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Effects of secondary plant metabolites and micronutrients on mitochondrial functions in a cellular model of early Alzheimer disease

Dissertation

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Abbreviation

AD	Alzheimer disease
ADP	adenosine diphosphate
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid beta
BACAE1	β-site amyloid precursor protein cleaving enzyme 1
BDNF	Brain-derived neurotrophic factor
CAT	catalase
CF	cafestol
Cof	caffeine
COX-2	cyclooxygenase-2
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferases
ETC	electron transport chain
FAD	familial Alzheimer disease
$FADH_2$	flavin adenine dinucleotide hydroquinone
FMN	flavin mononucleotide
Fol	folic acid
GPX	glutathione peroxidase
GST	glutathione s-transferase
HstP	hesperetin
HO	hydroxyl radical
HO-1	heme oxygenase-1
hTau	hyperphosphorylated tau
icv-STZ	intracerebroventricular administration of streptozocin
IL	interleukin
iNOS	inducible nitric oxide synthases
KCC	combination of kahweol, cafestol and caffeine
KW	kahweol
LDH	lactate dehydrogenase
LDHA	lactate dehydrogenase A
LOAD	late-onset Alzheimer disease

LSP	lipopolysaccharide
MAPK	mitogen activated protein kinase
MgOr	magnesium-orotate
MD	mitochondrial dysfunction
Mg	magnesium
MMP	mitochondrial membrane potential
mtDNA	mitochondrial DNA
$NADH/H^+$	nicotinamide adenine dinucleotide hydroquinone
NF-κB	nuclear factor k-light-chain-enhancer of actd B cells
NFTs	neurofibrillary tangles
NOX4	NADPH oxidase 4
Nrf-2	nuclear factor E2-related factor
PDH	pyruvate dehydrogenase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositide 3-kinase
PLPF	pyridoxal 5' phosphate
PMP	pyridoxamine 5' phosphate
PS	presenilin
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species
sAPP	soluble amyloid precursor protein
SC	super cocktail (of all used compounds)
SOD	superoxide dismutase
TCA	tricarbon acid
TNF-α	tumor necrosis factor alpha
TrkB	tropomyosin receptor kinase B
Vit B6	vitamin B6
WHO	World Health Organization

1. Introduction

1.1 General Introduction

Our society is changing at an increasing rate, presenting our healthcare system with ever greater challenges. Due to demographic change and the resulting increase in a cohort of people with increasing life expectancy, age-specific diseases in particular are on the rise. One of these age-specific diseases is Alzheimer disease (AD).

AD has now been placed on the WHO priority list of global health problems. Since its first description in 1906, there is still no cure for it [1]. AD is the most common form of dementia [2] and currently affects about 1.73% of the population in the European Union where by 2050 this number is expected to increase to 2%, totaling over 14.2 million people [3]. AD is characterized by a progressive loss of memory and language skills, personality and behavior changes and a reduced quality of life [2]. Neuropathological hallmarks of AD include the presence of amyloid beta (Aβ) which mainly accumulates extracellularly leading to plaque formation and intracellularly hyperphosphorylated tau protein [4, 5]. Furthermore, AD leads to impaired glucose metabolism [6] and increased reactive oxygen species (ROS) production, which in turn leads to increased oxidative stress and its consequences [7, 8]. One main reason for oxidative stress and another hallmark of of the disease is mitochondrial dysfunction (MD) [9]. Since there is no cure yet, it is necessary to delay the symptoms as long as possible or even prevent them from occurring. One such approach is physical activity, in which the performance of physical activity is negatively associated with the occurrence of AD [10, 11]. Another way would be to use pharmacaceutials, which tries to target different points namely the amyloids, the tau protein or the neuroinflammations. Although great progress is being made in understanding AD in this area, only a few of them achieve approval [12]. As a third way, the focus is on nutrition in the prevention or therapy of AD. Secondary plant compounds in particular play a decisive role here, including polyphenols and flavonoids [13–15], but also vitamins and minerals, which can also be called biofactors play a central role [16].

1.2 Alzheimer Disease

1.2.1 General

AD was first described by Alois Alzheimer in 1906. However, it took more than 70 years for AD to be declared a common cause of dementia and the leading cause of death among those suffering from it [17]. Dementia refers to a variety of clinical syndromes and heterogeneous disorders of the brain, in which AD takes the most common form [18, 2]. Dementia is characterized by memory loss, language problems, difficulties in coping with problems and other impairments in cognitive abilities [2]. These difficulties are due to damage or loss of nerves and neurons in the part of the brain where cognitive functions reside. In AD, not only these areas are affected, but also those in which the basic functions of the body, such as walking or swallowing, are laid out [19].

There are 50 million people living with dementia in the world (as of 2018). This number will nevertheless triple by the year 2050 [20]. In Germany, 1.7 million people are currently living with dementia (as of 2021), a large proportion of whom have AD [21]. The prevalence of getting AD increases with age, >65 years the mean prevalence in Europe increases to 8.5% [21]. The proportion of people with AD and dementia is higher in women than in men, which may be mainly due to the longer life expectancy of women [22].

1.2.2 Pathology

AD can be divided into two the main categories, sporadic late-onset AD (LOAD) and familial AD (FAD) [23]. While symptoms of sporadic generally appear between the ages of 60-65 years [19], familial AD is characterized by the onset of symptom before the age of 60 years [24]. In isolated cases, familial AD may also begin before 30 years of age [23]. The main genetic factor for LOAD is apolipoprotein E (ApoE) on chromosome 19, which has three variants: E2, E3 and E4. The most common form is E3. Individuals with the E4 form have a generally higher risk and those with E2 have a decreased risk for AD [24]. The greatest risk factor for LOAD is aging, which is the most common form of the disease [23].

Both sporadic and familial AD are associated with impaired Aβ homeostasis [24, 25]. The main cause of amyloid plaques is the elimination of amyloid precursor protein (APP) [25]. APP is an integral membrane protein and is expressed in many tissues, especially in the synapses of neurons. It plays an important role in many biological activities, such as

neuronal development, signaling and intracellular transport. The most abundant form of APP in the brain is APP_{695}, which is produced mainly by neurons [26]. This form differs from the other longer APP forms in that the ectodomain lacks a protease inhibitor sequence [27]. Normally, APP is cleaved close to the membrane by an extracellular protease called α-secretase. This releases a soluble fragment, sAPPα. A second cut is made through a complex within the membrane by a γ-secretase. The subunit of this secretase is a presenilin protein encoded by either presenilin (PS) 1 or 2. Presenilins are a family of related transmembrane proteins that form the subunits of the gamma-secretase protein complex [28]. The second cut releases an intracellular peptide, the amyloid intracellular domain and a small residual peptide. The pathway carried out by α-secretase is referred to as the non-amyloid pathway; no plaques are formed in this case [29]. In other situations, a pathogenic form may occur. The extracellular cut may occur slightly further away from the membrane. This process is carried out by a protease, the β-secretase. This is followed by the cutting of the γ-secretase. The amino acid fragment that remains between the two cuts is the amyloid-β [29]. This processing results in Aβ-peptides with 37 - 43 amino acids, where $Aβ_{42}$ is considered to be more neurotoxic [27].This pathway is also referred to as the amyloid pathway, both shown in **Figure 1**.

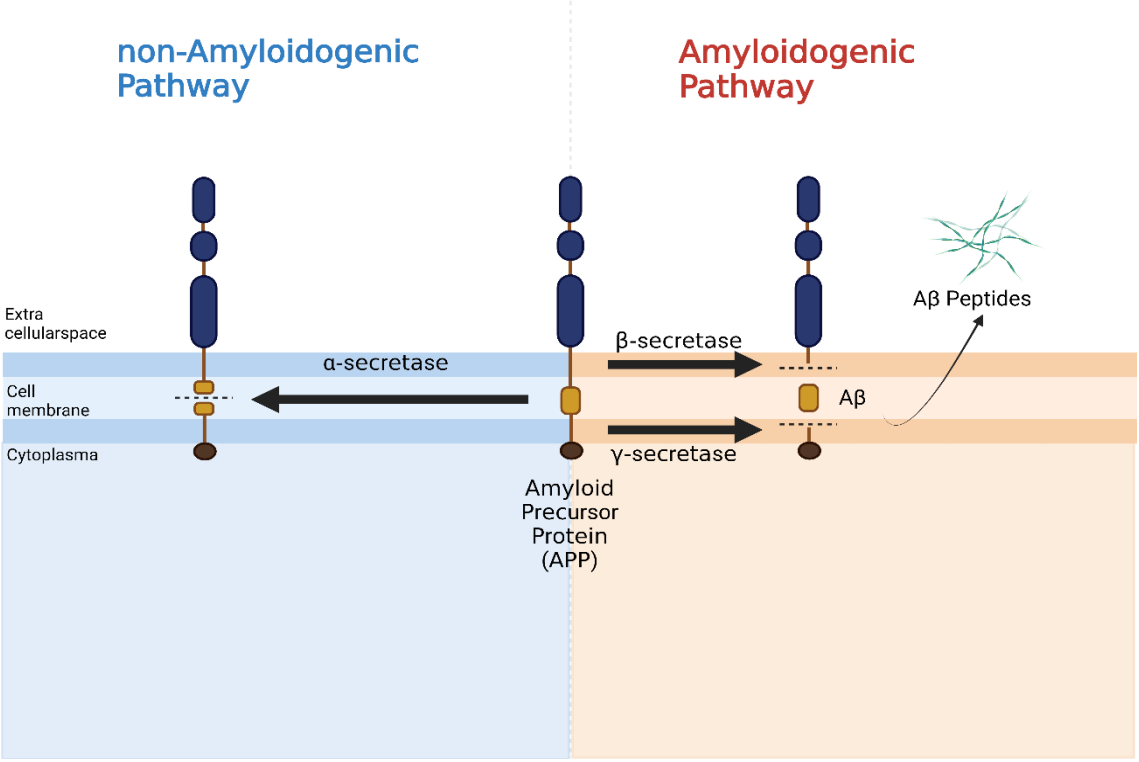

Figure 1: Illustration of the amyloid and non-amyloid pathway. On the left side, the non-amyloidicenic pathway is shown. Here, the amyloid precursor protein (APP) is cut by the α-secretase. This results in the formation of a soluble fragment (sAPPα). The amyloidogenic pathway is shown on the right. Here, a cut is first made further away at the membrane by β-secretase, followed by another cut by γ-secretase. This produces the amyloid-beta (Aβ) fragment. Created with Biorender.com

In addition to Aβ homeostasis, the formation of hyperphosphorylated tau (hTau) is also discussed in the context of AD. The tau protein serves the stability of axonal microtubules in the brain and is involved in the growth and regulation of axons. The binding of tau occurs via post translational modification by phosphorylation [30]. Once neurofibrillary tangles (NFTs) form from tau, they form a possible further basis for the development of AD [31]. The accumulation of phosphorylated and aggregated tau has since been suspected to cause AD and tautopathies [32, 33]. Hyperphosphorylation of tau renders it insoluble, reduces its affinity for microtubules and causes it to self-associate with filament structures [23]. Once hTau is present, it is more resistant to protease-induced degradation [24]. There is some overlap of tau aggregation and Aβ toxicity. While NFTs correlates with axonal transport loss and AD progression, it is not accompanied by neuronal loss. The resulting Aβ plaques and NFTs condition lesions in brain areas involved in learning and memory. These include the hippocampus, amygdala and frontal, temporal and parietal lobes [23, 32, 30].

1.3 Macro- and Micronutritions

Since there is still no cure for AD despite abundant efforts, the focus is on prevention and symptomatic therapy. In addition to exercise and sport, nutrition and the supplementation of certain nutrients have emerged as a possible starting point. Nutrition can be broadly divided into macro- and micronutrients. Macronutrients are divided into carbohydrates, fats and, proteins. The macronutrients protein, fat and carbohydrates provide energy and important components for our body. Protein consists of a collection of linked amino acids; fat consists of glycerol and fatty acids; and carbohydrates consist of either monosaccharides or their linkage into chains. These linkages can be hydrolyzed in the human intestine or are resistant to it, which makes them dietary fiber. A combination of these substances is necessary to maintain our health. [34]. By combining the proportions of macronutrients and omitting certain ones, a wide variety of diets can be created. All of these affect our health and health status in many ways. Certain diets can influence our sleep [35], reduce our cardiovascular risk [36], prevent obesity [37] and certain diseases [38], influence the immune system [39] and even prevent neurodegenerative diseases [40–42]. Micronutrients, on the other hand, are essential cofactors for the maintenance of metabolic functions, but do not provide any energy themselves. These include vitamins, minerals and trace elements [43]. Three micronutrients that were in the focus of the

current dissertation are highlighted below, including folic acid, magnesium-orotate and vitamin B6, as shown in **Figure 2**.

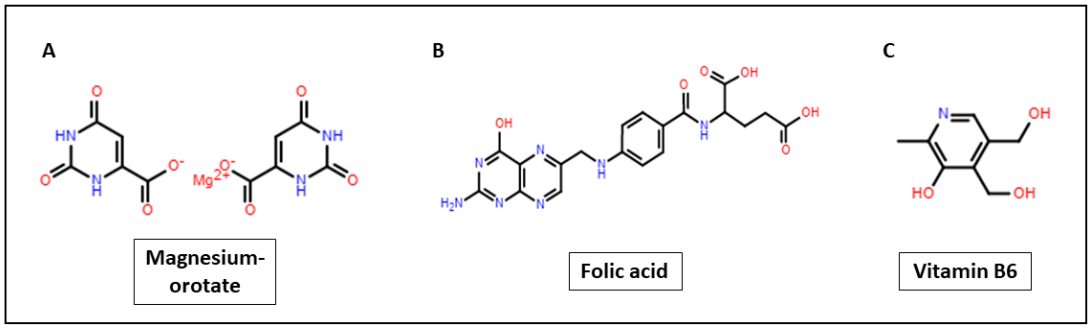

Figure 2: Chemical structure of (A) magnesium-orotate, (B) folic acid and (C) vitamin B6

1.3.1 **Folic Acid**

Folic acid (Fol) is a water-soluble synthetically produced substance, chemically composed of the structures of L-glutamic acid and pteroic acid, which in turn is derived from para-aminobenzoic acid. Folic acid is the synthetically produced variant with only one glutamate residue, whereas the term folate covers all forms with different numbers of glutamate residues [44, 45]. Folate is found in a variety of foods, including vegetables, especially green vegetable varieties, peas, seafood and cereals. Foods with the highest content are spinach, liver, asparagus and sprouts [46]. The daily amount of folate that should be ingested is 400µg [47]. In the human body, folate functions as a coenzyme in carbon transfer in the synthesis of nucleic acids and in protein metabolism [48, 46, 47]. One of the most important reactions that cannot take place without folate is the conversion of homocysteine to methionine in the synthesis of s-adenosyl-methionine, an important methyl donor. Another dependent metabolic pathway, without which cell division would not be possible, is the methylation of deoxyuridylate to thymidylate in the formation of DNA [46]. As described above, deficiencies in micronutrients lead to the occurrence of certain diseases. There seems to be a correlation between high homocysteine or low folic acid levels and the occurrence of dementia or Alzheimer's disease [49–53]. Elevated homocysteine level could negatively impact the brain through multiple mechanisms, including cerebrovascular ischemia leading to neuronal cell death, activation of tau kinases leading to tangle deposition and inhibition of methylation reactions [54]. However, it appears that supplementation with folic acid, while reducing homocysteine levels in some studies, has no effect on cognitive function or the incidence of AD [55–

58]. There is conflicting conjecture as to whether and how folate affects the progression of AD. Although, it appears that a deficit of folate is associated with the onset of AD. Further research is needed to investigate the effect of folate on AD pathology.

1.3.2 **Magnesium**

Magnesium (Mg) is the second most abundant mineral in the human body after calcium. It occurs naturally in many foods including green leaves, nuts, cerals, and cores. Furthermore, Mg is added to many foods or offered as a dietary supplement for addition. In this context, some health claims are made for Mg, such as "reduction of tiredness and fatigue " or „contribution to normal psychological functions" to name a few [59]. Mg-Ions (Mg^{2+}) are important cofactors for more than 300 enzymes that control various biochemical reactions, including protein synthesis, nerve and muscle functions and blood glucose control [60, 61, 46]. The content of Mg in the body is about 25g, with a large part bound in the bones, only about 1% is present outside cells [62, 63]. The daily intake of Mg should be 300mg/d for women and 350mg/d for males [64] and plant foods, including green vegetables, legumes and cereals are good sources of it [61, 60]. Magnesium deficiency is rather rare in our latitudes, however, the supplementation of Mg may help in the improvement of some diseases. There is hardly a medical problem in which Mg does not seem to play a role. High blood pressure can be lowered by a few points with the administration of Mg [65, 66] and there is a correlation between diets high in Mg and a reduced risk of diabetes mellitus type 2. One explanation for this could be the relationship between the role of Mg in the glucose metabolism [67, 68]. Furthermore, Mg is involved in the bone metabolism [69] and supplementation is positively related to bone density [70]. However, the evidence for these findings is relatively thin and the data is inconclusive and largely relates to retrospective studies, so further intensive research on the topic is needed.

Mg also may affect AD, although the mechanism is not yet fully understood. In mice, chronic undersupply with Mg has been shown to affect memory [71], whereas in a rat model of AD, supplementation with Mg resulted in improvement.[72]. There also appears to be evidence that cognitive function is improving in patients with dementia [73]. Since Mg is involved in many important metabolic pathways especially for neuronal properties, supplementation of Mg lowered Aβ in an AD mouse model while improving memory

[74]. Low levels of Mg have also been found in various tissues of AD patients [75, 76], it therefore seems to be some correlation between the prevention and the occurrence of AD and the supply or administration of Mg.

1.3.3 Vitamin B6

Vitamin B6 (Vit B6) is a water-soluble vitamin found in many foods, for instance fish, beef liver and other organ meats, potatoes and other starchy vegetables, and fruit. Vit B6 is an umbrella term for six different vitamins including pyrodoxine, an alcohol and pyridoxal, an aldehyde. In addition, there are two coenzyme active forms of Vit B6, pyridoxal 5' phosphate (PLP) and pyridoxamine 5' phosphate (PMP) [47, 77]. The coenzyme form of Vit B6 has numerous metabolic tasks and is involved in over 100 enzyme reactions, most of which are found in protein metabolism [47]. Furthermore, Vit B6 is essential for cognitive development, as it is involved in the synthesis of neurotransmitters and keeps homocysteine levels in the body at a normal level [46]. The daily recommended dose of Vit B6 is 1.6mg [78]. The highest amount are found in fish, beef liver, potatoes and starchy vegetables [46, 47]. Vit B6 deficit is rather rare and often occurs in a combination of low levels of other B vitamins [46]. Since the undersupply occurs rather rarely, supplementation for the prevention of certain diseases is discussed. Vit B6 has been shown in some studies to reduce the risk of cardiovascular disease by lowering homocysteine levels [44, 79], although the majority of studies have found no effect [80, 81]. Poor Vit B6 supply is suspected to play a role in cognitive decline, while high supply is associated with improved results in memory tests [82, 83]. However, there are no studies that show a direct correlation between Vit B6 supplementation and AD or its improvement of AD symptoms. It is often discussed whether the administration of B vitamins, Vit B6 usually in combination with other B vitamins, lowers homocysteine levels and thus leads to an improvement of symptoms [84, 85]. Whereby this is discussed controversially [55, 86].

1.4 Plant Secondary Metabolites

Besides micronutrients, many other substances are present in our food playing an important role for human physiology, including secondary plant ingredients. Plant secondary metabolism is defined as a term for metabolic pathways and small molecule products that are not essential for the survival of the organism. Among the most important

groups of substances are phenols, terpenes and nitrogen-containing compounds with which plants can interact with their environment [87]. They serve to defend against pathogens (iridoids, canabinoids), to repel herbivores (tannins, alkaloids), to protect against UV radiation (carotenoids, flavnoids, anthocyanins), to attract pollinators (monoterpenes) [87–90]. For humans they are especially discussed because of their possible health-promoting properties [91, 92]. Among the important secondary plant compounds discussed here are the alkaloid caffeine (Cof), the diterpenes cafestol (CF) and kahweol (KW) and the flavonoid hesperetin (HstP), shown in **Figure 3.**

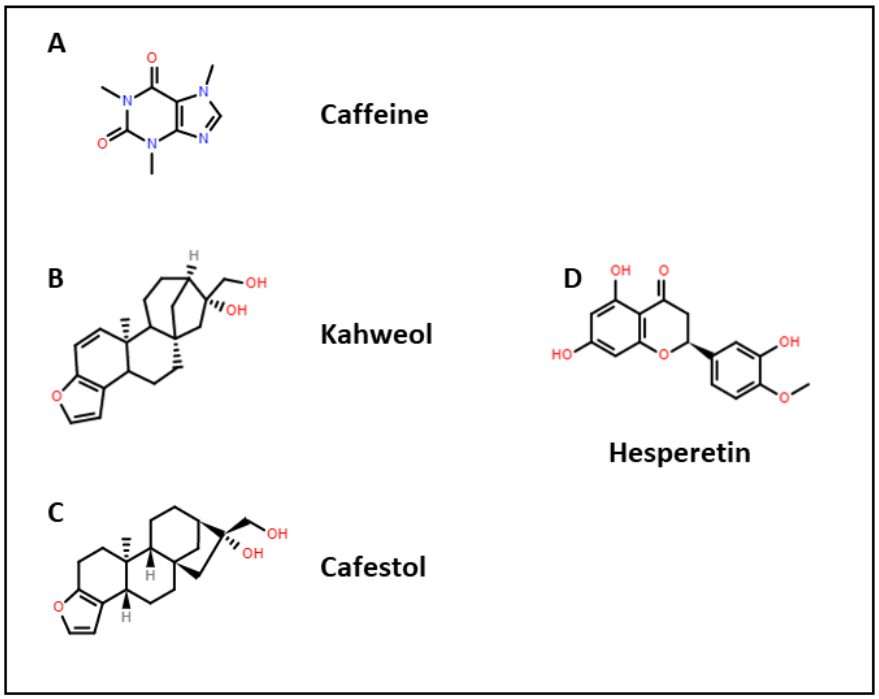

Figure 3: Chemical structure of (A) caffeine, (B) kahweol, (C) cafestol and (D) hesperetin

1.4.1 **Caffeine**

Caffeine (Cof) is a methylxanthine alkaloid and the most widely consumed psycho-stimulant worldwide [93]. It is found in over 60 plants and is present in everyday beverages such as coffee, tea, energy drinks, soft drinks and cocoa [94, 95]. A typical source of caffeine is coffee. An average cup of coffee has about 100 - 120mg of caffeine, depending on the preparation method, size, type and duration [96]. After oral intake, Cof is absorbed in the stomach and the small intestine within 30 minutes to about 99%. Cof reaches its peak in the bloodstream after 30 - 45 minutes [97]. Cof has a structural similarity to adenosine, which allows it to act antagonistically on the adenosine receptor. This gives caffeine its psychotropic and anti-inflammatory properties [98]. Due to its

lipophilic nature, Cof can be readily absorbed and cross barriers, including the blood-brain barrier. Regular consumption of increased amounts of Cof is associated with tolerance development [99].

It has been shown that consumption of moderate amounts is suspected to reduce AD risk compared to non-coffee drinkers, with a consumption around two cups a day [100, 101]. In animal models, Cof administration reduced Aβ deposition in the brain, as well as lowering $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels [102, 103]. Similarly, Aβ elimination in the mouse model was significantly increased by the administration of Cof [104]. Not only the biomarkers improved, but also the cognitive abilities [102, 103]. Other beneficial effects associated with AD include reduced oxidative stress, improved antioxidant capacity and lower AD prevalence in individuals with ApoE4 allele [99].

1.4.2 **Cafestol**

Cafestol (CF) is a naturally occurring diterpene in Arabica and Robusta coffee beans. Diterpenes consist of four isoprene units and belong to the group of tetracyclophetanes known as kauranes [105]. An average cup of coffee contains 3 - 4 mg of CF. The preparation must be based on a non-filtering method, as is the case with "French press" or "Turkish coffee" [106]. CF is thought to have anti-inflammatory, anti-angiogenic and anti-tumorigenic properties. It could be shown that CF has an increasing effect on the LDL level [107]. In cell experiments, CF demonstrated influences on the biochemical reaction pathways of transcription factors such as nuclear factor k-light-chain-enhancer of activated B cells (NF-κB) and on the upstream signaling cascade mitogen activated protein kinase (MAPK). The anti-inflammatory effect of CF is primarily mediated by the gene cyclooxygenase-2, which catalyzes prostaglandin E2 production [108]. As CF relates to AD, there are no studies to date that address this issue.

1.4.3 **Kahweol**

Kahweol (KW) belongs to the diterpenes and occurs naturally in Arabica coffee beans. It is considered a potent antioxidant with cytoprotective activity [105]. Compared to CF, KW differs in structure by an extra double bond [107]. There are 3 - 6 mg of KW in an average cup of coffee. Compared to CF, KW has a slightly higher bioavailability [106]. KW is also thought to have anti-inflammatory, anti-angiogenic and anti-tumorigenic properties. Like CF, KW also has an increasing effect on LDL levels [107]. The effect

induced by KW involves the transcription factor nuclear factor E2-related factor (Nrf-2) as well as the signaling cascades phosphoinositide 3-kinase (PI3K)/Akt and p38 MAPK. By means of this signaling cascade, in which the phase 2 enzyme detoxification is also activated, KW can have an influence on bioenergetics and mitochondrial dynamics [109, 110]. In cell experiments, KW was able to significantly reduce the externally added cell stress by means of hydrogen peroxide and thus protect the cell [109]. As with CF, there have been no studies on the relationship between AD and KW, or on its beneficial effects on AD.

1.4.4 **Hesperetin**

Hesperetin (HstP) belongs to the class of flavonoids called flavones and is found in the peels of citrus fruits, such as grapefruits or oranges [111]. Hesperetin is derived from the hydrolysis of its aglycone, hesperidin (hesperetin 7-rhammnoglucoside). HstP is a bioactive molecule that can act in multiple ways in the body. HstP has a lipid-lowering effect that despite feeding a high fat diet in a rat model, cholesterol and triacylglyceride levels decreased. Furthermore, the activities of HMG-CoA reductase and acyl-CoA cholesterol acyltransferase were decreased [112]. In addition to the lipid-lowering effect, HstP had a positive effect on the function of the heart in mouse models, where it protects against hypertension, fibrosis and dysfunction [113]. That HstP might not affect the lipid profile and blood pressure in randomized clinical trials is probably due to its quite low bioavailability, which is 15% [114, 115]. In an in vitro model, it was shown that HstP can cross the blood-brain barrier and is therefore a good molecule that can act on processes in the brain [116]. HstP is a very effective antioxidant [117, 118] and shows its abilities especially in the cell model. Here HstP protects cells from induced oxidative stress by hydrogen peroxide and thereby has a neuroprotective effect [119]. In addition, HstP was shown to protect cells from oxidative stress by several mechanisms, including receptor-mediated actions. HstP activates tropomyosin receptor kinase A and the estrogen receptor, which stimulate PGC-1α expression [120]. PGC-1α is an important factor for mitochondrial biogenesis and is known to protect against apoptosis, oxidative damage and Aβ-induced neurotoxicity [121]. With reference to AD, HstP shows that in rats induced AD by icv-STZ, memory recall and consolidation of recognition memory improved. So did the levels of antioxidative enzymes and glutathione. In this study, HstP in pure form was compared to a nanoform, with the nanoform performing better [122].

Nanoparticles are smaller than 1000nm, but larger than 100nm. Nano-sized formulations are particularly suitable for substances that are difficult to dissolve. Furthermore, the size of these newly formed compounds can increase the bioavailability of otherwise poorly available substances. Nanoparticles can increase the stability and absorption of secondary plant compounds. At the same time, the nanoform protects against too early degradation of the substances and thus prolongs their stay in the bloodstream [123, 124].

1.5 Mitochondria and Energy Metabolism

All the substances mentioned above have some effect on our metabolism. One of these effects concerns the energy metabolism (**Figure 4**). For this, the mitochondria are responsible, which will be described in more detail in the current chapter. Mitochondria have been known to produce energy from nutrients through oxidative phosphorylation for more than 50 years [125]. They are found in all eukaryotic cells and occupy at least 20% or more of the volume of the cell. The endosymbiont theory states that an ancestor of the mitochondrion evolved from an α-eukaryotic cell and was taken up by a host cell [125]. To protect itself from the outside world, the eubacterial progenitor cell repaired the host cell, thereby producing energy, which in turn was used by the host cell to grow. Thus, more than 1.5 billion years ago, a synergy occurred between the two cells [126].

1.5.1 Structure and Function

Mitochondria are independent cell organelles, which are half a micrometer to a few micrometers in size. They are dynamic organelles, constantly changing through fission and fusion [127]. Mitochondria occur in large networks or as individual oval compartments. Their size and number depends on the amount of energy required by the cell [128]. Mitochondria are characterized by a double membrane system. The outer mitochondrial membrane faces the cytosol and the inner mitochondrial membrane faces the matrix. The inner membrane is also called cristae [129]. The intermembrane space is the mitochondrial compartment located between the two membranes [129]. Within the mitochondrial matrix, the important metabolic pathways such as the β-oxidation of fats, the pyruvate dehydrogenase and other enzymes of the citrate cycle are anchored here. Further, the inner membrane is home to the complexes of the respiratory chain. These are important for the production of the energy carrier ATP [128].

Mitochondria have been primarily referred to as the "powerhouse of the cell" for a long time due to oxidative phosphorylation (OXPHOS), which produces energy needed for the cell. This is to the fact that mitochondria produce more than 90% of a cell's ATP requirements [130]. In addition, it is now recognized that mitochondria are also involved in other numerous physiological processes. These include ATP generation, ROS formation, intracellular calcium homeostasis and apoptosis. Furthermore, mitochondria provide important biomolecules for the cell [24].

