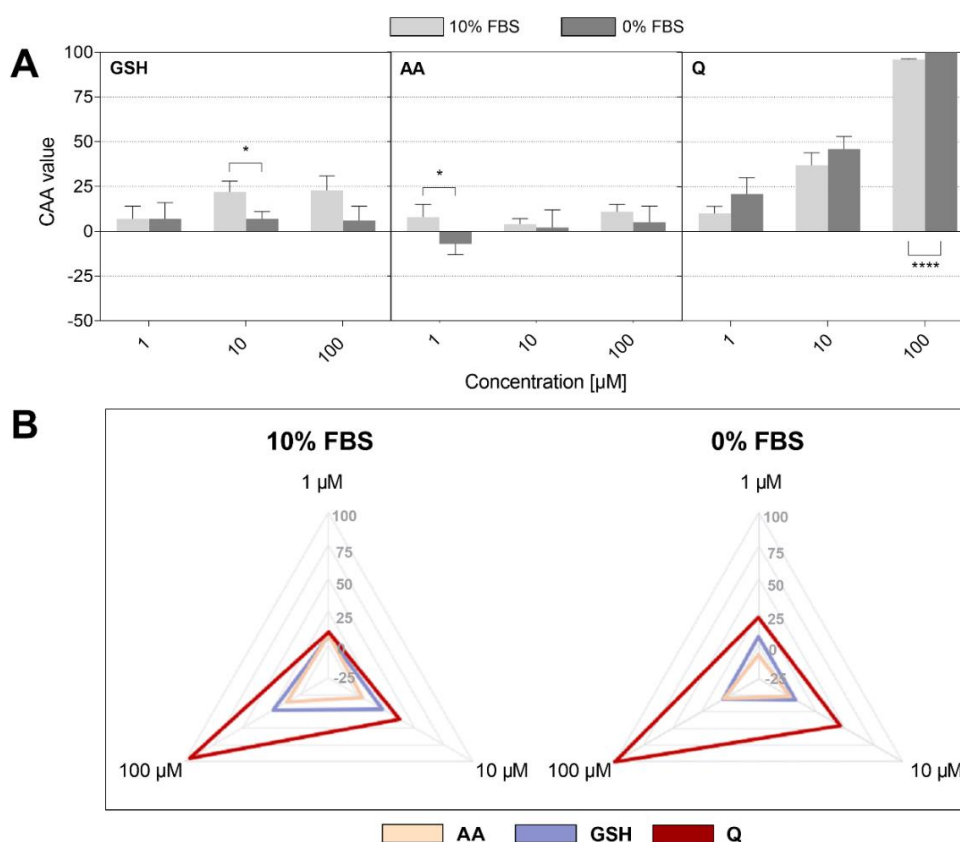


Figure 4.35. A – The impact of serum starvation of HT29 cells treated with L-ascorbic acid (AA) on cellular antioxidant activity; glutathione (GSH) and quercetin (Q) were included as reference compounds.

The results are expressed as means \pm SD of three independent experiments carried out in triplicate.

Statistical analysis was carried out with Student's t-test and the level of significance was set at $p < 0.05$.

The asterisks mark p values as follows: (*) – ≤ 0.05 , (****) – ≤ 0.0001 . Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before the experiment.

B – Comparison radar charts visualizing differences between CAA values of AA vs. reference compounds: GSH and Q determined in HT29 cells cultured under either normal (10% FBS) or serum deprived (0% FBS) conditions. The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$. All experimental details for Q and GSH were described in

4.2. and 4.3.1.2. sections of Results, respectively.

The impact of culture conditions on the antioxidant activity was examined only for AA that served as a representative compound for this group. As shown in Figure 4.35A, the reducing capacity of AA in serum-starved cells was not significantly affected besides the lowest tested concentration (1 μM), where a switch towards slightly pro-oxidative effects occurred. Compared to the same range of concentrations of GSH and Q, the impact of AA on reducing capacity of HT29 cells is more similar to that of GSH than that of Q regardless of culturing conditions of cells (Figure 4.35B). Under normal culturing conditions, AA, GSH and Q applied to cells at 1 μM concentration had the same impact on cellular antioxidant activity. At 10 μM concentration, AA was significantly weaker antioxidant than GSH (p value 0.0179) and Q (p value 0.0009), while GSH was markedly weaker than Q (p value 0.0385). At the highest tested concentration, AA and GSH affected CAA values similarly, and both were markedly poorer antioxidants compared to Q (p value < 0.0001). Under serum-starved conditions, the impact of AA on reducing capacity of cells was notably weaker compared to Q already at the lowest tested concentration (p value < 0.0131). Treatment of cells with Q resulted in significantly higher CAA values than in the case of AA and GSH at both 10 μM (p values 0.0008 and 0.0016, respectively) and 100 μM concentrations (p values < 0.0001).

4.4.1.3. Cytotoxicity by MTT test

As shown in Figure 4.36., shorter treatments of HT29 cells with AA, iAA, CaA and NaA did not significantly affect their growth in the range of concentrations reaching up to 1 mM. Only after 72 h the cell growth was slightly inhibited, however, by no more than 20%. In contrast, a notable cell growth stimulation was observed after shorter treatments with iNaA, which decreased to basal level after 72 h. All compounds applied to the cells at the highest dose, that is 10 mM, occurred to drastically reduce cell growth regardless of incubation time. The only exception was 10 mM AA, which after the shortest treatment only slightly inhibited cell growth. The treatment of cells with ABG at any concentration up to 1 mM tended to increase cell growth, particularly after prolonged treatments (24 and 72 h). The impact of AA derivatives on HT29 cell growth was similar to that of GSH and Q (Figure 4.1A), except for two compounds: iNaA and ABG, which markedly stimulated growth of HT29 for a wide range of concentrations.

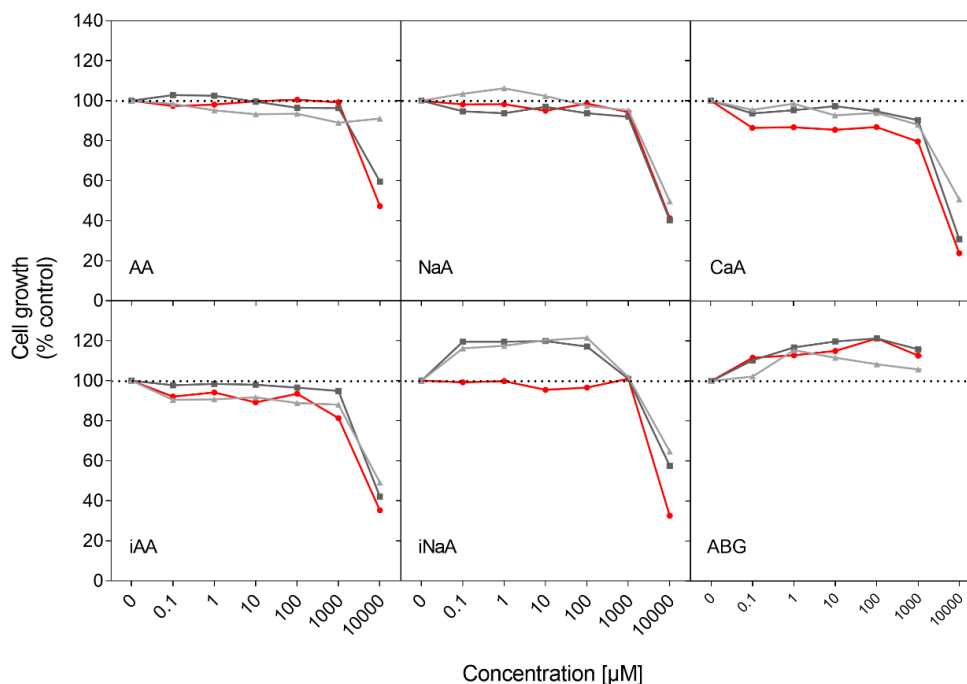


Figure 4.36. The impact of studied ascorbic acid derivatives on the growth of HT29 cells determined by MTT assay after 6 h (grey triangles), 24 h (dark grey squares) and 72 h (red circles) treatment with L-ascorbic acid (AA), sodium ascorbate (NaA), calcium ascorbate (CaA), D-isoascorbate (iAA), sodium D-isoascorbate (iNaA) and ascorbigen (ABG). Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

4.4.1.4. Genotoxicity and protection against oxidative DNA damage by comet assay

As it was previously described in the case of other studied groups of compounds, neither low, nor high concentrations of AA derivatives induced any DNA fragmentation in HT29 cells (Figure 4.37A). Compounds included in this group also did not protect cells against H₂O₂-induced DNA damage (Figure 4.37B). In both variants of comet assay, in the case of non-treated cells (negative control, C-), the damage did not exceed 3% of DNA in a comet tail. In a second variant of the test, pre-treatment of cells with higher concentrations of C-vitamins increased DNA fragmentation induced upon genotoxic treatment with H₂O₂, which in some cases reached statistical significance. Enhanced DNA fragmentation induced by H₂O₂ after pre-treatment of cells with antioxidants was not observed in any of already described groups of studied redox-active compounds, including reference antioxidants (GSH and Q, Table 4.2).



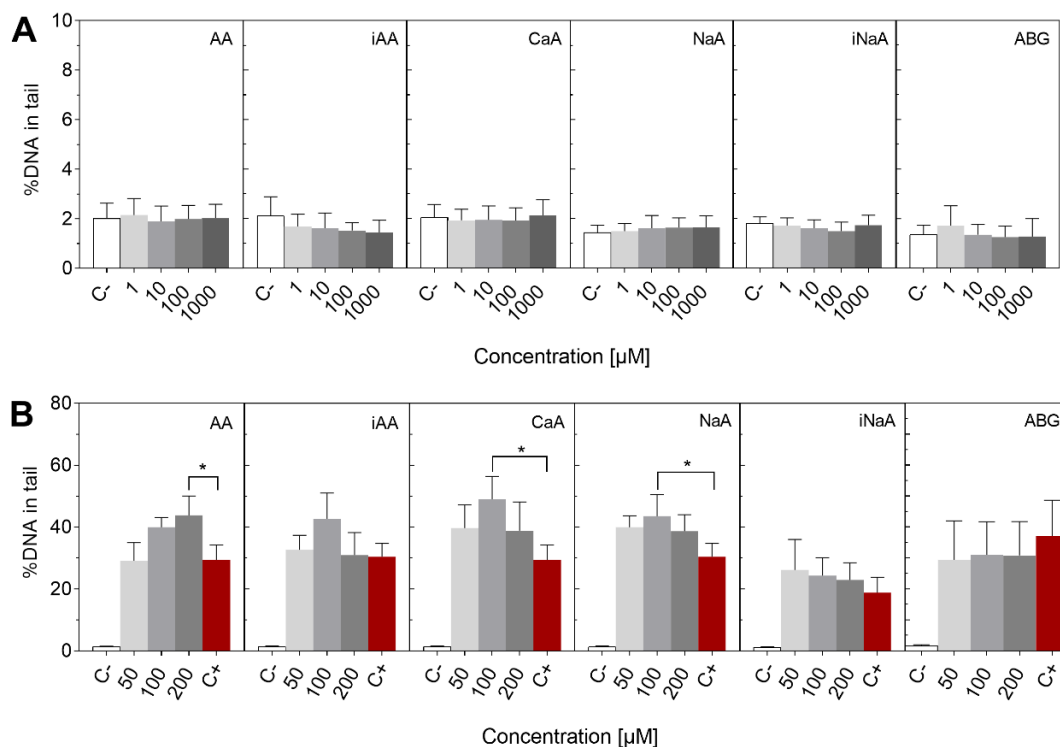


Figure 4.37. The genotoxicity of ascorbic acid derivatives towards HT29 cells – **A**, and their antigenotoxic impact in HT29 cells exposed to genotoxic treatment with H_2O_2 ($150 \mu\text{M}$, 1 h) used as a model genotoxic oxidant – **B**. The cells were treated with studied adenine derivatives for 24 h (genotoxicity) or for 24 h prior to 1 hour exposure to $150 \mu\text{M}$ H_2O_2 (antigenotoxic activity) and then submitted to comet assay procedure.

The results are expressed as mean percentage of DNA in comet tail \pm SD from three independent experiments carried out in duplicate and compared with C-, negative control (cells treated with solvent only) and C+, positive control ($150 \mu\text{M}$ H_2O_2). The statistical analysis was conducted by one way ANOVA with Dunnett's test with the level of significance set at $p < 0.05$. The abbreviations used refer to: AA, L-ascorbic acid; iAA, D-isoascorbic acid; CaA, calcium L-ascorbate; NaA, sodium L-ascorbate; iNaA, sodium D-isoascorbate; ABG, ascorbigen.

4.4.1.5. Genomic studies

Vitamin C is recognized as one of the most important physiological antioxidants. Though in terms of thermodynamics, it is a better electron donor than GSH, in chemical assays it displayed mediocre ability to scavenge ABTS and DPPH radicals. Moreover, as already discussed and shown in Figure 4.35, the impact of AA on reducing capacity of cells was rather poor, at least at concentrations up to $100 \mu\text{M}$, which reflect average physiological plasma content of ascorbate in a healthy person. Such results suggest that AA may perform its important role in maintenance of redox homeostasis through other mechanisms, such as modulation of expression of genes related to antioxidant defense and protection against oxidative stress. This hypothesis was investigated using RT-PCR-based genomic approach (Qiagen, USA).

The impact of ascorbic acid derivatives, including C-vitamins, on the expression of redox-related genes was studied with the use of microarrays consisting of 84 genes involved in oxidative

stress response and antioxidant defence systems (the list of genes is attached in Appendix C, Table C1). Such analysis was conducted previously in the case of reference antioxidants: GSH and Q [176,263]. The ascorbate antioxidants selected for genomic analysis were AA, CaA and ABG. AA was selected as a parent compound and representative of the whole group as well as a human exogenous antioxidant performing important endogenous functions. CaA was chosen as the strongest antioxidant according to n_{10} values and CAA values at 500 and 1000 μM . Finally, the selection of ABG as another AA derivative for genomic analysis was favoured by its activity that was particularly distinct from the rest of the group. The concentrations chosen for cell treatment reflect the physiologically relevant situation: 50 μM represents the average plasma concentration of AA in healthy individuals who consume fruits and vegetables daily, while 200 μM is the highest plasma concentration of AA that could be reached after the prolonged supplementation not exceeding maximum tolerated dose [414]. Besides, such a concentration could be easily achieved in the GI tract exposed to ingested food.

There were two restrictions to be taken into account during processing of data derived from experiments performed using RT-PCR microarrays. Firstly, genes whose expression was under detection limit during qPCR reaction, *i.e.* below cycle threshold cut-off value set at 36 cycles, were excluded from further analysis. Secondly, according to manufacturer's instructions, fold change values that are considered as biologically-relevant are equal or higher than 2 (for upregulated genes) or lower than 0.5 (for downregulated genes). However, in this work, we acknowledged all changes in gene expression, which were statistically significant (p value < 0.05), since these experiments were intended to spot even subtle changes in the patterns of gene expression. For the same reason, alterations in the expression of genes, which failed slightly to reach statistical significance (p values between 0.05 and 0.1) were also included in Figure 4.38 and were discussed as indicating a trend in gene expression patterns. The reason was that in cells treated with physiologically relevant concentrations of non-toxic dietary antioxidants, no big impact on transcriptome can be expected. In contrast, such major specific alterations in gene expression may be seen, for example, in drug research.

The treatment with the lower concentration (50 μM) of AA significantly downregulated the expression of only two genes, *CYBB* and *RNF7* by 3.7-fold and 1.8-fold, respectively. *CYBB* gene encodes β -chain of cytochrome b, which is one of membrane-spanning subunits required for functionality of enzymatic complex called NADPH oxidase [437], while *RNF7* encodes a component of ubiquitin ligase complex, called ring finger protein 7, which was reported to probably protect cells from redox-induced apoptosis [438]. These genes are involved in cellular response to oxidative stress as well as ROS-generation mechanisms of immune response, as shown in Figure 4.39B. Six other genes involved in protection against oxidative stress (*GPX1*, *GSTZ1*, *PDLIM1*, *PRDX5*, *PRDX6*, *PRNP*) showed a trend towards being downregulated. A statistically significant, nonetheless slight (1.3-fold) upregulation by 50 μM AA was observed only for *OXS1* that encodes protein called oxidative-stress responsive kinase 1. This protein belongs to Ser/Thr protein kinase family that is activated in response to environmental stress [439]. A trend towards upregulation was observed for four other genes

(*PRDX1*, *PRDX3*, *SOD1*, *TXN*) encoding proteins that indirectly or directly participate in H₂O₂ metabolism (Figure 4.39A). In turn, 50 μM CaA did not significantly affect the expression of any of the investigated genes. Only expression of two genes, *MGST3* and *PRDX4*, involved in xenobiotic metabolism and H₂O₂ reduction, showed a slight trend towards upregulation (1.4-fold). None of the genes was significantly affected by the treatment with the lower dose of ABG. Thus, despite the presence of common AA structure in all three investigated derivatives, their impact on transcription of redox-related genes in HT29 cells was very different at 50 μM treatment.