The mitochondrion has its own genome, which comprises only 37 genes, 13 of which are responsible for protein synthesis of the respiratory complexes. The remaining proteins important for the mitochondrion are encoded by the nucleus of the cell. They are synthesized in the cytosol of the cell and imported post-translationally into the mitochondrion [128, 126].

1.5.2 Glycolysis

Glycolysis is the first step of energy production from glucose metabolism performed by all prokaryotic and eukaryotic cells. This metabolic pathway proceeds in several steps, with one mole of glucose being metabolized into two moles of pyruvate. Glycolysis takes place in the cytosol of the cell [131]. In aerobic cells, pyruvate is further metabolized stepwise to CO_2 via the tricarbon acid (TCA) cycle [132]. This enables the cell to produce reducing equivalents, which are used in OXPHOS for ATP generation [133].

Another way of metabolizing pyruvate is reductive conversion to organic acids or alcohols [132]. This is done by anaerobic cells. Those cells further metabolize the resulting pyruvate to lactate. This effect also occurs in aerobic cancer cells and is called the Warburg effect [134]. Compared to OXPHOS, glycolysis produces only two moles of ATP, which seems relatively small compared to up to 28 moles of ATP. However, glycolysis proceeds much faster and thus enables a constant energy supply even in the case of a backlog in the TCA [133].

1.5.3 Tricarboxylic Acid Cycle

The citrate cycle, also known as the TCA cycle or Krebs cycle, is a biochemical cycle that occurs within the mitochondrial matrix [135]. Within this cycle, which consists of eight steps, citrate is first consumed and then regenerated. The TCA enables the

connection of all important metabolic pathways from carbohydrates, proteins and fats. The final product of these degradation pathways is acetyl-CoA [135]. The speed-determining enzyme is citrate synthase. By catalyzing the condensation of oxaloacetate and acetyl-CoA to citrate, it represents the first and most important step [136]. Acetyl-CoA enters the TCA and is oxidized, producing the reduction equivalents $NADH/H^+$ and $FADH_2$ [136]. These two eventually transfer electrons to the mitochondrial respiratory chain to initiate OXPHOS [135].

The TCA holds another important role in providing metabolic intermediates for gluconeogenesis, transamination, deamination and lipogenesis [137].

1.5.4 **Oxidative Phosphorylation**

The place where most energy is produced in the form of ATP is oxidative phosphorylation [127]. Here, an electrochemical gradient is established with the help of four complexes, which is used at the fifth complex to generate ATP [24]. The electron transport facilitated by complexes I, III and IV is coupled to proton transport. This transport conditions the proton gradient known as the mitochondrial membrane potential (MMP) [138]. Complex II does not transport protons and thus is not involved in the assembly of the MMP. The individual redox-active complexes (complex I - IV) transfer electrons up to the final acceptor, oxygen, to form water [135]. The reduction equivalents used ($NADH/H^+$, $FADH_2$) act as electron donors in the respiratory chain. The reduction equivalents originate from a variety of diverse metabolic pathways. These include glycolysis, citrate cycle, β-oxidation, pyruvate dehydrogenase complex and some degradation products of amino acids [138].

The first and largest complex of the electron transport chain (ETC) is NADH dehydrogenase (complex I). This complex transfers two electrons from NADH to the cofactor flavin mononucleotide, which in turn transfers them to ubiquinone via iron-sulfur clusters. During this electron transfer, four protons are transported from the mitochondrial matrix into the intermembrane space [139, 126]. This complex is responsible for about 40% of the total MMP and is thus indispensable for MMP assembly [140].

Complex II is called succinate dehydrogenase and is the smallest complex of the respiratory chain. This complex enables electron transfer from $FADH_2$ to succinate, which is thereby oxidized to fumarate. During this process, no protons are transported into the intermembrane space [139, 128, 126].

The third complex is cytochrome c reductase and further transfers the electrons of complexes I and II to cytochrome c (complex III). Reoxidation of ubiquinone releases two electrons, which originate from complex I and II. The transport of ubiquinone to cytochrome c occurs in two steps. First, one electron is transferred to cytochrome c, via cytochrome c1, with the help of an iron-sulfur cluster. Meanwhile, the other electron is used to oxidize ubihydroquinone. This reaction is also called the Q cycle. In the Q cycle, electrons are transferred through two ubihydroquinone oxidation centers. For each electron transferred, two protons are translocated across the mitochondrial membrane [126, 128, 139].

At complex IV, the cytochrome c oxidase, electrons are transferred to molecular oxygen. Oxygen is the final acceptor and is eventually reduced to H_2O with four electrons [138]. At the same time, four protons of the matrix are pumped to the cytoplasmic side of the inner mitochondrial membrane [126, 141]. This step can lead to the formation of ROS [142]. These include superoxide, hydrogen peroxide and HO compounds generated from them [139]. ATP generation is made possible by the last complex, ATP synthase (complex V). Here, the energy of the proton gradient is used to bind phosphate to ADP in an energy-rich manner. ATP synthase consists of an F0 and an F1 subunit. While the F0 subunit represents a proton channel and ensures the reflux of protons, the F1 subunit can use this energy flux by means of conformational changes to generate ATP [128, 126, 141].

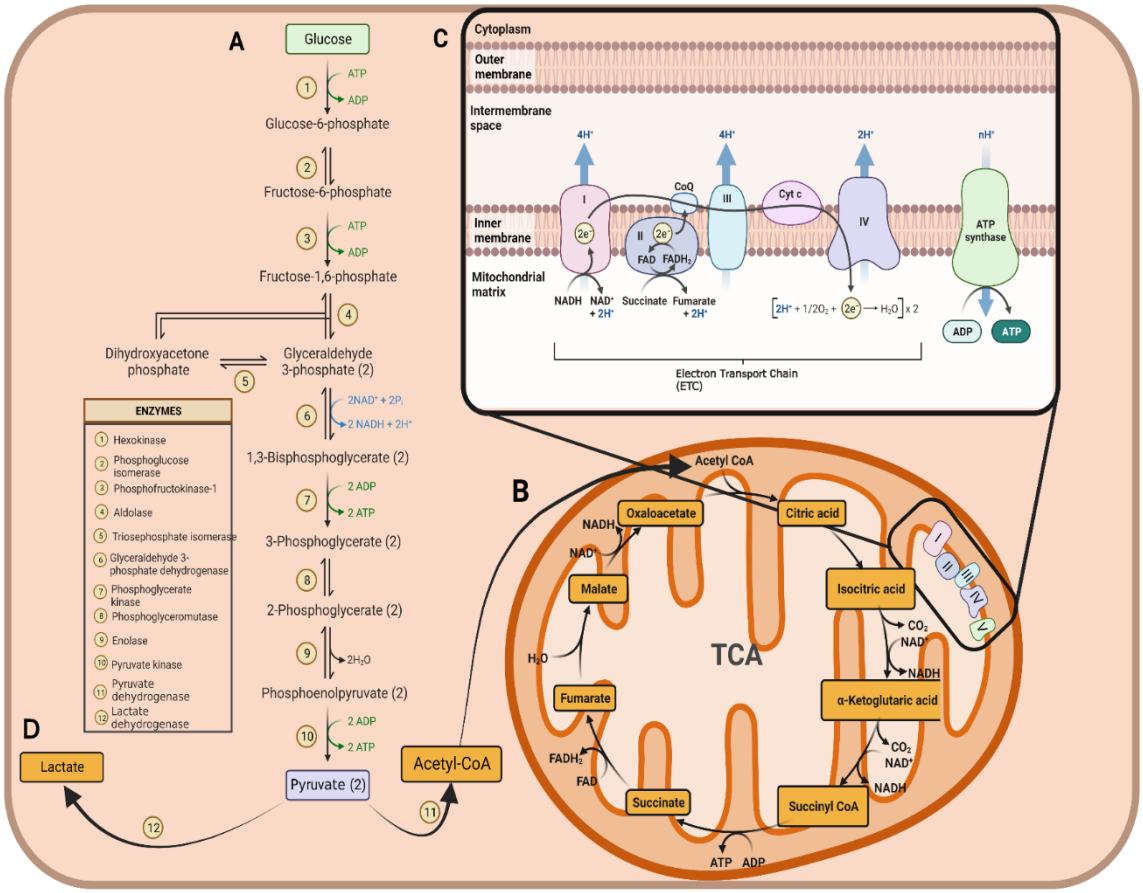

Figure 4: This figure shows aerobic and anaerobic glycolysis from glycolysis to oxidative phosphorylation in simplified form. A) shows the glycolytic degradation of glucose through several degradation steps to pyruvate. This pyruvate is degraded in B) in the tricarboxylic acid cycle (TCA) to the reducing equivalents. These are metabolized in C) by oxidative phosphorylation, producing ATP at the end. D) Represents the anaerobic pathway in which lactate is produced at the end. Created with Biorender.com

1.5.5 **Reactive Oxygen Species**

Oxygen, which is essential for life, is also converted to a small extent into reactive oxygen species (ROS), with superoxide anions, hydrogen peroxides and hydroxyl radicals being the most common representatives [143]. Mitochondria, in addition to providing energy, in the form of ATP, are also the largest cause in the generation of ROS [144]. In addition to mitochondria, there are a variety of other internal sources, such as NADPH oxidase, cytochrome P450, endoplasmic reticulum, peroxisomes and lysosomes. Exogenous sources include ultraviolet light, radiation, xenobiotics and other environmental factors [145].

In order to degrade or detoxify ROS, cells possess various defense mechanisms, which include enzymes as well as non-enzymatic antioxidants, such as glutathione, vitamins C and E, polyphenols and many more. The most important enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [146, 145]. Accordingly, a balance between ROS production and the antioxidant defense mechanisms designed for it is elementary. If this balance is disturbed, the accumulation of ROS can

be harmful to humans and contribute to the development of various diseases, including AD [147, 145, 148].

In higher organisms, as mentioned at the beginning, the mitochondria are one of the main producers of ROS due to the respiratory chain localized in them [149]. Eleven mitochondrial points have been identified where electrons are donated to oxygen, producing superoxide and/or hydrogen peroxide during substrate oxidation [150]. These are mainly found at complexes I and III [151]. In complex I, the production of superoxides is dependent on the reaction of oxygen with reduced FMN. The ratio of reduced to non-reduced FMN is determined by the ratio of NADH to NAD^+ [152]. If the respiratory chain is inhibited by damage, mutation, or a build-up of NADH due to low ATP consumption, the NADH/NAD^+ ratio increases and superoxide production occurs [144]. Complex III also produces superoxides, but these are rapidly converted to hydrogen peroxide. Inhibition of complex III appears to be the major cause of superoxide production. As soon as the binding of ubiquinone to the inner mitochondrial membrane, or the transfer of electrons in the Q cycle, is inhibited, increased ROS production occurs within complex III [148].

Potential toxic effects of ROS include DNA, RNA and protein [153]. In addition to proteins, the lipid membrane of cells is also attacked. This leads to lipid oxidation, in which further radicals are generated, resulting in a chain reaction and oxidation of further lipids within the membrane [128]. In particular, mitochondrial DNA (mtDNA) is also affected. mtDNA is ten times more damaged by ROS than nuclear DNA. This results in an increased mutation rate of mitochondrial DNA. One possible reason for this is the absence of histones, as well as the relative proximity of the mtDNA to the mitochondrial membrane [154].

Despite all negative effects, ROS also have positive physiological properties without which cells could not be [155, 153]. ROS function as signaling molecules to regulate and maintain normal physiological functions by interacting mainly with cysteine residues of proteins. This results in changes in protein function that affect transcription, phosphorylation and other important signaling events and/or alter metabolic fluxes and reactions in the cell by changing enzymatic properties [156–158]. In addition, ROS are required for the proliferation of cells [159, 157] as well as for the immune response. Release of the proinflammatory cytokines interleukin, tumor necrosis factor α and interferon β is required for the immune response [160, 161].

One consequence of unbalanced production and degeneration of ROS is the above-mentioned AD, which is related to mitochondrial dysfunction, which can be promoted by ROS. This connection will be discussed in the next chapter.

1.5.6 Link Between Mitochondrial Dysfunction and Alzheimer Disease

Since its formulation in 1992, the "amyloid cascade hypothesis" has dominated the field of AD. This hypothesis is based on the following two assumptions: The formation of extra neuronal senile plaques by Aβ-peptides and the presence of a mutation of the precursor protein APP. Due to the failure of all human clinical phase III trials, this hypothesis has increasingly fallen into the background [162, 163]. In 2004, a new hypothesis was put forward to explain the occurrence of sporadic AD. The so-called "mitochondrial cascade hypothesis" describes that mitochondrial dysfunction (MD) is the primary trigger of a cascade of events that ultimately leads to sporadic, late-onset AD [164, 162]. MD is an early-onset feature of AD in which almost all mitochondrial functions are affected [165, 9, 162].

This is reflected in decreased glucose metabolism at baseline and decreased glucose consumption are the first signs and a sensitive parameter to detect cognitive changes and functions [6]. The decrease in glucose metabolism is due to the reduced expression of coding subunits of the ETC. Furthermore, the activity of key enzymes of oxidative metabolism is reduced [166, 167]. The limited function of the ETC underlies the decline of complexes I and IV. This leads to a decrease in membrane potential and ATP production [168, 169]. These defects in turn lead to an increase in oxidative stress, resulting in mutations within the mtDNA, which is an early symptom of AD [170]. These defects in the mtDNA lead to reduced transcription of the important mitochondrial proteins and thus damage the function of the mitochondrion [171, 172]. This in turn causes more ROS, which simultaneously promotes the transcription of pro-inflammatory genes and the release of cytokines, such as interleukin-1, -6 or TNF-α. This leads to the loss of neurons and therefore more ROS, which, in combination with neuroinflammation, promotes the production of Aβ [173]. As a result, there is a cascade of inflammatory responses, increased oxidative stress and mitochondrial dysfunction, resulting in cell death and AD [174, 175].

AD is characterised by an imbalance of Aβ production and degradation. The question here is whether Aβ triggers mitochondrial dysfunction or whether mitochondrial

dysfunction triggers Aβ imbalance. Inhibition of cytochrome oxidase promotes amyloidgenic fragmentation of APP and that Aβ inhibits cytochrome oxidase [176–178]. For the sporadic late forms, the available data suggest that amyloidgenesis follows mitochondrial dysfunction [179]. In LOAD MD is more widespread than Aβ deposition, thus MD cannot be explained by Aβ alone [180].

Swerdlow et al. write that the mitochondrial cascade is only applicable to LOAD. They propose that dysfunction and mitochondrial ROS overproduction are a link between the mitochondrial cascade hypothesis in sporadic AD and the amyloid cascade hypothesis. The mitochondrial hypothesis and the amyloid cascade hypothesis differ in that LOAD is triggered by mitochondrial dysfunction. Furthermore, the increased Aβ production in LOAD could represent a compensatory event occurring in response to primary mitochondrial pathology, whereas Aβ production in FAD is exclusively a toxic phenomenon [179].

2. Aim of this work

The improvement and delay of neurodegenerative diseases is an important field in the scientific community. The difficulty that arises with a neurodegenerative disease such as AD is that there is no effective therapy, so it is important to take preventive measures early and at an early stage of the disease to delay the disease as long as possible.

Opportunities to support this are lifestyle changes, physical activity and a healthy diet. It is important to use substances that are available to everyone and that can be found in our daily diet. For this purpose, the substance hesperetin, which is found in citrus fruits and the juices made from them, has proven to be a suitable cannidate. The micronutrients magnesium and folic acid, which are found in many foods, as well as coffee, one of the world's most commonly consumed beverages, have been repeatedly discussed as possible candidates for preventive interventions.

Based on this, a total of four publications have emerged dealing with these substances and their effect on mitochondrial dysfunction, Aβ levels, oxidative stress and glycolysis in an early cell model of AD. In publication one, the effect of hesperetin was investigated. Study two examined the effects due to magnesium orotate and folic acid. Study three focused on the substances most commonly found in coffee, caffeine, kahweol and cafestol. Each of the investigated substances affected one field of early AD, so in study four we investigated a cocktail of all substances to see if all three fields were addressed simultaneously.

The aim of this work is to present a collection of possible potentially effective ingredients and to discuss them in the context of early AD and to put them in a context that was not possible in the previous publications.

3. Articles

3.1 Hesperetin Nanocrystals Improve Mitochondrial Function in a Cell Model of Early Alzheimer Disease

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Hesperetin Nanocrystals Improve Mitochondrial Function in a Cell Model of Early Alzheimer Disease

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Abstract: Mitochondrial dysfunction represents a hallmark of both brain aging and age-related neurodegenerative disorders including Alzheimer disease (AD). AD-related mitochondrial dysfunction is characterized by an impaired electron transport chain (ETC), subsequent decreased adenosine triphoshpate (ATP) levels, and elevated generation of reactive oxygen species (ROS). The bioactive citrus flavanone hesperetin (Hst) is known to modulate inflammatory response, to function as an antioxidant, and to provide neuroprotective properties. The efficacy in improving mitochondrial dysfunction of Hst nanocrystals (HstN) with increased bioavailability has not yet been investigated. Human SH-SY5Y cells harboring neuronal amyloid precursor protein (APP_{695}) acted as a model for the initial phase of AD. MOCK-transfected cells served as controls. The energetic metabolite ATP was determined using a luciferase-catalyzed bioluminescence assay. The activity of mitochondrial respiration chain complexes was assessed by high-resolution respirometry using a Clarke electrode. Expression levels of mitochondrial respiratory chain complex genes were determined using quantitative real-time polymerase chain reaction (qRT-PCR). The levels of amyloid β-protein ($A\beta_{1-40}$) were measured using homogeneous time-resolved fluorescence (HTRF). ROS levels, peroxidase activity, and cytochrome c activity were determined using a fluorescence assay. Compared to pure Hst dissolved in ethanol (HstP), SH-SY5Y-APP_{695} cells incubated with HstN resulted in significantly reduced mitochondrial dysfunction: ATP levels and respiratory chain complex activity significantly increased. Gene expression levels of RCC I, IV, and V were significantly upregulated. In comparison, the effects of HstN on SY5Y-MOCK control cells were relatively small. Pure Hst dissolved in ethanol (HstP) had almost no effect on both cell lines. Neither HstN nor HstP led to significant changes in $A\beta_{1-40}$ levels. HstN and HstP were both shown to lower peroxidase activity significantly. Furthermore, HstN significantly reduced cytochrome c activity, whereas HstP had a significant effect on reducing ROS in SH-SY5Y-APP_{695} cells. Thus, it seems that the mechanisms involved may not be linked to altered Aβ production. Nanoflavonoids such as HstN have the potential to prevent mitochondria against dysfunction. Compared to its pure form, HstN showed a greater effect in combatting mitochondrial dysfunction. Further studies should evaluate whether HstN protects against age-related mitochondrial dysfunction and thus may contribute to late-onset AD.

Keywords: Alzheimer disease; mitochondria; ROS; mitochondria dysfunction; nanoparticles; hesperetin; amyloid beta; peroxidase activity

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1. Introduction

To date, more than 50 million people have developed Alzheimer disease (AD), and by the end of 2050, over 152 million will be affected [1]. AD is the most common form of dementia and has its origin in various disorders of the brain [2]. Despite intensive

research, AD is not curable yet. It is only possible to treat symptoms, which can slow, but not stop, progression of the disease. One possible trigger of AD is overexpression of amyloid β-protein (Aβ). An imbalance in production and removal leads to accumulation and aggregation in the brain, resulting in inflammatory reactions, reactive oxygen species (ROS) production, and the loss of neurons, which lead to dementia [3]. However, clinical data on the efficacy of drugs that target Aβ are inconsistent with the Aβ hypothesis, and its general rejection is currently debated [4–6]. The multifactorial pathology of AD makes it difficult to develop viable therapies, and research should focus on novel targets. One promising target is mitochondrial dysfunction, which represents a final common pathway of brain aging and dementia [7].

Reports suggest that mitochondrial dysfunction is an early event in the development of AD [8,9]. Almost all mitochondrial functions are impaired in AD [7,10]. The first signs of mitochondrial dysfunction are reduced glucose consumption [11] and reduced activity of key enzymes in the oxidative metabolism [12,13]. The limited function of the electron transport chain (ETC) is due to decreased activity in complexes I and IV. This results in decreased membrane potential and ATP production [14,15]. These defects, in turn, lead to increased oxidative stress, which has a further negative effect on mitochondrial function [16].

One possible approach to prevent mitochondrial dysfunction is the use of polyphenols, especially flavonoids [17]. One of the flavonoids, Hst, is the aglycon of hesperedin, a major flavonoid in orange juice but also found in high amounts in the peel of oranges and other citrus fruits in [18,19]. HstP, which was first extracted from hesperidin by hydrolysis in 1928 [20], is a bioactive molecule that can act in the body in many different ways. In mice, HstP has a lipid-lowering effect by decreasing cholesterol and triacylglycerides, and it has a positive effect on mouse hearts, improving blood pressure and protecting against fibrosis [21,22]. That HstP might not affect the lipid profile and blood pressure in randomized clinical trials is probably due to its quite low bioavailability, which is 15% [23,24]. Various strategies are available to overcome poor solubility, and drug nanocrystals represent one of the most powerful formulation strategies to enhance the kinetic solubility and dissolution rate of poorly soluble drugs.

Hst is a natural flavonoid with high antioxidant activity, poor water solubility, and lipophilic characteristics [25]. Even though liposomes are rather crude biomimetic models of biological cell membranes, data have shown that liposome electrokinetic chromatography (LEKC) can be used to predict the behavior of compounds in living systems [26]. For hesperetin, studies have shown high experimentally determined distribution constants (log KD) of about 2.25–3.65. They claim that hesperetin belongs to the category of antioxidants that preferentially interact with hydrophobic phospholipid membrane [26]. It has been shown in an in vitro model that Hst may cross the blood–brain barrier [27]. Hst was also reported to act as a radical scavenger [28].

We recently reported high antioxidative capacity of Hst nanocrystals using a DPPH assay [25]. In a study using PC12 cells, HstP provided protection against H_2O_2-induced oxidative stress. Incubation with HstP reduced cell viability, protected against membrane destruction, intercepted ROS formation, increased catalase activity, and protected against H_2O_2-induced reduction in mitochondrial membrane potential [29]. Nanoparticles are smaller than 1000 nm, but larger than 100 nm [30]. Nano-sized formulations are particularly suitable for substances that are difficult to dissolve. Furthermore, the size of these newly formed compounds can increase the bioavailability of otherwise poorly available substances. A promising approach could be using nano-hesperetin (HstN) to improve the low bioavailability. In a study employing rats injected intracerebroventricularly with streptozotocin, HstN improved their memory and recognition. In addition, the antioxidative enzyme activity and glutathione levels were increased in contrast to HstP [31]. At the same time, the nano-form protects against premature degradation of the substance and thus prolongs its stay in the bloodstream [32,33]. In this study, we examined the effects of Hst in

both nano and pure form on mitochondrial function in SH-SY5Y-APP$_{695}$ cells, an in vitro model for the initial phase of AD [34,35].

2. Materials and Methods

2.1. Hesperetin Nanocrystals

Hst was purchased from Exquim S.A. (Spain). Alkyl polyglycosid (Plantacare 2000 UP®; PC) was used as a stabilizer. Production of hesperetin nanocrystals (HstN) was achieved by small-scale milling modified after Romeo et al. [30]. For this, coarse suspension was filled in a 2 mL glass vial with 3 stirring bars and yttria-stabilized zircon oxide milling beads (diameter 1 mm; Retsch, Haan, Germany) with a suspension-to-bead ratio of 1:1 (*v*/*v*). All vials were stirred on a magnetic stirring plate (IKA RCT standard, Haan, Germany) at 1200 rpm in ice water for 8 h. After production and before treatment of cells, particle size was strictly monitored. The average particle size was determined by photon correlation spectroscopy using a ZetaSizer NanoZS (Malvern-Panalytical, Kassel, Germany), laser diffraction using a Mastersizer 3000 (Malvern-Panalytical, Kassel, Germany), and light microscopy using an Olympus BX53 light microscope (Olympus Cooperation, Tokyo, Japan) equipped with an Olympus SC50 CMOS color camera (Olympus Soft Imaging Solutions GmbH, Nord, Germany) according to [25]. Since polyphenols have the potential to act as pan-assay interference compounds [36], we used PAINS-Remover at https://www.cbligand.org/PAINS/ (accessed on 25 May 2021) to check whether hesperetin would be likely to interfere with screening technologies [37]. We found no evidence that Hst would act as such a compound.

2.2. Cell Culture

Human neuroblastoma SH-SY5Y cells were cultured at 37 °C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (*v*/*v*) heat-inactivated fetal calf serum, 60 µg/mL streptomycin, 60 units/mL penicillin, 0.3 mg/mL hygromycin, MEM non-essential amino acids, and 1 mM sodium pyruvate 1%. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP695 (APP) or its empty expression vector pCEP4 (Invitrogen, Europe) alone as control (MOCK); for details; please refer to [35]. Cells were passaged every 3 days and were used for experiments when they reached 70–80% confluence.

2.3. Cell Treatment

Cells were incubated with concentrations of 0.01 to 10 µM HstN or HstP for 24 h after they reached confluence of 80%. Ethanol (1%) was used as a control for HstP and PlantaCare (PC) for HstN.

2.4. Homogeneous Membrane Integrity Measurement

A homogeneous membrane integrity assay was used to measure the number of nonviable cells after incubation with HstN 10 µM or HstP 10 µM. PC and EtOH served as controls. The measurement was performed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.5. ATP Measurement

A bioluminescence assay was used to measure ATP levels, which is based on the production of light from ATP and luciferin in the presence of luciferase. The measurement was performed using a ViaLight™ Plus Kit (Lonza, Basel, Switzerland) according to a previously published protocol [38]. Cells incubated with DMEM served as a control.

2.6. Cellular Respiration

Respiration in SH-SY5Y cells was assessed with an Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. The cells were treated according to a complex protocol developed by Dr. Erich Gnaiger [39]. Cells were incubated with different substrates, in-

hibitors, and uncouplers. First, cells were washed with PBS (containing potassium chloride 26.6 mM, potassium phosphate monobasic 14.705 mM, sodium chloride 1379.31 mM, and sodium phosphate dibasic 80.59 mM) and scraped into mitochondrial respiration medium (MiRO5) developed by Oroboros [39]. Afterwards, they were centrifuged, resuspended in MiRO5, and diluted to 10^6 cells/mL. After 2 mL of cell suspension was added to each chamber and endogenous respiration was stabilized, the cells were treated with digitonin (10 µg/10^6 cells) to permeabilize the membrane, leaving the outer and inner mitochondrial membrane intact. OXPHOS was measured by adding complex I and II the substrates malate (2 mM), glutamate (10 mM), and ADP (2 mM), followed by succinate (10 mM). Stepwise addition of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone showed the maximum capacity of the electron transfer system. To measure complex II activity, rotenone (0.1 mM), a complex I inhibitor, was injected. After that, oligomycin (2 µg/mL) was added to measure the leak respiration. Inhibition of complex III by the addition of antimycin A (2.5 µM) determined residual oxygen consumption, which was subtracted from all respiratory states. The activity of complex IV was measured by adding *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (0.5 mM) and ascorbate (2 mM). To measure the sodium autoxidation rate, azide (≥100 mM) was added. Afterwards, complex IV respiration was corrected for autoxidation. PC was used as control for HstN and ethanol for HstP.

2.7. Citrate Synthase Activity

A subsample of cell suspension from the respiratory measurement was immediately frozen in liquid nitrogen and stored at −80 °C. It was then measured according to a protocol described in a previous study [38]. PC was used as control for HstN and ethanol for HstP.

2.8. Real-Time qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. RNA was quantified using a Nanodrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 1 µg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). qRT-PCR was conducted using a CfX 96 Connect™ system (Bio-Rad, Munich, Germany). Primers were provided from Biomol (Hamburg, Germany). All primers are listed in Table 1. The cDNA aliquots were diluted 1:10 with RNase-free water (Qiagen, Hilden, Germany), and all samples were analyzed in triplicate. PCR cycling conditions were as follows: initial denaturation for 3 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, 58 °C for 30 s (or 56 °C for 45 s, depending on the primer), and 72 °C for 29 s. Expression was analyzed with −(2ΔΔCq) using Bio-Rad CfX manager software. Normalization factor was calculated based on the geometric mean of the levels of multiple control genes of *β-actin (ACTB)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, and *phosphoglycerate kinase 1 (PGK1)* according to the MIQE guidelines [40]. No-template control served as an assay control to exclude impurities.

2.9. Western Blotting

Samples (10 µg protein) were mixed with 20 mM Tris(hydroxymethyl)aminomethane buffer (pH 7.4), Laemmli Sample Buffer 2x (Bio-Rad, Hercules, CA, USA), and β-mercaptoethanol. After denaturation for 5 min at 95 °C, the samples were loaded on a Mini-PROTEAN TGX Gel 12% (Bio-Rad, Hercules, CA, USA) and separated by electrophoresis (35 min at 200 V). Gels were transferred onto a polyvinylidene fluoride (PVDF) membrane (90 min at 30 V). After incubation with blocking solution for 30 min, the membrane was washed 3 times with Tris-buffered saline containing Tween®20 (TBST) and incubated with primary antibodies overnight at 4 °C with constant shaking. After washing with TBST 3 times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem via Merck Millipore, Darmstadt, Germany)

for 10 min at 20 °C with constant shaking. After incubation, the membrane was again washed 3 times with TBST. Visualization was carried out with Luminata™ Western HRP Substrate (Merck Millipore, Darmstadt, Germany). GAPDH served as loading control. Band analysis was performed using a ChemiDoc XRS system (Bio-Rad, Munich, Germany). Detection of respiratory system complexes was carried out by MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (ab110413; Abcam, Cambridge, UK). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (MAB374; Merck Millipore, Darmstadt, Germany) was used to verify equal protein loading.

Table 1. Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR.

Primer	Sequence	Manufacturer	Product Size	Concentration (nM)
ß-Actin (ACTB) NM_001101.2	5′-ggacttcgagcaagagatgg-3′ 5′-agcactgtgttggcgtacag-3′	Biomol, Hamburg, Germany	234	200
ATP-synthase delta-subunit (ATP5D) NM_001687	5′-ggaagctcctcctcagcttt-3′ 5′-caggcttccgggtctttaat-3′	Biomol, Hamburg, Germany	198	200
COX subunit 5A (COX5A) NM_004255.3	5′-gcatgcagacggttaaatga-3′ 5′-agttcctccggagtggagat-3′	Biomol, Hamburg, Germany	152	200
Citrate synthase (CS) NM_004077	5′-ccatccacagtgaccatgag-3′ 5-ctttgccaacttccttctgc-3′	Biomol, Hamburg, Germany	186	400
NADH-dehydrogenase flavoprotein 1 (CI) NM_007103.3	5′-cgccacctagcgtctctatc-3′ 5′-tgaaaatccggtcttcatcc-3′	Biomol, Hamburg, Germany	213	200
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) NM_002046.2	5′-gagtcaacggatttggtcgt-3′ 5′-ttgattttggagggatctcg-3′	Biomol, Hamburg, Germany	238	200
Phosphoglycerate kinase 1 (PGK1) NM_000291.2	5′-ctgtgggggtatttgaatgg-3′ 5′-cttccaggagctccaaa-3′	Biomol, Hamburg, Germany	198	200

2.10. Aβ1-40 Measurement

After 24 h incubation, the medium in the cell culture flasks was collected and the cells were rinsed with cold PBS. The suspension was then centrifuged at 220× *g* for 5 min. Then, the supernatant was discarded and the cell pellet was resuspended in 1.5 mL PBS and protease inhibitor cocktail (Merck, Darmstadt, Germany). The suspension was then centrifuged at 112× *g* for 5 min and the supernatant was removed. The cell pellet was collected and 600 µL of cell extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated for 30 min. After that, the suspension was centrifuged at 13,000× *g* for 10 min. The supernatant was transferred to a new tube and stored at −80 °C. The supernatant was thawed on ice and pipetted onto a 384-well plate (Greiner Bio-One, Kremsmuenster, Austria). To measure the amyloid β-protein concentration, an HTRF-Amyloid-Beta 1-40 Kit (Cisbio, Codolet, France) was used, with samples treated according to the manufacturer's instructions. The optical density was then measured at 665 and 622 nm emission wavelengths. Aβ levels were normalized against protein content.

2.11. Protein Quantification

Protein content was determined using a Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

2.12. Peroxidase Activity

Peroxidase activation was measured using an Amplex™ Red Peroxidase Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cells were seeded in 96-well plates and incubated for 24 h.

2.13. Peroxidase Activity of SH-SY5Y-MOCK Cells in Presence of Aβ

$A\beta_{25\text{-}35}$ powder was dissolved and sonicated for 10 min in 5 M Tris-HCl buffer (pH 7.4) at 4 °C and aggregated at 37 °C for 16 h. Then SH-SY5Y-MOCK cells were incubated for 24 h with 10 µM and 1 µM Aβ. Peroxidase activation was measured using the Amplex™ Red Peroxidase Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Tris-HCl served as control.

2.14. Peroxidase Activity of Cytochrome c

To determine the peroxidase activity of cytochrome c, we used 2 µM cytochrome c from horse heart (Sigma-Aldrich, St. Louis, MO, USA) dissolved in Na_2HPO_4 buffer (100 mM, pH 7) combined with 25 mM guaiacol (Sigma-Aldrich, St. Louis, MO, USA). Then, 10 µM of HstN or HstP was applied, and the reaction was started by adding 10 mM H_2O_2 (Merck, Billerica, MA, USA). The reaction temperature was set to 25 °C. The resulting orange colored product was measured at 470 nm.

2.15. ROS Measurement

Cellular ROS production was determined using a DCFDA/H2DCFDA Cellular ROS Assay Kit (ab113851; Abcam, Cambridge, UK). Cells were incubated for 24 h with our substances, then the manufacturer's instructions were followed.

2.16. Statistics

Statistical analyses were performed by applying one-way ANOVA with Tukey´s multiple comparison post hoc test and Student's unpaired t-test (GraphPad Prism 9 software, San Diego, CA, USA). Statistical significance was defined for p values as follows: ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results

3.1. Mitochondrial Dysfunction in SH-SY5Y-APP_{695} Cells

SH-SY5Y-APP_{695} cells are an established model for the initial phase of AD [34,35]. Here, we confirm that SH-SY5Y-APP_{695} cells are deficient with regard to ATP levels and respiration but show an increase in Aβ levels compared to SH-SY5Y-MOCK cells. A significant reduction in respiration was measured across all complexes in SH-SY5Y-APP_{695} cells (Figure 1A). The significant lower respiration led to reduced ATP production in SH-SY5Y-APP_{695} cells compared with SH-SY5Y-MOCK cells (Figure 1B). In comparison, SH-SY5Y-APP_{695} cells produced significantly higher Aβ levels than SH-SY5Y-MOCK cells (Figure 1C). Figure 1D shows that the ROS levels in SH-SY5Y-APP_{695} cells were significantly higher than in SH-SY5Y-MOCK cells. There was no significant difference in peroxidase activity between the two models, as shown in Figure 1E. Figure 1F shows LDH release from SH-SY5Y-APP_{695} cells compared to SH-SY5Y–MOCK cells. There were no significant differences in the membrane integrity between the two cell lines.

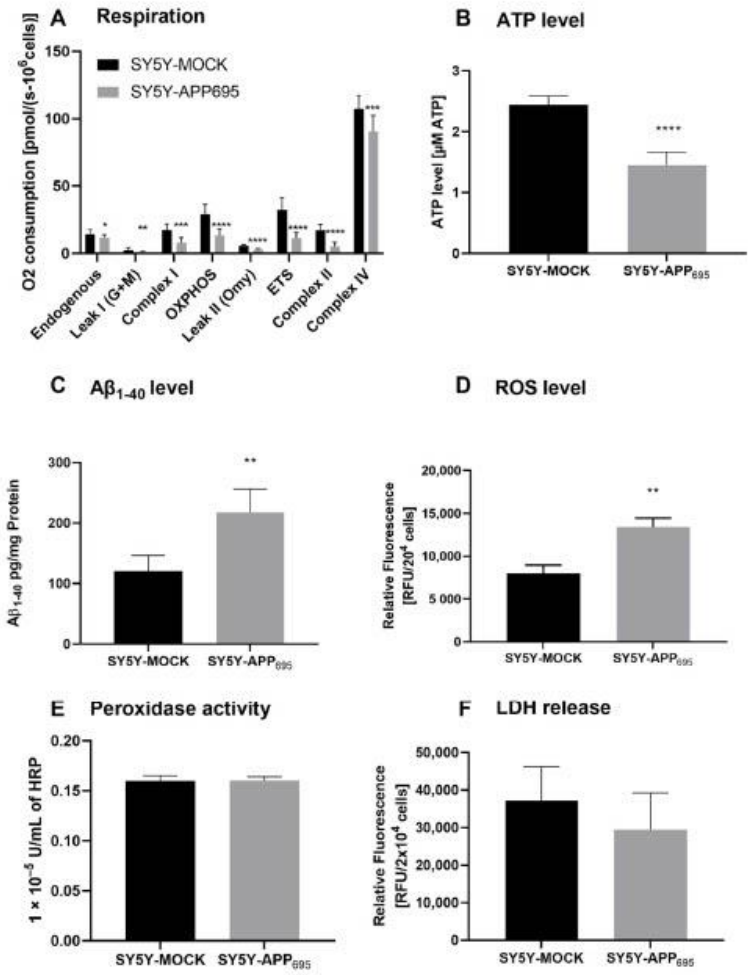

Figure 1. Respiration, ATP, $A\beta_{1\text{-}40}$ level, ROS level, and peroxidase activity of SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. (**A**) Respiration of SH-SY5Y-MOCK and -APP cells adjusted to cell count. Respective mean values ± SD are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and **** $p < 0.0001$. Significance was determined by Student's unpaired t-test, N = 12. (**B**) SH-SY5Y-APP_{695} cells exhibit reduced ATP levels compared to SH-SY5Y-MOCk control cells. ATP levels were determined after 24 h seeding by bioluminescence assay. Respective mean values ± SD are shown. Significance was determined by Student's unpaired t-test (****$p < 0.0001$), N = 8. (**C**) Concentration of $A\beta_{1\text{-}40}$ levels in SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. ** $p < 0.01$. Significance was determined by Student's unpaired t-test. N = 6. (**D**) ROS levels in RUF/20^4 cells in SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. Respective mean values ± SD are shown. Significance was determined by Student's unpaired t-test (*** $p < 0.001$), N = 8. (**E**) Peroxidase activity in SH-SY5Y-MOCK cells compared to -APP_{696} cells. Respective mean values ± SD are shown. Significance was determined by Student's unpaired t-test, N = 8. (**F**) Membrane integrity by LDH release level in RUF/20^4 cells in SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. Respective mean values ± SD are shown, N = 4.

3.2. Effect of HstN and HstP on Membrane Integrity

To examine the influence of HstN and HstP on cell viability, a membrane integrity assay was performed. Both substances, HstN (Figure 2A) and HstP (Figure 2B), had lowering effects but no significant effect on LDH release in SH-SY5Y-APP$_{695}$ cells compared to the control.

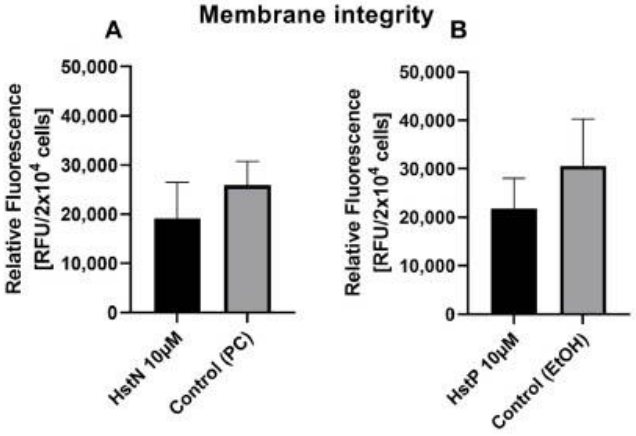

Figure 2. Effect of HstN and HstP on membrane integrity of SH-SY5Y-APP$_{695}$ cells. (**A**) Effect on membrane integrity after incubation with HstN. PC served as control. (**B**) Effect on membrane integrity after incubation with HstP. EtOH served as control. Respective mean values ± SD are shown, N = 4. Significance was determined by unpaired Student's t-test. HstN, hesperetin nanocrystal; HstP, hesperetin in pure form; PC, PlantaCare; EtOH, ethanol.

3.3. Effect of Hesperetin and Its Nanocrystals on Mitochondrial Functions

To investigate the effect of Hst, we incubated SH-SY5Y-APP$_{695}$ and -MOCK cells with HstN and HstP. First, we measured the effects of HstN and HstP (10 µM) on O_2 consumption in the two types of cells after incubation for 24 h. Then, citrate synthase activity was measured as an established mitochondrial mass marker [41], and we evaluated the impact of different concentrations of HstN and HstP on ATP levels in SH-SY5Y-APP$_{695}$ and -MOCK control cells.

Incubation of HstN had a significant increasing effect on complex I ($p = 0.0155$) of the O_2 flux in SH-SY5Y-MOCK cells adjusted to the cell count (Figure 3A). HstN had a significant effect of increasing the activity of all complexes of the respiratory chain (Figure 3B). The activity of complex I ($p = 0.053$), OXPHOS (complex I + II; $p = 0.0241$), and complex IV ($p = 0.0468$) as well as ETC ($p = 0.0162$) and leak II respiration ($p = 0.0168$) were significantly enhanced. Complex II showed the greatest changes compared to the control ($p < 0.0001$) when O_2 flux was adjusted to the citrate synthase activity (CS). To establish whether the increased activity was a result of an enhanced mitochondrial mass or higher activity of the complexes, CS was measured. CS represents an established marker for mitochondrial mass [42]. We found no significant effects between the incubation of HstN and control in both cell lines, as shown in Figure 3C,D.

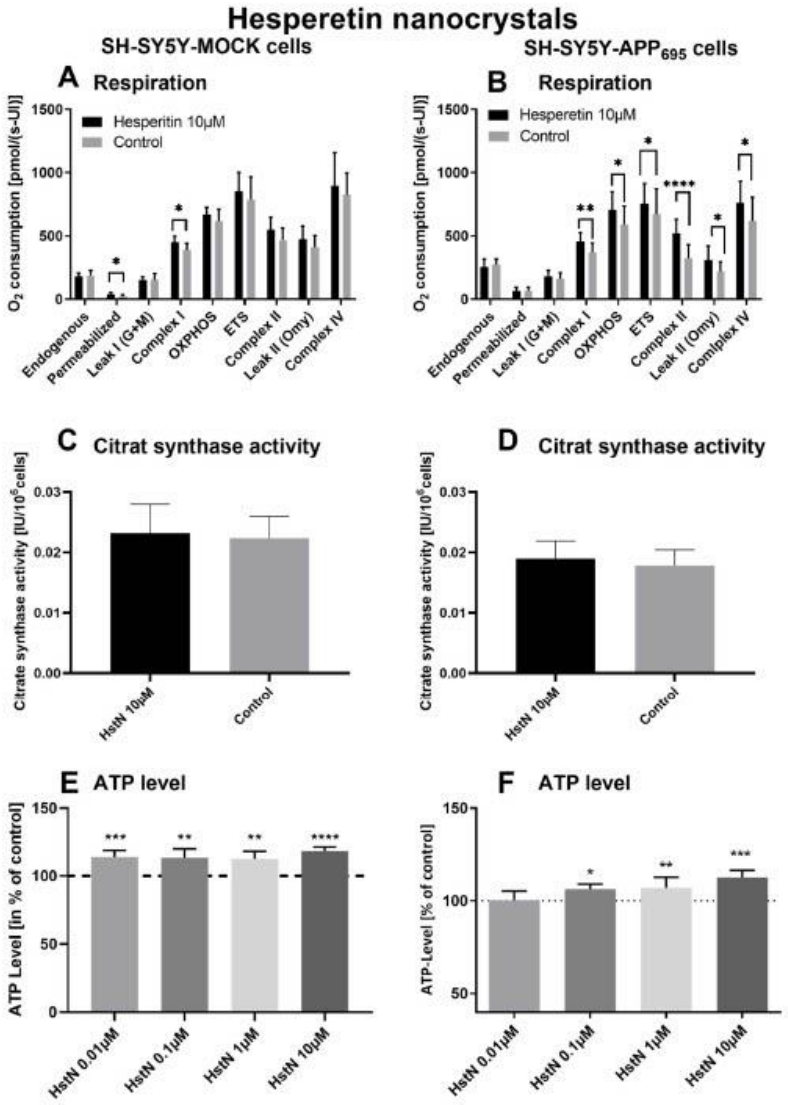

Figure 3. Respiration, citrate synthase activity, and ATP level of SH-SY5Y cells after incubation with hesperitin nanocrystals (HstN). Cells were incubated for 24 h with 10 µM HstN or PlantaCare as control, N = 12. Respective mean values ± SD are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Significance was determined by Student's unpaired t-test. Respiration of (**A**) SH-SY5Y-MOCK and (**B**) SH-SY5Y-APP_{695} cells adjusted to international units (IU) of citrate synthase activity. Citrate synthase activity of (**C**) SH-SY5Y-MOCK cells and (**D**) SH-SY5Y-APP_{695} cells. Values are given as IU of citrate synthase activity. ATP level of (**E**) SH-SY5Y-MOCK cells and (**F**) SH-SY5Y-APP_{695} cells after 24 h incubation with 0.01–10 µM HstN. Cells treated with cell culture medium served as control (100%).

To test the effect of HstN on ATP levels, SH-SY5Y-MOCK and -APP$_{695}$ cells were incubated with different concentrations for 24 h (Figure 3E,F). The 10 µM HstN concentration had the greatest influence ($p < 0.0001$). SH-SY5Y-MOCK cells showed increased levels of ATP after incubation with HstN compared to the control. On average, the ATP levels of cells incubated with HstN were 15% higher than those of the control group. SH-SY5Y-APP$_{695}$ cells showed increased ATP levels after incubation with 0.01 to 10 µM HstN. The 10 µM concentration had the greatest impact ($p = 0.008$). On average, the ATP levels were 6% higher compared to controls.

In the next step, we examined the effect of HstP on the complex activity of the respiratory chain (Table 2). It turned out that HstP had no significant effect on SH-SY5Y-MOCK and -APP$_{695}$ cells. To investigate whether HstP had an effect on the mitochondrial mass, we measured the citrate synthase activity. There were no significant differences between HstP and the solvent group with regard to the citrate synthase activity in both SH-SY5Y cell lines.

Table 2. Respiration, citrate synthase activity, and ATP levels of SH-SY5Y cells after incubation with HstP and HstN. For respiration, cells are incubated for 24 h with 10 µM HstP and HstN or EtOH and PC as control, N = 10 for HstP and N = 12 for HstN. Respective mean values are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared to control. Significance was determined with Student's unpaired t-test. Respiration of SH-SY5Y-MOCK and -APP$_{695}$ cells was adjusted to international units (IU) of citrate synthase activity. ATP levels are given in % of control and were measured after 24 h of incubation with 0.01–10 µM HstP. Cells treated with DMEM served as a control. HstP, hesperetin in pure form; HstN, hesperetin nanocrystals.

Experiment		*SH-SY5Y-MOCK*		*SH-SY5Y-APP$_{695}$*		*SH-SY5Y-MOCK*		*SH-SY5Y-APP$_{695}$*	
		HstP 10 µM	Control	HstP 10 µM	Control	HstN 10 µM	Control	HstN 10 µM	Control
Oxygraph O_2 consumption (pmol/(s-UI))	Endogenous	318 ± 45	311 ± 77	301 ± 47	322 ± 42	180 ± 26	187 ± 40	254 ± 62	273 ± 45
	Permeabilized	18 ± 11	23 ± 20	32 ± 14	37 ± 12	40 ± 15 *	24 ± 13	65 ± 30	69 ± 30
	Leak I (G+M)	102 ± 32	116 ± 22	155 ± 25	174 ± 32	151 ± 27	153 ± 51	179 ± 48	159 ± 51
	Complex I	686 ± 96	643 ± 140	546 ± 140	547 ± 155	452 ± 45 *	389 ± 53	454 ± 73 **	370 ± 74
	OXPHOS	1185 ± 163	1091 ± 358	758 ± 286	865 ± 346	669 ± 57	619 ± 94	710 ± 140 *	588 ± 149
	ETC	1395 ± 219	1275 ± 449	874 ± 274	1064 ± 240	854 ± 148	787 ± 181	753 ± 134 *	672 ± 182
	Complex II	958 ± 112	883 ± 138	600 ± 220	739 ± 179	549 ± 100	468 ± 94	522 ± 110 ****	324 ± 107
	Leak II (Omy)	910 ± 171	850 ± 297	608 ± 242	608 ± 261	477 ± 101	410 ± 93	311 ± 110 *	219 ± 78
	Complex IV	1519 ± 190	1446 ± 232	411 ± 248	415 ± 223	898 ± 258	824 ± 173	761 ± 172 *	619 ± 186
Citrate synthase activity (IU/10^6 cells)		0.01378 ± 0.02	0.0138787 ± 0.004	0.01699 ± 0.001	0.0191789 ± 0.002	0.0231924 ± 0.005	0.0223699 ± 0.004	0.018955 ± 0.003	0.0178201 ± 0.003
ATP levels (% of contral)	0.01 µM	105 ± 11		104 ± 3		114 ± 5 ***		100 ± 5	
	0.1 µM	108 ± 6		108 ± 3		114 ± 7 **		106 ± 3 *	
	1 µM	109 ± 7 *		109 ± 2 *		113 ± 6 **		107 ± 6 **	
	10 µM	100 ± 10		107 ± 6		118 ± 3 ****		113 ± 4 ***	

To investigate the effects of HstP on the ATP level, SH-SY5Y-MOCK cells were incubated for 24 h with different concentrations of HstP (Table 2). Significant elevating effects were observed with an HstP concentration of 1 µM, with ATP levels on average 9% higher than in the control group.

3.4. qRT-PCR and Western Blot Analysis

The results of the relative mRNA expression are shown in Table 3. HstN had no effect on the relative mRNA expression of citrate synthase (*CS*), *COX subunit 5A* (*COX5A*), *NADH-dehydrogenase flavoprotein 1* (*CI*), or *ATP-synthase delta-subunit* (*ATP5D*) in SH-SY5Y-MOCK cells. On the other hand, HstN had a significant boost effect on the expression of genes in SH-SY5Y-APP$_{695}$ cells. Expression of the complex I gene showed significantly increased values between HstN and PC ($p = 0.0012$). There were also significant elevated mRNA values of *COX5A* ($p = 0.0275$) and *ATP5D* ($p = 0.0229$). HstN-related increased gene expression did not result in enhanced protein levels of subunit *NDUF88* (complex I), *COX-II* (complex IV), or *ATP5D* (complex V) (Figure 4).

Table 3. Effect of HstN at relative normalized mRNA expression levels in SH-SH-SY5Y-MOCK and -APP$_{695}$ cells after incubation with HstN for 24 h, determined using quantitative real-time PCR compared to SH-SY5Y cells incubated with PlantaCare (PC). mRNA expression of control cells incubated with PC is set as 100%. Respective mean values ± SD are shown. Significance was determined with unpaired Student's t-test (* $p < 0.05$, ** $p < 0.01$). Calculation of normalization factor based on geometric mean of multiple control genes levels of *ß-actin* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and *phosphoglycerate kinase 1* (*PGK1*). HstN, hesperetin nanocrystals.

Complex	SH-SY5Y-MOCK Cells	SH-SY5Y-APP$_{695}$ Cells
CS	108.0 ± 12.44	121.1 ± 45.81
CI	66.38 ± 20.32	207.0 ± 27.35 **
COX5A	86.27 ± 12.89	195.6 ± 39.51 *
ATP5D	89.54 ± 13.9	184.5 ± 33.59 *

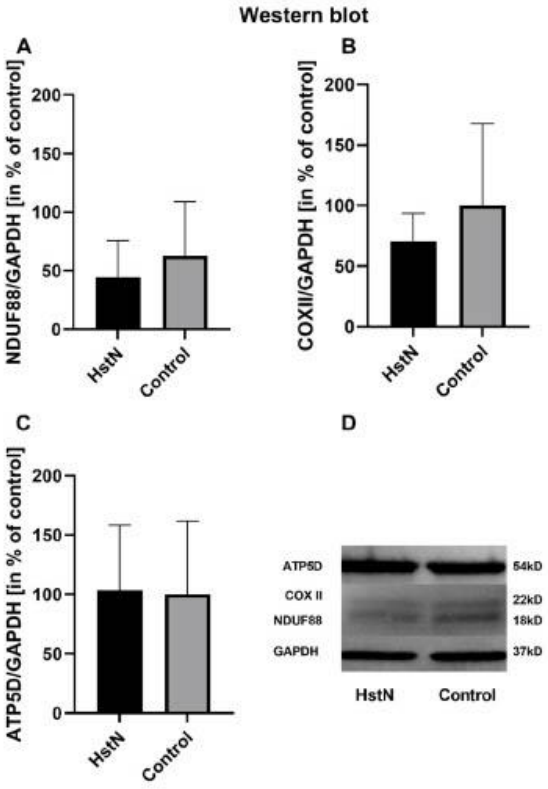

Figure 4. Western blot analysis of mitochondrial respiratory chain complexes of five experiments. Protein levels of (**A**) complex I subunit *NDUF88*, (**B**) complex IV subunit *COXII*, and (**C**) complex V subunit *ATP5D* in % of control after incubation with 10 µM HstN or PC (control) in SH-SY5Y-APP$_{695}$ cells. (**D**) Lower part: representative Western blots of one experiment. GAPDH served as loading control. Respective mean values ± SD are shown. Significance was determined by Student's unpaired t-test. HstN, hesperetin nanocrystal.

3.4. Production of $A\beta_{1-40}$

To investigate whether HstN or HstP had an effect on $A\beta_{1-40}$ levels, SH-SY5Y-APP_{695} cells were incubated for 24 h with the Hst preparations or solvent control PC or EtOH. The results in Figure 5 show that HstN and HstP had no significant effect on $A\beta_{1-40}$ levels compared to their respective solvent control in SH-SY5Y-APP_{695} cells. However, is obvious that incubation with HstN increased the expression of $A\beta_{1-40}$ numerically by about 50% (Figure 5A).

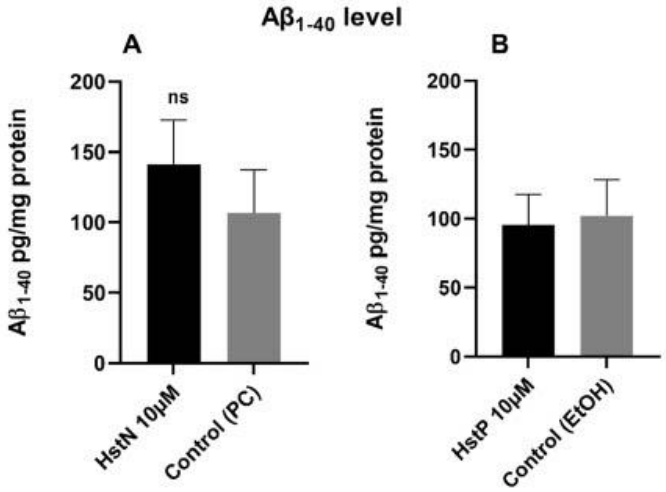

Figure 5. Effect of HstN and HstP on Aβ1-40 levels in SH-SY5Y-APP_{695} cells. Concentration of $A\beta_{1-40}$ in SH-SY5Y-APP_{695} cells: (**A**) incubated for 24 h with HstN, with PC as control, and (**B**) incubated for 24 h with HstP, with ethanol as control. Values are adjusted to protein concentrations. Respective mean values ± SD are shown, N = 6. Significance was determined with unpaired Student's t-test. ns, not significant; HstN, hesperetin nanocrystal; HstP, hesperetin in pure form; PC, PlantaCare; EtOH, ethanol.

3.5. Peroxidase Activity

In order to investigate the effects of HstN and HstP on peroxidase activity, SH-SY5Y-APP_{695} cells were incubated for 24 h with the appropriate substances. HstN (Figure 6A) had a significant lowering effect on peroxidase activity compared to the control ($p < 0.0001$). HstP (Figure 6B) had an even stronger lowering influence on peroxidase activity compared to the control ($p < 0.0001$).

3.6. Peroxidase Activity of SH-SY5Y-MOCK Cells Incubated with Aβ

To determine whether peroxidase activity is a result of Aβ, we incubated SH-SY5Y-MOCK cells with different concentrations of aggregated Aβs (Figure 7). The 10 µM Aβ concentration had a significant ($p = 0.012$) lowering effect on the peroxidase activity in comparison to the control. The 1 µM concentration had no significant effect on peroxidase activity in SH-SY5Y-MOCK cells.

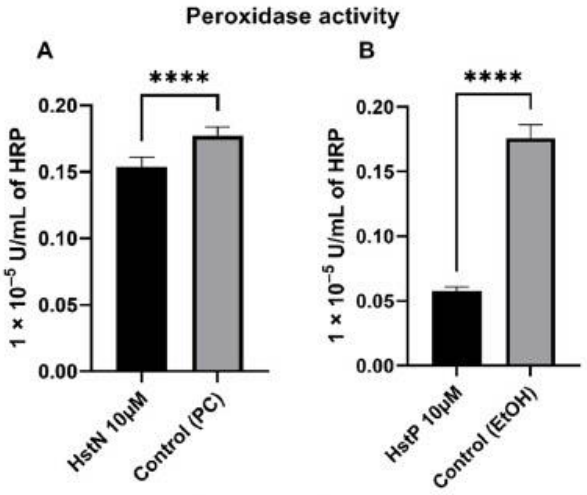

Figure 6. Effect of HstN and HstP on peroxidase activity in SH-SY5Y-APP$_{695}$ cells. Peroxidase activity in SH-SY5Y-APP$_{695}$ cells: (**A**) incubated for 24 h with HstN, with PC as control, and (**B**) incubated for 24 h with HstP, with ethanol as control. Respective mean values ± SD are shown, N = 10. Significance was determined by unpaired Student's t-test. **** $p < 0.0001$. HstN, hesperetin nanocrystal; HstP, hesperetin in pure form; PC, PlantaCare; EtOH, ethanol.

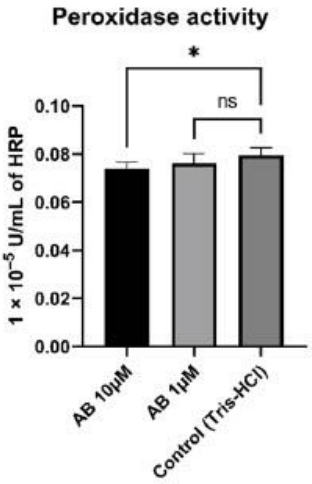

Figure 7. Effect of Aβ on peroxidase activity in SH-SY5Y-MOCK cells incubated with different concentrations of Aβ for 24 h. Respective mean values ± SD are shown, N = 7. Significance was determined by one-way ANOVA. * $p < 0.05$. Aβ, amyloid beta.