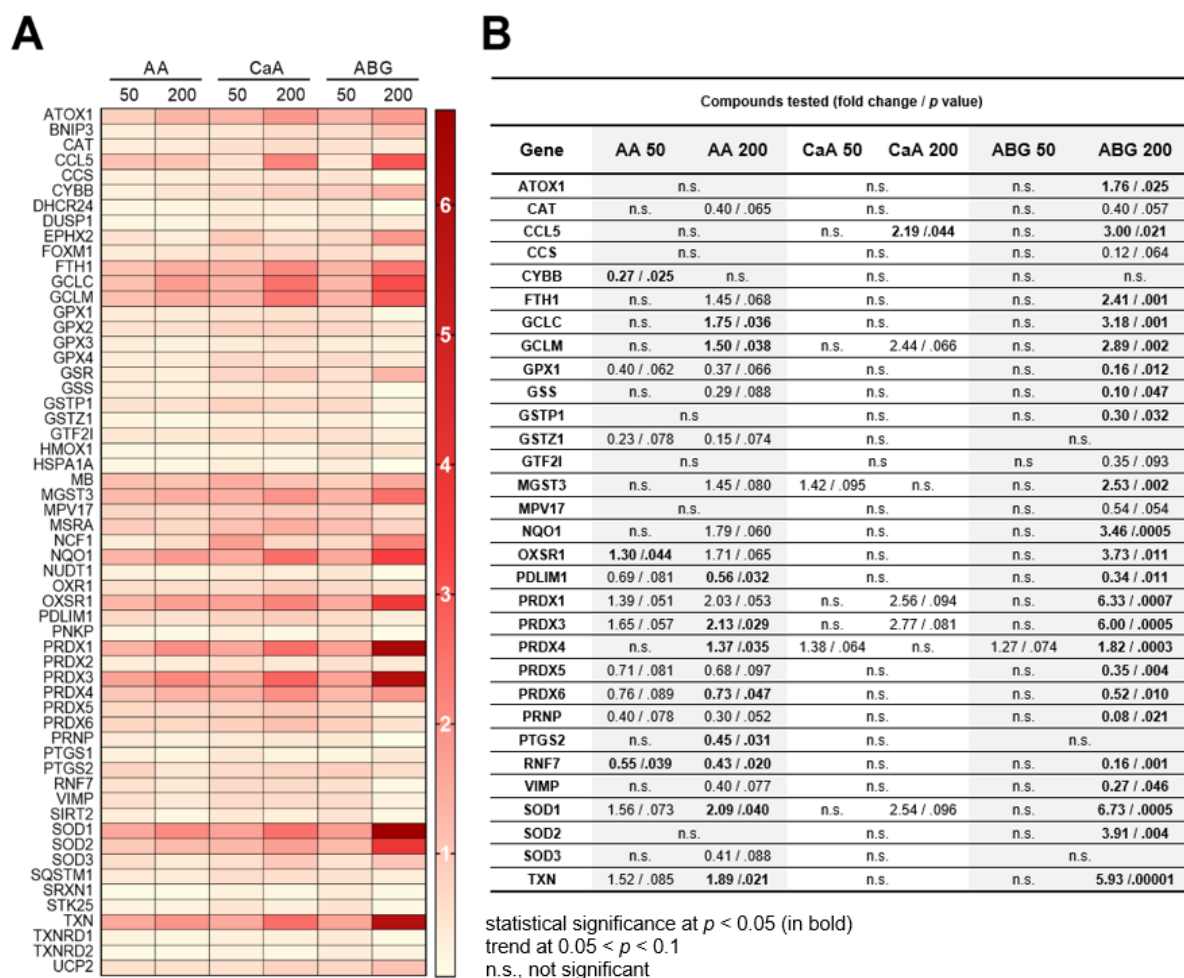


Figure 4.38. Heatmap presenting modulation of 84 oxidative stress response and antioxidant defence genes in HT29 cells after 24 h treatment with ascorbic acid (AA), calcium ascorbate (CaA) and ascorbigen (ABG) at 50 or 200 μM concentrations – **A**. Relevant changes in the expression of genes observed following the indicated treatment – **B**. The results are calculated based on three independent experiments.

The treatment of HT29 cells with the higher concentration (200 μM) of AA had a greater impact on downregulation of *RNF7* gene (2.3-fold) compared to the exposure to lower concentration described above. Other downregulated genes were *PTGS2* (2-fold decrease), whose increased expression is associated with inflammation [440]. Also *PDLIM1* and *PRDX6* involved in protection against oxidative stress, were downregulated by 1.7-fold and 1-4 fold, respectively. The upregulation upon cell treatment with 200 μM AA was observed for *GCLC*

and *GCLM* (1.5-fold and 1.75-fold, respectively), which encode two subunits of glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis [441] as well as for *PRDX4* (peroxiredoxin 4) and *TXN* (thioredoxin), indirectly involved in recycling of GSH from GSSG, were upregulated by 1.4-fold and 1.9-fold, respectively (Figure 4.39A). The connection between the latter two genes relies on the joint protection against oxidative stress that is provided by *PRDX4*, which exhibits peroxidase activity, thus neutralizes H_2O_2 , in a thioredoxin-dependent manner [442]. The expression of genes encoding cytosolic *SOD1* and mitochondrial *PRDX3* was increased 2.1-fold each, both of which support the removal of ROS. In the case of 200 μM CaA treatment, *CCL5*, encoding a protein that mediates inflammatory processes [443] was the only gene significantly upregulated (2.2-fold). Other genes that showed a trend towards upregulation (approximately 2-fold) were *GCLM*, *PRDX1*, *PRDX3* and *SOD1*, all involved in ROS detoxification.

The treatment of HT29 cells with 200 μM ABG had the most prominent impact on the expression of investigated panel of redox-related genes: transcription of 13 and 9 genes was significantly upregulated or downregulated, respectively. Compared to 200 μM AA, the effects of ABG were always stronger and involved the pool of 19 shared genes: *FTH1*, *GCLC*, *GCLM*, *GPX1*, *GSS*, *MGST3*, *NQO1*, *OXS1*, *PDLIM1*, *PRDX1*, *PRDX3*, *PRDX4*, *PRDX5*, *PRDX6*, *PRNP*, *RNF7*, *VIMP*, *SOD1*, *TXN*. The same trend occurred also in the case of *CCL5* upregulation compared to 200 μM CaA. Besides, ABG changed also the expression of a few antioxidant genes that were affected neither by AA nor by CaA (*ATOX1*, *GSTP1*, *SOD2*). *ATOX1* gene, which was upregulated, encodes ATX antioxidant protein 1 that is involved in the maintenance of copper homeostasis and therefore, in the activation of copper-dependent enzymes [444]. Another antioxidant gene that ABG upregulated was the mitochondrial superoxide dismutase, *SOD2*. In turn, the expression of *GSTP1*, involved in the metabolism of xenobiotics, was downregulated [445].

As shown in Figure 4.39, the treatment of HT29 cells with AA derivatives did not support cellular redox homeostasis unidirectionally. The treatment of cells with AA caused downregulation of *CYBB*, which makes one of subunits of ROS-generating enzyme – NADPH oxidase. Such results would suggest a decrease in generation of $O_2^{\cdot-}$, and thus alleviation of oxidative stress. On the other hand, decreased expression of *SOD3*, which acts in extracellular milieu, might suggest either lowered antioxidant protection or no need to even induce it. At the same time, the expression of *SOD1*, which converts $O_2^{\cdot-}$ to less reactive H_2O_2 , was upregulated. The possible enhanced production of H_2O_2 was not countbalanced by changed expression of other genes. For example, two other genes encoding antioxidant enzymes such as *CAT* and *GPX1*, which both neutralize H_2O_2 , were downregulated. This would suggest the increased preservation of H_2O_2 , which besides being one of ROS, is known to mediate intracellular signalling. Moreover, downregulation of *GPX1* might decrease utilization of GSH, which is being oxidized upon enzymatic reduction of H_2O_2 . It is commonly known that there is a strong interplay between AA and GSH in plants and animals, including humans, in terms of the maintenance of redox homeostasis [446,447]. Increased expression of *GCL*, one of subunits of enzyme participating

in the synthesis of GSH, may also suggest that AA induced enhanced preservation of GSH pool. However, in contrast, the expression of GSS, which is another enzyme involved in GSH biosynthesis, tended to be downregulated. Upregulation of *TXN* may also indirectly affect regene-

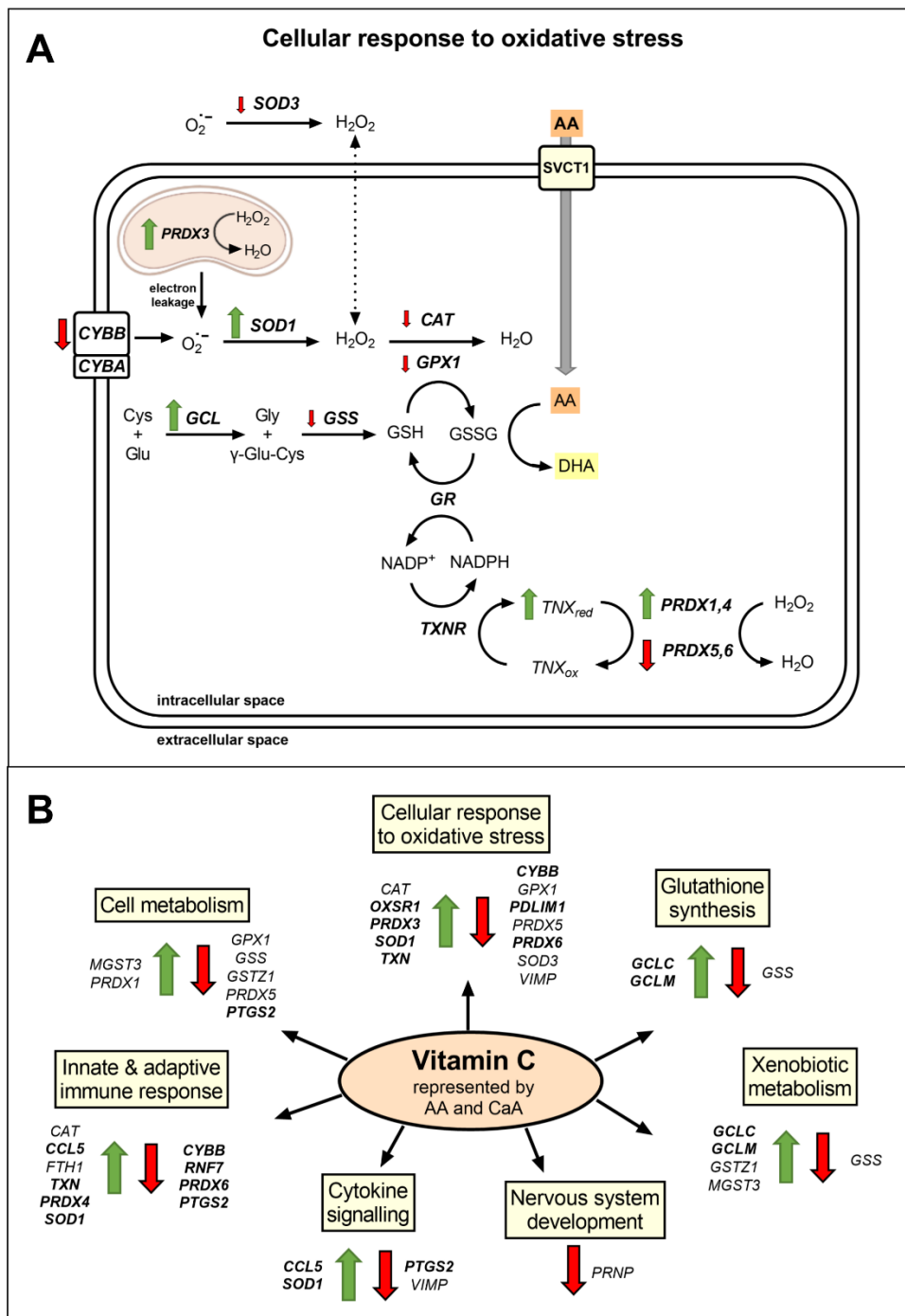


Figure 4.39. Cellular response to oxidative stress upon treatment with vitamin C represented by L-ascorbic acid (AA) and calcium ascorbate (CaA) can be modulated at the level of gene expression - **A**. Genes affected by treatment of HT29 cells with AA and CaA are involved in numerous pathways related to ROS neutralization, antioxidant protection and cellular metabolism – **B**. Genes whose expression was significantly changed in response to vitamin C treatments are written in bold (p value < 0.05), other affected genes refer to p values between 0.05 and 0.1.

-ration of GSH. TXN is utilized by PRDXs, which are other enzymes engaged in neutralization of H₂O₂. Here, mitochondrial *PRDX3* as well as cytosolic *PRDX1* and *PRDX4* were upregulated, while other cytosolic isoforms – *PRDX5* and *PRDX6*, were downregulated, as it was the case with *GPX1* and *CAT*. Based on the obtained results, it is hard to notice any clear pattern of changes in gene expression, which would suggest definite more antioxidant or pro-oxidant effects. The observed diversified cellular response to the treatment with AA derivatives rather points to the fine-regulation of endogenous systems of antioxidant protection preventing both oxidative and reductive stress occurrence.

In Table 4.3., the impact of AA on the modulation of redox-related genes in HT29 cells was compared to the effects induced by the treatment of cells with reference antioxidants – GSH and Q. Interestingly, the modulation of common genes by AA vs. GSH or Q was opposite in most cases. Precisely, genes upregulated by AA were downregulated by GSH and/or Q, while genes downregulated by AA were upregulated by GSH and/or Q. Common genes affected by AA, GSH and/or Q are listed in Figure 4.40. *TXN*, which is involved in one of the major intracellular antioxidant systems, was the only gene, whose expression was significantly changed by each of selected antioxidants: GSH, AA and Q, though, only in the case of higher concentrations. However, the difference between these antioxidants was that AA caused almost 2-fold upregulation of *TXN*, while GSH and Q – almost 2-fold downregulation. *TXN* downregulation may indirectly affect GSH recycling (Figure 4.39A). Both addition of exogenous GSH as well as Q, which kinetically acts as a strong antioxidant, may lead to downregulation of *TXN* as these antioxidants may overtake its impact on redox homeostasis. At the same time, AA lead to upregulation of *TXN*, which may be related to enhanced H₂O₂-mediated intracellular signalling. GSH and AA significantly affected the expression of only 2 common genes (*PRDX3* and *SOD1*), both of which are involved in neutralization of excessive amounts of ROS. Again, higher concentration of GSH caused statistically significant, however slight downregulation of *PRDX3* and *SOD1*, while higher concentration of AA caused 2-fold upregulation of these genes. The expression of *GSTZ1*, involved in metabolism of xenobiotics, was upregulated by both GSH and Q, while AA treatment showed trend to its downregulation. AA and Q did not affect any common genes, besides already described *TXN*. Taking into consideration changes in the expression of genes, which turned out not to be statistically significant, however, indicating some trends, still AA and Q did not affect any common genes, while the number of genes affected by GSH and AA raised up to 7. The number of genes affected by all three antioxidants increased up to 3 and included *TXN*, *SOD1* and *GSTZ1*.



Table 4.3. Fold changes in the expression of redox-related genes in HT29 cells affected by 24 h treatment with major endogenous antioxidant – glutathione (GSH), human dietary antioxidant performing vital physiological functions – L-ascorbic acid (AA), and dietary polyphenol – quercetin (Q). Statistically significant results (p value < 0.05) were written in bold; changes for which and p values were maintained between 0.05 and 0.1 were included and interpreted as indicating a trend. The results are calculated based on three independent experiments.