3.7. Peroxidase Activity of Cytochrome c

To test whether the lower peroxidase activity was a result of reduced activity of cytochrome c, which can act in a similar way to a peroxidase, we measured the activity of cytochrome c in the presence of HstN (Figure 8A) and HstP (Figure 8B). The results show

that HstN significantly decreased the activity of cytochrome c as a peroxidase compared to the control ($p = 0.0225$). HstP had a lowering effect but not a significant one.

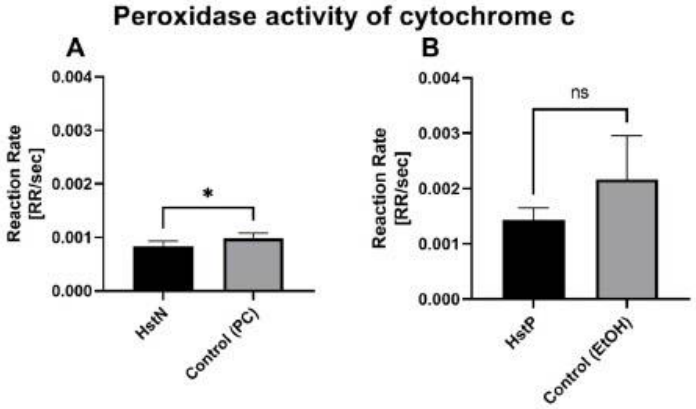

Figure 8. Effects of HstN and HstP on peroxidase activity of cytochrome c: (**A**) incubated with HstN, with PC as control (N = 6), and (**B**) incubated with HstP, with ethanol as control (N = 5). Respective mean values ± SD are shown. Significance was determined with unpaired Student's t-test. * $p < 0.05$. HstN, hesperetin nanocrystal; HstP, hesperetin in pure form; PC, PlantaCare; EtOH, ethanol.

3.8. ROS Measurement

In the next step, we examined the effects of HstN and HstP on general ROS expression in SH-SY5Y-APP$_{695}$ cells. We found a small lowering effect of ROS after incubation with HstN (Figure 9A). In contrast, HstP (Figure 9B) had a significant decreasing effect on ROS production compared to the control ($p = 0.045$).

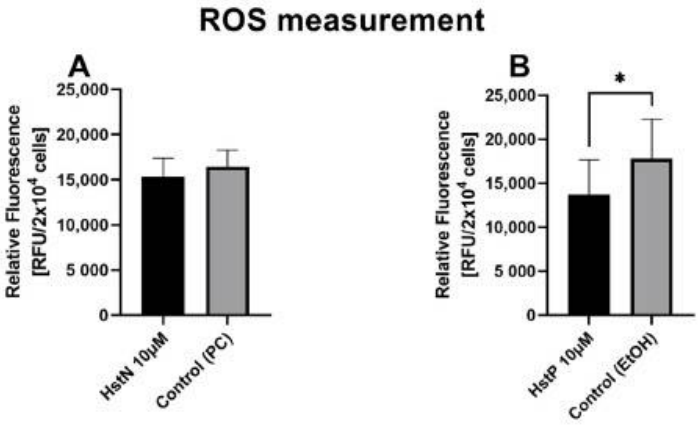

Figure 9. Effect of HstN and HstP on expression of ROS production in SH-SY5Y-APP$_{695}$ cells: (**A**) incubated for 24 h with HstN, with PC as control, and (**B**) incubated for 24 h with HstP, with ethanol as control. Respective mean values ± SD are shown, N = 10. Significance was determined with unpaired Student's t-test. * $p < 0.05$. HstN, hesperetin nanocrystal; HstP, hesperetin in pure form; PC, PlantaCare; EtOH, ethanol.

4. Discussion

In the present work, we examined the effects of HstN and HstP in a cellular model of early Alzheimer's disease, focusing on mitochondrial function in SH-SY5Y cells. Mitochondrial dysfunction is an early indicator of AD [10].

Effect of Hesperetin on Mitochondrial Function in SH-SY5Y-MOCK and -APP$_{695}$ Cells

Mitochondria are the powerhouses of cells and responsible for energy production in the form of ATP. To investigate the effects on mitochondrial function, we incubated SH-SY5Y-MOCK cells with HstN and measured the ATP levels (Figure 3E). All concentrations of HstN significantly enhanced ATP levels. The 10 µM concentration had the greatest effect. Biesemann et al., who incubated human muscle cells with HstP, reported similar results. They pointed out that 10 µM HstP had the highest effect on increasing cellular ATP levels [43]. Mitochondria are not only the main producers but also targets of ROS [44,45]. Mitochondrial ROS production has a negative effect on ATP levels [45]. A possible explanation for the effect of HstP is that this flavonoid [46] has scavenging effects and protects the cells against ROS-induced damage. Previous work showed that HstP functions as an antioxidant and protects cells against oxidative stress [29,47]. This effect could lead to increased production of ATP compared to untreated controls. Dissolving compounds in solvents is commonly performed when testing them in cell cultures; however, harmful effects such as precipitation, induced oxidative stress and complications must also be considered when applying them in vivo. To circumvent this, we developed a nano-formulation of Hst and compared the effects with its pure form (dissolved). SH-SY5Y-MOCK and -APP$_{695}$ cells were incubated with different concentrations of HstP. Regarding ATP levels, only the 1 µM concentration of HstP had a significant enhancing effect in both cell types (Table 2). A possible explanation could be the particle size of the nanocrystals. In a previous study, we determined that Hst with smaller particles had a greater effect on elevating ATP levels in SH-SY5Y-APP$_{wt}$ cells compared to larger ones [25]. Similar, most studies have shown that the particle size has an impact on cell functions [48–50]. Thus, HstP may have had a lower effect on ATP levels due to its particle size.

To investigate whether HstN or HstP would influence cell membrane integrity, we incubated SH-SY5Y-APP$_{695}$ cells with both substances (Figure 2). LDH release is a good marker for cell viability [51], and here, HstN and HstP seemed to improve cell viability and lower LDH release in the surrounding medium but not significantly.

To test the impact of Hst on O_2 flux, we incubated SH-SY5Y-MOCK and -APP$_{695}$ cells with HstN and HstP. Incubation of both types of cells with HstP did not significantly alter O_2 consumption (Table 2). Moreover, it did not affect citrate synthase activity, which is a good biomarker for mitochondrial mass [42].

When SH-SY5Y-MOCK cells were incubated with HstN and adjusted to citrate synthase activity, complex I showed significant improvement O_2 consumption compared to the control (Figure 3A). As complex I activity plays an integral part in creating the proton gradient necessary for energy production, a significant increase in its activity is reflected in increased ATP levels (Figure 3E). The increased complex activity cannot be explained by increased mitochondrial mass, as citrate synthase activity was unchanged (Figure 3C). In SH-SY5Y-APP$_{695}$ cells incubated with HstN, all complexes showed increased activity compared to controls (Figure 3B). To study whether the increased complex activity was a result of increased mitochondrial mass, we measured the citrate synthase activity, which again showed no difference between the two groups (Figure 3D). The Aβ deposition resulting from AD could have a negative effect on the electron transport chain, most notably on complex I and IV [52,53]. This leads to lower activity of the respiratory chain and reduced energy metabolism [54]. Damaged or inhibited respiratory chain complexes produce more ROS, leading to increased oxidative stress [55]. These effects seem to be mitigated by HstN, so the incubated groups had higher complex activity. Kheradmand et al. and Moghaddam et al. showed that HstP and HstN upregulate antioxidative mechanisms; for example, catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRx), and glutathione

(GSH) levels were upregulated in a rat model of AD [31,56]. Thus, HstN has a greater effect compared to HstP. Due to the activation of those mechanisms, the antioxidative effect of Hst may not only be a result of scavenging ROS but also be mediated by the activation of antioxidative systems, which, in turn, would lead to enhanced complex activity compared to control cells.

Furthermore, we studied the effects of HstN on the expression of genes encoding the production of key protein complexes of the respiratory chain. The expressions of *CS*, *COX5A*, *CI*, and *ATP5D* were determined after incubation of SH-SY5Y-MOCK and -APP_{695} cells with HstN (Table 3). HstN had no significant effect on the mRNA levels in SH-SY5Y-MOCK cells. Incubation of SH-SY5Y-APP_{695} cells with Hst significantly increased the expression of *CI*. HstN also significantly upregulated the gene expression of *COX5A* and *ATP5D*. Mastroeni et al. reported that the gene expressions of complexes I, IV, and V were reduced in patients with AD [57]. Biesemann et al. observed that 10 µM of HstP increased the expressions of complexes I, III, and IV in human muscle cells [43]. Similarly, in our study, HstN increased the mRNA levels of all three investigated complexes, suggesting increased production of the complexes. However, despite elevated mRNA levels, no increased complex protein levels were found (Figure 4). It has been shown that elevated gene expression is not necessarily associated with increased protein production [58]. Thus, the increased complex activity and the resulting elevated ATP levels in SH-SY5Y-APP_{695} cells after HstN incubation might not be explained by higher amounts of protein.

Next, we examined the effects of Hst on the production of Aβ in SH-SY5Y-APP_{695} cells (Figure 5). Hst in its pure and nano forms had no significant impact on Aβ1-40 levels. Thus, the advantageous effects of HstN on mitochondrial function might be independent of the Aβ pathway. Similarly, we recently reported that olesoxime, a modulator of the mitochondrial permeability transition pore, improved mitochondrial dysfunction and increased Aβ levels in Thy1-$AβPP_{SL}$ mice and HEK_{293} cells [59]. As Hst also interacts with membranes [60,61], future studies should explore whether this interaction affects APP processing at the plasma membrane [62].

Then, we investigated the effects of HstN and HstP on peroxidase activity. Both substances had a lowering effect on peroxidase activity (Figure 6). We hypothesize that the lower peroxidase activity is a result of reduced peroxidase activity of cytochrome c. Cytochrome c can act in a similar way to a peroxidase, which will then catalyst the oxidation of cardiolipin. This promotes an early stage of apoptosis and the release of cytochrome c, which will accelerate cell death [63–65]. HstN had a significant reducing effect on cytochrome c activity (Figure 8A). HstP seemed to reduce the activity, but this was not significant (Figure 8B). Potentially higher peroxidase activity may be a result of complex formation from Aβ with heme, which can act in a similar way to a peroxidase [66–68]. To investigate this, we incubated SH-SY5Y-MOCK cells with low levels of Aβ with different concentrations of aggregated Aβ (Figure 7). Contrary to our original assumption, Aβ actually decreased peroxidase activity instead of increasing it. A possible explanation could be the aggregation condition of the Aβ peptides. Yuan et al. found that non-aggregated Aβ had higher peroxidase activity compared with aggregated [67]. A second possible explanation could be the Aβ peptide used. Here, we used $Aβ_{25-35}$, which is more neurotoxic than $Aβ_{1-40}$ or $Aβ_{1-42}$ [69] but maybe does not bind as well as those other peptides to heme to increase peroxidase activity [67].

In the last step, we measured the ROS level in SH-SY5Y-APP695 cells (Figure 9) to confirm our hypothesis that Hst had a protective effect against ROS, which would explain the increased ATP levels and complex activity. We found that HstP had a significantly greater effect on reducing ROS compared to HstN. This contradicts our theory that the increased ATP levels and complex activity were due to increased protection by ROS. Thus, the effects shown are not solely due to the protective effect against ROS in HstN.

We conclude that HstN has a larger influence on mitochondrial functions, especially in the SH-SY5Y-APP_{695} AD cell model. Similarly, Kheradmand et al. showed greater effects for HstN in a Wistar AD rat model [31].

At this point, it should be noted that the cell model used, which was described as a model of early Alzheimer's disease, is essentially an Aβ overexpression model that does not, of course, reflect the complex pathophysiological processes of neurodegeneration. Nevertheless, our data may help to understand the molecular basis of the effects of Hst on the disease process.

5. Conclusions

In this study, we investigated the effects of Hst in its pure and nanocrystal forms on mitochondrial functions in SH-SY5Y-APP$_{695}$ cells. Hst nanocrystals had a superior beneficial impact on mitochondrial dysfunction compared to the pure form in a cellular model of early AD. The beneficial results in mitochondrial function may be linked to effects on gene expression but not on the expression of complex protein levels, including Aβ production. Furthermore, HstN and HstP reduce peroxidase activity, and especially HstN reduces the activity of cytochrome c as a peroxidase.

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3.2 Effects of Combining Biofactors on Bioenergetic Parameters, Aβ Levels and Survival in Alzheimer Model Organisms

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Effects of Combining Biofactors on Bioenergetic Parameters, Aβ Levels and Survival in Alzheimer Model Organisms

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Abstract: Increased amyloid beta (Aβ) levels and mitochondrial dysfunction (MD) in the human brain characterize Alzheimer disease (AD). Folic acid, magnesium and vitamin B6 are essential micronutrients that may provide neuroprotection. Bioenergetic parameters and amyloid precursor protein (APP) processing products were investigated in vitro in human neuroblastoma SH-SY5Y-APP695 cells, expressing neuronal APP, and in vivo, in the invertebrate *Caenorhabditis elegans* (CL2006 & GMC101) expressing muscular APP. Model organisms were incubated with either folic acid and magnesium-orotate (ID63) or folic acid, magnesium-orotate and vitamin B6 (ID64) in different concentrations. ID63 and ID64 reduced Aβ, soluble alpha APP (sAPPα), and lactate levels in SH-SY5Y-APP695 cells. The latter might be explained by enhanced expression of lactate dehydrogenase (LDHA). Micronutrient combinations had no effects on mitochondrial parameters in SH-SY5Y-APP695 cells. ID64 showed a significant life-prolonging effect in *C. elegans* CL2006. Incubation of GMC101 with ID63 significantly lowered Aβ aggregation. Both combinations significantly reduced paralysis and thus improved the phenotype in GMC101. Thus, the combinations of the tested biofactors are effective in pre-clinical models of AD by interfering with Aβ related pathways and glycolysis.

Keywords: Alzheimer disease; mitochondria; mitochondria dysfunction; folic acid; vitamin B6; magnesium-orotate; amyloid beta; *C. elegans*; biofactor

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1. Introduction

At present, 50 million people are suffering from Alzheimer's disease (AD) and this number will rise to approximately 152 million in 2050 [1]. Unfortunately, there is no cure for AD yet. Approved drugs only treat symptoms [2]. There are several hypotheses regarding the etiology of Alzheimer's disease, but the causes of the disease are unknown. Previous research has focused on amyloid and tau, which has not yet led to major breakthroughs. Therefore, there is a trend towards multifactorial treatments and, among other things, energy metabolism with regard to mitochondrial functions. Two of the hallmarks of AD are mitochondrial dysfunction (MD) [3] and overproduction of beta-amyloid (Aβ) [4]. The first signs of beginning MD are a reduction of glucose consumption [5] and a reduced activity of key enzymes of the oxidative metabolism [6,7]. Almost all mitochondrial functions are impaired in AD [8,9]. The limited function of the electron transport chain (ETC) is the reason for the decrease in complexes IV and I. This results in a decreased mitochondrial membrane potential (MMP) and ATP production [10]. Another important characteristic of AD is that Aβ is cleaved of from a much larger amyloid precursor protein (APP) [11]. APP is cleaved via two pathways, a non-amyloid and an amyloidogenic pathway. The APP is spliced by the different types of protease, namely α-, β- and γ-secretase [11,12]. Depending on which protease cleaves the APP, Aβ peptides are produced. The α-protease cleaves the APP closer to the membrane, resulting in a shorter fragment in the membrane, which is then further cleaved by the γ-protease to a non-amyloidogenic product. However, when β-protease cleaves the protein, larger fragments are produced, which are then cleaved by

γ-protease to form different large Aβ proteins [12] including $A\beta_{1-40}$ and $A\beta_{1-42}$ which may be the main triggers for AD [2,11,12]. It appears that $A\beta_{1-42}$ has the greater neurotoxic potential compared to the $A\beta_{1-40}$ form. Furthermore, $A\beta_{1-42}$ tends to aggregate more, which can lead to plaque formation. In addition, the relationship between $A\beta_{1-40}$ and $A\beta_{1-42}$ is important, as the two influence each other [13–15]. There is evidence that sAPPα and sAPPβ share some properties [16], where sAPPα can be neuroprotective, whereas sAPPβ lacks most of the neuroprotective properties and has a rather negative effect [17]. Another early sign of AD is impaired glucose metabolisation which leads to MD and an increase in oxidant production [18,19]. The glycolysis pathway represents a way to ensure sufficient energy production and bypass Aβ induced impairment of mitochondria [20,21]. The enzymes pyruvate dehydrogenase kinase 1 (PDK1) and LDHA are of interest here. PDK1 phosphoylates pyruvate dehydrogenase and inactivates it. LDHA converts pyruvate into lactate. Both are markers of aerobic glycolysis. If a change occurs here, conclusions can be drawn about energy production [22,23].

There is evidence that specific biofactors [24], which are defined as substances required by the body for its normal physiological functioning and/ or with health-beneficial and/ or disease-preventive biological activities, may interfere with pathophysiological processes leading to AD [25–27]. A cocktail containing some of our tested compounds had a positive effect on AD symptoms in a TgF344-AD rat model. This could raise the mitochondrial function of the transgenic rats to the level of the wild-type rats [28]. Here, folic acid, magnesium-orotate and vitamin B6 in different combinations were tested in cellular and an invertebrate model of AD. The synthetically produced water-soluble folic acid, which consists of pterin, p-aminobenzoic acid and L-glutamic acid, belongs to the vitamin B complexes. Folate (also known as vitamin B9) is used as an umbrella term for the various derivatives of tetrahydrofolate (THF), with the synthetically produced form being referred to as folic acid [29,30]. Folate deficiency impairs DNA as well as mtDNA synthesis and stability and causes oxidative stress in the form of ROS, which, as already listed, is also associated with AD pathogenesis. In this context, neuronal impairment and increased cell death occur in AD. In addition, a deficiency of folate leads to a decrease in the methylation of enzymes and promoter regions of genes that are presumably also involved in AD pathogenesis [31,32]. Magnesium-orotate (MgOr), which is very poorly soluble in water, is the magnesium salt of orotic acid. As a source of magnesium (Mg), MgOr is used for the oral treatment of Mg deficiency. Orotic acid is a key intermediate in the biosynthetic pathway of pyrimidines and improves energy status by stimulating, among other things, the synthesis of glycogen and ATP [33]. Mg^{2+} is the fourth most abundant mineral as well as the second most abundant intracellular divalent cation in the human body and acts as a cofactor [34–36]. Mg is involved in protein synthesis, cellular energy production and storage, reproduction, DNA and RNA synthesis, and mitochondrial membrane potential [35]. Mg is also involved in the maintenance of physiological nerve and muscle function, cardiac excitability, and neuromuscular conduction [35,36]. Several pathological mechanisms in AD are discussed on which Mg might have a positive influence. Mg appears to inhibit the activity of γ-secretase and the proinflammatory TNF-α (tumor necrosis factor α) produced by microglia. Mg also inhibited IL-1β (interleukin-1β) and Aβ-induced, which all together induced inflammation. In addition, Mg has been reported to decrease the influx of Aβ across the blood-brain barrier [34]. High Mg concentrations have been shown to promote APP processing towards α-secretase due to the upregulation of transcription factors such as CREB [37]. Mg deficiency may be a risk factor for ADs and that possible supplementation may be a potentially valuable adjunct treatment for AD [38]. The water soluble vitamin B6 (Vit B6) is used as an umbrella term for various derivates from which pyridoxine is the most common form. It is an enzymatic cofactor required for more than 140 biochemical reactions, including transaminations, α-decarboxylations and replacement reactions [39]. Through the application of Vit B6, the oxidative stress induced by Aβ could be inhibited [40]. Furthermore, Vit B6 reduces the plasma levels of Aβ [41] and prevents the grey matter atrophy related to AD [42].

The present work investigated the effects of different B vitamins and MgOr on MD and the processing of APP in SH-SY5Y-APP$_{695}$ cells a cellular model of early AD. Furthermore, the effects of the substances were tested in CL2006 and GMC101, both invertebrate models of AD.

2. Results

2.1. General Overview of Tests and Results

In Table 1 below, all tests and results are listed to provide a general overview of the subsequent tests and results. Here, the substance under investigation is shown against the control. For more detailed insights, the results are described in the respective chapters.

Table 1. General overview of all tests and results of all biofactors. Combinations ID63 and ID64 and the single substances compared to the control.

	ID63 vs. CTR	ID64 vs. CTR	MgOr vs. CTR	Fol vs. CTR	Vit B6 vs. CTR
Aβ_{1-40}	Significant lower	Significant lower	Significant lower	Significant lower	No significant change
Aβ_{1-42}	No significant change	Significant lower	Significant lower	Significant lower	Significant lower
sAPPα	Significant lower	Significant lower	Not tested	Not tested	Not tested
sAPPβ	No significant change	No significant change	Not tested	Not tested	Not tested
ATP level	No significant change	No significant change	Not tested	Not tested	Not tested
MMP level	No significant change	No significant change	Not tested	Not tested	Not tested
Respiration	No significant change	No significant change	Not tested	Not tested	Not tested
Citrate synthase activity	No significant change	No significant change	Not tested	Not tested	Not tested
Lactate level	Significant lower	Significant lower	Not tested	Not tested	Not tested
Pyruvate level	No significant change	No significant change	Not tested	Not tested	Not tested
Lactate/Pyruvate Ratio	No significant change	Significant lower	Not tested	Not tested	Not tested
Gen expression PDK1	No significant change	No significant change	Not tested	Not tested	Not tested
Gen expression LDHA	No significant change	Significant higher	Not tested	Not tested	Not tested
Lifespan *C. elegans* in %	No significant change	Significant higher	Significant higher	Significant higher	Significant higher
Mean survival *C. elegans*	Significant higher	Significant higher	Significant higher	Significant higher	Significant higher
Paralysis *C. elegans*	Significant lower	Significant lower	Not tested	Not tested	Not tested
Aβ_{1-42} *C. elegans*	No significant change	No significant change	Not tested	Not tested	Not tested
Aβ_{1-42} aggreation *C. elegans*	Significant lower	No significant change	Not tested	Not tested	Not tested

2.2. Aβ_{1-40} Production

First, we tested different concentrations of biofactors on Aβ_{1-40} production in SH-SY5Y-APP$_{695}$ cells. Cells were incubated for 24 h with zinc orotate (ZnO), magnesium-orotate (MgO), benfotiamine (vitamin B1), folic acid (Fol), cholecalciferol (Vit D3), cobalamin (Vit B12), and pyridoxine (Vit B6) to select possible hit substances for further experiments (data not shown). Potential hit substances were identified, from which finally Fol 10 µM, MgOr 200 µM and Vit B6 100 nM turned out to be the most promising ones, which we applied in two different combinations.

To investigate the effect on the Aβ_{1-40} production, SH-SY5Y-APP$_{695}$ cells were incubated with both combinations (MgOr 200 µM & Fol 10 µM = ID63//MgOr 200 µM & Fol 10 µM & Vit B6 100 nM = ID64) and the single compounds for 24 h (Figure 1). ID63 had a significant lowering effect on the Aβ_{1-40} level ($p > 0.0001$). The ID63 combination even had an over additive effect compared to the single substances MgOr ($p = 0.0028$) and Fol ($p > 0.0001$). The ID64 combination had a significantly decreasing effect on the Aβ

levels compared to the control ($p > 0.0001$). Furthermore, ID64 had a significantly reducing effect in comparison to the single substances MgOr ($p = 0.0497$), Fol ($p > 0.0001$) and Vit B6 ($p > 0.0001$).

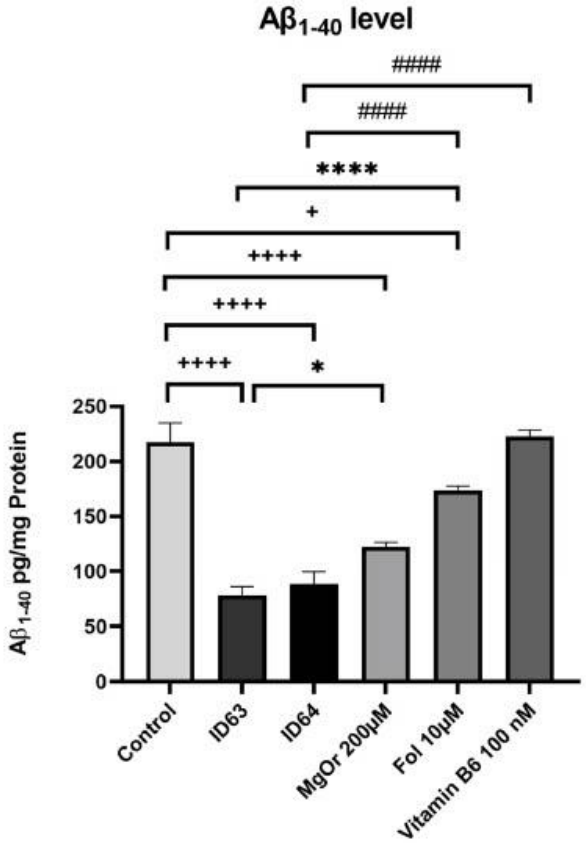

Figure 1. Effect of ID63 and ID64 in SH-SY5Y$_{695}$ cells compared to the control or their single substances on the Aβ_{1-40} level after 24 h incubation. N = 6. Aβ_{1-40} levels were adjusted to the protein content. Significance was determined by Student's unpaired *t*-test and one-way ANOVA. + significant against control; * significant against ID63; # significant against ID64. * $p < 0.05$, **** $p < 0.0001$, #### $p < 0.0001$ and + $p < 0.05$, $^{++++}$ $p < 0.0001$. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

2.3. *Aβ_{1-42} Production*

To study the production of Aβ_{1-42}, SH-SY5Y-APP$_{695}$ cells were incubated for 24 h with ID63, ID64 or the single compounds (Figure 2). In comparison to the control, the single compounds MgOr ($p = 0.0024$) and folic acid ($p = 0.0004$), as well as the combination ID 64 ($p = 0.0039$) had a significant lowering effect on Aβ_{1-42} levels, while the combination ID63 had a slight reducing effect on the Aβ_{1-42} levels, although not a significant one. ID64 also showed significantly lower Aβ_{1-42} levels in comparison to B6 ($p = 0.0009$). However, folic acid alone, reduced the levels to a higher extent than any combinations.

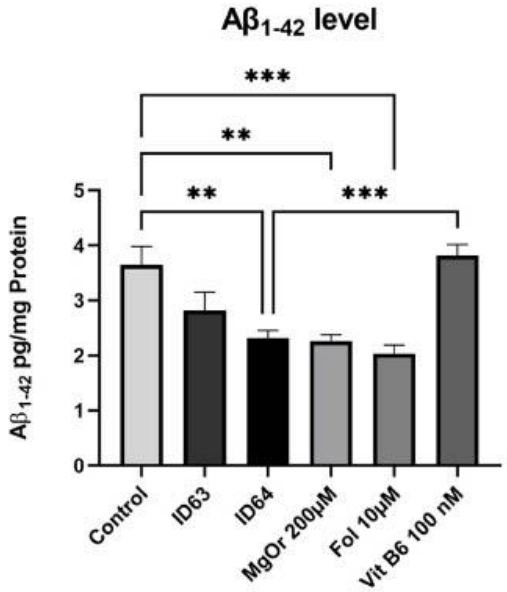

Figure 2. Effect of ID63 and ID64 in SH-SY5Y$_{695}$ cells compared to the control or their single substances on the Aβ_{1-42} level after 24 h incubation. N = 6. Aβ_{1-42} levels were adjusted to the protein content. Significance was determined by Student's unpaired *t*-test and one-way ANOVA. ** $p < 0.01$ and *** $p < 0.001$. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

2.4. *sAPPα and sAPPβ Level*

The α- and β-secretase cleaving products of APP, sAPPα (Figure 3A) and sAPPβ (Figure 3B), respectively, were determined after incubation with either ID63 or ID64 for 24 h. Figure 4A shows that ID63 had a significantly lowering effect on the sAPPα ($p = 0.0214$) compared to the control. ID64 had an even greater effect on the reduction of sAPPα fragments ($p > 0.0001$). In contrast to the sAPPα fragment production, the sAPPβ fragments (Figure 3B) were lowered compared to the control though not significantly. ID64 had a greater effect than ID63. It should be noted that basal levels of sAPPβ were approximately one hundredfold lower compared with sAPPα (Figure 3).

2.5. *Effect on the Mitochondrial Function*

To investigate the effect of ID63 and ID64 on mitochondrial function, we incubated SH-SY5Y-APP$_{695}$ cells for 24 h with ID63 or ID64. Respiration under O_2 consumption through the respiratory chain builds up the mitochondrial membrane potential, which allows ATP to be generated with the help of ATP synthase. First, we measured the ATP level after incubation with ID63 or ID64. Afterwards, the MMP was examined as well as the O_2 consumption and citrate synthase activity (Figure 4).

Neither ID63 nor ID64 had an increased effect on the ATP level (Figure 5A,B) or an effect on the MMP level (Figure 4C,D). ID63 had a slightly increasing effect on the complex activity of complex I, II and IV compared to the control (Figure 4E). In contrast, ID64 had a slightly decreasing effect on the complex activity of complex II and IV (Figure 4F). Whereas ID63 had no effect on the citrate synthase activity compared with the control (Figure 4G),

and ID64 even had a slightly decreasing effect compared with the control (Figure 4H). However, none of these effects is statistically significant.