Gene	Fold change / p value					
	GSH 1 μ M	GSH 10 μ M	AA 50 μ M	AA 200 μ M	Q 1 μ M	Q 10 μ M
ALOX12					0.59 / 0.092	
BNIP3	2.84 / 0.058	3.81 / 0.030				
CAT				0.40 / 0.065		
CCL5	2.36 / 0.056					
CYBB			0.27 / 0.025			
FTH1				1.45 / 0.068		
GCLC				1.75 / 0.036		
GCLM		0.56 / 0.063		1.50 / 0.038		
GFT2I					1.40 / 0.033	
GPX1		0.82 / 0.074	0.40 / 0.062	0.37 / 0.066		
GPX4						1.23 / 0.074
GSR					1.78 / 0.040	1.82 / 0.047
GSS		1.20 / 0.074		0.29 / 0.088		
GSTZ1	2.24 / 0.006	1.98 / 0.002	0.23 / 0.078	0.15 / 0.074		1.44 / 0.031
HSPA1A					2.37 / 0.043	2.44 / 0.050
MGST3				1.45 / 0.080		
MT3					0.52 / 0.090	
NCF1		5.57 / 0.001				
NOS2		2.79 / 0.013				
NOX5	5.11 / 0.016	3.62 / 0.019				
NQO1				1.79 / 0.060		
OXSR1			1.30 / 0.044	1.71 / 0.065		
PDLIM1	1.33 / 0.072	1.40 / 0.057	0.69 / 0.081	0.56 / 0.032		
PRDX1		0.69 / 0.043	1.39 / 0.051	2.03 / 0.053		
PRDX2	1.61 / 0.018	1.57 / 0.006				
PRDX3	0.69 / 0.094	0.61 / 0.017	1.65 / 0.057	2.13 / 0.029		
PRDX4				1.37 / 0.035		
PRDX5			0.71 / 0.081	0.68 / 0.097		
PRDX6			0.76 / 0.089	0.73 / 0.047		
PRNP	1.77 / 0.029	1.30 / 0.002	0.40 / 0.078	0.30 / 0.052		
PTGS1	3.89 / 0.069				2.17 / 0.005	1.84 / 0.032
PTGS2				0.45 / 0.031		
RNF7			0.55 / 0.039	0.43 / 0.020		
SEPP1	3.95 / 0.030	2.83 / 0.012				
SIRT2						1.57 / 0.072
SOD1		0.81 / 0.045	1.56 / 0.073	2.09 / 0.040		0.59 / 0.068
SOD2					0.71 / 0.082	0.52 / 0.063
SOD3				0.41 / 0.088		
STK25						1.96 / 0.048
TXN	0.77 / 0.061	0.66 / 0.002	1.52 / 0.085	1.89 / 0.021	0.61 / 0.080	0.54 / 0.046
TXNRD1					2.02 / 0.019	2.06 / 0.009
TXNRD2	2.13 / 0.009	1.99 / 0.026				
VIMP				0.40 / 0.077		

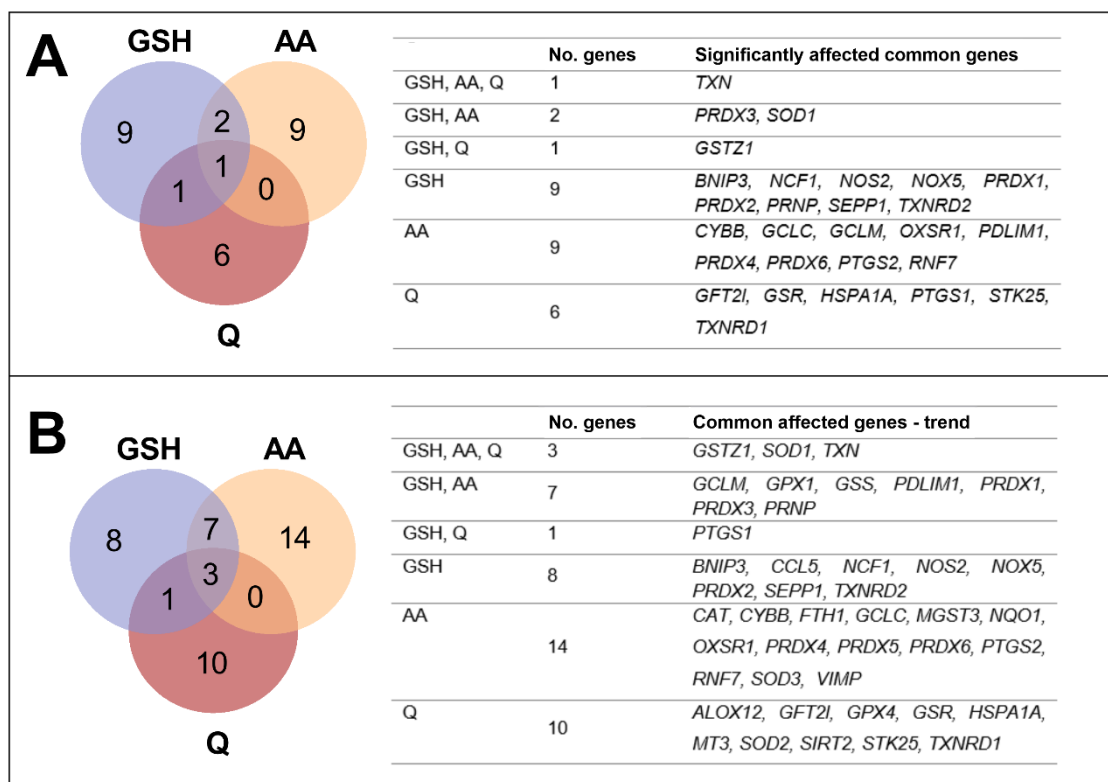


Figure 4.40. Venn diagrams showing changes in the expression of common genes regulated by glutathione (GSH), L-ascorbic acid (AA) and/or quercetin (Q) at p values < 0.05 – **A**, and p values between 0.05 and 0.1 – **B**.

4.4.1.6. Ascorbic acid and its derivatives – recapitulation

The antioxidant properties of studied AA derivatives are summarised in Figure 4.41. According to standard reduction potentials E^0 , CaA was the strongest antioxidant among the studied derivatives of AA (Table 4.1). This was in line with ABTS and DPPH n_{10} series (Figure 4.41) and CAA value for the highest tested concentration of CaA – 1000 μM (Figure 4.34). However, its value of formal reduction potential $E^{0'}$, which refers to physiological pH 7.4, imply that its ability to donate electrons was lower than that of other AA derivatives, GSH and Q. The only exception was ABG, whose high $E^{0'}$ value points rather to more pro-oxidant activity, which was actually observed at the highest tested concentration. Accordingly, ABG occurred to be an extremely poor scavenger of ABTS and DPPH radicals. Nevertheless, again somewhat contradictorily, E^0 value of ABG indicates that it is the second best electron donor right after CaA. These observations suggest that pH can dramatically affect reducing ability of AA derivatives. According to $E^{0'}$ values, the strongest antioxidant behaviour among tested AA derivatives was exhibited by iNaA, however, it was not confirmed by n_{10} values calculated based on ABTS and DPPH tests. All tested AA derivatives at 100 μM concentration had similar impact on cellular antioxidant activity of HT29 cells. Compared to GSH, these compounds turned out to be slightly weaker cellular antioxidants, while compared to Q – even up to 20 times weaker.

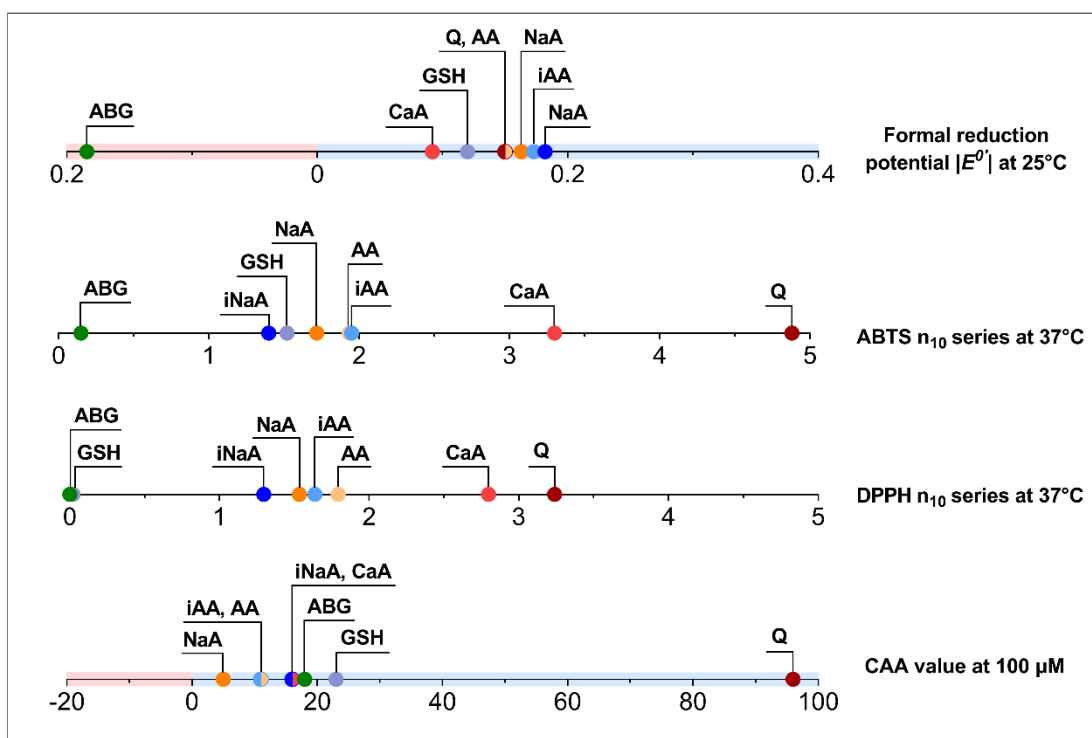


Figure 4.41. Axes comparing redox properties of ascorbic acid derivatives ordered according to: formal reduction potential (E° [V], shown as absolute values); n_{10} values calculated based on ABTS and DPPH tests; the cellular antioxidant activity (CAA) values measured in cells cultured in complete medium and treated with 100 μM concentration of investigated compounds (AA, L-ascorbic acid; NaA, sodium L-ascorbate; CaA, calcium L-ascorbate; iAA, D-isoascorbic acid; iNaA, sodium D-isoascorbate; ABG, ascorbigen; GSH, glutathione; Q, quercetin). Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity.

AA derivatives did not have notable impact on the growth of HT29 cells at wide range of concentrations with the exception of iNaA and ABG, which stimulated cell growth by around 20%. High concentrations (10 mM) of AA derivatives resulted in significant, up to 80% decrease in cell growth, probably as a result of reductive stress induction. To compare, the same concentration of GSH resulted only in 20% decrease in cell growth. At concentrations up to 100 μM , the impact of AA derivatives (except for iNaA and ABG, as previously mentioned) on cell growth was in line with that of GSH and Q (Figure 4.1). As in the case of other groups of studied compounds, AA derivatives were not genotoxic to HT29 cells. However, pre-treatment with C-vitamins followed by genotoxic treatment with H_2O_2 resulted in significantly increased DNA fragmentation, which was not observed in the case of any other compound studied in this thesis.

AA and ABG affected the expression of a great variety of redox-related genes, especially at higher concentration (200 μM); the impact of ABG was repeatedly more powerful compared to AA. Treatment of HT29 cells with higher concentration of CaA upregulated the expression of only one gene involved in inflammatory response – *CCL5*, which was also upregulated by higher concentration of ABG. Such an unexpected lack of genomic impact of CaA on endogenous antioxidant systems may suggest that their induction was not needed, because CaA is an



antioxidant strong enough by itself to prevent oxidative stress, as it was described in the section 4.1 in the case of catechins and Q [176,263].

Compared to reference antioxidants, AA affected greater pool of genes, many of which were common with GSH rather than with Q. This suggests that at the genomic level, AA acts more like endogenous rather than exogenous antioxidant. However, the impact of AA on the expression of redox-related genes in HT29 cells was very often opposite to that of both GSH and Q, *i.e.* a gene was downregulated by AA but upregulated by GSH and Q, or the other way round. Moreover, it is worthy of noting that the number of genes downregulated by AA was significantly higher than in the case of GSH and Q, both of which tended mostly to rather upregulate redox-related genes.

Table 4.4. Summary of upregulated and downregulated genes upon 24 h treatment of HT29 cells with glutathione (GSH), L-ascorbic acid (AA) and quercetin (Q). Genes that induced statistically significant changes in expression (p value < 0.5) are written in bold.

Treatment	Upregulated genes	Downregulated genes
GSH 1 μ M	<i>BNIP3, CCL5, GSTZ1, NOX5, PDLIM1, PRDX2, PRNP, PTGS1, SEPP1, TXNRD2</i>	<i>PRDX3, TXN</i>
GSH 10 μ M	<i>BNIP3, GSS, GSTZ1, NCF1, NOS2, NOX5, PDLIM1, PRDX2, PRNP, SEPP1, TXNRD2</i>	<i>GCLM, GPX1, PRDX1, PRDX3, SOD1, TXN</i>
AA 50 μ M	<i>OXS1, PRDX1, PRDX3, SOD1, TXN</i>	<i>CYBB, GPX1, GSTZ1, PRDX5, PRDX6, PRNP, RNF7</i>
AA 200 μ M	<i>FTH1, GCLC, GCLM, MGST3, NQO1, OXS1, PRDX1, PRDX3, PRDX4, SOD1, TXN</i>	<i>CAT, GPX1, GSS, GSTZ1, PDLIM1, PRDX5, PRDX6, PRNP, PTGS2, RNF7, SOD3, VIMP</i>
Q 1 μ M	<i>GFT2I, GSR, HSPA1A, PTGS1, TXNRD1</i>	<i>ALOX12, MT3, SOD2, TXN</i>
Q 10 μ M	<i>GPX4, GSR, GSTZ1, HSPA1A, PTGS1, SIRT2, STK25, TXNRD1</i>	<i>SOD1, SOD2, TXN</i>

4.5. Summary of results

The antioxidant properties of investigated redox-active compounds are summarised in Figure 4.42. It can be easily noticed that according to formal reduction potentials $E^{0'}$, half of the studied compounds turned out to be thermodynamically better electron acceptors, thus could be expected to act rather as pro-oxidants, while the other half (including reference antioxidants) embraced better electron donors that could play the role of antioxidants. This is not surprising, because most of the compounds marked on the axis highlighted in red, even if commonly regarded as antioxidants, were actually used in their oxidized form (e.g. CoQ₁₀, LA) or both oxidized and reduced forms (GSSG/GSH, NAD⁺/NADH). All adenine derivatives are characterized by positive $E^{0'}$ values, thus thermodynamically are poor antioxidants. H₂O₂ is situated right to Ade derivatives, which means that it is slightly less oxidizing. However, in the case of purine derivatives and MT, it must be emphasized that only one-electron reduction potentials (radical form/reduced form) were available in the literature. This was in contrast to other compounds studied, for which two-electron reduction potentials (oxidized form/reduced form) were used for comparison whenever available. Moving towards more reducing activity, MT is followed by another purine derivative – UA. Even though UA is known to be an important physiological antioxidant, in the light of presented data, this activity does not seem to derive from its thermodynamic properties. $E^{0'}$ values of NAD⁺ and LA are similar, thus thermodynamically they both are weak pro-oxidants, yet LA displays well documented antioxidant activity in cellular setting. Last three compounds, whose $E^{0'}$ values fall between 0 and 0.2 on the axis representing oxidizing activity are ABG, GSSG and CoQ₁₀. This means that, in terms of electrochemical properties, all of them are weak oxidants. Even those first data suggest that thermodynamic properties cannot be the only factors underlying redox behaviour of so far discussed compounds. Those with more unequivocal reductive properties described below seem to be less problematic. According to $E^{0'}$ values CA (or more precisely α -ketoglutarate/isocitrate redox pair) appears to be the best reductor. However, it must be stressed that oxidation of isocitrate to α -ketoglutarate is enzymatically catalysed by isocitrate dehydrogenase with the simultaneous reduction of NAD⁺ and as such, it cannot be directly compared to other redox pairs, for which only the change in the level of molecule's oxidation is described by $E^{0'}$ value. The second best reducing agent is NADH. NADH is followed by Cys, which is a key endogenous antioxidant involved in the redox signalling, as it was described in the introductory section to thiol antioxidants (section 4.3.1.). Notably, there is a significant difference in reducing activity between Cys and the subsequent redox-active compounds. Values of $E^{0'}$ falling between 0.1 and 0.2 V embraced seven redox-active compounds: AA derivatives, NAC and reference antioxidants – GSH and Q. Thermodynamically, Q is almost equally strong antioxidant as AA, while GSH is a weaker one. $E^{0'}$ value of CaA suggests that it is the weakest antioxidant among compounds studied. When this order of reductive properties based on thermodynamics is compared with the results obtained for the same compounds by other methods of antioxidant activity evaluation, it is clearly seen that reduction potential does not play the decisive role.