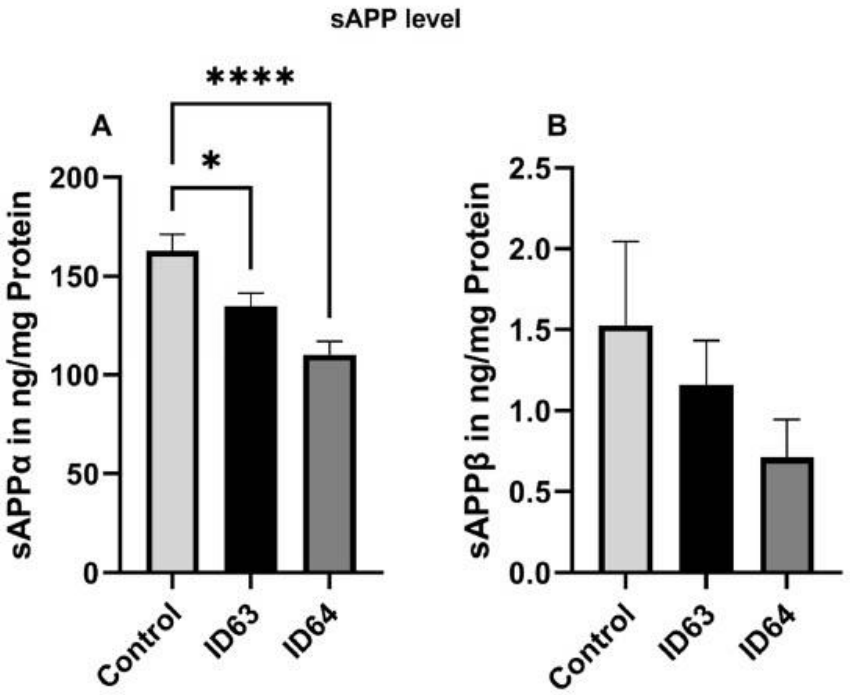

Figure 3. Effect of the incubation with ID63 or ID64 on the human soluble amyloid precursor protein α (sAPPα) and β (sAPPβ) after 24 h of incubation. (**A**) sAPPα level of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. (**B**) sAPPβ level of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. N = 6. sAPP levels were adjusted to the protein content. Significance was determined by one-way ANOVA. * $p < 0.05$ and **** $p < 0.0001$. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

2.6. Lactate and Pyruvate Level

To investigate if the glycolysis is affected by ID63 or ID64, the lactate and pyruvate levels were measured. As seen in Figure 6, only lactate is significantly reduced by ID63 ($p = 0.0328$) and ID64 ($p > 0.0001$) compared to the control while pyruvate levels were not influenced. The ratio of lactate/pyruvate was significantly affected by ID64 ($p = 0.0057$) (Figure 5C).

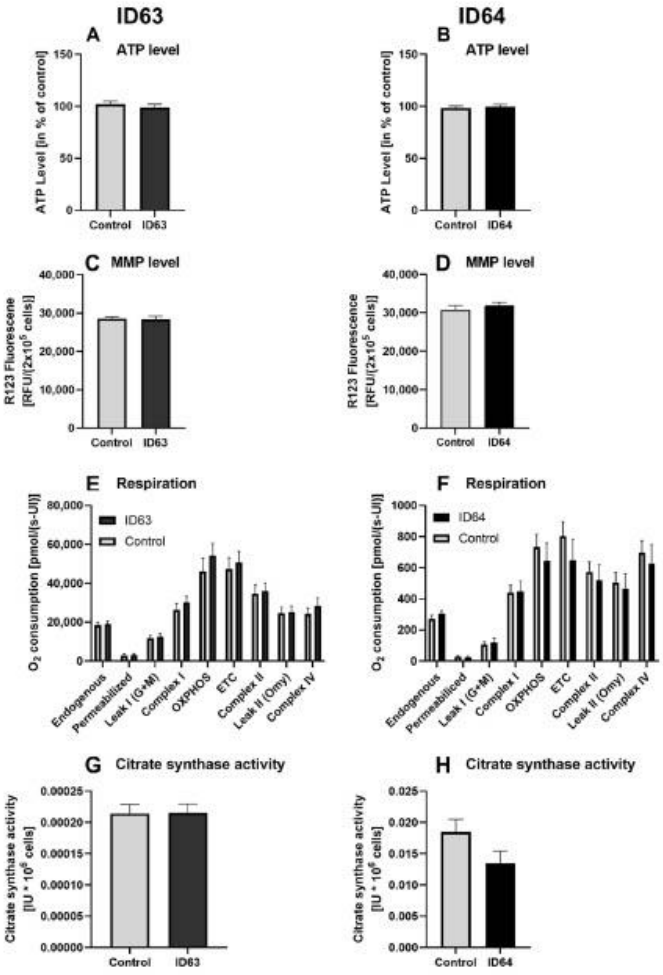

Figure 4. ATP level, MMP level, respiration and citrate synthase activity of SH-SY5Y-APP$_{695}$ cells incubated for 24 h with ID63 or ID64. (**A**) ATP level of SH-SY5Y-APP$_{695}$ cells incubated with ID63 and (**B**) ATP level of incubation with ID64 compared to the control. Cells treated with cell culture medium served as control (100%). N = 12. (**C**) MMP level of 2×10^5 SH-SY5Y-APP$_{695}$ cells incubated with ID63 and (**D**) MMP level of incubation with ID64 compared to the control. N = 16. (**E**) Respiration of SH-SY5Y-APP$_{695}$ cells incubated with ID63 and (**F**) incubation with ID64 compared to the control. SH-SY5Y-APP$_{695}$ cells adjusted to international units (IU) of citrate synthase activity. N = 15. (**G**) Citrate synthase activity of SH-SY5Y-APP$_{695}$ cells incubated with ID63 and (**H**) Citrate synthase activity incubated with ID64 compared to control. N = 12. Significance was determined by Student's unpaired *t*-test. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6. Leak I (G + M) = leak respiration with glutamate and malate; OXPHOS = oxidative phosphorylation system; ETC = electron transport chain; Leak II (Omy) = leak respiration with oligomycin.

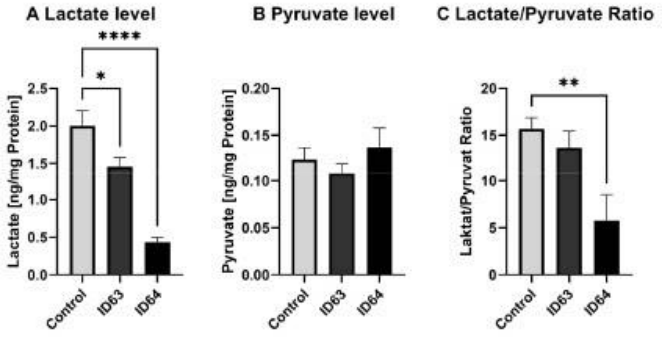

Figure 5. Effect of the incubation with ID63 or ID64 on lactate and pyruvate level after 24 h of incubation. (**A**) Lactate level of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. (**B**) Pyruvate level of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. (**C**) Lactate to pyruvate ratio. N = 6. Levels were adjusted to the protein content. Significance was determined by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

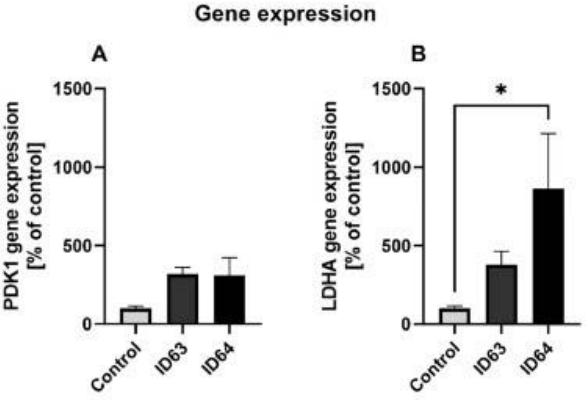

Figure 6. Effect of the incubation with ID63 or ID64 on the gene expression after 24 h of incubation. (**A**) Gene expression of pyruvate dehydrogenase kinase 1 (PDK1) of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. (**B**) Gene expression of lactate dehydrogenase A (LDHA) of SH-SY5Y$_{695}$ cells after the incubation with ID63 or ID64 compared to the control. N = 8. Significance was determined by one-way ANOVA. * $p < 0.05$. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

2.7. qPCR

To investigate the molecular basis of altered lactate and pyruvate levels, the gene expression of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) were examined after 24 h incubation using qRT-PCR. ID63 and ID64 had no significant effect on PDK1 gene expression compared to the control (Figure 6). Both combinations increased LDHA mRNA levels (Figure 6B), with ID64 ($p = 0.0148$) showing a significant increase in gene expression.

2.8. Effect on the Lifespan of C. elegans in Heat Stress Survival Assay

To test the effect of the combinations in vivo two invertebrate AD-models were used. *C. elegans* CL2006 were incubated with the same compounds but in different concentrations. The single compounds Fol 50 µM ($p > 0.0001$), Vit B6 100 µM ($p = 0.0397$) and MgOr 100 µM ($p = 0.001$) had a significant life-extending effect compared to the control (see Figure 7A,B). As shown in Figure 7A, the $ID63_{worm}$ extended lifespan of CL2006 compared to the control by trend ($p = 0.0502$), whhereas in Figure 7B, it can be seen that $ID64_{worm}$ had a significant life-prolonging effect ($p = 0.0002$) compared to the control. Subsequently, the mean survival of the nematodes after the incubations of the combinations and single substances was assessed. Thereby Figure 7C shows that $ID63_{worm}$ ($p = 0.0196$), Fol 50 µM ($p < 0.0001$), MgOr 100 µM ($p = 0.0004$), $ID64_{worm}$ ($p < 0.0001$) and Vit B6 ($p = 0.0107$) had a significant increasing effect on the mean survival of the nematodes compared to the control. Folic acid alone was numerically more effective than the combinations or the other single compounds.

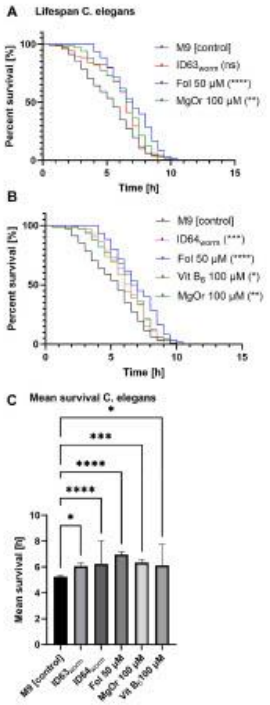

Figure 7. The lifespan under heat-stress of *C. elegans* after treatment with either $ID63_{worm}$, $ID64_{worm}$ or their single substances in CL2006. (**A**) The single substances lead to an increase in heat-stress resistance of the nematodes although the combination of them does not have a significant impact. (**B**) After combination with vitamin B_6, the resulting treatment leads to a significant increase in heat-stress resistance. For heat-stress experiments, the survival was assessed according to the penetration of SYTOX Green nucleic acid stain into dead cells. N > 60. log-rank (MantelCox) test. (**C**) Mean Survival of *C. elegans* after treatment with either $ID63_{worm}$, $ID64_{worm}$ or their single substances in CL2006. Significance was determined by one-way ANOVA. p * < 0.05, p ** < 0.01, p *** < 0.001 and p **** < 0.0001. $ID63_{worm}$ = Fol 50 µM and MgOr 100 µM; $ID64_{worm}$ Fol 50 µM, MgOr 100 µM and Vit B6 100 µM.

2.9. Effect on the Paralysis

To investigate the effect of combining biofactors on paralysis induced by Aβ, *C. elegans* GMC101 were incubated for 24 h at 25 °C with either $ID63_{worm}$ or $ID64_{worm}$. Both combinations ($p = 0.0243$ for $ID63_{worm}$ and $p = 0.0149$ for $ID64_{worm}$) were able to significantly decrease the paralysis induced by Aβ (Figure 8). Thus, the phenotype of this AD-worm was significantly enhanced by both biofactor combinations to a comparable extent.

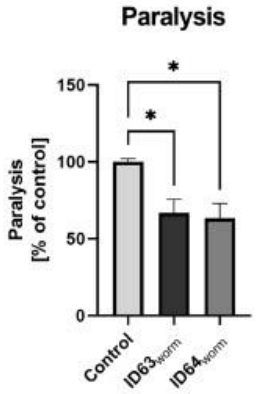

Figure 8. The Aβ induced paralysis after treatment with either $ID63_{worm}$ or $ID64_{worm}$ in GMC101. Both the feeding of both treatment combinations leads to significant reduction of Aβ induced paralysis after incubation of the nematodes for 24 h at 25 °C. N = 4. Mean ± SEM. One-way ANOVA with Tukey's comparison post hoc test. p * < 0.05 $ID63_{worm}$ = Fol 50 µM and MgOr 100 µM; $ID64_{worm}$ Fol 50 µM, MgOr 100 µM and Vit B6 100 µM.

2.10. $A\beta_{1-42}$ Production in GMC101

To study the production of $A\beta_{1-42}$ in nematodes, GMC101 was incubated for 24 h at 25 °C with both combinations and then the Aβ level was examined. There was no effect on the $A\beta_{1-42}$ levels compared to the control (Figure 9). Since $A\beta_{1-40}$ is not produced in GMC101 this amyloid peptide was not investigated.

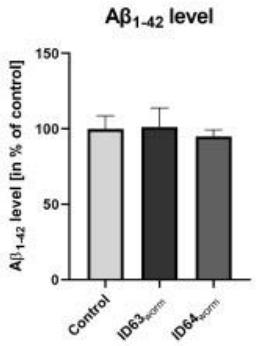

Figure 9. Effect of ID63 and ID64 in transgene nematodes GMC101 compared to the controls on the $A\beta_{1-42}$ level after 48 h incubation. Neither ID63 nor ID64 did not lead to a significant alteration of $A\beta_{1-42}$. N = 8. $A\beta_{1-42}$ levels were adjusted to the protein content. N = 8. Mean ± SEM. Student's *t*-test. $ID63_{worm}$ = Fol 50 µM and MgOr 100 µM; $ID64_{worm}$ Fol 50 µM, MgOr 100 µM and Vit B6 100 µM.

2.11. $A\beta_{1-42}$ Aggregation

To investigate the aggregation of Aβ in nematodes, GMC101 was incubated at 25 °C for 24 h and stained with thioflavine (ThT), which labels β-sheet structures of aggregated peptides. There was a significant reduction of Aβ-aggr by the combination $ID63_{worm}$ ($p = 0.0324$) compared to the control, whereas $ID64_{worm}$ was without an effect (Figure 10).

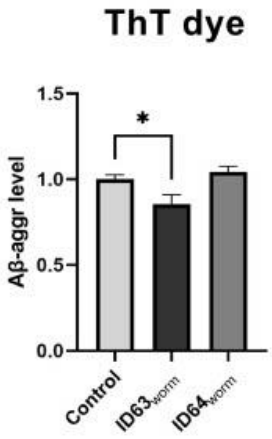

Figure 10. The effect of either $ID63_{worm}$ or $ID64_{worm}$ on the accumulation of aggregated Aβ (Aβ-aggr) in GMC101 after 48 h incubation. The treatment of the nematodes with ID63 leads to a significant reduction of accumulated Aβ. Whereby the treatment with ID64 combination did not lead to an alteration of Aβ levels; N = 8; mean ± SEM; one-way ANOVA with Tukey's comparison post hoc test. * $p < 0.05$. $ID63_{worm}$ = Fol 50 µM and MgOr 100 µM; $ID64_{worm}$ Fol 50 µM, MgOr 100 µM and Vit B6 100 µM.

3. Discussion

In the present work, we examined the effect of MgOr, Fol and Vit B6 in different combinations on bioenergetic parameters including mitochondrial function and glycolysis, as well as Aβ production in cellular and invertebrate models of AD. We wanted to create a combination product that achieves an optimal result by combining several biofactors. The hit compounds used in the current study were selected after a screening of seven substances of interest whose concentrations used in the experiments were based on known literature values [37,43–47]. We selected the concentrations because they were mostly tested in our cell model, related to AD or cell survival and they were used in relative physiological concentrations. This resulted in the combinations ID63 and ID64. The combinations ID63 and ID64 significantly reduced $A\beta_{1-40}$ levels in SH-SY5Y-APP_{695} cells compared to the control (Figure 1). Even when compared to the individual components of the combinations, ID63 was able to show an over additively reducing effect on Aβ levels. Similar results were obtained by Li et al. who found a dose-dependent decrease of $A\beta_{1-40}$ levels by incubation with Fol, modulating DNA methyltransferase activity [48]. In a study with AD patients, an intervention with Fol significantly reduced Aβ levels and increased the concentration of s-adenosyl methionine (SAM) [49]. Low SAM levels are a risk factor for AD, whereby incubation with SAM led to a decrease in Aβ levels in SK-N-SH cells [50]. Fol and Vit B6 are essential for the SAM cycle [51]. It seems that in our SH-SY5Y model the additional administration of Vit B6 shows no additional effect on $A\beta_{1-40}$ levels. Furthermore, lower Mg^{2+} are associated with the occurrence of AD which negatively affects brain energy metabolism [52]. In addition, low Mg levels are also negatively correlated with the occurrence of $A\beta_{1-40}$ and $A\beta_{1-42}$ [53]. As a result, an administration of Mg^{2+}

leads to a decrease in Aβ levels of N2a-APP cells [37]. After the incubation with ID63, SH-SY5Y-APP$_{695}$ cells showed reduced levels of Aβ_{1-42} compared to the control, although not significantly. In contrast, the incubation with ID64 showed a significant reduction of Aβ_{1-42} (Figure 2) compared to the control. Administration of a Fol-rich diet significantly reduced Aβ_{1-42} levels in APP/PS1 mice compared to the standard diet [54]. Similarly, the incubation with Mg significantly decreased Aβ_{1-42} production in Na2 neuroblastoma cells and transgenic mice [37,55,56]. In our work, we could not reproduce these described effects shown despite the administration of both substances in SH-SY5Y-APP$_{695}$ cells. However, it seems that Vit B6 has a crucial role in the reduction of Aβ_{1-42} levels (ID64) even if Vit B6 alone showed no effects. In contrast, it did not provide any benefit at the Aβ_{1-40} level (Figure 1). Next, we examined sAPPα and -β levels after incubation with ID63 and ID64. The reducing effects on Aβ_{1-42} levels by ID64 in SH-SY5Y-APP$_{695}$ cells could not be confirmed in transgene nematodes GMC101. On the one hand, incubation decreased the sAPPα levels significantly compared to the control (Figure 3A). On the other hand, both ID63 and ID64 had a reducing but not significant effect on the sAPPβ levels compared to the control (Figure 3B). Whereas it should be noted that the concentration of sAPPβ is 100 times lower than sAPPα. Studies showed that the concentration of sAPPα is generally higher than that of sAPPβ [57,58]. An application of Mg^{2+} increased the amount of sAPPα in APP/PS1 transgenic mice and simultaneously decreased the concentration of sAPPβ [37]. The results shown in this study [37], where both sAPPα and -β were decreased, could not be reproduced in our work. We assume that this was due to a general decrease in APP production, since the application of Fol can reduce APP processing [59,60], which in turn can affect sAPPα and -β, resulting in generally lowered levels.

Next, we tested ID63 and ID64 on MD in SH-SY5Y-APP$_{695}$ cells. SH-SY5Y-APP$_{695}$ cells show reduced ATP level, MMP and O_2 consumption compared to their non-transfected SH-SY5Y-MOCK cells [61]. Mg and Fol are vital compounds for enzymes and ATP production in cells [31,33,36]. A deficit leads to the reduced production of ATP [31,34]. In our case, which is not a deficit model, we did not observe any improvement in ATP level, MPP, O_2 consumption or citrate synthase activity in SH-SY5Y-APP$_{695}$ cells (Figure 4). In a study by Viel et al. using a similar cocktail consisting of some of our compounds, the mitochondrial complex activity in a transgenic rat model was increased to that of wild type rats [28]. This effect is not found in our case, possibly due to the additional substances contained in the cocktail, which had been the decisive factor here.

It has been shown that in the brains of AD patients there is a switch from aerobic respiration to glycolytic metabolism [62,63]. This is accompanied by an increase in lactate and pyruvate values [64,65], which results from insufficient utilization in oxidative phosphorylation [66]. Both biofactor combinations used in our investigations were able to significantly reduce the lactate values compared to the untreated control cells. The switch to increased oxidative phosphorylation could not be shown in our work, because there was no effect on ATP, MMP, OXPHOS or citrate synthase activity in the respiratory chain. To investigate the impact of the two combinations on the glycolytic genes, mRNA levels of PDK1 and LDHA were determined (Figure 6). There was an increase in the expression of PDK1 by both combinations compared to the control. PDK1 phosphorylates pyruvate dehydrogenase and inactivates it. This may lead to a reverse transport of pyruvate from the mitochondrion into the cytosol, where it is used for glycolytic energy production via lactate [67,68]. Both combinations were able to increase the expression of LDHA, while ID64 did so significantly. LDHA converts pyruvate to lactate and vice versa [23,69], although the expression was increased there were decreased lactate levels with both ID63 and ID64 (Figure 5). By upregulating PDK1 and LDHA expression, the cell may reduce the effects of Aβ toxicity and ROS production by shifting from mitochondrial to glycolytic energy production [23,69]. Both combinations appear to support this process, resulting in an upregulation of expression relative to control.

Based on the results obtained, especially those related to Aβ, we wanted to test our compounds in another model of AD. For this purpose, we adjusted the concentrations

and tested them in two invertebrate models of AD. CL2006 and GMC101 both express human Aβ in their muscle cells. While CL2006 produces Aβ continuously at 20 °C [70], GMC101 needs a temperature shift to 25 °C to initiate Aβ production [71]. Phenotypically, both are identified like wild-type N2 [72]. $ID63_{worm}$ had no significant life-prolonging effect on CL2006 compared to the control. Whereas the single substances and $ID64_{worm}$ had a significant effect on the lifespan (Figure 7A,B). In contrast, all tested combinations and single substances, have a significantly increased effect on mean survival (Figure 7C). CL2006 continuously produces Aβ, which has a toxic effect on the lifespan [70,73]. In particular, it has effects on DAF-16, which is expressed in the nucleus during stress and has an effect on life span extension [74]. DAF-16 is notable for being responsible for activating genes involved in longevity, lipogenesis, heat shock survival and oxidative stress responses [75,76], homologs being found in *C. elegans*, humans and mice [77]. It was demonstrated that incubation with Fol extended the lifespan by increasing the expression of DAF-16 [78]. It seems that the substances used have a similar effect on lifespan, whereas the single substance Fol seems to be superior. Furthermore, it was investigated whether the substances can reduce the toxic effect caused by Aβ. For this purpose, it was examined whether the paralysis in GMC101 changes because of the administration (Figure 8). Both combinations were able to reduce the paralyzes, indicating the significantly reduced toxicity of Aβ. Similar effects have been shown with other substances, which reduced the paralysis and thus the toxicity of Aβ [73,79–81]. The reduced toxicity could be a consequence of the reduction of Aβ levels in *C. elegans*. It could be shown that the administration of single substances leads to a reduction of the Aβ levels [37,48,50,54,55]. To verify this, the $Aβ_{1–42}$ levels were determined in GMC101 (Figure 9). Here, we found no significant effect in reduced Aβ level after incubation with both combinations. It should be noted here that the effects on $Aβ_{1–42}$ were also relatively moderate in the cells (see above). $Aβ_{1–40}$ was not measured in GMC101 because the worm does not produce these peptides. To examine the aggregation of Aβ after incubation with both combinations, samples were stained with ThT, which labels Aβ-structures in proteins (Figure 10). A significant reduction in Aβ-aggr was observed after incubation with $ID63_{worm}$. The combination with additional vitamin B6 did not reduce aggregation further. A similar effect was found in a study by Yu et al., in which the aggregation of Aβ in mice was reduced by incubation with magnesium [82].

It remains to be considered that there are both advantages and disadvantages to administering the substances as a single or combination preparation. Combined administration could lead to synergistic effects that enhance the positive effects and thus lead to an additive effect, as shown in the $Aβ_{1–40}$ values Figure 1. However, it cannot be ruled out that negative effects may occur when more than one substance is administered, as the substances may influence each other. Complex formation or competition for transport systems into the cell could occur. Especially when moving from an in vitro to an in vivo experiment. In order to exclude these effects, further experiments will have to be carried out in the future.

4. Materials and Methods

4.1. Cell Culture

The cultivation of SY5Y cells was performed in 75 cm^2 cell culture flasks under sterile conditions. To ensure optimal growth, cells were split several times per week once a cell density of approximately 70–80% was reached. All cells were cultured in an incubator at 37 °C and 5% CO_2 in DMEM supplemented with 10% (*v*/*v*) heat-inactivated fetal calf serum, 60 µg/mL streptomycin, 0.3 mg/mL hygromycin, 1% MEM non-essential amino acids, and 1 mM sodium pyruvate 1%, 60 units/mL penicillin. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP_{695} (APP_{695}) and were kindly donated by A. Eckert (Basel, Switzerland) (for details; please refer to [83]).

4.2. Cell Treatment

First of all, cells were incubated for 24 h with different concentrations of zinc orotate (ZnOr), magnesium-orotate (MgOr), benfotiamine (vitamin B1), folic acid (Fol), cholecalciferol (Vit D3), cobalamin (Vit B12), and pyridoxine (Vit B6) for possible hit substances. After the hit substances were determined, cells were incubated with 200 µM magnesium-orotate (MgOr) and folic acid 10 µM (Fol) (combination ID63) or 200 µM MgOr, 10 µM folic acid and 100 nM Vit B6 (combination ID64). We have derived the concentrations used here from the literature [37,43–47]. The solution medium NaOH diluted 1:8 with water served as a control. The ratio had the best dissolving properties without being toxic to the cells.

4.3. ATP Measurement

A bioluminescence assay was used to determine the ATP levels, which is based on the production of light from ATP and luciferin in the presence of luciferase. The test was performed using the ATPlite Luminescence Assay System (PerkinElmer, Rodgau, Germany) according to the previously published protocol [84].

4.4. MMP Measurement

Mitochondrial membrane potential (MMP) was measured using the fluorescence dye rhodamine-123 (R123). Cells were incubated for 15 min with 0.4 µM R123 and centrifuged at 750× *g* for 5 min before being washed with Hank's Balanced Salt Solution (HBSS) buffer supplemented with Mg^{2+}, Ca^{2+}, and HEPES. The cells were suspended with fresh HBSS before they were evaluated by measuring the R123 fluorescence. The excitation wavelength was set to 490 nm and the emission wavelength to 535 nm witch CLARIOstar (BMG Labtech, Ortenberg, Germany).

4.5. Cellular Respiration

Respiration in SH-SY5Y_{695} cells was assessed using an Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. The cells were treated according to a complex protocol developed by Dr. Erich Gnaiger [85]. They were incubated with different substrates, inhibitors and uncouplers. First, cells were washed with PBS (containing potassium chloride 26.6 mM, potassium phosphate monobasic 14.705 mM, sodium chloride 1379.31 mM and sodium phosphate dibasic 80.59 mM) and scraped into mitochondrial respiration medium (MiRO5) developed by Oroboros [85]. Afterwards, the cells were centrifuged, resuspended in MiRO5, and diluted to 10^6 cells/mL. After 2 mL of cell suspension was added to each chamber and endogenous respiration was stabilized, the cells were treated with digitonin (10 µg/10^6 cells) to permeabilize the membrane, leaving the outer and inner mitochondrial membrane intact. OXPHOS was measured by adding the complex I and II substrates malate (2 mM), glutamate (10 mM) and ADP (2 mM), followed by succinate (10 mM). Gradual addition of carbonyl cyanide-4- before it is evaluated by measuring the R123 fluorescence (trifluoromethoxy) phenylhydrazone showed the maximum capacity of the electron transfer system. Rotenon (0.1 mM) was added to measure the activity of complex II. To investigate the leak respiration, oligomycin (2 µL/mL) was injected. To inhabitation of complex III to determined residual oxygen consumption, antimycin A (2.5 µM) was added. This value was subtracted from all respiratory states. Adding N measured N, N′, N′-tetramethyl-p-phenylenediamine (0.5 mM) and ascorbate (2 mM) the activity of complex IV. To measure the sodium autoxidation rate, azide (≥100 mM) was added. Afterwards, complex IV respiration was corrected by subtracting the autoxidation rate of azid. NaOH served as control.

4.6. Citrate Synthase Activity

Cell samples from respirometry measurements were frozen and stored at −80 °C for the determination of citrate synthase activity. Samples were thawed while the reaction mix (0.1 mM 5,5′-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 0.5 mM oxaloacetate, 50 µM EDTA, 0.31 mM acetyl coenzyme A, 5 mM triethanolamine hydrochloride, and 0.1 M Tris-HCl)

was mixed and heated at 30 °C for 5 min. Afterwards, 40 µL of samples were submitted in triplets and mixed with 110 µL of the reaction mix. The absorption was measured at 412 nm.

4.7. $A\beta_{1-40}$ Measurement

After 24 h incubation, the $A\beta_{1-40}$ levels were determined in SH-SY5Y-APP_{695} cells using HTRF Amyloid-Beta 1–40 kit (Cisbio, Codolet, France). The protocol was the same as described earlier [61]. Aβ concentrations were normalized against protein content.

4.8. $A\beta_{1-42}$ Measurement

After 24 h incubation, the $A\beta_{1-42}$ levels were determined in SH-SY5Y-APP_{695} cells and nematodes GMC101 using the Human-$A\beta_{1-42}$ ELISA Kit (Invitrogen™, Waltham, MA, USA). The protocol was performed according to the manufacturer's instructions. Aβ concentrations were normalized against protein content.

4.9. Protein Quantification

Protein content was determined using a Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

4.10. Quantification of Human Soluble Amyloid Precursor Protein α (sAPPα)

The sample preparation was the same according to the $A\beta_{1-40}$ quantification. The sAPPα levels were determined using the Human Soluble Amyloid Precursor Protein α (sAPPα) ELISA Kit (Cusabio, Wuhan, China). The process was carried out according to the manufacturer's instructions. Levels were normalized to the protein content.

4.11. Quantification of Human Soluble Amyloid Precursor Protein β (sAPPβ)

The sample preparation was the same according to the $A\beta_{1-40}$ quantification. The sAPPβ levels were determined using the Human Soluble Amyloid Precursor Protein β (sAPPβ) ELISA Kit (BT LAB, Zhejiang, China). The process was carried out according to the manufacturer's instructions. Levels were normalized to the protein content.

4.12. Pyruvate and Lactate Content

Frozen cells, which were previously harvested and incubated for 24 h, were thawed at room temperature. Pyruvate and lactate concentrations were assessed using a pyruvate assay kit (MAK071, Sigma Aldrich, Darmstadt, Germany) and a lactate assay kit (MAK064, Sigma Aldrich, Darmstadt, Germany) according to the manufacturer's instructions.

4.13. Real-Time qRT-PCR

To isolate RNA, we used the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Nanodrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify RNA. The TURBO DNA-free™ kit was used according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) to remove residual genomic DNA. cDNA was synthesized from 1 µg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). qRT-PCR was conducted using a CfX 96 Connect™ system (Bio-Rad, Munich, Germany). All used primers are listed in Table 2. The cDNA aliquots were analyzed in triplicate and diluted 1:10 with RNase-free water (Qiagen, Hilden, Germany). PCR cycling conditions were as follows: initial denaturation for 3 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, 58 °C for 30 s (or 56 °C for 45 s, depending on the primer), and 72 °C for 29 s. Expression was analyzed with $-(2\Delta\Delta Cq)$ using Bio-Rad CfX manager software. To normalize the values a factor was calculated based on the geometric mean of the levels of multiple control genes of *β-actin (ACTB)*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, and *phosphoglycerate kinase 1 (PGK1)* according to the MIQE guidelines [86]. RNA free water served as an assay control to exclude impurities.

Table 2. Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR.

Primer	Sequence	Manufacturer	Product Size	Concentration (nM)
ß-Actin (ACTB) NM_001101.2	5′-ggacttcgagcaagagatgg-3′ 5′-agcactgtgttggcgtacag-3′	Biomol, Hamburg, Germany	234	200
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) NM_002046.2	5′-gagtcaacggatttggtcgt-3′ 5′-ttgattttggagggatctcg-3′	Biomol, Hamburg, Germany	238	200
Phosphoglycerate kinase 1 (PGK1) NM_000291.2	5′-ctgtgggggtatttgaatgg-3′ 5′-cttccaggagctccaaa-3′	Biomol, Hamburg, Germany	198	200
Pyruvate dehydrogenase kinase, isozyme 1 (PDK1) NM_002610	5′-atacggatcagaaaccgaca-3′ 5′-cagacgcctagcattttcat-3′	Biomol, Hamburg, Germany	291	100
Human lactate dehydrogenase A like 6B (LDHA) NM_033195	5′-ggtgtccctttgaaggatct-3′ 5′-tgcagtcacttctttgtgga-3′	Biomol, Hamburg, Germany	*87*	400

4.14. Nematode and Bacterial Strain

C. elegans strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, MN, USA) and included CL2006 [Punc-54::human A-beta 3–42; pRF4 (rol-6(su1006))] and GMC101 [(Punc-54::A-beta::unc-54 3Prime UTR; Pmtl2::GFP)]. The strain GMC101 expresses the full-length human $A\beta_{1-42}$ peptide in body-wall muscle cells that aggregates in vivo. Shifting L4 or young adult animals from 20 °C to 25 °C could induce the expression of Aβ and cause paralysis. The strain CL2006 constitutively expressed Aβ when cultured at 20 °C.

Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with the bacterial *E. coli* strain OP50. According to standard protocols, the seeded plates were stored at 20 °C [87]. Synchronous populations were generated for all experiments by using a standard bleaching protocol [88].

4.15. Cultivation and Treatment

Post-bleaching generated larvae were washed twice in M9-buffer and the number of larvae in 10 µL were adjusted to 10 larvae. Afterward, the synchronized larvae were raised in cell culture flasks (Sarstedt, Nümbrecht, Germany) in either an amount of 1000 or 5000 nematodes, depending on the experiments. OP50-NGM was added to the flasks as a standardized source of food. The larvae were maintained under shaking at 20 °C until they reached young adulthood within 3 days. The micronutrients were dissolved in advance in M9 buffer. For each micronutrient observed in this study, we generated a series of concentrations as follows. Folic acid (Fol) 50 µM, magnesium orotate (MgOr) 0.1 mM (together $ID63_{worm}$) and Fol 50 µM, MgOr 0.1 mM and vitamin B6 (Vit B6) 100 µM (together $ID64_{worm}$). After reaching adulthood (48 h before the experiment), the micronutrients were added to the flasks. Pure M9-buffer was used as a control. Then, 24 h before the experiment amyloid aggregation was proceeded by upshifting young adult GMC101 from 20 °C to 25 °C.

4.16. Paralyze Assay

Cell culture flasks containing approximately 1000 adult amyloid beta producing nematodes were incubated for several hours to achieve Aβ induced paralysis (GMC101 24 h at 25 °C). On a NGM Agar Plate, 25 nematodes were placed and by physically touching with a platinum tip the paralysis status was recorded. Nematodes which normally act

after being touched by the wire are recognized as "not paralyzed" whereas uncoordinated movements or just head movements were recorded as "paralyzed".

4.17. Heat-Stress Survival Assay

After 48 h of incubation of CL2006 with the mentioned effectors, the time till death was determined using a microplate thermotolerance assay [89]. In preparation, the nematodes were washed out of the flasks with M9-buffer into 15 mL tubes followed by 3 additional washing steps. Each well of a black 384-well low-volume microtiter plate (Greiner Bio-One, Frickenhausen, Germany) was prefilled with 6.5 µL M9-buffer/Tween®20 (1% *v*/*v*). In the following step, one nematode in 1 µL M9-buffer was transferred and immersed in the well under a stereomicroscope (Breukhoven Microscope systems, Netherlands). SYTOX™ Green (Life Technologies, Karlsruhe, Germany) in a final concentration of 1 µM was added to reach a final volume of 15 µL in the well. SYTOX™ Green creates a fluorescent signal after binding to DNA. The plates were sealed with a Rotilab sealing film (Greiner Bio-One, Frickenhausen, Germany). The heat-shock was applied and the fluorescence was measured every 30 min for 17 h at 37 °C following the protocol previously described [90]. The excitation was set at 485 nm and the emission was detected at 538 nm.

4.18. ThT Dying of Aβ Aggregates

Detection and quantification of Aβ aggregates (Aβ-aggr) in GMC101 were performed using the fluorescent dye thioflavin T (ThT) according to a previously described method with minor modifications [91]. Synchronized and heat incubated nematodes were washed out of the cell culture flasks with M9-buffer/Tween®20 (1% *v*/*v*) and separated from larvae. After centrifugation, 200 µL of a thick pellet of nematodes was transferred into a microcentrifuge tube and were frozen in liquid nitrogen. Samples were thawed with 500 µL of PBS including proteinase inhibitor. Afterward, the samples were homogenized with a sonifier 3 × 20 s on ice. Protein contents in the homogenate were assessed according to the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin was used as a standard. Finally, fluorescence was measured in a black 96-well plate by adding 1 mM ThT (final concentration 20 mM). The volume in each well was 100 µL by adding M9. To determine the fluorescence of ThT, samples were measured by excitation at 440 nm and emission at 482 nm.

4.19. Statistics

Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Tukey's multiple comparison post-hock test, log-rank (Mantel-Cox) test and student's unpaired *t*-test (Prism 9.1 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for *p* values ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

5. Conclusions

In the present study, we reported that different combinations of folic acid, magnesium orotate, and vitamin B6 had significant effects on glycolysis, Aβ production, and Aβ aggregation in SH-SY5Y-APP$_{695}$ cells and *C. elegans*. The phenotype of the in vivo model was significantly improved, highlighting the potential of the tested biofactor combinations as candidate therapeutics in AD. However, since the data did not consistently show a benefit of either combination, this study does not allow a clear statement as to whether vitamin B6 is required in addition to the combination of folic acid and magnesium orotate.

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3.3 The Effect of Selected Coffee Constituents on Mitochondrial Dysfunction in an Early Alzheimer Disease Cell Model

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Article

THE EFFECT OF SELECTED COFFEE CONSTITUENTS ON MITOCHON-DRIAL DYSFUNCTION IN AN EARLY ALZHEIMER DISEASE CELL MODEL

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THE EFFECT OF SELECTED COFFEE CONSTITUENTS ON MITOCHONDRIAL DYSFUNCTION IN AN EARLY ALZHEIMER DISEASE CELL MODEL

Alzheimer disease (AD) is an emerging medical problem worldwide without any cure yet. By 2050, more than 152 million people will be affected. AD is characterized by mitochondrial dys-function (MD) and increased amyloid beta (Aβ) levels. Coffee is one of the most commonly consumed beverages. It has many bioactive and neuroprotective ingredients of which caffeine (Cof), kahweohl (KW) and cafestol (CF) shows a variety of pharmacological properties such as anti-inflammatory and neuroprotective effects. Effects of Cof, KW, and CF were tested in a cel-lular model of AD on MD and Aβ. SH-SY5Y-APP695 cells were incubated with 50µM Cof, 1µM CF and 1µM KW for 24h. The energetic metabolite ATP was determined using a luciferase-catalyzed bioluminescence assay. The activity of mitochondrial respiration chain complexes was assessed by high-resolution respirometry using a Clarke electrode. Expression levels genes were deter-mined using quantitative real-time polymerase chain reaction (qRT-PCR). The levels of amyloid β-protein ($A\beta_{1-40}$) were measured using homogeneous time-resolved fluorescence (HTRF). ROS levels, cAMP levels, and peroxidase activity were determined using a fluorescence assay. The combination of Cof, KW and CF significantly increased ATP levels. The combination had neither a significant effect on MMP, on activity of respiration chain complexes, nor on $A\beta_{1-40}$ levels. cAMP levels were slightly increased after incubation with the combination, but not the peroxi-dase activity. Pyruvate levels and the lactate-pyruvate-ration but not lactate levels were signifi-cantly enhanced. No effect was seen on the expression level of lactate dehydrogenase and py-ruvate dehydrogenase kinase. In some experiments we have tested the single substances. They showed significant results especially in ATP, lactate and pyruvate values compared to the con-trol. The combinations have a lesser effect on mitochondrial dysfunction in cells and none on Aβ production. Whereas ATP levels and pyruvate levels were significantly increased. This suggests a change in glycolysis in neuronal cells harbouring human genes relevant for AD.

Keywords: Alzheimer disease; mitochondria; mitochondria dysfunction; caffeine; cafestol; kahweol; amyloid beta;

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease belonging to the class of dementias. It manifests itself through a progressive loss of function of neurons of the central nervous system [1]. Dementia affects 5-7% of people over 60 in developed countries [2], of which Alzheimer's disease, discovered by German physician Alois Alzheimer in 1906, is the most common manifestation, accounting for 60% [1]. Clinical symptoms include memory loss as well as language and orientation problems [2]. One possible cause of AD is a manifestation of mitochondrial dysfunction (MD) as an early event of AD [3,4]. Almost all mitochondrial functions are impaired in AD [5,6]. First signs of MD are a reduction in key enzymes of in the oxidative metabolism [7,8] and glucose consumption [9]. The limited function of the electron transport chain (ETC) is the reason for the decrease activity of complexes IV and I. These complications lead to a lower membrane potential (MMP) and to reduced ATP levels [10,11]. A further possible trigger of the emergence of AD is the overexpression of amyloid-beta (Aβ) [12]. An imbalance between production and removal leads to accumulation and aggregation in the brain, this leads to inflammatory responses, production of reactive oxygen species (ROS) and loss of neurons, this leads to MD, dementia and Alzheimer disease [13]. An important antioxidant enzyme involved in AD is peroxidase, which catalyzes the oxidation of organic and non-organic substances using H2O2. In this process, it is supposed to protect against the harmful effects of physiological accumulation of ROS. A change in peroxidase activity could induce effects on ROS levels. Another impairment in AD is cyclic adenosine monophosphate (cAMP), which is one of the nucleotides and serves as a second messenger in the body for signaling metabolic pathways and hormone effects. [14,15]. The signaling cascade consisting of cAMP/PKA/CREB is considered to be generally important for the processes and

functioning in learning and memory [16]. Performance deficits may be mediated by deficits in the signaling cascade. One trigger of this impairment could be Aβ toxicity [17]. Up to now, there is no cure for it, which is why prevention and therapy are in the focus of attention. A possibility for this offer might be coffee.

Today, there is a high consumption of coffee worldwide, which in Germany is 150 l per capita per year [18]. Due to a high diversity of bioactive ingredients, the beverage has been the subject of research for many years regarding its influence on human health. Caffeine has been the focus of attention due to its stimulant effects. In addition, many other secondary plant compounds with bioactive effects are now known in coffee. These potentially health-promoting effects are based on cardioprotective, hepatoprotective and neuroprotective properties [19]. Roasted coffee contains a variety of bioactive ingredients. Besides caffeine, coffee contains other diterpenes in the form of fatty acid esters, with cafestol and kahweol making up the largest proportion [20]. Depending on the coffee variety, the composition of cafestol and kahweol differs. Arabica coffee contains cafestol and kahweol, whereas robusta contains cafestol and small amounts of kahweol [21]. The effects of caffeine are due to mechanisms of action on nerve cells in the brain, where the pathological changes during AD also occur [22]. Coffee and caffeine intake is associated with a lower risk of developing dementia and AD through a wide variety of mechanisms [23–25]. The data situation on kahweol and cafestol is limited, but there are some evidence that both substances also have a neuroprotective effect [26–28]. In this study, we focused on the combination of caffeine, kahweol and cafestol in small doses on an early AD cell model.

2. Materials and Methods

2.1 Cell culture

Human neuroblastoma SH-SY5Y cells were cultured at 37 °C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 60µg/mL streptomycin, 60units/mL penicillin, 0.3mg/mL hygromycin, MEM non-essential amino acids, and 1mM sodium pyruvate 1%. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP_{695} (APP_{695}) (for details; please refer to [29]). Cells were passaged every 3 days and were used for experiments when they reached 70-80% confluence.

2.2 Cell treatment

Cells were incubated with 1µM kahweol (KW), 1µM cafestol (Caf) and 50µM caffeine (Cof) (KCC) for 24h. The solution medium DMSO served as control.

2.3 ATP Measurement

A bioluminescence assay was used to determine the ATP levels, which is based on the production of light from ATP and luciferin in the presence of luciferase. The test was performed using the ATPlite Luminescence Assay System (PerkinElmer, Rodgau, Germany) according to the previously published protocol [30].

2.4 MMP Measurement

Mitochondrial membrane potential (MMP) was measured using the fluorescence dye rhodamine-123 (R123). Cells were incubated for 15 min with 0.4 µM R123 and centrifuged at 750 x g for 5 min before being washed with Hank's Balanced Salt

Solution (HBSS) buffer supplemented with Mg^{2+}, Ca^{2+}, and HEPES. The Cells were suspended with fresh HBSS before they were evaluated by measuring the R123 fluorescence. The excitation wavelength was set to 490 nm and the emission wavelength to 535 nm.

2.5 Cellular Respiration

Respiration in SH-SY5Y_{695} cells was assessed using an Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. The cells were treated according to a complex protocol developed by Dr Erich Gnaiger [31]. They were incubated with different substrates, inhibitors and uncouplers. First, cells were washed with PBS (containing potassium chloride 26.6 mM, potassium phosphate monobasic 14.705 mM, sodium chloride 1379.31 mM and sodium phosphate dibasic 80.59 mM) and scraped into mitochondrial respiration medium (MiRO5) developed by Oroboros [31]. Afterward, they were centrifuged, resuspended in MiRO5, and diluted to 10^6 cells/mL. After 2mL of cell suspension was added to each chamber and endogenous respiration was stabilized, the cells were treated with digitonin (10µg/10^6 cells) to permeabilize the membrane, leaving the outer and inner mitochondrial membrane intact. OXPHOS was measured by adding the complex I and II substrates malate (2mM), glutamate (10mM) and ADP (2mM), followed by succinate (10 mM). Gradual addition of carbonyl cyanide-4- before it is evaluated by measuring the R123 fluorescence (trifluoromethoxy) phenylhydrazone showed the maximum capacity of the electron transfer system. To measure complex II activity, rotenone (0.1mM), a complex I inhibitor, was injected. After that, oligomycin (2 µg/mL) was added to measure the leak respiration. Inhibition of complex III by the addition of antimycin A (2.5 µM) determined residual oxygen consumption, which was subtracted from all respiratory

states. The activity of complex IV was measured by adding *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine (0.5 mM) and ascorbate (2 mM). To measure the sodium autoxidation rate, azide (≥100mM) was added. Afterward, complex IV respiration was corrected for autoxidation. DMSO served as control.

2.6 Citrate Synthase Activity

Cell samples from respirometry measurements were frozen and stored at -80°C for the determination of citrate synthase activity. Samples were thawed while the reaction mix (0.1 mM 5,5′-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 0.5 mM oxaloacetate, 50 µM EDTA, 0.31 mM acetyl coenzyme A, 5 mM triethanolamine hydrochloride and 0.1 M Tris-HCl) was mixed and heated at 30°C for 5 min. Afterward, 40 µl of samples were submitted in triplets and mixed with 110µl of the reaction mix. The absorption was measured at 412nm.

2.7 $A\beta_{1-40}$ Determination

After 24 h incubation, the $A\beta_{1-40}$ levels were determined in SH-SY5Y-APP_{695} cells using HTRF Amyloid-Beta 1-40 kit (Cisbio, Codolet, France). The protocol was the same as recently described [32]. Aβ concentrations were normalized against the protein content.

2.8. Peroxidase Activity

Peroxidase activation was measured using an AmplexTM Red Peroxidase Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cells were seeded in 96-well plates and incubated for 24 h.

2.9 Protein Quantification

Protein content was determined using a Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

2.10. ROS Measurement

Cellular ROS production was determined using a DCFDA/H2DCFDA Cellular ROS Assay Kit (ab113851; Abcam, Cambridge, UK). Cells were incubated for 24h with our substances and then the manufacturer's instructions were followed.

2.11 Pyruvate and Lactate Content

Frozen cells, which were previously harvested and incubated for 24h, were thawed at room temperature. Pyruvate and lactate concentrations were assessed using a pyruvate assay kit (MAK071, Sigma Aldrich, Darmstadt, Germany) and a lactate assay kit (MAK064, Sigma Aldrich, Darmstadt, Germany) according to the manufacturer's instructions.

2.12 Real-Time qRT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. RNA was quantified using a Nanodrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA).

189 Complementary DNA was synthesized from 1 µg total RNA using an iScript cDNA
190 Synthesis Kit (Bio-Rad, Munich, Germany). qRT-PCR was conducted using a CfX 96
191 Connect™ system (Bio-Rad, Munich, Germany). Primers were provided from Biomol
192 (Hamburg, Germany). All primers are listed in Table 1. The cDNA aliquots were
193 diluted 1:10 with RNase-free water (Qiagen, Hilden, Germany), and all samples were
194 analyzed in triplicate. PCR cycling conditions were as follows: initial denaturation for 3
195 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, 58 °C for 30 s (or 56 °C for 45 s,
196 depending on the primer), and 72 °C for 29 s. Expression was analyzed with $-(2\Delta\Delta Cq)$
197 using Bio-Rad CfX manager software. The normalization factor was calculated based
198 on the geometric mean of the levels of multiple control genes of *ß-actin (ACTB),*
199 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, and *phosphoglycerate kinase 1*
200 *(PGK1)* according to the MIQE guidelines [33]. No-template control served as an assay
201 control to exclude impurities.
202
203 **Table 1.** Oligonucleotide primer sequences, product sizes, and primer concentrations
204 for quantitative real-time PCR.

Primer	Sequence	Manufacturer	Product size	Concentration (nM)
ß-Actin (ACTB) *NM_001101.2*	5′-ggacttcgagcaagagatgg-3′ 5′-agcactgtgttggcgtacag-3′	Biomol, Hamburg, Germany	234	200
Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* *NM_002046.2*	5′-gagtcaacggatttggtcgt-3′ 5′-ttgattttggagggatctcg-3′	Biomol, Hamburg, Germany	238	200
Phosphoglycerate kinase 1 *(PGK1)* *NM_000291.2*	5′-ctgtgggggtatttgaatgg-3′ 5′-cttccaggagctccaaa-3′	Biomol, Hamburg, Germany	198	200
Pyruvate dehydrogenase kinase, isozyme 1 *(PDK1)* *NM_002610*	5′-atacggatcagaaaccgaca-3′ 5′-cagacgcctagcattttcat-3′	Biomol, Hamburg, Germany	291	100
Human lactate dehydrogenase A like 6B *(LDHA)* *NM_033195*	5′-ggtgtccctttgaaggatct-3′ 5′-tgcagtcacttctttgtgga-3′	Biomol, Hamburg, Germany	87	400

206
207

208 ***2.13 cAMP level***

209 Cellular peroxidase activity was determined using a cAMP Direct Immunoassays Kit
210 (Abcam, Cambridge, UK). Cells were incubated for 24h with our substances and then
211 the manufacturer's instructions were followed.

212

213 ***2.14 Statistics***

214 Unless otherwise stated, values are presented as mean ± standard error of the mean
215 (SEM). Statistical analyses were performed by applying one-way ANOVA with

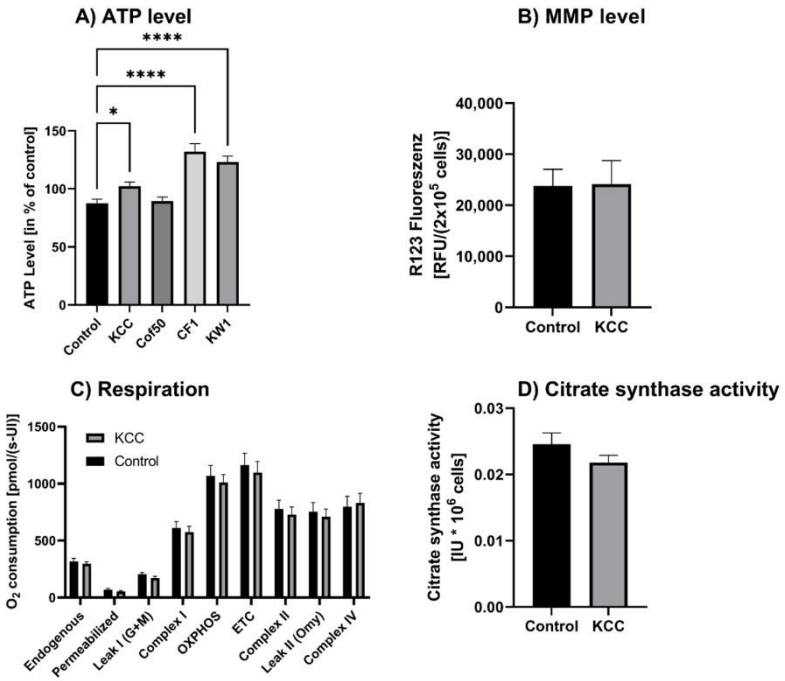
Tukey´s multiple comparison post-hock test and student's unpaired t-test (Prism 9.1 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for *p* values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

3.1 Mitochondrial functions

To find the right concentrations for our combination we tested different concentrations of Cof, CF and KW (SupFigure 1), as well as combinations of the three mentioned substances, for the best combination, for the effects on the ATP levels (SupFigure 2). The concentration of kahweol 1µM, cafestol 1µM and caffeine 50µM (KCC) contain all of our three substances and turned out to be the best. There the highest ATP values for a combination were achieved. We tested KCC on the mitochondrial functions, we incubated SH-SY5Y-APP695 cells for 24h with all three of the substances combined. Respiration under O2 consumption through the respiratory chain builds up the mitochondrial membrane potential, which is used by the ATP synthase to produce ATP. We measured the ATP level, MMP level, respiration and citrate synthase activity (Fig. 1). The KCC combination had a significant increasing effect ($p = 0.0151$) on the ATP level compared to the control (Fig. 1A). Also the single substances CF1 and KW1 ($p = 0.0001$) had a significant increasing effect compared to the control. Whereas the incubation with KCC had no effect on the MMP level, respiration or citrate synthase activity. Caffeine alone does not increase or decrease ATP levels. Only the combination with one or both diterpenes shows a significant increase at 50 µM (SupFigure 2). We chose the concentration of 50µM caffeine to be closer to the doses that are realistic when consuming coffee without damaging the cells or causing a loss of ATP.

240



241

242 Figure 1: ATP level, MMP level, respiration and citrate synthase activity of SH-SY5Y-
243 APP_{695} cells incubated for 24h with KCC. (A) ATP level of SH-SY5Y-APP_{695} cells
244 incubated with KCC. Cells treated with cell culture medium served as control (100%).
245 N = 8. B) MMP level of $2x10^5$ SH-SY5Y-APP_{695} cells incubated with N = 16. (C)
246 Respiration of SH-SY5Y-APP_{695} cells incubated with KCC compared to the control.
247 SH-SY5Y-APP_{695} cells adjusted to international units (IU) of citrate synthase activity.
248 N = 18. (G) Citrate synthase activity of SH-SY5Y-APP_{695} cells incubated with KCC
249 compared to control. N = 18. Significance was determined by Student's unpaired t-test.
250 Data are displayed as the mean ± SEM. *P > 0.05, ****p> 0.0001. KCC = kahweol
251 1µM, cafestol 1µM and caffeine 50µM. Concentrations are given in µM

3.2 $A\beta_{1\text{-}40}$ Production

To measure the effect of KCC on the amyloid beta (Aβ) level SH-SY5Y-APP695 cells were incubated for 24h. Here we found no effect of KCC on the Aβ1-40 level compared to the control (Fig. 2).

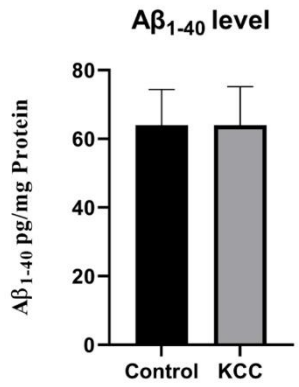


Figure 2: Effect of KCC on the $A\beta_{1\text{-}40}$ level of SH-SY5Y-APP_{695} cells incubated for 24h. N = 10. $A\beta_{1\text{-}40}$ levels were adjusted to the protein content. Significance was determined by Student's unpaired t-test. Data are displayed as the mean ± SEM. KCC = kahweol 1µM, cafestol 1µM and caffeine 50µM.

3.3 Peroxidase

In order to investigated the effect of KCC or the single substances on the peroxidase activity in SH-SY5Y-APP695 cells we incubated them for 24h. Here we found no differences between incubations and the control with regard to the peroxidase activity (Fig. 3).

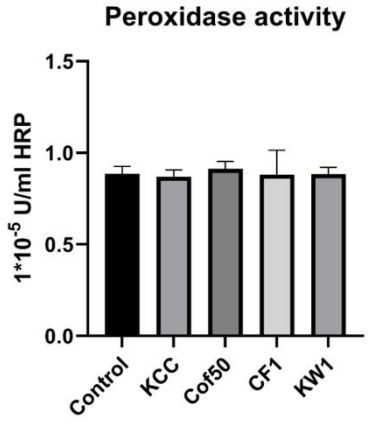


Figure 3: Effect of KCC and the single substances on the peroxidase activity of SH-SY5Y-APP$_{695}$ cells incubated for 24h. N = 14. Significance was determined by Student's unpaired t-test. Data are displayed as the mean ± SEM. KCC = kahweol 1µM, cafestol 1µM and caffeine 50µM. Concentrations are given in µM.

3.4 ROS measurement

In the next step, we examined the ROS level in SH-SY5Y-APP695 cells after the incubation with KCC or the single substances for 24h. Here, we found no different between both treatments (Fig. 4).

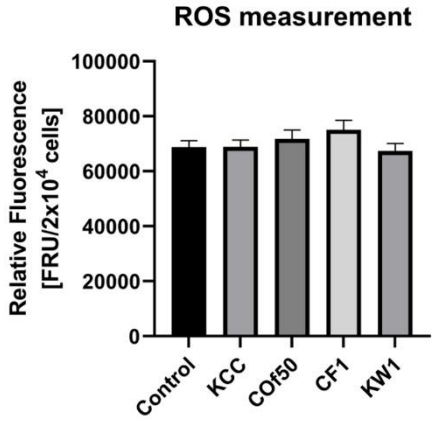


Figure 4: ROS measurement of SH-SY5Y-APP$_{695}$ cells after incubation with KCC for 24h. N = 12. Significance was determined by Student's unpaired t-test. Data are displayed as the mean ± SEM. KCC = kahweol 1µM, cafestol 1µM and caffeine 50µM. Concentrations are given in µM.

3.5 cAMP

To test the effect of KCC on the cAMP level, we incubated SH-SY5Y-APP$_{695}$ cells for 24h. Here we had an increase, but not a significant, of cAMP level after the incubation with KCC compared to control (Fig. 5).

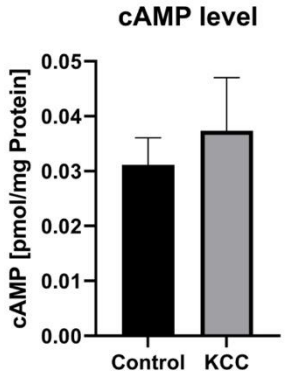
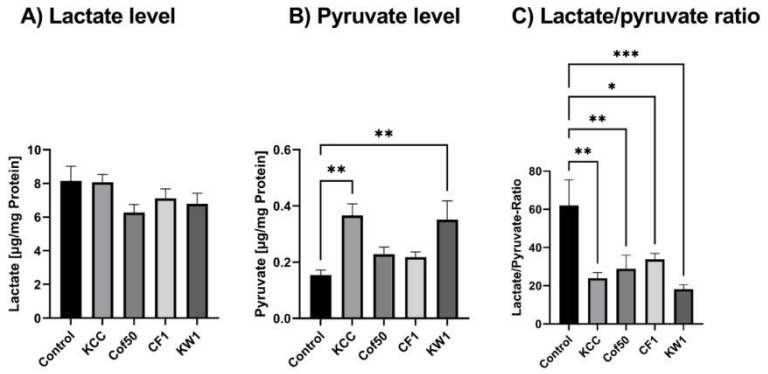


Figure 5: cAMP level of SH-SY5Y-APP$_{695}$ cells after incubation with KCC for 24h compared to the control. N = 8. Significance was determined by Student's unpaired t-test. Data are displayed as the mean ± SEM. KCC = kahweol 1µM, cafestol 1µM and caffeine 50µM.