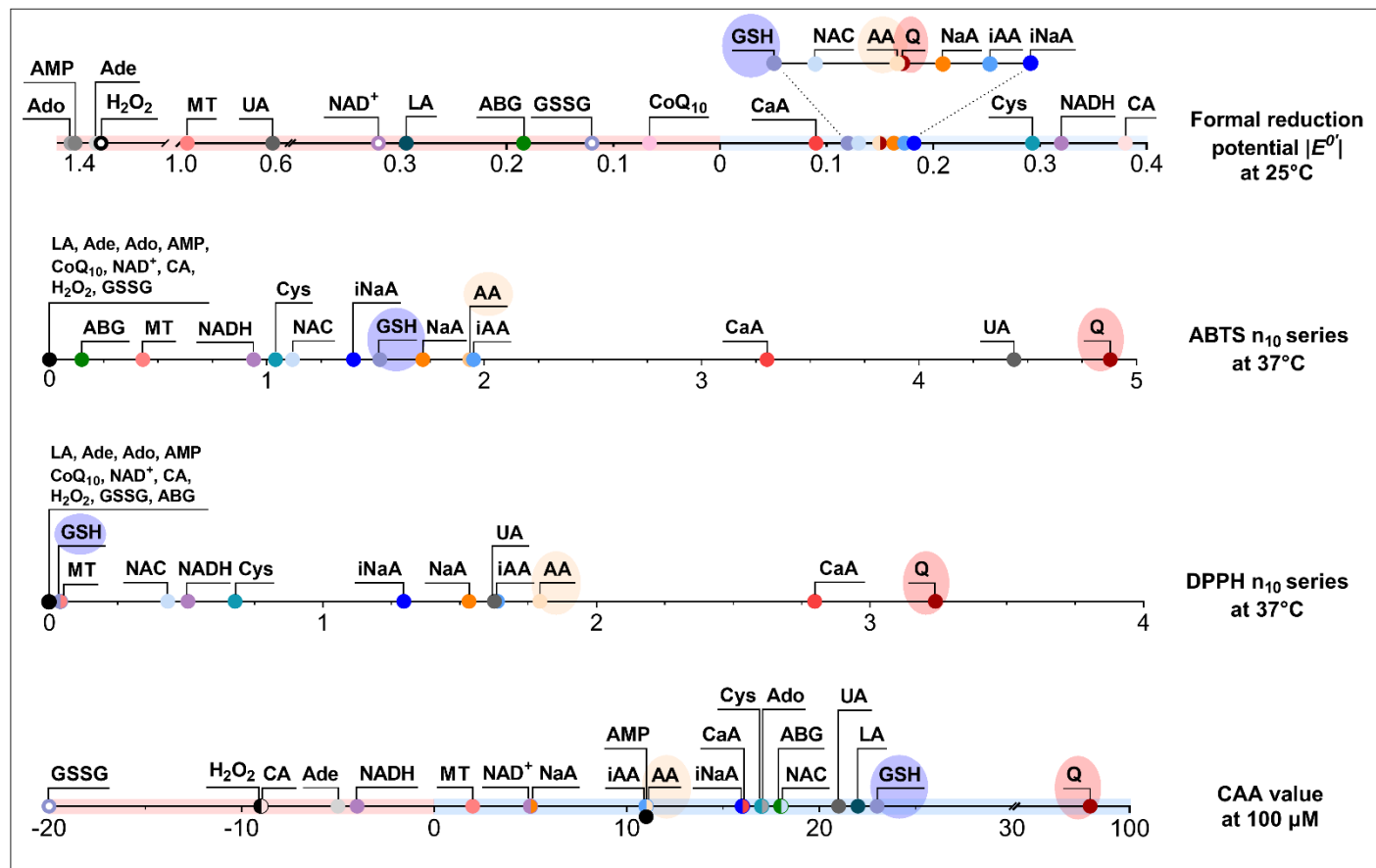


Figure 4.42. Axes comparing redox properties of investigated redox-active compounds ordered according to: formal reduction potential (E^0 [V], shown as absolute values); n_{10} values calculated based on ABTS and DPPH tests; the cellular antioxidant activity (CAA) values, measured in cells cultured in complete medium and treated with $100 \mu\text{M}$ concentration of investigated compounds. Colour highlights along axes mark reducing (blue) and oxidizing (red) ranges of compounds' activity. Glutathione (GSH) and quercetin (Q) are highlighted respectively as representatives of endogenous and exogenous antioxidants, whereas L-ascorbic acid (AA) is highlighted as a representative of exogenous antioxidant required for proper physiological functions.

As can be easily seen in Figure 4.42, majority of the compounds characterized by high $E^{0'}$ values were also too poor radical scavengers to give any response in chemical tests. Therefore, as shown in Figure 4.43A and B, r^2 value calculated for the linear regression line that describes the relationship between $E^{0'}$ and n_{10} series illustrating the ability to scavenge ABTS or DPPH radicals is extremely low. The reactivity of studied compounds towards these two synthetic radicals was not identical, nevertheless the results of both chemical tests show strong interdependence ($r^2 = 0.82$, Figure 4.43D). In both chemical tests, Q turned out to be the strongest antioxidant, followed closely by UA and CaA. Much weaker, however still satisfactory radical scavenging activity in cell-free system was exhibited by the group of AA derivatives, which confirms that indeed AA derivatives are correctly used in industry to prevent oxidative spoilage of foods.

When only reference compounds and AA are taken into account, the order of scavenging activity, which is as follows: GSH < AA < Q, is in line with their electrochemical properties. It can be presumed that other polyphenols could be even better radical scavengers than Q representing this group of phytochemicals, which was the most effective radical scavenger in chemical tests. The scavenging activity of AA derivatives was higher than that of thiol antioxidants (including GSH) and NADH; the latter being the only efficient radical scavenger among tested mitochondrial redox-active agents. The redox-active compounds that exhibited activity in chemical tests were better scavengers of ABTS radical than of DPPH radical.

In accord with the results of chemical tests, Q turned out to also exhibit the strongest antioxidant activity in the cellular setting. This polyphenol profoundly surpassed the impact on reducing capacity of cells exhibited by any other investigated compound. Q was stronger than three most important endogenous antioxidants – GSH, LA and UA. CAA values determined for the latter compounds at 100 μM concentration were almost 5 times lower. Such vast differences in antioxidant activity between endogenous compounds and Q were not observed in chemical assays. Interestingly, electrochemical properties of LA and the lack of ability to scavenge ABTS and DPPH radicals pointed to its rather oxidizing activity. Thus, it can be presumed that the high cellular antioxidant activity of LA might be a result of its intracellular conversion to reduced form – DHLA.

As shown in Figure 4.42, any clearcut relationship between chemical structure and cellular antioxidant activity cannot be found; the already mentioned four antioxidants are stronger than other thiol antioxidants, and both Ado as well as AA derivatives, all of which exhibited almost the same impact on reducing capacity of HT29 cells. Two mitochondrial redox-active agents – NAD^+ and MT – barely had any impact on CAA values. NAD^+ and NADH affected reducing capacity of cells in opposite way; NAD^+ turned out to be slightly reducing while NADH – slightly pro-oxidizing. In the light of what has been already described in the recapitulation of results for mitochondrial redox active agents (section 4.3.3.5), it seems electrochemically justified that NADH is maintained in its reduced form only at the proper NAD^+/NADH ratio; otherwise NADH overabundance might lead to reductive stress and cause disruption of cellular redox signalling network. The slightly pro-oxidative effects suggested by CAA values determined for Ade, CA and



H₂O₂ seem to reflect their chemical behaviour characterized by the lack of ability to scavenge ABTS and DPPH radicals as well as high $E^{0'}$ values. The only exception was CA, whose $E^{0'}$ value suggests rather strong reducing activity, however, its conversion into α -ketoglutarate with concomitant reduction of NAD⁺ requires enzymatic catalysis as already mentioned. The strongest pro-oxidant in CAA test turned out to be GSSG, which could not be predicted based on its relatively low $E^{0'}$ value. Therefore, the results of CAA test, which reflect the impact of investigated compounds on the cellular antioxidant status after their absorption by intestinal HT29 cells, seem to mirror neither $E^{0'}$ nor the results of chemical tests, at least according to CAA values obtained as a result of cell treatment with 100 μ M concentration of studied compounds. Nonetheless, as shown in Figure 4.43C, $E^{0'}$ and CAA values turned out not to be correlated at all ($r^2 = 0.04$), which implies that thermodynamics in the case of cellular oxidoreductive processes is not a decisive factor underlying biological behaviour of redox-active compounds. Much stronger linear relationships were found for CAA values and n_{10} series obtained by both ABTS and DPPH tests ($r^2 = 0.47$ and $r^2 = 0.40$, respectively; Figure 4.43E and F). In contrast to $E^{0'}$, the chemical tests involved measurements performed after fixed time of the reaction, thus they considered, to certain extent, also the kinetic aspects of radical reduction. The latter seems to have decisive impact on antioxidant activity of redox-active compounds in cellular setting. The importance of kinetics for the reducing capacity of polyphenols, e.g. Q, has been already shown in the former studies carried out in the frame of the same research project applying the same cellular models and methodology [176].

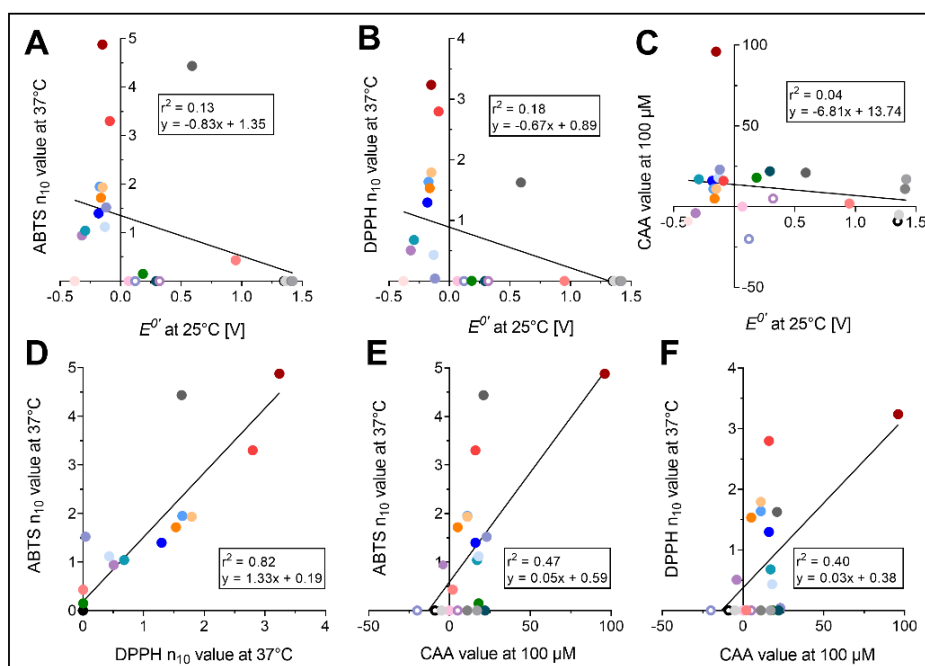


Figure 4.43. The relationships between the studied redox properties of investigated redox-active compounds. Correlations between formal reduction potential ($E^{0'}$) vs. n_{10} values calculated based on ABTS or DPPH tests – **A** and **B**, respectively; $E^{0'}$ vs. cellular antioxidant activity (CAA) values – **C**; n_{10} values calculated based on ABTS vs. DPPH tests – **D**; CAA values vs. n_{10} values calculated based on ABTS or DPPH tests – **E** and **F**, respectively.



To summarise the redox-related part of this study, none of the examined endogenous redox-active compounds was even close to display as strong antioxidant activity as that of Q – a polyphenol found ubiquitously in dietary sources. A major endogenous antioxidant – GSH, well recognized as “one of the most powerful antioxidants” may be powerful in terms of the multitude of its functions, which ensure the maintenance of redox homeostasis, however, it turned out to be a rather moderate reducing agent according to the results of applied chemical and biological tests. In terms of thermodynamic behaviour, AA – human exogenous antioxidant required for proper physiological function – was equally effective electron donor as Q, and stronger than GSH. However, such favourable reducing properties of AA were not reflected in the cellular setting, where it turned out to be a relatively weak antioxidant compared to the reference compounds. The most striking picture coming out of these results is that most of endogenous antioxidants display rather weak reducing activity, especially when compared to Q. However, as discussed in the previous recapitulations dedicated to individual studied groups of redox-active compounds, in most cases the impact of low concentrations of Q (not exceeding 10 μM) on reducing capacity of cells did not significantly differ from other investigated compounds. This observation suggests that such low concentrations of Q, achievable in the bloodstream, did not affect redox homeostasis of HT29 cells. Only in the case of higher concentrations, achievable in the GI tract, this polyphenol enhanced the cellular antioxidant barrier substantially more effectively than endogenous redox-active compounds or vitamin C derivatives. Similar dose-dependence was shown in the same cellular model after treatment with catechins, whose low concentrations were not able to override cellular redox buffering capacity, and concentrations higher than 10 μM were necessary to bring about substantial enhancement of cellular antioxidant status [263].

On top of typical determinations of antioxidant activity, the impact of investigated redox-active compounds on cell growth was applied as one of the indicators of cellular redox status since initiation of proliferation is dependent on ROS signalling [448]. However, cell growth may be also inhibited in the situation of ROS abundance, which is characteristic feature of cells with high rate of metabolism e.g. cancer cells [449]. Overall, most of the studied redox-active compounds did not show any notable impact on cell growth with a few exceptions that are described below. Stimulation of cell growth observed after treatment with low to moderate concentrations of NAC, ABG and iNaA, all of which displayed similar impact on reducing capacity of cells, suggest that these compounds might have alleviated oxidative stress, which is an inherent feature of metabolism of HT29 cells employed in this research. In the case of Ade, the observed stimulation of cell growth might be a result of its use as a substrate for DNA replication rather than its impact on cellular redox homeostasis, since Ade occurred to display rather pro-oxidative activity. Moreover, enhanced proliferation of cells generates excessive ROS, which might have been reflected by pro-oxidative CAA values of Ade. Similar impact on cell growth may be expected in the case of Ado and AMP, as both of them may serve as precursors of DNA biosynthesis. Indeed, Ado also stimulated cell growth and exhibited weak antioxidant impact on cellular antioxidant activity, which was almost equal to that of already mentioned NAC, ABG and



iNaA. The last Ade derivative – AMP – also enhanced cell growth, however, its antioxidative effects were slightly weaker than those displayed by Ado.

Inhibition of cell growth observed after prolonged treatment with high concentrations of the reducing agents (e.g. millimolar concentrations of GSH, Cys and AA derivatives as well as high micromolar concentrations of LA) might be a consequence of disrupted redox signalling caused by too high antioxidant load, which pushed cells towards reductive stress. Such inhibitory effects were also observed in the same cellular model after exposure of cells to high concentrations of catechins [263]. On the other hand, extreme decrease in cell growth caused by high concentrations of endogenous pro-oxidants (GSSG, H₂O₂) is a well-known phenomenon that might have been caused by exacerbated oxidative stress leading to apoptosis and cell death [450]. Finally, high concentrations of both NADH and NAD⁺ also led to significant inhibition of cell growth. The adequate ratio of these compounds is required for proper function of metabolic enzymes, thus excessive amounts of either reduced or oxidized form of NAD disrupt not only redox homeostasis but also impair vital cellular metabolic pathways [395,451].

In the situation of additional oxidative challenge, it turned out that hardly any of investigated redox-active compounds provided protection against H₂O₂-induced DNA fragmentation in comet assay. The only exceptions were Q and LA applied to HT29 cells at high, 100 µM concentration. In the case of Q, the observed protection could be explained by its strong antioxidant activity proven in a number of assays included in this thesis. Protective effects caused by LA might have resulted from its intracellular conversion to DHLA, which is characterized by low *E*⁰ indicating its strong reducing properties comparable to those of Cys and NADH [265,312]. However, since thermodynamics of oxidoreductive processes did not appear to be a decisive factor determining biological activity of the studied redox-active compounds, such hypothesis would require further investigation. The opposite effects were observed upon treatment of cells with high concentrations of C-vitamins, *i.e.* 200 µM AA as well as 100 µM NaA and CaA, which aggravated the level of DNA fragmentation following the genotoxic treatment with H₂O₂. The increased level of DNA fragmentation in cells pre-treated with ascorbates and then exposed to H₂O₂ was probably a result of Fenton reaction. It is well documented that AA reduces Fe(III) to Fe(II), which can subsequently react with either molecular oxygen or H₂O₂ and generate O₂^{•-} or HO[•], respectively. The latter molecule is a strong genotoxic agent, while O₂^{•-} is known as a source of other ROS, including H₂O₂, which only aggravates generation of HO[•] and its damaging effects to biomolecules such as DNA.