3.6 Lactate and pyruvate level

To examine the effect of KCC on the lactate and pyruvate level we incubated SH-SY5Y-APP$_{695}$ cells for 24h. There were no different between KCC compared to the control for the lactate level (Fig. 6A). However, the incubation with KCC significant increased the pyruvate value compared to the control ($p = 0.0027$) (Fig. 6B). Likewise, the lactate/pyruvate ratio (Fig. 6C) was significantly reduced by KCC compared to the control ($p = 0.0141$). To investigate whether the increased pyruvate levels were caused by a single substance. We determined the values of the individual substances. This showed that the pyruvate values were significantly increased by KW ($p = 0.0062$, Fig 6B). All the individual substances (Fig 6C) also significantly reduced the lactate/pyruvate ratio (Cof $p = 0.0097$, CF $p = 0.0321$, KW $p = 0.0008$).

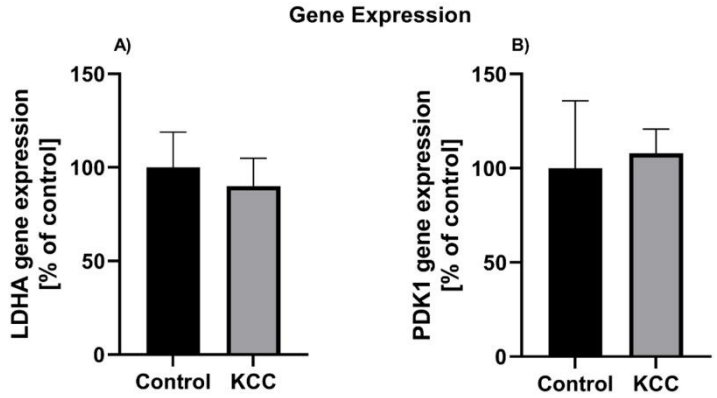


302

303 Figure 6: Effect of the on lactate and pyruvate level after 24h of incubation with
304 caffeine (Cof), cafestol (CF) and kahweol (KW) or the combination KCC. (A) Lactate
305 level of SH-SY5Y-APP$_{695}$ cells after the incubation compared to the control. (B)
306 Pyruvate level of SH-SY5Y-APP$_{695}$ cells after the incubation with KCC compared to
307 the control. (C) Lactate to pyruvate ratio. N = 8. Levels were adjusted to the protein
308 content. Concentrations are given in µM. Significance was determined by one-way
309 ANOVA. * $p < 0.05$ and ** $p < 0.01$, ***$p > 0.001$. Data are displayed as ±SEM.
310 Concentrations are given in µM.

311 ***3.7 qPCR***

312 To investigate the molecular basis of altered lactate and pyruvate levels, the gene
313 expression of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A
314 (LDHA) were examined after 24 h incubation using qRT-PCR. KCC had no significant
315 effect on LDHA or PDK1 gene expression compared to control (Fig.7).



316

317 Figure 7: Effect on the incubation with KCC on the gene expression after 24h of
318 incubation. (A) Gene expression of lactate dehydrogenase A (LDHA) of SH-SY5Y$_{695}$
319 cells after the incubation with KCC compared to the control. (B) Gene expression of
320 pyruvate dehydrogenase kinase 1 (PDK1) of SH-SY5Y$_{695}$ cells after the incubation with
321 KCC compared to the control. N = 8. The normalization factor was calculated based on
322 the geometric mean of the levels of multiple control genes of *ß-actin (ACTB),*
323 *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, and *phosphoglycerate kinase 1*
324 *(PGK1)* according to the MIQE guidelines [33]. Significance was determined by
325 Student's unpaired t-test. Data are displayed as the mean ± SEM. KCC = kahweol 1µM,
326 cafestol 1µM and caffeine 50µM.

327 **4. Discussion**

328 In the present work, we aimed to investigate different bioactive substances present in
329 coffee. For this purpose, we selected three molecules that occur in larger concentrations
330 in coffee - namely, caffeine, kahweol and cafestol. We tested various concentrations and
331 combinations of these three compounds, of which 1µM kahweol, 1µM cafestol and
332 50µM caffeine (KCC) together were most promising. The compounds were tested in a
333 SH-SY5Y-APP$_{695}$ cells, an established model for early AD.

First, we investigated the effects of the single coffee compounds and its combination (KCC) on the mitochondrial dysfunction present occurring in SH-SY5Y-APP_{695} cells. The incubation with KCC significantly increased the ATP levels compared to the control (Fig. 1A). In addition, KW1 and CF1 increase the ATP levels significant. Caffeine alone does not increase or decrease ATP levels. Only the combination with one or both diterpenes shows a significant increase at Cof 50µM (SupFigure 2). In a transgenic mouse model of AD that oral treatment with caffeine intake of 0.6mg/d raised the ATP level significantly [34]. According to our result, kahweol was able to significantly rise ATP levels in SH-SY5Y cells a model [28]. It seems a combination of both compounds has equally positive effects on ATP levels. Due to the lack of knowledge it is unclear to what extent CF affects ATP levels. We found no indication that increased ATP levels were a result of enhanced MMP in consequence of an accelerated complex activity through the respiratory chain. In studies on SH-SY5Y cells with KW, no change in MMP or oxygen consumption could be measured by single administration, but KW was able to attenuate the effect by the stressor H_2O_2 [26,28]. This suggests that KW in combination with the other investigated substances has a positive effect on the maintenance of the MMP and the complex activity of the respiratory chain. In addition, the increased ATP level were not a result of a boosted mitochondrial mass (Fig. 1D), which leads to the assumption that these come from a different source. Another parameter related to mitochondrial quality and functionality are reactive oxygen species (ROS) that are produced in excess due to an insufficient proton transfer through the mitochondrial respiration chain. Due to their ability to interact with cellular components such mitochondrial membrane lipids or DNA, they can thus cumulatively cause oxidative damage that shortens lifespan and leads to impaired mitochondrial function [35]. However, in our study the tested

359 compounds and their combination showed no antioxidant activity. Studies have shown
360 that the addition of caffeine can lower ROS levels and thus have anti-inflammatory
361 effects. Overall, caffeine is thought to reduce oxidative stress. Caffeine has been shown
362 to inhibit the activity of NF-$_k$B and increase that of superoxide dismutase [36]. CF is
363 also thought to have a suppressive function of NF-$_k$B [37]. Peroxidase, which catalyses
364 the oxidation of organic and non-organic substances using H_2O_2, is considered an
365 important antioxidant enzyme. In this process, it is supposed to protect against the
366 harmful effects of physiological accumulation of ROS. Caffeine has been shown to
367 increase the activity of antioxidant enzymes in the liver of mice or to increase overall
368 antioxidant [38,39]. Glutathione peroxidase as well as superoxide dismutase, both
369 antioxidant enzymes, benefit from increased activity after coffee addition in human
370 experiments [40]. We examined the peroxidase activity that represents an important
371 detoxification mechanism and found no significant differences by incubation with the
372 single substances or its combination. The pathological appearance of AD is
373 characterized by a deposition of oligomeric Aβ in the brain. For this purpose, we
374 measured $A\beta_{1-40}$ levels in our experiments (Fig. 2). There were no significant changes
375 due to the incubations compared to the control. However, there is evidence that there is
376 a caffeine-induced protective effect on the internalization of APP processing. Thus,
377 $A\beta_{1-40}$ concentration was significantly decreased due to the addition of caffeine [41]. In
378 animal experiments with mice, it was shown that both a single and long-term
379 administration of caffeine significantly reduced plasma Aβ levels. Increased cognitive
380 performance and decreased Aβ deposition in the brain were also observed [42]. Studies
381 in humans showed that moderate coffee consumption was associated with lower Aβ
382 levels. This potential neuroprotective effect is consistent with other studies that suggest
383 coffee consumers are at lower risk for AD [43–45]. It is possible that a longer

384 incubation period with the three components would have had a greater effect on Aβ
385 production.
386 Cyclic adenosine monophosphate (cAMP) belongs to the nucleotides and serves
387 as a second messenger in the body. Its tasks are signal transmission in the context of
388 metabolic pathways and hormonal effects, in which a signal cascade is induced by
389 cAMP. Its main mode of action is to activate the cAMP-dependent protein kinase A
390 enzyme family. One consequence of protein kinase A (PKA) being activated in this way
391 is that it influences gene transcription by means of phosyphorylation of the cAMP
392 response element binding protein (CREB) [14,15]. CREB affects neuron growth, neuron
393 differentiation, synaptic plasticity and neurogenesis. In diseases such as Parkinson's
394 disease, Huntington's disease and AD, an impairment of CREB has been observed [46].
395 The cAMP signaling cascades thus altered have the ability to modulate long-term
396 memory. This is based on the interaction of phosphorylated CREB with target genes.
397 Thus, it is postulated that cAMP has a beneficial effect on cognitive performance in the
398 context of short-term and long-term memory [47]. Cognitive performance deficits
399 occurring in AD is mediated, among other things, by the cAMP/PKA/CREB signaling
400 cascade. Aβ toxicity may represent one trigger of this impairment [17]. Furthermore,
401 pathological Aβ concentration shows to cause a rapid and sustained decrease in PKA
402 activity and inhibition of CREB phosphorylation [48]. Similarly, oxidative stress
403 inhibits PKA activity and phosphorylation of CREB have. Administration of
404 antioxidants has been shown to protect neurons from Aβ-induced inactivation by PKA
405 [16]. In our study, Figure 5 shows an increase in cAMP levels by incubation with KCC
406 compared with the control, although not a significant one. Studies have shown that
407 caffeine has an increasing effect on cAMP concentration [49,50]. Furthermore, caffeine
408 has shown a beneficial effect on PKA activity in animal models of AD [48]. Overall,

409 many benefits lie in increasing the cAMP signalling cascade, which includes activation
410 of CREB, sirtuin 1, and Nrf2. In the Nrf2 signalling pathway, potential modes of action
411 of caffeine, cafestol, and kahweol overlap. Studies suggest that CF and KW may
412 condition health-promoting effects via Nrf2, but unlike caffeine, without exerting
413 influence on cAMP levels. There is no known cAMP modulation by CF or KW [51–
414 53].

415 Glucose is the primary source of energy for brain activity. It is suspected that
416 due to this, an impairment of glucose metabolism is associated with neurodegenerative
417 diseases [54]. Lactate is the end product of anaerobic glycolysis catalysed by lactate
418 dehydrogenase (LDH). Lactate is considered as an important bioenergetic metabolite
419 formed in the absence of oxygen by fermentation or in the presence of oxygen by
420 aerobic glycolysis [55]. Lactate is the link between the glycolytic and aerobic metabolic
421 pathways [56]. It has been observed that when ATP production is impaired, lactate
422 production for short- and long-term energy provision is stimulated. Thus, increased
423 ATP levels may decrease cellular lactate demand [57]. In our work, despite increased
424 ATP levels, there was no decrease in lactate values (Fig. 6A). Pyruvate is the end
425 product of glycolysis and is considered a key molecule for numerous metabolic
426 pathways. These include mitochondrial ATP generation and several biosynthetic
427 pathways that cross the citrate cycle [58]. In general, neurons have an increased
428 pyruvate demand due to a high energy turnover [59,60]. The significantly increased
429 pyruvate concentration may indicates a relevant influence on the metabolism of
430 pyruvate [61]. In our study, incubation with the combination of compounds significantly
431 increased pyruvate levels (Fig. 6B). This effect might relay on KW, which as a single
432 substance significantly increased the pyruvate levels, whereas the other single
433 substances could only partly raise the levels. Other studies have shown that increased

434 pyruvate levels can protect neurons from damage. In this case, the damage triggered by
435 a stressor was annulated by an increased pyruvate concentration and at the same time
436 pyruvate specifically protects the mitochondria from oxidative stress [62,63]. With
437 regard to the lactate/pyruvate ratio (L/P), the results obtained indicate that there is a
438 lowered ratio in favour of pyruvate. An increase in L/P is accompanied by an increase
439 in $NADH/NAD^+$. In our experiments, there should be a decreased ratio of $NADH/NAD^+$
440 corresponding to L/P [56]. A shift in this ratio could have an impact on glycolysis, as
441 there is inhibition of glycolysis when the $NADH/NAD^+$ ratio is increased [56,64]. This
442 may suggest that increased glycolysis may be present in the experiments performed here
443 due to a decreased $NADH/NAD^+$ ratio. Gene expression analysis of lactate
444 dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) performed in
445 our study (Fig. 7) showed no significant differences from control. There was a slight
446 decrease in LDA and a slight increase in PDK1 expression. In an AD model with
447 Drosophila melanogaster, downregulation of LDHA was shown to stimulate
448 neuroprotective effects. Consistent with this, overexpression of LDHA showed a
449 shortened lifespan as well as brain dysfunction. Based on this, LDHA can be considered
450 a modulator of aging processes [65–67]. PDK1 inhibits pyruvate dehydrogenase (PDH)
451 via reversible phosphorylation. PDH catalyses the oxidative decarboxylation of
452 pyruvate to acetyl-CoA and CO_2 in the mitochondrial matrix [63]. Shift in PDK activity
453 may result in changes in mitochondrial function as well as neurological activity. With
454 age, there is a decrease in PDH via increased PDK activity, which shifts energy
455 production from aerobic to anaerobic glycolysis [68]. Overall, synergistically, the slight
456 decrease in LDHA in combination with the slightly increased PDK1 could explain the
457 increased pyruvate concentration. Thus, the increased ATP levels could be the result of
458 increased glycolysis. This would go hand in hand with the observations made of the

increased pyruvate concentration. Thus, an increased glucose metabolism up to the generation of pyruvate can be assumed. If an increased metabolism of glucose persists, need to be addressed by future studies.

5. Conclusion

In the present study, we observed the effect of a combination of different coffee components, kahweol, caffestol, and caffeine, on early AD symptomatology in a cell model of early AD. ATP levels were increase independent from mitochondrial function. Despite the lack of change in gene expression and the lack of change in mitochondrial complex activity, we suspect a change within glycolysis due to the change in pyruvate level. Further investigations have to address if coffee components affect glycolysis.

Supplementary Materials: The following supporting information can be downloaded at:

Author Contributions; investigation, L.B, M.T.L.; writing—original draft preparation, L.B.; supervision, G.P.E. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest

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3.4 Combination of secondary plant metabolites and micronutrients against Alzheimer disease in a SH-SY5Y-APP$_{695}$ cell model

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Combination of Secondary Plant Metabolites and Micronutrients Improves Mitochondrial Function in a Cell Model of Early Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is characterized by excessive formation of beta-amyloid peptides (Aβ), mitochondrial dysfunction, enhanced production of reactive oxygen species (ROS), and altered glycolysis. Since the disease is currently not curable, preventive and supportive approaches are in the focus of science. Based on studies of promising single substances, the present study used a mixture (cocktail, SC) of compounds consisting of hesperetin (HstP), magnesium-orotate (MgOr), and folic acid (Fol), as well as the combination (KCC) of caffeine (Cof), kahweol (KW) and cafestol (CF). For all compounds, we showed positive results in SH-SY5Y-APP$_{695}$ cells—a model of early AD. Thus, SH-SY5Y-APP$_{695}$ cells were incubated with SC and the activity of the mitochondrial respiration chain complexes were measured, as well as levels of ATP, Aβ, ROS, lactate and pyruvate. Incubation of SH-SY5Y-APP$_{695}$ cells with SC significantly increased the endogenous respiration of mitochondria and ATP levels, while Aβ$_{1–40}$ levels were significantly decreased. Incubation with SC showed no significant effects on oxidative stress and glycolysis. In summary, this combination of compounds with proven effects on mitochondrial parameters has the potential to improve mitochondrial dysfunction in a cellular model of AD.

Keywords: hesperetin; magnesium-orotate; folic acid; caffeine; kahweol; cafestol; ROS; Alzheimer's disease; mitochondrial dysfunction; amyloid-beta

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1. Introduction

Alzheimer's disease (AD) has now been placed on the WHO's list of global health priorities. Since its first description in 1906, there is still no effective treatment for the disease [1]. In 2018, 1.73% of the population in the European Union was living with dementia and this figure is expected to reach 2.0% by 2050 [2]. AD is the most common form of dementia and is on the rise due to an aging population [3]. AD is characterized by a progressive loss of memory, speech, personality and behavior, which is accompanied by limitations in the quality of life [3]. Signs including the presence of amyloid beta (Aβ), which accumulates extracellularly, leading to plaque formation and hyperphosphorylation of tau protein characterize AD [4,5]. Furthermore, AD leads to impaired glucose metabolism [6] and increased reactive oxygen species (ROS) production, which in turn leads to increased oxidative stress [7,8]. Mitochondrial dysfunction (MD) was identified as playing a central role in the etiology of AD, leading to reduced metabolism, increased ROS, lipid peroxidation and finally to apoptosis [9,10]. The first sign of MD is a decrease of enzymes from the oxidative metabolism [11,12], leading to a limited function of the electron transport chain (ETC) and thus to a reduced complex activity of complexes I and IV. This in turn leads to a lower membrane potential and to lower ATP levels [13,14]. These defects lead to increased ROS production and defects within the mitochondrion, which contributes to an increase in mitochondrial dysfunction [15].

One way to support the various defects in AD is through the use of secondary plant compounds, especially flavonoids [16–18]. The flavonoid hesperetin (HstP) is an aglycon of hesperedin and is mainly found in the peel of oranges and other citrus fruit [19]. The bioavailability of HstP is limited; only about 20% of it is absorbed. On the other hand Hesperedin, the glycosylated form, is almost completely absorbed [20,21]. However, its possible ability to cross the blood–brain barrier makes it a potential candidate for neurodegenerative diseases [22]. HstP was shown to have a protective effect on neurons previously damaged by Aβ [23]. Hesperetin improve cognitive abilities by increasing BDNF levels in rats. Furthermore, it increases the activities of CAT, SOD, GRX and GPX, and cytoprotective effects of hesperetin against Aβ-induced impairment of glucose transport have been reported [24]. We recently have shown that HstP reduces ROS levels in SH-SY5Y-APP_{695} cells without affecting mitochondrial function or $Aβ_{1–40}$ level [25]. In another study, we showed that magnesium-orotate (MgOr) and folic acid (Fol) reduced Aβ, soluble alpha APP (sAPPα) and lactate levels in SH-SY5Y-APP_{695} cells [26].

Folate is an important product in metabolism, and when deficient, leads to insufficient DNA and mtDNA synthesis and stability, which is why it can lead to oxidative stress, a process associated with Alzheimer's disease. This leads to the worsening of the neuronal state and increased cell death. Deficiency leads to impaired methylation of enzymes and promoter regions of genes involved in AD. [25,26]. MgOr is the magnesium salt of orotic acid, showing enhanced bioavailability. Orotic acid is a key substance in biosynthetic pathways, as well as in the synthesis of glycogen and ATP [27]. Mg is involved in many synthesis processes, as well as in maintaining physiological nerve and muscle function [28–30]. Many different mechanisms related to Mg and AD are discussed. It is thought to positively influence various inflammatory pathways and to influence APP processing towards α-secretase, as well as preventing Aβ from crossing the blood–brain barrier [29,31]. Another product discussed in connection with AD are ingredients of coffee, particular caffeine, kahweol and cafestol. Coffee is one of the most commonly consumed beverages [32]. Coffee is supposed to have cardio-, hepato- and neuroprotective properties [33]. The consumption of coffee and caffeine is associated with a lower risk of AD [34–36]. After oral intake, caffeine is 99% absorbed and peaks in the bloodstream after 30–45 min [37]. Cafestol is absorbed at about 70%, while kahweol has a slightly higher bioavailability [38]. All three substances show the ability to cross the blood–brain barrier [39]. Data on kahweol and cafestol are scarce, but there is evidence that both are neuroprotective [40–42]. Our preliminary data show that a combination of caffeine, kahwol and cafestol (KCC) affects glycolysis in SH-SY5Y-APP_{695} cells (unpublished data).

Based on the previous experiments on various dietary compounds in SH-SY5Y-APP695 cells, which showed different beneficial effects, the hypothesis was tested whether a combination of these compounds would also have beneficial activity and possibly show synergistic effects. HstP improves mitochondrial dysfunction [43], MgOr and Fol reduce Aβ levels within cells [26] and caffeine, kahwol and cafestol (KCC) affects glycolysis (unpublished data). A combination of all compounds (SC) in meaningful doses was tested in the SH-SY5Y-APP_{695} cell model regarding mitochondrial function, oxidative stress, Aβ production and glycolysis.

2. Results

2.1. Characterization of the Cellular Model

SH-SY5Y-APP_{695} cells are an established model for early phase AD [44,45]. SH-SY5Y-APP_{695} cells show significantly decreased ATP levels and respiration compared to SH-SY5Y-MOCK cells (Figure 1A,B). Furthermore, in SH-SY5Y-APP_{695} cells, Aβ levels are significantly increased (Figure 1C) and ROS production is significantly increased (Figure 1D) compared to control cells.

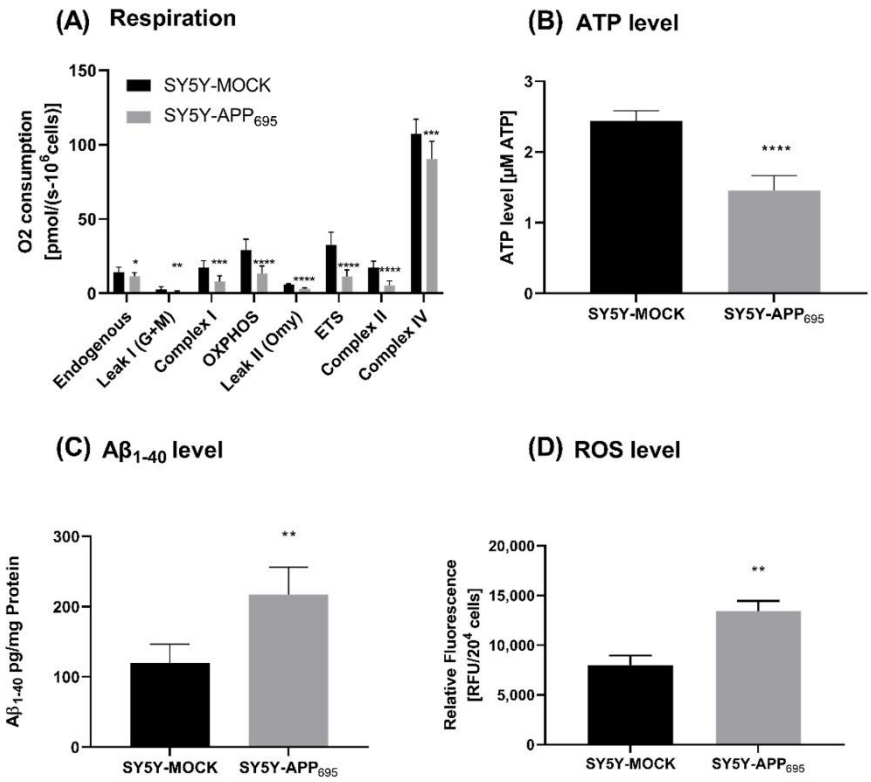

Figure 1. Respiration, ATP, $A\beta_{1\text{-}40}$ level and ROS level of SH-SY5Y-APP695 cells compared to SH-SY5Y-MOCK cells. (**A**) Respiration of SH-SY5Y-MOCK and -APP cells adjusted to cell count. Respective mean values ± SD are shown. Significance was determined using Student's unpaired *t*-test, N = 12. (**B**) SH-SY5Y-APP_{695} cells exhibit reduced ATP levels compared to SH-SY5Y-MOCk control cells. ATP levels were determined after 24 h seeding by bioluminescence assay. Respective mean values ± SD are shown. Significance was determined using Student's unpaired *t*-test, N = 8. (**C**) Concentration of $A\beta_{1\text{–}40}$ levels in SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. Significance was determined using Student's unpaired *t*-test. N = 6. (**D**) ROS levels in RUF/20^4 cells in SH-SY5Y-APP_{695} cells compared to SH-SY5Y-MOCK cells. Respective mean values ± SD are shown. Significance was determined using Student´s unpaired *t*-test, N = 8. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

2.2. Overview

The SC used in this study is a combination of different compounds, which have been previously tested as single substances or combinations in SH-SY5Y-APP_{695} cells. The Table 1 compares the effects of the individual substances and their concentrations with the results of the SC reported herein.

Table 1. Effects of the combination of dietary compounds tested herein compared with the effects of the individual components in the SH-SY5Y-APP$_{695}$ cells.

	HstP	MgOr + Fol	Cof + KW + CF	SC
ATP level	↑	=	↑	↑
OXPHOS	=	=	=	↑
ROS	↓	=	=	=
Aβ level	=	↓	=	↓
Lactate	=	↓	=	=
Pyruvate	=	=	↑	=
L/P Ratio	=	=	↓	=
Source	[43]	[26]	[25]	

↑ = significant increase; ↓ = significant decrease; = no significant change; L/P = lactate-pyruvate-ratio; Concentration of tested substances were HstP (10 µM), MgOr (200 µM), Fol 10 µM), Cof (50 µM). KW (1 µM), CF (1 µM) and SC (all concentration combined).

2.3. Mitochondrial Parameter

First, we examined the cells for various mitochondrial functions, including ATP levels, respirometry and related citrate synthase activity. Incubation with SC resulted in a significant increase in ATP levels compared to control ($p = 0.0031$, Figure 2A). Since the increased ATP levels may be due to a change in the activity of respiration chain complexes, respiration was investigated next. A significant increase in endogenous respiration of SC compared to SCTR was found ($p = 0.0314$). Except for complex II and leak II, the O_2 consumption of SC tended to be higher than that of SCTR (Figure 2B). However, the changes were not significantly different. Moreover, there was no difference in the mitochondrial mass marker citrate synthase activity between the groups (Figure 2C).

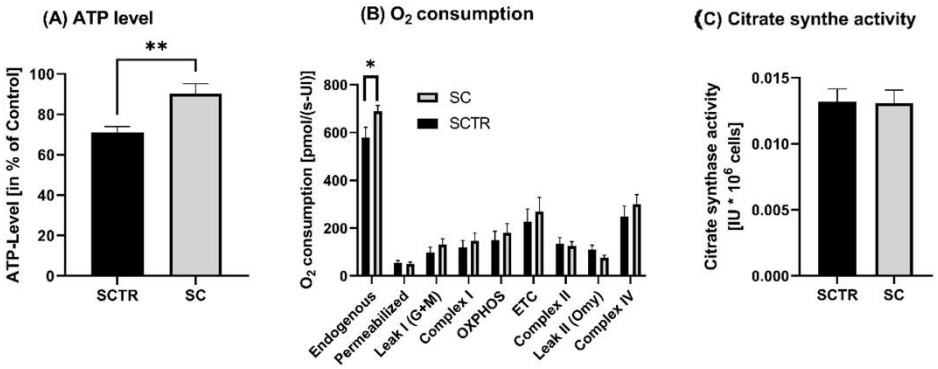

Figure 2. ATP level, respiration and citrate synthase activity of SH-SY5Y-APP$_{695}$ cells incubated for 24 h with SC. (**A**) ATP level of SH-SY5Y-APP$_{695}$ cells incubated with SC. Cells treated with cell culture medium served as control (100%). N = 11. (**B**) Respiration of SH-SY5Y-APP$_{695}$ cells incubated with SC compared to the control. SH-SY5Y-APP$_{695}$ cells adjusted to international units (IU) of citrate synthase activity. N = 16. (**C**) Citrate synthase activity of SH-SY5Y-APP$_{695}$ cells incubated with SC compared to control. N = 16. Significance was determined using Student's unpaired *t*-test. Data are displayed as the mean ± SEM. * $p > 0.05$, ** $p > 0.01$, SC = cocktail, SCTR = control.

2.4. $A\beta_{1–40}$ Level

To investigate the Aβ levels, the cells were incubated with SC or SCTR for 24 h (Figure 3). SC significantly decreased the Aβ levels of the cells compared to the control ($p = 0.0217$).

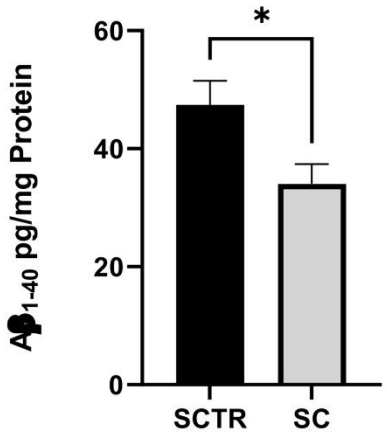

Figure 3. Effect of SC on the $A\beta_{1–40}$ level of SH-SY5Y-APP_{695} cells incubated for 24 h. N = 9. $A\beta_{1-40}$ levels were adjusted to the protein content. Significance was determined using Student's unpaired *t*-test. Data are displayed as the mean ± SEM. * $p > 0.05$ SC = cocktail, SCTR = control.

2.5. ROS

To investigate the effects of incubation on ROS levels, cells were incubated with the respective substance for 24 h (Figure 4). No significant differences were found.