The general conclusion that may be drawn based on the presented results is that endogenous redox-active compounds can modulate cellular redox homeostasis via different mechanisms, which are not necessarily electrochemically determined. However, their impact on antioxidative cellular barrier does not seem sufficient to protect cells against ROS damage in case of the occurrence of oxidative stress.



5. DISCUSSION

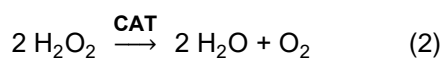
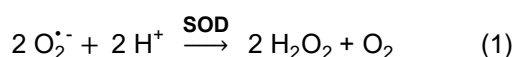
Oxidative stress, understood as a disturbance of organism's homeostasis due to overabundance of reactive oxygen species (ROS), is known to be implicated in the development of numerous diseases such as diabetes, obesity, cardiovascular and neurodegenerative diseases as well as cancer. Whether oxidative stress is a cause or a consequence of mentioned diseases, so far has not been fully understood. This is because the knowledge on the role of ROS as well as other related reactive species is still evolving. Initially, ROS were recognized solely as deleterious oxidizing agents, which when in excess may lead to intracellular damage of biomolecules, while antioxidants – as ROS scavengers – seemed to be perfect remedies preventing the detrimental effects of ROS. Indeed, diets rich in antioxidants have been well-known to be associated with long-term beneficial health effects and longevity [234–237,239,452]. On the grounds of promising results of epidemiological studies, the subject of antioxidants has been extensively investigated in order to find efficient therapeutic strategies that could be implemented in the treatment of diseases, in the pathogenesis of which oxidative stress has been known to occur. However, the long-term beneficial effects of antioxidant-rich foods consumption mostly failed to be replicated in clinical trials when antioxidants (mainly vitamin A, C, E and β -carotene) were administered to study participants in a form of isolated compounds [177,188,225,232,242–244]. These negative results made some researchers become sceptical if extra doses of antioxidants, at least in a form of dietary supplements, can provide any real health benefits. Later, an interest in antioxidant vitamins was replaced by the research on polyphenols that are the most powerful plant antioxidants. Their therapeutic application also turned out to be challenging due to limited bioavailability; plasma concentrations of polyphenols reach at most a low micromolar range [453]. As a consequence, clinical effectiveness of antioxidants, especially those that are of plant origin, has become also often questioned since most of promising data, majority of which was derived from *in vitro* studies, were obtained for high, physiologically irrelevant doses. However, as correctly pointed by Halliwell *et al.* [232,454], such high concentrations of phytochemicals could be reached in the gastrointestinal tract, where these compounds indeed might display their health-promoting effects.

Several issues, which could have contributed to this disappointing ineffectiveness of dietary antioxidants were discussed in detail in the Introduction, section 1.4. (subsection entitled “Efficacy of antioxidants”). In short, firstly, administration of antioxidant nutrients, such as vitamin A, C, E and β -carotene, may be expected to be effective when their deficiency is diagnosed, however, not necessarily in well-nourished populations. Secondly, the assumption that antioxidants would delay the occurrence of diseases if taken in high doses was proven to be either ineffective or even harmful [227,246]. Lastly, supplementation of study participants with mixtures of isolated dietary antioxidants, believed to exert additive or synergistic effects, also ended up with disappointing results [5,6]. To make things worse, the research approach implemented in those investigations did not provide any chemical, cellular or molecular data that could constitute the convincing evidence explaining why purified antioxidants failed to provide meaningful health

benefits despite such promising results of preclinical studies. Research conducted in the framework of this thesis was aimed at identifying chemico-biological determinants that might shed some light on the reasons of this discrepancy.

In 2018, Davies and Holt, with the aid of modelling techniques (based on Gillespie's Stochastic Simulation Algorithm and Markov chain), demonstrated that low-molecular-weight antioxidants are not likely to be effective in oxidative damage prevention when compared to enzymatic endogenous antioxidants [184]. Indeed, reaction rate constants for enzymatic antioxidants are incomparably higher than those determined for non-enzymatic reducing agents [455]. For example, a constant rate of superoxide radical ($O_2^{\bullet-}$) dismutation catalysed by superoxide dismutase (SOD) is $\sim 2 \times 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$, which is considered to be the fastest known enzymatic reaction [186]. In contrast, reaction rate constants of redox reactions between ascorbate or α -tocopherol and $O_2^{\bullet-}$ are $3.3 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ and $4.9 \times 10^3 \text{ M}^{-1}\cdot\text{s}^{-1}$, respectively [456]. Even lower rate constants of $O_2^{\bullet-}$ reduction were determined for thiol antioxidants (N-acetyl-L-cysteine, L-cysteine, glutathione), which turned out to be up to seven orders of magnitude lower compared to SOD-catalysed reaction [285]. However paradoxically, in spite of high velocity of enzymatic reactions, endogenous antioxidant systems, apparently are insufficient to prevent the occurrence of oxidative damage *in vivo* [183,457]. Otherwise, there would be no oxidative damage to proteins or DNA occurring, since any ROS would be immediately neutralized before it could do any harm. This is however not the case; oxidative damage to biomolecules is omnipresent [457].

There are two main limitations of antioxidant enzymes that explain their ineffectiveness in protection against oxidative stress. Firstly, antioxidant enzymes as any enzymes are substrate specific, thus they are able to neutralize only specific types of ROS. The multitude of ROS occurring in organisms exceeds the diversity of antioxidant enzymes, at least to the best of current knowledge. Secondly, even the important and quickest enzymes neutralizing ROS, often catalyse reactions that convert one specific type of ROS into another. Reactions catalysed by SOD and CAT, (1) and (2) respectively, may serve as perfect examples:



Dismutation of $O_2^{\bullet-}$ catalysed by SOD gives rise to less reactive H_2O_2 , which nevertheless is still another reactive form of oxygen. H_2O_2 can be further converted by CAT into even less reactive triplet oxygen (3O_2) and water. Unfortunately, 3O_2 easily undergoes either one-electron reduction to $O_2^{\bullet-}$, or excitation to singlet oxygen (1O_2) as a result of e.g. UV light exposure [458]. Thus, antioxidant enzymes, despite high reaction rate constants of the processes catalysed by them, are not able to fully eliminate ROS. Their activity still generates other, only less reactive types of ROS. At this point, it seems reasonable to conclude that systems alleviating oxidative challenges require more complex, multi-level solutions to ensure truly effective antioxidant protection.

Indeed, antioxidant enzymes are supported by endogenous systems of protection against oxidative stress that include also low-molecular-weight compounds, such as GSH. Depending on

tissue, intracellular concentrations of GSH are maintained within millimolar range (up to 10 mM), which reflects the importance of this “redox buffer” [206]. The fundamental role of GSH is to act as an antioxidant. This redox-active thiol can either directly scavenge ROS or be used as a substrate by enzymatic antioxidants such as glutathione peroxidases (GPx). GSH is the main, but not the only endogenous antioxidant. There is an array of other redox-active compounds, which perform their functions through different mechanisms. As an example may serve α -lipoic acid (LA), which is thermodynamically stronger reducing agent than GSH, thus it is able to recycle the oxidized GSSG back to its reduced, functional form. Uric acid (UA) contributes to 2/3 of plasma reducing capacity as well as provides efficient protection against lipid peroxidation in human blood, however, only in the presence of ascorbic acid and other thiols [335,336]. Another important redox-active compound is coenzyme Q₁₀ (CoQ₁₀), which is the only endogenous lipophilic antioxidant in humans. It is anchored in cellular membranes, where it can protect phospholipid bilayer from lipid peroxidation [338,339]. Besides, CoQ₁₀ was shown to exhibit the ability to restore reduced forms of α -tocopherol and ascorbate from α -tocopheroxyl and ascorbyl radicals, respectively [344], [345]. L-Ascorbic acid (AA) may also serve as an example of endogenously relevant antioxidant, even though it is food-borne exogenous compound, at least in the case of humans, yet it performs indispensable physiological functions. Hence, the network of endogenous antioxidants is varied in terms of chemical structures, their physicochemical properties and mechanisms of action. Nonetheless, the question how effective antioxidant barrier is built by endogenous antioxidants, including cooperation with antioxidant vitamins, has not been answered so far.

As it has been already described in detail in the “Framework and scope of the research” (section 4.1.), former results from our lab showed that GSH, a major endogenous antioxidant, turned out to be a rather weak cellular antioxidant *per se* and that it relies also on inducible endogenous antioxidant systems to ensure the protection against oxidative stress as demonstrated by transcriptomic analysis [263]. In contrast, exogenous antioxidants such as polyphenols, provide protection against oxidative stress rather by direct ROS scavenging, without the necessity of involving additional cellular antioxidant defense systems [176]. The aim of this thesis was to investigate whether endogenous antioxidants are capable of efficient maintenance of redox balance without support from exogenous dietary antioxidants, when cells are challenged by oxidative stress. This goal was addressed by finding out how strong is the impact of endogenous antioxidants applied exogenously as if they were derived from dietary sources, on the cellular antioxidant capacity and other markers of protection against oxidative stress. Twenty compounds studied represented five differing in biological function groups of redox-active agents: thiol antioxidants contributing to the maintenance of proper thiol-redox balance (N-acetyl-L-cysteine, L-cysteine, α -lipoic acid); purine derivatives involved among others in cellular bioenergetics (adenine, adenosine, adenosine-5'-monophosphate, uric acid); mitochondrial redox-active agents required for proper course of metabolic processes (coenzyme Q₁₀, melatonin, nicotinamide adenine dinucleotide reduced and oxidized, citric acid); endogenous pro-oxidants formed as a result of ROS neutralization (glutathione disulphide, hydrogen peroxide) and already



described ascorbic acid derivatives including C-vitamins (L-ascorbic acid, D-isoascorbic acid, sodium L-ascorbate, calcium ascorbate, sodium D-isoascorbate, ascorbigen).

The research was conducted using two different methodological approaches: chemical and biological. Chemical methods assessing the antioxidant activity included two spectrophotometric tests applying ABTS and DPPH synthetic radicals. Results of these tests were expressed as n_{10} values, which can be regarded as a parameter that takes into account also reduction reaction kinetics. Biological approach included determination of the impact of investigated redox-active compounds on the reducing capacity of human colon adenocarcinoma HT29 cells, which served as a model of gastrointestinal tract, where intestinal cells are in direct contact with ingested food. This aspect was investigated with the aid of Cellular Antioxidant Activity (CAA) test, which is recognized as superior to already mentioned cell-free spectrophotometric antioxidant activity assays, since CAA test takes into account the uptake of compounds by cells and their cellular metabolism. Moreover, the impact of studied redox-active compounds on cellular redox status was investigated by the assessment of how they affect cell growth as well as by the determination of their ability to protect DNA from oxidative damage. Finally, the results of antioxidant activity, assessed with the aid of chemical and biological tests, were discussed in the light of their previously established thermodynamic properties described by formal reduction potential $E^{0'}$.

Since protection provided solely by enzymatic endogenous antioxidants is not sufficient to avoid oxidative damage to biomolecules, antioxidant enzymes need to be supported by other endogenous low-molecular-weight reducing agents. As already mentioned, owing to the multitude of its functions, GSH plays a role of the major "redox buffer", however, according to the results of applied chemical and biological tests, this antioxidant turned out to be at most a moderate reducing agent, despite the additional support from a variety of induced redox-related genes [263]. The concerted action of GSH and other endogenous antioxidants appears to be sufficient to maintain cellular redox homeostasis under eustress. The question to be answered was whether under conditions of oxidative stress, this intracellular antioxidant barrier would be able to maintain proper redox balance and to prevent oxidative damage to biomolecules.

One of the fundamental aspects defining oxidoreductive properties of redox-active compounds is electrochemistry. Formal reduction potential $E^{0'}$ is an electrochemical parameter, which describes the tendency of a compound to accept electrons. As such, the higher the $E^{0'}$ values, the more likely is a compound to become reduced, which is a feature of strong oxidants. By analogy, the more negative $E^{0'}$ values, which reflect better electron donation, the stronger reducing agents. Electrochemical data, which were either determined by Klaudia Suliborska, PhD, or available in literature, were used to order biologically relevant redox-active compounds from the strongest oxidants to the strongest antioxidants, as presented in Figure 5.1A. Most common oxidants and reactive oxygen species were also included to reflect the oxidative challenges, to which cells (and organisms) are constantly exposed. As it can be presumed based on the difference between the $E^{0'}$ values for possible oxidant/antioxidant pairs, most of the listed



oxidizing agents are capable to react with, and thus to oxidize the investigated reducing agents, regardless of whether they are of endogenous or exogenous origin.

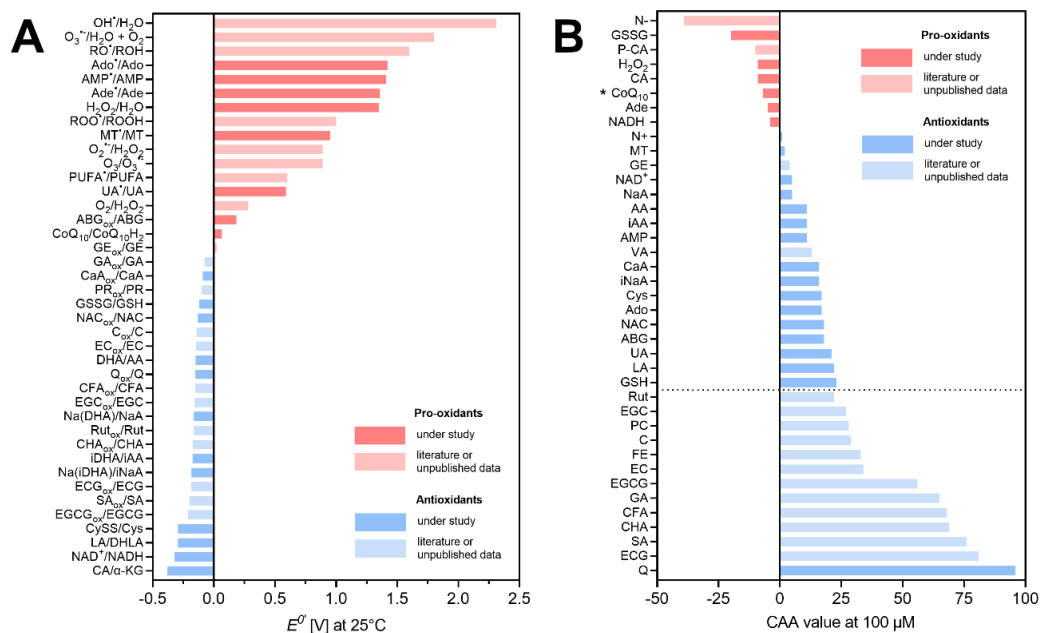


Figure 5.1. The comparison of oxidoreductive properties of the most important antioxidants and pro-oxidants that affect cellular redox homeostasis ordered according to formal reduction potential $E^{0'}$ – **A**, or ordered according to their impact on cellular antioxidant activity in HT29 cells exposed to 100 μ M concentration – **B**. CoQ₁₀ is marked with an asterisk (*) since this was the only compound that could not be tested at 100 μ M concentration, thus its CAA value on the figure corresponds to its highest tested concentration – 5 μ M. The $E^{0'}$ data are either borrowed from [459], or were kindly provided by Suliborska from her doctoral dissertation [264]. References for compounds under study are reported in Table 4.1 in 4.1. section of Results. Dotted line marks the borderline between the strongest endogenous antioxidant – glutathione (GSH) and dietary polyphenols exhibiting strong antioxidant activity.

For example, HO[•] known to be the most reactive molecule among ROS, is thermodynamically 6 times stronger oxidant than the strongest reductant studied – CA, an intermediate formed in the course of metabolic and bioenergetic processes. The second strongest reducing agent, in terms of thermodynamics, is NADH, an important coenzyme indispensable in numerous catabolic reactions. Taking into account biological importance of the two mentioned above compounds for the maintenance of vital cellular functions such as catabolism providing substrates and energy ensuring cell growth, these reducing agents are not very likely to act in the front line of antioxidant defense system. Especially, that in the reaction with strong oxidants, such as HO[•], they could be easily not only oxidized, but even degraded. This could limit their availability and lead to the serious impairment of cellular bioenergetic capacity [460].

Another issue revealed by the present research on endogenous redox-active compounds is the discrepancy between $E^{0'}$ values and the actual antioxidant status measured in cells exposed to them. As has been already published as well as confirmed by Pearson's coefficients

calculated for this research results (Table 5.1), thermodynamics not necessarily reflects the antioxidant behaviour of studied compounds under cellular conditions since the correlation between CAA and $E^{0'}$ values is extremely poor. The kinetics – thus velocity – of ROS neutralization seems to play a decisive role under cellular setting, as demonstrated also in earlier investigations [176].

Table 5.1. Pearson's coefficients showing correlations between parameters describing redox properties ($E^{0'}$, formal reduction potential at 25°C; n_{10} series calculated based on results of ABTS and DPPH tests; the cellular antioxidant activity (CAA) values) of investigated redox-active compounds.

	$E^{0'}$ [V]	ABTS n_{10} values	DPPH n_{10} values	CAA 100 μ M
$E^{0'}$ [V]		-0.355	-0.419	-0.191
ABTS n_{10} values	-0.355		0.904	0.686
DPPH n_{10} values	-0.419	0.904		0.624
CAA 100 μ M	-0.191	0.686	0.624	

Figure 5.1B presents CAA values established for the compounds either investigated in this thesis, these are marked with the more intense colour, or studied by Monika Baranowska, PhD [176,263 & unpublished data]. The CAA values determined in this study for endogenous redox-active compounds compared to those previously established for different dietary polyphenols suggest that the former are rather weak antioxidants, at least at 100 μ M concentration (Figure 5.1B). For example, GSH displayed the strongest impact on reducing capacity of HT29 cells among endogenous antioxidants (CAA value = 23), whereas cell treatment with all tested catechins, quercetin as well as some phenolic acids resulted in CAA values higher by from 17% (EGC) up to 400% (Q). Moreover, in the current study none of the endogenous antioxidants – with the sole exception of α -lipoic acid at non-physiological concentration 100 μ M – was able to prevent DNA fragmentation induced by H_2O_2 , a medium strength ROS judging by $E^{0'}$ established for H_2O_2/H_2O redox pair [459]. In the case of ascorbic acid derivatives, considered as vital physiological antioxidants, those with vitamin C activity (*i.e.* AA, CaA and NaA) instead of preventing DNA fragmentation, led to its increased levels. It follows that endogenous antioxidants may fail to ensure sufficient antioxidant barrier against such strong oxygen radicals as HO^\bullet or lipid radicals characterized by very high $E^{0'}$ values (CAA values are not available for these radicals so they could not be used for comparison). This implies that the efficient multi-level protection against oxidative stress may require the cooperation of endogenous antioxidants with those of dietary origin, whose impact on reducing capacity of HT29 cells was significantly greater.

In contrast, strong antioxidant activity displayed by exogenous dietary reductants (Figure 5.1B) suggests that they could play a role of “the first line of antioxidant defense”, at least in terms of direct, efficient ROS scavenging. The potential oxidative degradation of exogenous antioxidants as a consequence of reaction with strong oxidants would not affect any vital cellular functions, as it could be the case with *e.g.* NADH or antioxidant vitamins [462]. Indeed, the notion that endogenous antioxidant systems need support from dietary antioxidants has been already suggested by other researchers [188,197,412]. Nevertheless, so far no systematic investigations



have been performed to explain which chemical properties and/or biological effects make the difference. It is also important to emphasize in this context that concentration of an antioxidant is not decisive when it comes to direct reaction with oxidants. More important is the difference between their reduction potentials. However, as have been already pointed, the reduction of oxidant must be quick enough to prevent alternative reactions such as oxidation of other cellular molecules or detoxification of the exogenous antioxidants. Thus, the interplay between thermodynamic and kinetic factors decides about the effectiveness of the antioxidant. Therefore, although GSH can reach millimolar range inside cells or relatively high plasma concentrations of vitamin C or uric acid [414,461,462], the significantly lower bioavailability of polyphenols may still be sufficient to ensure their better antioxidant effectiveness [453]. Taking into account that endogenous antioxidants display significantly weaker reducing activity compared to exogenous antioxidants, the limited bioavailability of the latter may still secure proper cellular redox homeostasis. Otherwise, high concentrations of such powerful plant-derived antioxidant xenobiotics could impair redox balance of cells towards reductive stress [263].

To conclude, the obtained results of chemical and biological assays show that endogenous antioxidants are not sufficient to provide cells with effective protection against oxidative stress. This implies that it is necessary to support endogenous antioxidant barrier with the exogenous dietary antioxidants *e.g.* of plant origin. In the view of constant exposure to ubiquitous oxidants and other reactive species, the growing frequency of oxidative stress-related diseases as well as the necessity of antioxidant protection, it might be worth to return to the once coined term “phytamins” to emphasize nutritional importance of food-derived antioxidants required for support and thus efficient function of endogenous antioxidant systems.



6. SUMMARY

Endogenous antioxidants are recognized as the body's first line of defense against excessive amounts of pro-oxidants, which may disrupt redox homeostasis, and thus induce oxidative stress. However, it has been suggested that these physiological reducing agents may not be sufficient to provide protection against excessive oxidative challenge, thus they may need the support of exogenous antioxidants such as polyphenols, which are commonly found in foods and beverages. The former studies have shown that catechins, belonging to the group of polyphenols, when applied at high concentrations, *i.e.* matching those in edible plants, display strong impact on reducing capacity of HT29 cells applied as *in vitro* cellular model of the alimentary tract that is in direct contact with ingested food. On the contrary, exogenously applied glutathione (GSH) turned out to have significantly weaker impact on reducing capacity of HT29 cells. However, the differences that were observed at the genomic level between catechins and GSH has given a hint on the mechanisms of antioxidant action. Catechins had barely any effect on the antioxidant gene expression, whereas GSH significantly affected the expression of a broad pool of redox-related genes. These results led to the formulation of the hypothesis that endogenous antioxidants, being chemically weaker reductants, rely more on endogenous antioxidant system than stronger plant-derived dietary antioxidants. The aim of the current research was to verify the above hypothesis by determining the impact of 20 redox-active compounds, which can be produced endogenously, but also provided with food, on redox homeostasis in the above mentioned cellular model of the gastrointestinal tract. The activities investigated for the tested compounds were compared to those exhibited by two selected reference compounds: GSH, a representative of endogenous antioxidants, and quercetin (Q), recognized as a strong reducing polyphenol, thus it served as a representative of exogenous antioxidants.

The evaluation of the antioxidant properties of the tested compounds was carried out with the use of chemical and biological methods. The chemical methods included two spectrophotometric tests using ABTS and DPPH radicals, which are commonly used in determining the antioxidant activity of food and drink samples. The biological method for assessing the antioxidant activity of the tested compounds was CAA (Cellular Antioxidant Activity) test performed with the use of HT29 cell line, a cellular model of the gastrointestinal tract in *in vitro* studies. The influence of both the investigated redox-active and reference compounds on redox homeostasis was carried out by assessing the cell growth, which is inherently related to ROS signalling, with the aid of the MTT test. The ability of tested compounds to provide protection against DNA fragmentation induced by treatment of cells with genotoxic concentration of H₂O₂ was determined by comet test. The obtained results are discussed in relation to the electrochemical properties of the investigated redox-active compounds.



The results obtained in this thesis showed that:

- Neither electrochemical properties nor chemical structures of investigated redox-active compounds may be regarded as predictors of antioxidant activity in the cellular setting. This suggests that the mechanism underlying redox behaviour of endogenous antioxidants is not based solely of their tendency to donate electrons as was also previously observed for plant polyphenols.
- In contrast to formal reduction potentials, the results of chemical tests employing ABTS and DPPH radicals expressed as n_{10} values occurred to be well correlated with values of cellular antioxidant activity. Since n_{10} values are calculated based on measurements taken after fixed reaction time, they reflect also the kinetic aspects of radical reduction, the velocity of redox reaction seems to play a decisive role how the redox-active compounds will affect cellular redox homeostasis.
- According to the results of applied chemical and biological tests, a major endogenous antioxidant – GSH, turned out to be a rather moderate reducing agent, however, it was still the strongest among investigated endogenous low-molecular-weight antioxidants.
- The investigated endogenous redox-active compounds displayed significantly weaker antioxidant activity than exogenous polyphenol – Q, in both chemical and biological assays.
- In general, most of the studied redox-active compounds did not show any notable impact on cell growth with a few exceptions. The inhibition of cell growth observed after prolonged treatment with high concentrations of the reducing agents could be a result of reductive stress occurring as a consequence of disrupted redox signalling caused by too high antioxidant load.
- In the situation of additional oxidative challenge, the investigated endogenous redox-active compounds did not provide protection against H_2O_2 -induced DNA fragmentation. The only exceptions was α -lipoic acid applied to HT29 cells at high (100 μ M) concentration. The same effect was observed also in the case of a reference exogenous antioxidant – Q.
- The obtained results suggest that the endogenous system of antioxidant protection of the organism is not sufficient to provide effective defense against oxidative stress, therefore it seems necessary to provide plant-derived exogenous antioxidants, *i.e.* polyphenols with daily diet. Based on these results it might be worth considering the implementation of the term “phytamins” to reflect the necessity of consumption of plant-derived antioxidants required for efficient function of endogenous antioxidant systems

The novelty of the presented research lies in that it provides chemical and biological evidence that differentiates redox behaviour of endogenous redox-active compounds versus apparently stronger exogenous plant-derived polyphenols. So far, no such systematic investigations evaluating the antioxidant activity, in terms of chemical properties and biological effects, for a broad range of redox-active compounds have been performed. Moreover, these investigations were conducted with the use of unified toolbox of methods enabling direct comparison of the obtained data and relating them to former studies. The results showed that

kinetics of redox reactions is an important factor underlying reducing behaviour of antioxidants in the cellular setting, while such a possibility was verified negatively in the case of thermodynamic properties of investigated compounds. In terms of thermodynamics, endogenous antioxidants turned out to be relatively weak reducing agents to confront known oxidants, to which cells may be exposed. This observation, in the light of the rather weak impact of endogenous antioxidants versus strong effects of polyphenols on cellular antioxidant activity supports the notion that cooperation of both endogenous and exogenous antioxidants is necessary to ensure proper protection against oxidative challenges.

7. REFERENCES

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Appendix A

I. Spectrophotometric assays

Table A1. Stoichiometric values n_{10} calculated based on the results of DPPH and ABTS assays carried out in triplicate at 25, 37 and 41 °C.

Compound	Stoichiometric value n_{10}					
	ABTS test			DPPH test		
	25 °C	37 °C	41 °C	25 °C	37 °C	41 °C
NAC	1.153 ± 0.032	1.118 ± 0.040	1.137 ± 0.033	0.433 ± 0.025	0.371 ± 0.021	0.428 ± 0.029
Cys	0.992 ± 0.027	1.040 ± 0.043	0.999 ± 0.053	0.679 ± 0.037	0.742 ± 0.038	0.696 ± 0.028
LA	0	0	0	0	0	0
Ade	0	0	0	0	0	0
Ado	0	0	0	0	0	0
AMP	0	0	0	0	0	0
UA	4.437 ± 0.153	4.353 ± 0.139	4.178 ± 0.094	1.627 ± 0.056	1.637 ± 0.083	1.717 ± 0.119
CoQ ₁₀	0	0	0	0	0	0
MT	0.330 ± 0.011	0.430 ± 0.025	0.430 ± 0.017	-	0.002 ± 0.000	-
CA	0	0	0	0	0	0
NADH	0.938 ± 0.033	0.940 ± 0.028	0.973 ± 0.030	0.656 ± 0.045	0.506 ± 0.062	0.618 ± 0.057
NAD ⁺	0	0	0	0	0	0
GSSG	0	0	0	0	0	0
H ₂ O ₂	0	0	0	0	0	0
AA	1.805 ± 0.069	1.934 ± 0.061	1.959 ± 0.054	1.859 ± 0.042	1.793 ± 0.034	1.763 ± 0.023
NaA	1.677 ± 0.035	1.718 ± 0.014	1.675 ± 0.027	1.551 ± 0.025	1.534 ± 0.022	1.441 ± 0.021
CaA	3.069 ± 0.109	3.301 ± 0.040	2.961 ± 0.062	2.752 ± 0.063	2.798 ± 0.040	2.785 ± 0.036
iAA	1.986 ± 0.042	1.951 ± 0.040	1.943 ± 0.043	1.606 ± 0.033	1.638 ± 0.031	1.653 ± 0.033
iNaA	1.316 ± 0.038	1.399 ± 0.031	1.435 ± 0.023	1.248 ± 0.029	1.296 ± 0.021	1.317 ± 0.023
ABG	0.146 ± 0.008	0.149 ± 0.005	0.153 ± 0.006	0.004 ± 0.000	0.004 ± 0.000	0.004 ± 0.000

II. MTT test

- Endogenous thiol antioxidants

Table A2. The impact of studied endogenous thiol antioxidants on the growth of HT29 cells determined by MTT assay after 6, 24 or 72 h treatment. Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

Concentration [μM]	Cell growth (% of control)		
	Cys		
	6 h	24 h	72 h
0.1	103.0 \pm 8.0	101.8 \pm 4.1	100.5 \pm 5.7
1	103.5 \pm 5.4	102.6 \pm 4.3	96.2 \pm 6.8
10	103.4 \pm 3.4	103.9 \pm 5.1	99.1 \pm 5.8
100	104.4 \pm 5.5	100.1 \pm 8.8	97.6 \pm 6.6
1,000	105.1 \pm 7.1	101.0 \pm 5.2	96.4 \pm 6.4
5,000	98.2 \pm 5.0	94.7 \pm 7.6	84.5 \pm 8.3
Concentration [μM]	NAC		
	6 h	24 h	72 h
	0.1	111.5 \pm 4.1	110.1 \pm 4.2
1	119.5 \pm 6.7	111.4 \pm 7.4	112.4 \pm 3.8
10	118.7 \pm 8.9	113.8 \pm 4.4	111.4 \pm 5.0
100	120.9 \pm 8.9	113.3 \pm 9.0	113.6 \pm 4.0
1,000	120.2 \pm 9.4	110.0 \pm 8.2	107.7 \pm 5.1
5,000	107.4 \pm 12.1	102.4 \pm 12.6	97.4 \pm 11.0
Concentration [μM]	LA		
	6 h	24 h	72 h
	0.1	111.1 \pm 8.3	112.2 \pm 5.0
1	107.9 \pm 7.5	111.6 \pm 6.1	104.0 \pm 8.0
10	111.7 \pm 9.9	106.7 \pm 11.3	106.3 \pm 7.1
100	108.8 \pm 9.4	112.4 \pm 7.3	102.7 \pm 6.1
250	105.4 \pm 4.5	107.3 \pm 3.4	68.9 \pm 5.0
500	107.7 \pm 7.8	105.1 \pm 6.2	28.7 \pm 6.3
1,000	106.9 \pm 9.4	101.2 \pm 10.0	17.3 \pm 2.2

- Purine derivatives

Table A3. The impact of studied purine derivatives on the growth of HT29 cells determined by MTT assay after 6, 24 or 72 h treatment. Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

Concentration [μM]	Cell growth (% of control)		
	Ade		
	6 h	24 h	72 h
0.1	125.9 \pm 14.3	109.9 \pm 10.7	102.3 \pm 7.3
1	124.9 \pm 11.0	112.7 \pm 8.1	105.3 \pm 7.3
10	125.7 \pm 14.2	111.7 \pm 8.4	104.2 \pm 7.8
100	124.5 \pm 11.2	110.8 \pm 6.5	102.1 \pm 7.5
500	121.2 \pm 11.3	108.8 \pm 7.9	94.8 \pm 7.1
Concentration [μM]	Ado		
	6 h	24 h	72 h
0.1	116.6 \pm 11.8	116.7 \pm 5.7	106.5 \pm 3.5
1	116.4 \pm 9.3	114.2 \pm 4.6	104.3 \pm 5.4
10	118.8 \pm 11.3	114.7 \pm 5.4	105.8 \pm 3.2
100	115.0 \pm 14.0	112.5 \pm 3.3	105.7 \pm 2.7
1,000	112.7 \pm 11.0	101.4 \pm 7.5	83.1 \pm 6.9
Concentration [μM]	AMP		
	6 h	24 h	72 h
0.1	113.4 \pm 7.3	114.7 \pm 4.6	109.7 \pm 5.6
1	112.7 \pm 6.0	116.4 \pm 4.8	109.6 \pm 7.1
10	112.8 \pm 6.6	115.7 \pm 5.4	109.4 \pm 6.6
100	114.6 \pm 5.8	115.4 \pm 3.5	109.7 \pm 5.8
1,000	107.6 \pm 6.2	111.5 \pm 6.2	90.9 \pm 13.6
Concentration [μM]	UA		
	6 h	24 h	72 h
10	102.5 \pm 12.5	99.0 \pm 6.6	96.5 \pm 9.5
50	99.2 \pm 6.8	98.2 \pm 4.7	97.6 \pm 5.6
100	97.9 \pm 8.3	94.7 \pm 4.0	95.8 \pm 6.2
200	95.3 \pm 8.9	95.3 \pm 6.5	96.0 \pm 5.7
300	91.8 \pm 5.7	90.4 \pm 7.3	96.4 \pm 4.7
400	94.1 \pm 9.5	85.9 \pm 7.4	85.4 \pm 5.9

- Mitochondrial redox-active agents

Table A4. The impact of studied mitochondrial redox-active agents on the growth of HT29 cells determined by MTT assay after 6, 24 or 72 h treatment. Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

Concentration [μM]	Cell growth (% of control)		
	CA		
	6 h	24 h	72 h
0.1	99.6 \pm 5.2	102.2 \pm 2.2	100.9 \pm 2.7
1	103.5 \pm 6.4	102.7 \pm 4.6	98.8 \pm 4.0
10	104.6 \pm 3.4	102.4 \pm 2.2	98.8 \pm 3.9
100	104.6 \pm 5.1	101.3 \pm 4.3	99.8 \pm 2.7
1,000	103.8 \pm 3.3	100.1 \pm 3.5	95.3 \pm 4.5
Concentration [μM]	CoQ ₁₀		
	6 h	24 h	72 h
0.01	102.8 \pm 5.0	102.2 \pm 4.1	102.3 \pm 4.0
0.1	102.1 \pm 6.1	101.8 \pm 3.2	102.3 \pm 5.8
1	101.5 \pm 5.6	103.0 \pm 3.0	105.4 \pm 3.9
2.5	102.3 \pm 5.2	99.0 \pm 7.6	102.9 \pm 6.6
5	100.6 \pm 8.5	100.6 \pm 6.1	104.5 \pm 6.6
Concentration [μM]	MT		
	6 h	24 h	72 h
0.01	103.4 \pm 8.2	107.7 \pm 9.3	101.5 \pm 4.2
0.1	105.1 \pm 8.9	109.6 \pm 9.7	102.7 \pm 4.9
1	108.8 \pm 12.3	112.3 \pm 10.6	103.9 \pm 5.5
10	106.3 \pm 14.4	112.6 \pm 9.7	103.7 \pm 4.5
100	100.4 \pm 14.6	104.7 \pm 8.3	100.6 \pm 6.5
Concentration [μM]	NADH		
	6 h	24 h	72 h
0.1	104.8 \pm 11.7	106.6 \pm 14.3	105.0 \pm 7.4
1	107.3 \pm 12.3	108.3 \pm 14.7	105.3 \pm 7.5
10	106.2 \pm 9.9	104.4 \pm 14.3	104.6 \pm 6.9
100	105.5 \pm 10.8	103.8 \pm 11.8	99.0 \pm 7.9
1,000	104.0 \pm 11.0	98.1 \pm 14.3	65.5 \pm 3.8
Concentration [μM]	NAD ⁺		
	6 h	24 h	72 h
0.1	99.6 \pm 4.9	102.7 \pm 3.3	99.5 \pm 3.9
1	99.8 \pm 5.4	101.5 \pm 3.8	98.0 \pm 5.8
10	100.8 \pm 3.7	102.0 \pm 3.8	100.5 \pm 4.4
100	92.4 \pm 7.1	94.6 \pm 6.3	88.6 \pm 4.1
1,000	94.4 \pm 4.8	95.0 \pm 7.0	63.1 \pm 4.3

- Endogenous pro-oxidants

Table A5. The impact of studied endogenous pro-oxidants on the growth of HT29 cells determined by MTT assay after 6, 24 or 72 h treatment. Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

Concentration [μM]	Cell growth (% of control)		
	GSSG		
	6 h	24 h	72 h
0.1	99.4 \pm 3.7	101.4 \pm 2.4	100.1 \pm 3.2
1	101.4 \pm 4.4	102.5 \pm 2.2	99.3 \pm 5.9
10	102.1 \pm 3.3	99.7 \pm 6.9	98.9 \pm 6.1
100	101.4 \pm 3.9	103.2 \pm 2.4	97.8 \pm 5.9
500	105.6 \pm 8.4	96.3 \pm 4.5	77.1 \pm 9.7
1,000	104.1 \pm 7.7	97.5 \pm 7.5	51.3 \pm 11.2
Concentration [μM]	H_2O_2		
	6 h	24 h	72 h
0.1	99.4 \pm 5.7	102.2 \pm 4.2	101.8 \pm 2.5
1	100.3 \pm 4.9	99.6 \pm 3.4	100.3 \pm 3.1
10	101.0 \pm 4.0	102.6 \pm 4.3	102.4 \pm 3.0
100	101.8 \pm 6.7	102.3 \pm 3.6	100.7 \pm 4.8
1,000	15.1 \pm 3.2	13.3 \pm 1.2	13.8 \pm 4.6



- Ascorbic acid and its derivatives

Table A6. The impact of studied ascorbic acid and its derivatives on the growth of HT29 cells determined by MTT assay after 6, 24 or 72 h treatment. Cell growth is expressed relative to control non-treated cells whose growth is regarded as 100%. Results are means of three independent experiments carried out in quadruplicate (SD < 15%).

Concentration [μM]	Cell growth (% of control)		
	6 h	24 h	72 h
	AA		
0.1	98.30 \pm 5.43	99.20 \pm 5.22	92.15 \pm 6.15
1	95.18 \pm 5.93	94.93 \pm 12.95	93.10 \pm 10.53
10	93.33 \pm 6.82	95.86 \pm 8.11	94.45 \pm 6.47
100	93.47 \pm 5.69	92.78 \pm 4.39	95.06 \pm 6.68
1,000	91.25 \pm 6.13	92.10 \pm 5.53	93.42 \pm 5.74
10,000	91.03 \pm 3.99	57.44 \pm 3.99	44.82 \pm 11.06
	NaA		
0.1	103.48 \pm 5.81	94.61 \pm 4.93	98.18 \pm 5.98
1	106.13 \pm 3.83	93.75 \pm 7.94	98.30 \pm 4.25
10	102.36 \pm 5.48	96.64 \pm 6.00	95.03 \pm 5.63
100	97.54 \pm 4.48	93.79 \pm 4.42	98.54 \pm 5.33
1,000	95.24 \pm 5.38	91.91 \pm 4.67	94.28 \pm 3.38
10,000	49.80 \pm 2.99	40.49 \pm 6.06	41.38 \pm 6.70
	CaA		
0.1	97.11 \pm 4.81	93.58 \pm 6.44	85.97 \pm 6.84
1	100.25 \pm 8.88	94.92 \pm 5.90	86.26 \pm 6.42
10	94.23 \pm 4.93	93.75 \pm 13.25	81.34 \pm 9.56
100	95.53 \pm 5.12	91.73 \pm 9.99	85.95 \pm 4.82
1,000	86.68 \pm 9.85	90.21 \pm 6.45	79.43 \pm 5.30
10,000	48.83 \pm 10.33	31.17 \pm 3.16	23.31 \pm 4.37
	iAA		
0.1	95.58 \pm 4.70	97.81 \pm 6.80	90.89 \pm 6.76
1	96.15 \pm 4.91	98.65 \pm 6.00	93.33 \pm 7.10
10	97.35 \pm 5.07	98.00 \pm 6.13	88.43 \pm 6.52
100	94.30 \pm 5.27	96.56 \pm 4.46	92.54 \pm 3.71
1,000	91.84 \pm 6.83	94.80 \pm 4.64	80.47 \pm 5.14
10,000	54.74 \pm 6.13	40.13 \pm 10.50	35.15 \pm 3.73
	iNaA		
0.1	116.65 \pm 14.27	124.76 \pm 8.84	103.98 \pm 9.69
1	117.34 \pm 12.59	120.42 \pm 14.32	105.59 \pm 6.41
10	115.73 \pm 13.53	117.14 \pm 11.67	104.88 \pm 9.26
100	115.87 \pm 10.43	116.46 \pm 13.86	104.09 \pm 9.41
1,000	105.76 \pm 13.79	110.78 \pm 13.67	91.94 \pm 14.76
10,000	53.16 \pm 14.71	58.34 \pm 7.15	28.71 \pm 14.02
	ABG		
0.1	102.95 \pm 8.25	110.38 \pm 7.84	109.34 \pm 5.30
1	119.23 \pm 8.93	115.32 \pm 6.68	113.00 \pm 5.27
10	112.70 \pm 7.60	120.54 \pm 6.16	113.41 \pm 8.20
100	108.40 \pm 8.11	121.95 \pm 9.56	110.87 \pm 9.29
1,000	109.24 \pm 5.30	115.90 \pm 5.29	107.38 \pm 6.72

III. CAA assay

- Endogenous thiol antioxidants

Table A7. The impact of serum starvation of HT29 cells treated with endogenous thiols on cellular antioxidant activity (CAA). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Concentration [μ M]	CAA value	
	Cys	
	0% FBS	10% FBS
0.1	20 \pm 4	4 \pm 5
1	24 \pm 6	22 \pm 6
10	20 \pm 13	23 \pm 9
100	35 \pm 5	20 \pm 4
Concentration [μ M]	NAC	
	0% FBS	10% FBS
	0.1	1 \pm 9
1	-3 \pm 9	20 \pm 11
10	-1 \pm 9	21 \pm 13
100	-7 \pm 8	18 \pm 8
Concentration [μ M]	LA	
	0% FBS	10% FBS
	0.1	14 \pm 1
1	14 \pm 14	23 \pm 7
10	8 \pm 12	20 \pm 5
100	-17 \pm 7	22 \pm 7

- Purine derivatives

Table A8. The impact of serum starvation of HT29 cells treated with purine derivatives on cellular antioxidant activity (CAA). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Concentration [μ M]	CAA value	
	Ade	
	0% FBS	10% FBS
0.1	14 \pm 9	6 \pm 8
1	31 \pm 16	2 \pm 9
10	32 \pm 8	-2 \pm 9
100	29 \pm 4	-5 \pm 3
Concentration [μ M]	Ado	
	0% FBS	10% FBS
	0.1	26 \pm 4
1	37 \pm 7	17 \pm 7
10	37 \pm 9	8 \pm 9
100	31 \pm 18	17 \pm 20
Concentration [μ M]	AMP	
	0% FBS	10% FBS
	0.1	4 \pm 12
1	8 \pm 5	27 \pm 6
10	15 \pm 8	12 \pm 7
100	22 \pm 13	11 \pm 12
Concentration [μ M]	UA	
	0% FBS	10% FBS
	50	7 \pm 6
100	4 \pm 8	21 \pm 5
200	3 \pm 10	35 \pm 5
300	28 \pm 5	19 \pm 21
400	34 \pm 9	13 \pm 17

- Mitochondrial redox-active agents

Table A9. The impact of serum starvation of HT29 cells treated with mitochondrial redox-active agents on cellular antioxidant activity (CAA). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Concentration [μ M]	CAA value	
	CA	
	0% FBS	10% FBS
0.1	1 \pm 6	7 \pm 5
1	-8 \pm 2	6 \pm 8
10	-4 \pm 4	-5 \pm 5
100	-9 \pm 17	-9 \pm 9
Concentration [μ M]	CoQ ₁₀	
	0% FBS	10% FBS
	0.1	6 \pm 6
1	0 \pm 7	-14 \pm 2
2.5	-9 \pm 7	-12 \pm 5
5	-8 \pm 8	-7 \pm 9
Concentration [μ M]	MT	
	0% FBS	10% FBS
	0.1	4 \pm 12
1	7 \pm 12	1 \pm 8
10	-1 \pm 3	4 \pm 10
100	-1 \pm 8	2 \pm 8
Concentration [μ M]	NADH	
	0% FBS	10% FBS
	0.1	-7 \pm 11
1	-9 \pm 9	-4 \pm 3
10	-4 \pm 10	-4 \pm 6
100	4 \pm 12	-4 \pm 10
Concentration [μ M]	NAD ⁺	
	0% FBS	10% FBS
	0.1	3 \pm 6
1	-2 \pm 2	9 \pm 6
10	5 \pm 3	13 \pm 5
100	-1 \pm 7	5 \pm 6

- Endogenous pro-oxidants

Table A10. The impact of serum starvation of HT29 cells treated with endogenous pro-oxidants on cellular antioxidant activity (CAA). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Concentration [μ M]	CAA value	
	GSSG	
	0% FBS	10% FBS
0.1	4 \pm 8	-1 \pm 5
1	-6 \pm 4	-2 \pm 5
10	1 \pm 8	-12 \pm 3
100	-8 \pm 6	-20 \pm 10
Concentration [μ M]	H ₂ O ₂	
	0% FBS	10% FBS
	0.1	-15 \pm 7
1	-13 \pm 11	6 \pm 8
10	-18 \pm 7	4 \pm 9
100	-24 \pm 3	-9 \pm 6
150	-34 \pm 8	-16 \pm 4

- Ascorbic acid and its derivatives

Table A11. The impact of serum starvation of HT29 cells treated with ascorbic acid derivatives on cellular antioxidant activity (CAA). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Compound	CAA value					
	Concentration [μ M]					
	1	10	100	200	500	1000
AA	8 \pm 7	4 \pm 3	11 \pm 4	12 \pm 5	31 \pm 7	57 \pm 6
CaA	9 \pm 6	-2 \pm 9	16 \pm 9	24 \pm 5	60 \pm 11	83 \pm 5
NaA	10 \pm 4	-2 \pm 9	5 \pm 10	8 \pm 9	23 \pm 9	72 \pm 7
iAA	5 \pm 1	9 \pm 7	11 \pm 10	15 \pm 7	22 \pm 15	52 \pm 4
iNaA	2 \pm 7	9 \pm 3	16 \pm 3	12 \pm 11	32 \pm 9	59 \pm 9
ABG	11 \pm 3	18 \pm 8	16 \pm 10	15 \pm 3	17 \pm 12	-3 \pm 9

Table A12. Cellular antioxidant activity of ascorbic acid cultured in serum-free medium (0% FBS). The results are means \pm SD of three independent experiments carried out in triplicate. Cells were cultured under either standard nutritional conditions (10% FBS) or in serum-free medium (0% FBS) for 20 h before and 1 h during treatment.

Concentration [μ M]	CAA value
	AA 0% FBS
1	-7 \pm 6
10	2 \pm 10
100	5 \pm 9

IV. Comet assay

- Endogenous thiol antioxidants

Table A14. The impact of 24 h treatment of HT29 cells with endogenous thiol antioxidants on DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments carried out in duplicate.

Compound	% DNA in a tail			
	Concentration [μ M]			
	C-	1	10	100
Cys	1.5 \pm 0.2	1.5 \pm 0.3	1.6 \pm 0.2	1.6 \pm 0.3
GSH	1.4 \pm 0.3	1.4 \pm 0.4	1.3 \pm 0.5	1.2 \pm 0.3
NAC	1.5 \pm 0.2	1.7 \pm 0.3	1.4 \pm 0.2	1.5 \pm 0.2
LA	1.0 \pm 0.1	1.1 \pm 0.3	1.1 \pm 0.2	1.0 \pm 0.1

Table A15. The impact of 24 h pre-treatment of HT29 cells with endogenous thiol antioxidants on the level of H₂O₂-induced DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail				
	150 μ M H ₂ O ₂	C-	Concentration [μ M]		
			1	10	100
Cys	36.9 \pm 13.5	2.3 \pm 0.9	33.3 \pm 6.9	38.6 \pm 10.8	42.3 \pm 7.7
NAC	36.9 \pm 13.5	2.3 \pm 0.9	31.6 \pm 15.4	42.9 \pm 8.8	34.6 \pm 12.7
LA	17.8 \pm 4.2	1.6 \pm 0.7	18.3 \pm 4.4	10.1 \pm 1.8	5.6 \pm 2.1

- Purine derivatives

Table A16. The impact of 24 h treatment of HT29 cells with purine derivatives on DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail			
	C-	Concentration [μ M]		
		1 / 100*	10 / 250*	100 / 400*
Ade	1.2 \pm 0.3	1.3 \pm 0.2	1.4 \pm 0.3	1.6 \pm 0.4
Ado	1.2 \pm 0.3	1.1 \pm 0.2	1.5 \pm 0.2	1.4 \pm 0.3
AMP	1.2 \pm 0.3	0.9 \pm 0.3	1.6 \pm 0.4	1.6 \pm 0.4
UA*	1.5 \pm 0.3	1.6 \pm 0.2	1.7 \pm 0.3	1.6 \pm 0.3

*UA was tested at 100, 250 and 400 μ M concentrations, which reflect its physiological concentrations

Table A17. The impact of 24 h pre-treatment of HT29 cells with purine derivatives on the level of H₂O₂-induced DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail				
	150 μ M H ₂ O ₂	Concentration [μ M]			
		0	1 / 100*	10 / 250*	100 / 400*
Ade	29.1 \pm 4.9	4.2 \pm 1.7	26.6 \pm 3.6	26.1 \pm 5.3	19.9 \pm 2.9
Ado	29.1 \pm 4.9	4.2 \pm 1.7	26.1 \pm 2.6	25.0 \pm 4.7	20.4 \pm 6.0
AMP	29.1 \pm 4.9	4.2 \pm 1.7	26.8 \pm 6.6	26.9 \pm 3.9	27.9 \pm 2.4
UA*	18.1 \pm 6.9	1.1 \pm 0.3	13.1 \pm 4.4	14.4 \pm 4.7	17.3 \pm 4.2

*UA was tested at 100, 250 and 400 μ M concentrations, which reflect its physiological concentrations



- Mitochondrial redox-active agents

Table A18. The impact of 24 h treatment of HT29 cells with mitochondrial redox-active compounds on DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail			
	Concentration [μ M]			
	C-	1	10 / 2.5	100 / 5
CoQ ₁₀ *	1.4 \pm 0.6	1.2 \pm 0.4	1.3 \pm 0.3	1.4 \pm 0.3
MT	1.4 \pm 0.4	1.4 \pm 0.4	1.2 \pm 0.2	1.1 \pm 0.4
CA	1.4 \pm 0.4	1.3 \pm 0.2	1.4 \pm 0.3	1.4 \pm 0.5
NADH	1.2 \pm 0.3	1.5 \pm 0.4	1.6 \pm 0.7	1.5 \pm 0.6
NAD ⁺	1.2 \pm 0.3	1.2 \pm 0.3	1.2 \pm 0.1	1.3 \pm 0.3

*CoQ₁₀ was tested at 1, 2.5 and 5 μ M concentrations, which reflect its physiological concentrations

Table A19. The impact of 24 h pre-treatment of HT29 cells with mitochondrial redox-active compounds on the level of H₂O₂-induced DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail				
	150 μ M H ₂ O ₂	Concentration [μ M]			
		0	1	10 / 2.5	100 / 5
CoQ ₁₀ *	19.0 \pm 3.5	1.0 \pm 0.1	19.2 \pm 2.9	20.0 \pm 3.9	19.9 \pm 4.1
MT	27.3 \pm 7.9	1.7 \pm 0.5	24.3 \pm 6.7	25.6 \pm 4.5	20.2 \pm 2.7
CA	32.2 \pm 8.3	1.0 \pm 0.2	34.9 \pm 9.4	26.7 \pm 3.8	33.3 \pm 4.7
NADH	34.9 \pm 5.3	1.3 \pm 0.2	28.3 \pm 9.5	29.0 \pm 3.1	29.0 \pm 11.7
NAD ⁺	34.9 \pm 5.3	1.3 \pm 0.2	34.7 \pm 11.5	25.3 \pm 8.9	25.1 \pm 11.2

*CoQ₁₀ was tested at 1, 2.5 and 5 μ M concentrations, which reflect its physiological concentrations

- Endogenous pro-oxidants

Table A20. The impact of 24 h treatment of HT29 cells with endogenous thiol antioxidants on DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail			
	Concentration [μ M]			
	C-	1	10	100
GSSG	1.4 \pm 0.4	1.3 \pm 0.3	1.5 \pm 0.4	1.0 \pm 0.3
H ₂ O ₂	1.4 \pm 0.4	1.5 \pm 0.2	1.8 \pm 0.6	1.3 \pm 0.4

Table A21. The impact of 24 h pre-treatment of HT29 cells with endogenous thiol antioxidants on the level of H₂O₂-induced DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as a negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail				
	150 μ M H ₂ O ₂	Concentration [μ M]			
		0	1	10	100
CoQ ₁₀ *	34.2 \pm 5.9	0.9 \pm 0.1	33.9 \pm 4.4	38.7 \pm 6.0	40.3 \pm 5.0

- Ascorbic acid and its derivatives

Table A22. The impact of 24 h treatment of HT29 cells with ascorbic acid and its derivatives on DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as negative control (C-). The results are means \pm SD of three independent experiments.

Compound	% DNA in a tail				
	C-	Concentration [μ M]			
		1	10	100	1000
AA	2.0 \pm 0.6	2.2 \pm 0.7	1.9 \pm 0.6	2.0 \pm 0.5	2.0 \pm 0.6
iAA	2.1 \pm 0.8	1.7 \pm 0.5	1.6 \pm 0.6	1.5 \pm 0.3	1.4 \pm 0.5
CaA	2.0 \pm 0.5	1.9 \pm 0.5	1.9 \pm 0.6	1.9 \pm 0.5	2.1 \pm 0.6
NaA	1.4 \pm 0.3	1.5 \pm 0.3	1.6 \pm 0.5	1.6 \pm 0.4	1.7 \pm 0.5
iNaA	1.8 \pm 0.3	1.7 \pm 0.3	1.6 \pm 0.3	1.5 \pm 0.4	1.7 \pm 0.4
ABG	1.3 \pm 0.4	1.7 \pm 0.8	1.4 \pm 0.4	1.3 \pm 0.5	1.3 \pm 0.7

Table A23. The impact of 24 h pre-treatment of HT29 cells with ascorbic acid and its derivatives on the level of H₂O₂-induced DNA damage expressed as a percentage of DNA in a comet tail. Cells treated with an appropriate solvent only served as negative control (C-). The results are means of three independent experiments \pm SD.

Compound	% DNA in a tail				
	150 μ M H ₂ O ₂	C-	Concentration [μ M]		
			50	100	200
AA	27.9 \pm 5.6	1.2 \pm 0.3	29.1 \pm 6.0	39.9 \pm 3.1	43.8 \pm 6.2
iAA	30.4 \pm 4.4	1.2 \pm 0.3	32.7 \pm 4.6	42.6 \pm 8.4	30.9 \pm 7.3
CaA	27.9 \pm 5.6	1.2 \pm 0.3	39.6 \pm 7.5	49.0 \pm 7.3	38.8 \pm 9.3
NaA	30.4 \pm 4.4	1.2 \pm 0.3	40.0 \pm 3.6	43.5 \pm 7.0	38.7 \pm 5.3
iNaA	18.8 \pm 5.0	1.1 \pm 0.2	26.1 \pm 9.9	24.3 \pm 5.8	22.9 \pm 5.5
ABG	47.4 \pm 11.5	1.6 \pm 0.4	29.4 \pm 12.5	31.0 \pm 10.7	30.9 \pm 10.9

Appendix B

I. Flow cytometry – materials and methods

HT29 cells were seeded in a petri dish (15 x 60 mm) at the density of 300,000 cells in 15 mL of McCoy's 5A medium supplemented with 10% FBS and 1% penicillin and streptomycin and were left to grow for 24 hours in a humidified atmosphere (37°C, 5% CO₂) in the Smart cell incubator. The next day, complete medium was removed, cells were washed with PBS and fresh serum and antibiotics-free medium was added. Cells were starved for 16 and 24 hours. When incubation time was finished, medium was removed and cells were detached using trypsin. Afterwards, trypsin was inactivated with serum-free medium and cells were centrifuged at 200 x g for 5 min at 4°C. Next, the supernatant was removed, cells were washed with 5 mL of cold PBS and centrifuged again. Then, most of the supernatant was removed and cells were resuspended in the residual PBS. Afterwards, 5 mL of cold 80% ethanol was added, the obtained solution was mixed using vortex and centrifuged at 400 x g for 5 min at 4°C. Next, ethanol was removed and mix of 0.5 mL of propidine iodine (1 µg/ mL) with RNase was added and left for 1 hour in the darkness at 4°C. Cell suspension was analysed with the use of Guava easyCyte 8 flow cytometer (Merck Millipore, USA). Data were processed with FlowJo™ v10.8.0 Software (BD Life Sciences, USA).

II. Results

HT29 cells were subjected to 16 and 24 h serum-starvation to induce the synchronization of cell cycle. As shown in Figure B1, both incubation times significantly induced the cell cycle arrest at G₀/G₁ phase, which shows that the homogenous population of cells was successfully obtained.

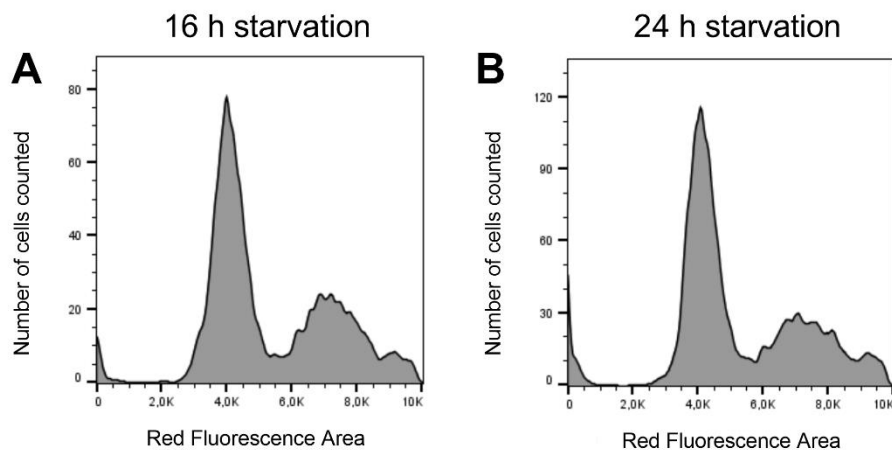


Figure B1. Histograms showing DNA content distribution in HT29 cells cultured under serum-deprived conditions for 16 h – **A**, or 24 h – **B**. Cells were stained propidine iodine (1 µg/mL) for 1 h before the analysis.

Appendix C

Table C1. List of human redox-related genes included in the 96-well RT2 Profiler PCR arrays (Qiagen, USA) used for genomic studies.

No.	RefSeq number	Symbol	Description
1	NM_000477	ALB	Albumin
2	NM_000697	ALOX12	Arachidonate 12-lipoxygenase
3	NM_001159	AOX1	Aldehyde oxidase 1
4	NM_000041	APOE	Apolipoprotein E
5	NM_004045	ATOX1	ATX1 antioxidant protein 1 homolog (yeast)
6	NM_004052	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
7	NM_001752	CAT	Catalase
8	NM_002985	CCL5	Chemokine (C-C motif) ligand 5
9	NM_005125	CCS	Copper chaperone for superoxide dismutase
10	NM_000397	CYBB	Cytochrome b-245, beta polypeptide
11	NM_134268	CYGB	Cytoglobin
12	NM_014762	DHCR24	24-dehydrocholesterol reductase
13	NM_175940	DUOX1	Dual oxidase 1
14	NM_014080	DUOX2	Dual oxidase 2
15	NM_004417	DUSP1	Dual specificity phosphatase 1
16	NM_001979	EPHX2	Epoxide hydrolase 2, cytoplasmic
17	NM_000502	EPX	Eosinophil peroxidase
18	NM_021953	FOXM1	Forkhead box M1
19	NM_002032	FTH1	Ferritin, heavy polypeptide 1
20	NM_001498	GCLC	Glutamate-cysteine ligase, catalytic subunit
21	NM_002061	GCLM	Glutamate-cysteine ligase, modifier subunit
22	NM_000581	GPX1	Glutathione peroxidase 1
23	NM_002083	GPX2	Glutathione peroxidase 2 (gastrointestinal)
24	NM_002084	GPX3	Glutathione peroxidase 3 (plasma)
25	NM_002085	GPX4	Glutathione peroxidase 4 (phospholipid hydroperoxidase)
26	NM_001509	GPX5	Glutathione peroxidase 5 (epididymal androgen-related protein)
27	NM_182701	GPX6	Glutathione peroxidase 6 (olfactory)
28	NM_015696	GPX7	Glutathione peroxidase 7
29	NM_000637	GSR	Glutathione reductase
30	NM_000178	GSS	Glutathione synthetase
31	NM_000852	GSTP1	Glutathione S-transferase pi 1
32	NM_001513	GSTZ1	Glutathione transferase zeta 1
33	NM_001518	GTF2I	General transcription factor Iii
34	NM_002133	HMOX1	Heme oxygenase (decycling) 1
35	NM_005345	HSPA1A	Heat shock 70kDa protein 1A
36	NM_006121	KRT1	Keratin 1
37	NM_006151	LPO	Lactoperoxidase
38	NM_005368	MB	Myoglobin
39	NM_000242	MBL2	Mannose-binding lectin (protein C) 2, soluble
40	NM_004528	MGST3	Microsomal glutathione S-transferase 3
41	NM_000250	MPO	Myeloperoxidase
42	NM_002437	MPV17	MpV17 mitochondrial inner membrane protein
43	NM_012331	MSRA	Methionine sulfoxide reductase A
44	NM_005954	MT3	Metallothionein 3
45	NM_000265	NCF1	Neutrophil cytosolic factor 1
46	NM_000433	NCF2	Neutrophil cytosolic factor 2
47	NM_000625	NOS2	Nitric oxide synthase 2, inducible
48	NM_016931	NOX4	NADPH oxidase 4
49	NM_024505	NOX5	NADPH oxidase, EF-hand calcium binding domain 5
50	NM_000903	NQO1	NAD(P)H dehydrogenase, quinone 1
51	NM_002452	NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1

No.	RefSeq number	Symbol	Description
52	NM_181354	OXR1	Oxidation resistance 1
53	NM_005109	OCSR1	Oxidative-stress responsive 1
54	NM_020992	PDLIM1	PDZ and LIM domain 1
55	NM_007254	PNKP	Polynucleotide kinase 3'-phosphatase
56	NM_002574	PRDX1	Peroxiredoxin 1
57	NM_005809	PRDX2	Peroxiredoxin 2
58	NM_006793	PRDX3	Peroxiredoxin 3
59	NM_006406	PRDX4	Peroxiredoxin 4
60	NM_181652	PRDX5	Peroxiredoxin 5
61	NM_004905	PRDX6	Peroxiredoxin 6
62	NM_020820	PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1
63	NM_183079	PRNP	Prion protein
64	NM_000962	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
65	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
66	NM_012293	PXDN	Peroxidasin homolog (Drosophila)
67	NM_014245	RNF7	Ring finger protein 7
68	NM_182826	SCARA3	Scavenger receptor class A, member 3
69	NM_203472	VIMP	Selenoprotein S
70	NM_005410	SEPP1	Selenoprotein P, plasma, 1
71	NM_003019	SFTPD	Surfactant protein D
72	NM_012237	SIRT2	Sirtuin 2
73	NM_000454	SOD1	Superoxide dismutase 1, soluble
74	NM_000636	SOD2	Superoxide dismutase 2, mitochondrial
75	NM_003102	SOD3	Superoxide dismutase 3, extracellular
76	NM_003900	SQSTM1	Sequestosome 1
77	NM_080725	SRXN1	Sulfiredoxin 1
78	NM_006374	STK25	Serine/threonine kinase 25
79	NM_000547	TPO	Thyroid peroxidase
80	NM_003319	TTN	Titin
81	NM_003329	TXN	Thioredoxin
82	NM_003330	TXNRD1	Thioredoxin reductase 1
83	NM_006440	TXNRD2	Thioredoxin reductase 2
84	NM_003355	UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)
85	NM_001101	ACTB	Actin, beta
86	NM_004048	B2M	Beta-2-microglobulin
87	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
88	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
89	NM_001002	RPLP0	Ribosomal protein, large, P0
90	SA_00105	HGDC	Human Genomic DNA Contamination
91-93	SA_00104	RTC	Reverse Transcription Control
94-96	SA_00103	PPC	Positive PCR Control