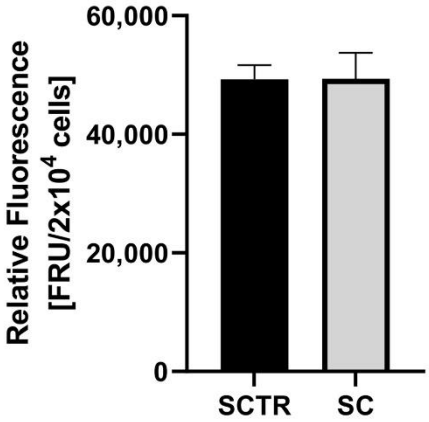

Figure 4. ROS measurement of SH-SY5Y-APP_{695} cells after incubation with SC for 24 h. N = 8. Significance was determined using Student's unpaired *t*-test. Data are displayed as the mean ± SEM.

2.6. Glycolysis

To investigate the effects of the incubations on glycolysis metabolism, the lactate and pyruvate values and their ratio were determined (Figure 5). There were no significant differences, although the lactate values of the SC tended to be higher than those of the control ($p = 0.0983$). In contrast, the pyruvate values of the SC tended to be lower than those of the control. This results in a lower ratio for the control ($p = 0.0509$).

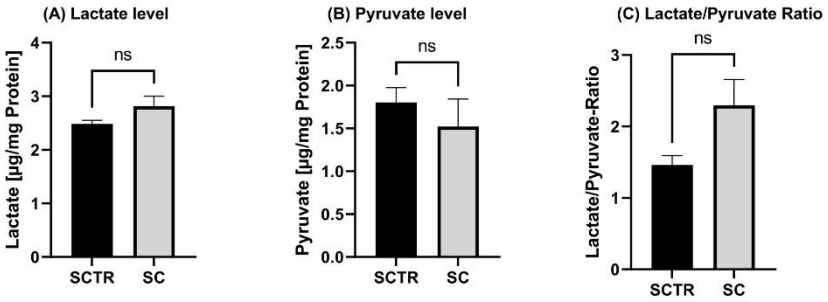

Figure 5. Effect of the on lactate and pyruvate level after 24 h of incubation with SC or the control. (**A**) Lactate level of SH-SY5Y-APP$_{695}$ cells after the incubation compared to the control. (**B**) Pyruvate level of SH-SY5Y-APP$_{695}$ cells after the incubation with SC compared to the control. (**C**) Lactate to pyruvate ratio. N = 10. Levels were adjusted to the protein content. Significance was determined using Student's unpaired *t*-test. ns = not significant. Data are displayed as ±SEM.

3. Discussion

In the following manuscript, we investigate the effects of HstP, MgOr, Fol, Cof, KW and CF as a cocktail in a human cell model of early AD. All of the previously listed compounds have shown effects in the area of mitochondrial dysfunction, Aβ levels and glycolysis in previous studies we conducted. Our question in this regard was whether all the substances, combined in a cocktail, address all the areas mentioned. The easy availability of the substances offers a good opportunity to intervene in early AD symptoms.

Mitochondrial dysfunction is one of the core issues in the development of AD, as described earlier, and is characterized by a decrease in ATP levels [13,14,46]. HstP elevated ATP levels in SH-SY5Y-APP$_{695}$ cells in our former studies [43]. Similar findings were reported in differentiated human myotubes by Biesemann et al. [47]. Mg and Fol are also involved in the provision of ATP. Deficiencies of these nutrients leads to a decrease in ATP production [29,48]. Deficits are also evident in AD, in which Mg and Fol levels are found to be reduced in brain tissues of patients [49–52]. In our studies using SH-SY5Y-APP$_{695}$ cells as an AD model, no increase in ATP levels was observed from the single administration of MgOr and Fol [26] The combination of the three coffee ingredients (Cof, KW, and CF) increased ATP levels [25], but Cof as single compound had no effect on ATP levels as shown in our experiments. However, it has been described in the literature that Cof affects ATP levels in HepG2 cells and smooth muscle cells [53,54].

The OXPHOS in mitochondria is built up by the activity of respiration chain complexes and is the main cellular source of ATP production [55,56]. The administration of SC significantly increased endogenous respiration in mitochondria. Furthermore, the activity of the individual complexes of the respiration chain was increased compared to the control. Increased respiration could be explained by the individual substances in the mixture (SC) or by the interaction of them. Activation of the Nrf-2 pathway by HstP represents one conceivable option [57,58]. Reduction of Nrf-2 results in decreased MMP, decreased ATP levels and impaired mitochondrial respiration [59]. A reduction of Aβ levels by MgOr and Fol could also be a possible explanation [26]. This could reduce the impairment of

complexes by Aβ. In our former experiments, KCC had no effect on OXPHOS, so the described effect for SC could be due to the other substances in the mixture.

Next, we investigated whether the combination of all tested compounds had any effect on ROS levels in SH-SY5Y cells (Figure 4). It was found that the ROS levels of the cocktail did not decrease. HstP had no ROS-reducing effect, as shown in our other study [43]. It is possible that the amount of the substances means that no ROS-reducing effect occurred. Fol and MgOr had no effect on ROS levels (Figure 3), whereas the literature suggests a ROS reducing effect from Mg [60], Fol [61], Cof [62], KW [63] and CF [64]. However, the described effects are difficult to compare, either because they are deficit models or because models other than our SH-SY5Y cell model were used.

Next, we took a closer look at Aβ levels (Figure 3). We found that SC significantly lowered $A\beta_{1-40}$ compared to the control. Considering the SC results, there is an additive effect on $A\beta_{1-40}$ levels. Of the compounds we tested, only MgOr and Fol had the potential to significantly lower Aβ levels [26], but in combination as SC, all substances lowered Aβ levels. Indeed, all our tested substances show the same potential in the literature [57,65–67]. The question now is whether the effect we observed is due to the combination of MgOr and Fol alone, or whether all substances are exerting their potential. It is possible that the combined effect, similar to the effect of the individual substances [66,67], results in suppression of BACE1 and APP expression and a greater reduction in β-secretase activity than we observed with MgOr and Fol [26]. This should be confirmed in further experiments.

As a final point, SC was examined for the effects on glycolysis (Figure 5). Although data did not reach significance, the incubation tended to increase lactate levels, with a concomitant decrease in pyruvate levels and an L/P in favor of lactate. Based on this observation, it can be assumed that the cells tend to increased anaerobic respiration due to the SC. It should be noted that MgOr and Fol, reduced Aβ levels, but did not affect glycolysis in our former experiments [26]. It has been shown that cells protect themselves from Aβ by increasing anaerobic respiration, initiating the Warburg effect [68–70]. This manifests itself in an increase in anaerobic respiration and an increase in lactate and a reduction in OXPHOS when the cells switch from aerobic to anaerobic respiration [71]. However, in our experiments OXPHOS increased.

In summary, SC has the potential to have a positive effect on AD, especially the increased ATP levels in combination with the lowered Aβ levels. Since SC consists of a variety of substances, it would be interesting for future studies to create combinations of two or three substances tested here and to investigate their effects on our parameters.

4. Materials and Methods

4.1. Cell Culture

Human SH-SY5Y-APP_{695} cells were incubated at 37 °C in DMEM in an atmosphere of 5% CO_2. DMEM was mixed with 10% heat inactivated fetal calf serum, streptomycin, penicillin, hygromycin, non-essential amino acids and sodium pyruvate. The cells were split on average every 3 days and used for experiments when they reached 70–80% growth.

4.2. Cell Treatment

Cells were incubated with a cocktail for 24 h each unless indicated. This cocktail contains a mixture of hesperetin 10 µM (HstP), magnesium orotate 200 µM (MgOr), folic acid 10 µM (Fol), caffeine 50 µM (Cof), cafestol 1 µM (CF) and kahweol 1 µM (KW) (together SC). The control (SCTR) was a mixture of solvents in which the substances were dissolved ethanol, DMSO and NaOH.

4.3. ATP Assay

To determine ATP levels, an ATP luciferase assay was performed. Light is generated by ATP and luciferin in the presence of luciferase. The test was performed using the ATPlite Luminescence Assay System (PerkinElmer, Rodgau, Germany).

4.4. Cellular Respiration

Respiration in SH-SY5Y$_{695}$ cells was measured using an Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. Cells were treated according to a complex protocol developed by Dr. Erich Gnaiger [72]. They were incubated with different substrates, inhibitors and uncouplers. First, cells were washed with PBS (containing potassium chloride 26.6 mM, potassium phosphate monobasic 14.705 mM, sodium chloride 1379.31 mM and sodium phosphate dibasic 80.59 mM), then scraped into mitochondrial respiratory medium (MiRO5) developed by Oroboros [72]. Cells were then centrifuged, resuspended in MiRO5 and diluted to 106 cells/mL. After 2 mL of cell suspension was added to each chamber and endogenous respiration was stabilized, cells were treated with digitonin (10 µg/106 cells) to permeabilize the membrane, leaving the outer and inner mitochondrial membranes intact. OXPHOS was measured by addition of complex I and II substrates malate (2 mM), glutamate (10 mM) and ADP (2 mM), followed by succinate (10 mM). The stepwise addition of carbonyl cyanide-4-, before being evaluated by measuring R123 fluorescence (trifluoromethoxy) phenylhydrazone, showed the maximum capacity of the electron transfer system. Rotenone (0.1 mM), a complex I inhibitor, was injected to measure complex II activity. Oligomycin (2 µg/mL) was then added to measure leak respiration. Inhibition of complex III via the addition of antimycin A (2.5 µM) was used to determine residual oxygen consumption, which was subtracted from all respiration states. The activity of complex IV was measured via the addition of N, N, N′-tetramethyl-p-phenylenediamine (0.5 mM) and ascorbate (2 mM). To measure the sodium autoxidation rate, azide ($\geq$100 mM) was added. Subsequently, complex IV respiration was corrected for autoxidation.

4.5. Citrate Synthase Activity

Cell samples from respirometry were frozen away at −80 °C for citrate synthase determination. Samples were spiked and mixed with the reaction mix (0.31 mM acetyl coenzyme A, 0.1 mM 5,5′-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 50 µM EDTA, 0.5 mM oxaloacetate, 5 mM triethanolamine hydrochloride and 0.1 M Tris-HCl) and warmed to 30 °C for 5 min. Samples of 40 µL each were applied as a triplet, 110 µL of reaction mix was added and the absorbance was measured.

4.6. Protein Quantification

To determine the protein content of the samples, a PierceTM Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used and the experiment was performed according to the manufacturer's instructions, using bovine serum albumin as the standard.

4.7. $A\beta_{1–40}$ Measurement

After 24 h incubation, A$\beta_{1–40}$ was determined using an HTRF amyloid beta 1–40 kit (Cisbio, Codolet, France). The protocol has been described previously [43]. A$\beta_{1–40}$ levels were normalized against protein levels.

4.8. ROS Quantification

A DCFDA/H2DCFDA kit was used to determine ROS levels (ab113851; Abcam, Cambridge, UK). Cells were seeded in 96-well plates and incubated for 24 h. The experiment was then performed according to the manufacturer's instructions.

4.9. Lactate and Pyruvate Measurement

Frozen cell samples, which were previously incubated for 24 h, were thawed at room temperature. An assay kit (MAK071, Sigma Aldrich, Darmstadt, Germany) was used to determine pyruvate levels. For the determination of lactate values, a lactate assay kit was used (MAK064, Sigma Aldrich, Darmstadt, Germany). The values were normalized to the protein content.

4.10. Statistics

Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical significance was defined for p values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Statistical analyses were performed by applying student's unpaired t-test and one-way ANOVA with Tukey´s multiple comparison post hoc test (Prism 9.5 GraphPad Software, San Diego, CA, USA).

5. Conclusions

In our former experiments using SH-SY5Y-APP$_{695}$ cells, we showed that HstP lowered the ROS level, MgOr and Fol lowered Aβ levels, and the combination of coffee ingredients affected glycolysis. In the current study, we addressed the question of whether a combination of these compounds have additive effects on the tested parameters. We found that the cocktail increased ATP levels and decreased Aβ levels. However, no advantage of the combination over the individual substances was found.

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4. Discussion

On the following pages, the data collected in the individual studies are discussed in the context of three major subfields. All studies presented herein, essentially addressed the three major sub-areas of mitochondrial dysfunction, Aβ symptomatology and glycolysis. For this purpose, each study used a different flavonoid, micronutrient, alkaloid or diterpene found in food. To check whether these substances also trigger the same effects in combination or perhaps even address all three major topics, a combination of the active compounds tested before was investigated as so called "super cocktail" (SC) in study number four. A brief overview of the respective results can be found in **Table 1**.

All experiments were performed in the same SH-SY5Y cell model of early AD [181] (LIT). SH-SY5Y were cloned from a SK-N-SH [182, 183] derived from a bone marrow biopsy of a 4-year-old suffering with neuroblastoma [182]. SH-SY5Y cells are a cells widely used today to demonstrate neuronal function and neurotoxicity [181, 184]. The cells we used were transfected with the human APP_{695} gen. This leads to increased levels of Aβ and other restrictions, which affect the mitochondria and result in a dysfunction which is not only described by us but also by others [184–187].

Table 1: Overview of the experiments discussed in this paper and their respective effects.

[188]	**HstP**	**MgOr + Fol**	**Cof + KW + CF**	**SC**
ATP level	↑	-	↑	↑
OXPHOS	-	-	-	↑
ROS	↓	-	-	-
Aβ level	-	↓	-	↓
Lactate	-	↓	-	-
Pyruvate	-	-	↑	-
L/P Ratio	-	-	↓	-
Source	[186]	[189]	[190]	[188]

↑ = significant increase; ↓ = significant decrease; - = no significant change; L/P = lactate-pyruvate-ratio; concentration of tested substances were HstP (10 µM), MgOr (200 µM), Fol 10 µM), Cof (50 µM). KW (1 µM), CF (1 µM) and SC (all concentration combined)

4.1 Micronutrients and Secondary Plant Constituents Against Mitochondrial Dysfunction

Mitochondrial dysfunction is one of the core issues in the development of AD, as described earlier and is characterized by a decrease in ATP [168, 169, 191]. In our studies, the first starting point when examining the effects on AD in the cell model was that of

ATP levels. ATP levels are not only a marker for how well the cell produces energy, they are also a marker for cell viability [192, 193].

Our studies showed that, with the exception of MgOr, Fol and Vit B6, all the substances we used increased ATP levels compared to the control in SH-SY5Y-APP$_{695}$ cells. The SC also increased the ATP levels when compared to the control. However, the values did not reach those of the medium control, which suggests that the large number of substances and solvents used behaved in a contraindicated manner, which was, however, compensated for in comparison with the SC control.

The increased ATP levels by HstP cannot be explained by changes within the miochondrial markers we tested, such as complex activity or mitochondrial mass. The other markers altered in AD will be discussed later. Furthermore, there are few to no sources that address the effects of HstP and ATP levels in the context of AD. *Biesemann et al*. have shown that HstP could increase ATP levels by 33%, but they refer to a model of muscle cells, so a comparison to neuronal cells is challenging. The authors attribute this to increased complex activity and decreased ROS levels [194]. The increase of ATP, as in *Bisemann et al*., due to reduced ROS levels could also occur in our case, this point will be discussed in the next chapters.

Next, we tested MgOr and Fol in our SH-SY5Y-APP$_{695}$ cell model. With the combination of MgOr and Fol, there were no changes in ATP levels. Mg and Fol are important micronutrients for ATP synthesis [195, 196]. Deficiencies of these nutrients leads to a decrease in ATP production [196, 197]. These deficits are also evident in AD, in which Mg and Fol levels are found reduced in brain tissues of patients [76, 75, 49, 51]. However, our cell model is not one in which there is a deficit of Mg and Fol. The medium of the cells is sufficiently enriched with Mg and Fol. The amount we have additionally administered to the cells does not seem to have had any effect on the total concentration, which is why the additional amount probably did not lead to an increase in ATP levels.

Next, we tested effects of Cof, KW, and CF as a combination (KCC) on ATP levels, which significantly increased them. Interestingly, the ATP increasing effect of KCC seems to come from the two diterpenes, as Cof alone had no effect. A higher dose of caffeine also had no effect, which would confirm that in our model the effect is not mediated by Cof but by KW and CF. This is shown by the fact that KW and CF alone significantly increased ATP levels. In the context of our results, Cof is able to increase ATP content, so Cof resulted in increased ATP content in smooth muscle cells [198] and in HepG2 cells [199]. *Katsuragi et al.* suggested than a higher energy potential caused an

acceleration of ATP synthesis by affecting the $Ca2^{+}$ signaling cascade [198]. *Riedel et al.* shows that a higher energy charge potential occurs after caffeine exposure. This suggests an energy recovery by attenuation of the energy consuming signaling pathways. [199]. Since there is currently no literature describing the effects of the diterpenes on ATP levels, we are the first to describe the effects individually and in combination in a SH-SY5Y cell model of AD.

To explain the enhanced ATP levels in some cases, we measured OXPHOS after incubation with the individual substances or with the SC. The OXPHOS is a pathway where ATP is produced, so an increase in the activity of the complexes could result in an increase in ATP [127, 128].

Among our tested substances, only SC had a significant effect on endogenous cellular respiration. The combination of MgOr, Fol and SC showed increased activity regarding the individual complexes of the respiration chain compared with the control, however the observed effects were not statistically significant. However, the increased ATP values cannot necessarily be explained by the improved or non-existing improvement in complex activity.

In AD, impairment of complexes I and IV in particular occurs, resulting in a lower membrane potential, leading to lower ATP levels [200, 163]. We [186] and *Rhein et al*. [185] have also shown this to be the case in the same cell model that we used, in which the complexes of the respiratory chain are present in reduced activity.

HstP, through its ROS protective effect, as described in more detail later in the chapter, probably ensured that the respiratory chain was less impaired and thus contributed more to the increase in ATP levels. *Biesemann et al*. comes to a similar conclusion, since incubation with HstP leads to an increase in complex respiration but also to an increased expression of respiratory complex genes [194]. However, the statements must be considered with caution since this is a completely different model and therefore a comparison can be difficult. It is possible that Nrf-2 plays a role in the increased activity of the complex by HstP. Reduction of Nrf-2 results in decreased MMP, decreased ATP levels and impaired mitochondrial respiration [201]. HstP is able to activate the NrF-2 pathway [202, 203] and thus contribute to an improved OXPHOS.

Fol and MgOr also slightly increased the complex activity compared to the control. This effect could be due to the Aβ-lowering effect of MgOr and Fol, which will be described

in more detail in the following chapter. As a result, the complexes of OXPHOS could operate better. However, this did not result in an increase in ATP levels.

The combination on Cof, CF and KW had no effect on OXPHOS and tended to have higher respiration than the combination by the control. There is evidence suggesting increased OXPHOS by caffeine, however it is hypothesized that this is due to increased mitochondrial mass [204]. This could not be shown in our experiment, as the mitochondrial mass – measured as citrate synthase activity - remained unchanged compared to the control, but it is also possible that this is due to the administration of the combination. In further studies, KW incubation was able to protect the OXPHOS activity of SH-SY5Y cells from an added stressor. Administration of KW alone did not cause any change, but was able to maintain the complex activity of I and II in the presence of H_2O_2 and methylglyoxal [109, 110].

The administration of SC significantly increased endogenous respiration. Furthermore, the respiration of the individual complexes was increased compared to the control and the leak respiration was reduced. This could be an indication for a lower ROS production, which occurs during leak respiration [144]. Possible reasons for the increased respiration could be the effects arising from the individual compounds. Activation of the Nrf-2 pathway by HstP, or reduction of Aβ levels by MgOr and Fol, would be a possible option. This could reduce the impairment of complexes by Aβ. The increased respiration would be a possible reason for the increased ATP levels observed by the combination with the cocktail.

One reason for the increased ATP levels may be a reduction in the damaging effects caused by ROS. Mitochondria are the main reason for the formation of ROS [144] because of the respiratory chain and are therefore also particularly susceptible respectively worthy of protection. ROS is generated during transport, in the process electrons escape and interact with molecular oxygen to form superoxide at the flavin mononucleotide site of complex I and the Q-cycle of complex III, which are the main sources of superoxide and hydrogen peroxide in mitochondria [205].

Here, HstP in particular has been shown to be an effective protector against ROS, with incubation with HstP significantly reducing ROS levels in our cell model. In a transgenic mouse model, HstP significantly reduced ROS levels in certain brain regions. At the same time, HstP counterbalanced the decreased Nrf-2 and heme oxygenase-1 (HO-1) protein expression after administration of lipopolysaccharide (LSP) [206]. Nrf-2 is a factor

responsible for the expression of antioxidant, anti-inflammatory and detoxifying proteins. HO-1 and its products have beneficial effects by protecting against oxidative damage, regulating apoptosis, modulating inflammation and contributing to angiogenesis. On the other hand, disturbances in HO-1 levels are associated with the pathogenesis of some age-related diseases such as neurodegeneration or cancer [207]. The antioxidant effect by HstP could be confirmed by *Kheramed et al*. who used an AD-induced rat model in which the administration of HstP significantly increased the activity of antioxidant enzymes and thus reduced the effects of oxidative stress [122]. It has been shown that flavonoids, of which HstP is a family member, can increase the expression of Nrf-2 and HO-1 to enhance the antioxidant effect [208]. HstP related hesperidin increases cellular antioxidant via ERK/Nrf2 signalling the cellular antioxidant defense capacity and the content of antioxidant enzymes such as CAT, SOD and GST by oxidative stress [209]. Activation via the Nrf-2 pathway was also confirmed in HepG2 cells. Here, oleic acid triggered overproduction of ROS was induced. HstP increased the activity of antioxidant levels by activating the Nrf-2 pathway and was able to reduce the induced oxidative stress [203]. In a study by *Ikram et al.*, injection of $A\beta_{1-42}$ into the brain of rats induced a neurodegenerative disorder similar to AD. Administration of HstP, which activated the Nrf-2 pathway, also attenuated the ROS production and oxidative stress induced by the injection [202]. In addition to Nrf-2, HstP also affects the NF-kB signaling pathway. NF-κB is a redox-regulated transcription factor that regulates inflammatory responses and cell injury [210]. NF-κB induces the expression of proinflammatory cytokines (IL-1, IL-6, TNF-α), COX-2, iNOS, vascular adhesion molecules [210]. From a functional perspective, Nrf-2 antagonizes NF-κB signaling. Nrf-2 inhibits oxidative stress-mediated NF-κB activation by decreasing intracellular ROS levels [211]. HstP shows exactly the same effect in mouse and cell models by activating Nrf-2 and suppressing $Nf\text{-}_kB$ [203, 202]. HstP appears to obtain its antioxidant properties via the upregulation of antioxidant enzymes. These are facilitated by the activation and expression of the NrF-2 and HO-1 pathways and down regulation of $Nf\text{-}_kB$. This minimizes the effects of ROS, which in turn may explain the ATP-enhancing effect of HstP.

Fol and MgOr were also evaluated for the effect on ROS. Here, the single administration of Fol or MgOr increased the ROS levels compared to the control. The administration of the combination was also higher than the control. The combination reached the same levels as the single dose of Fol. In contrast to our results, in an HT22 cell model treated with $A\beta_{25-35}$, ROS levels and apoptosis were significantly decreased by the administration

of Mg. Likewise, in the APP/PS1 mouse model used in the same study, NOX4, the marker responsible for oxidative stress, was decreased by Mg administration [212]. Presumably, suppression of the PI3K/Akt pathway results in increased occurrence of oxidative stress and apoptosis. This effect could be reduced by the administration of Mg [212]. The PI3K/Akt pathway is an important cellular signaling pathway that plays a central role in mediating cell apoptosis [213]. ROS production induced suppression of PI3K/Akt signaling pathway leads to cell apoptosis in cell model [214]. Another study showed that inhibition of apoptosis was correlated with ROS-mediated PI3K/Akt signalling in a streptozotocin-treated INS-1 cell model [215].

Fol showed no effect on ROS levels in our experiments. In contrast, *Bagherieh et al*. demonstrated in HepG2 cells that incubation with Fol significantly reduced ROS levels previously induced with palmitate. The authors suggested that this effect occurs via the NF-$_k$B pathway [216]. In SH-SY5Y cells cultured in folate-free medium, supplementation with Fol decreased the previously increased ROS levels. ROS levels were triggered by Aβ [217]. However, these results are difficult to compare with ours, because this is a deficit model, which is not the case in ours. In a study *by Dhitavat et al.*, incubation with Fol in differentiated SH-SY5Y5 cells reduced the effect of Aβ-induced enhancement [218]. In PIG1 cells, Fol reduced oxidative stress induced by H_2O_2. Here, oxidative protection was shown to be induced by activation of the Nrf-2 pathway. As soon as this pathway was switched off, the oxidative protection was no longer present [219]. Also, Fol was able to reduce lead poisoning by activating the Nrf-2 pathway in rats, which had elevated ROS levels and reduced antioxidant enzyme levels due to lead poisoning [220]. MgOr and Fol, similar to HstP, also seem to induce their antioxidant potential via activation of the Nrf-2 pathway, PI3K/Akt pathway and suppression of the Nf-$_k$B pathway, respectively. At least as far as the literature is concerned. In our experiments there was no ROS reducing effect.

Besides HstP, Fol and MgOr, we investigated the effects of Cof, CF, KW, or the combination KCC on ROS levels in SH-SY5Y cells. There was no impact on ROS levels except for the single dose of CF, which was significantly increased. In contrast to our experiments, Cof significantly reduced the effect of aluminium incubation and $A\beta_{25-35}$ in a SH-SY5Y cell model by *Giunta et al.* Here, the administration of Cof blocked the Nf-$_k$B signaling pathway and thus decreased apoptosis and ROS production [221]. Similar effects were observed in a C57BL/6N mouse model injected with LPS. Administration of

LPS increased ROS levels and expression of Nf-kB. Furthermore, it reduced the expression of Nrf-2 and HO-1. Daily injection of Cof reduced ROS levels and Nf-$_k$B expression and increased Nrf-2 expression [222]. The coffee diterpenes kahweol and cafestol also show ROS inhibitory properties in the literature. KW was able to cushion the ROS formation by the neurotoxin 6-hydroxydopamine in SH-SY5Y cells. At the same time, KW upregulated HO-1 expression and induced PI3K activation, which induces the Nrf-2 pathway and thereby affects ROS formation [223]. Similarly, *Fürstenau et al*. demonstrated that KW in SH-SY5Y cells reduced oxidative stress induced by H_2O_2. The effect was abolished when the transcription of Nrf-2 and the expression of HO-1 were prevented and PI3K/Akt was blocked. This indicates that KW also exerts its oxidative protection via the PI3K/Akt and Nrf2/HO-1 axis [109]. Similar results were also shown by *Oliveira et al*. Here, SH-SY5Y cells were also protected from ROS, which were not damaged by H_2O_2 but by methylglyoxal. The protective effect of KW was found to be mediated by the PI3K/Akt and Nrf-2 pathway [110]. Like KW, CF shows its antioxidant properties via activation of the Nrf-2 pathway and expression of HO-1. Cells were incubated with urotensin-II, an initiator of ROS and pre-treatment with CF reduced this effect [224]. In our case, the incubation of CF significantly increased ROS levels, but had no effect on the combined administration of KCC. It is possible that KW and CF played off each other, which is why the ROS levels remain similarly high as in the control. Overall, KW is considered to have a higher antioxidant potency than CF, as it is more sensitive to electrophilic influences and oxidizing reactions due to the extra double bond [107].

It was interesting for us to see how a combination of all the substances we tested affected the ROS levels in SH-SY5Y cells and whether the ROS reducing effect of HstP also mattered here. It was shown that the ROS levels of the cocktail did not. Thus, could not reach its potential. It is possible that the number of substances leads to the fact that no ROS reducing effect has occurred.

In addition, it would be interesting to see whether the activation of the Nrf-2 pathway, the expression of HO-1 and the reduction of Nf-kB described in the literature can also be detected in our model by our compounds. These pathways are not addressed resulting in the lack of ROS lowering effect in our experiments. In conclusion, the increased ATP levels of HstP may have resulted from the reduction of ROS levels, possibly via the Nrf-2 pathway or the suppression of Nf-$_k$B. For all other substances, other factors and signalling pathways may have been involved.

4.2 Flavonoid, Micronutrients, Caffeine and Diterpenes Against the Amyloid Beta Cascade

In addition to MD, Aβ levels are also repeatedly associated with the occurrence of AD. However, according to *Swerdlow et al*. it is not entirely clear whether the MD is caused by the Aβ levels or whether the MD leads to an overproduction and a poor clearance of Aβ [179, 180]. Nevertheless, the appearance of Aβ is associated with the occurrence of AD [24, 25]. In the context of this, we have examined $A\beta_{1\text{-}40}$ in all studies and $A\beta_{1\text{-}42}$ in some studies. $A\beta_{1\text{-}40}$ is the soluble form, whereas $A\beta_{1\text{-}42}$ tends to aggregate and form plaques [225]. The latter is considered the more neurotoxic of the two [226].

In our study, incubation of HstP of SH-SY5Y cells did not alter $A\beta_{1\text{-}40}$ levels. In contrast, there is evidence in the literature that HstP affects the deposition of Aβ. Administration of HstP significantly decreased expression elevation of APP, β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and Aβ in mice and in an HT22 cell model [202]. It has been shown that BACE1 levels and activity are increased under AD and stress conditions. BACE1 is the rate determining enzyme and the first enzyme in Aβ production [227, 228]. In a study by *Lee et al*. it was shown that HstP acts as a non-competitive inhibitor against BACE1. Thus, HstP could inhibit the production of Aβ and therefore act as a preventive and therapeutic agent against AD [229]. In a model using APP/PS1 mice, administration of hesperidin significantly reduced Aβ deposition and plaque-associated APP expression in mouse brain [230]. In this study, hesperidin is used instead of HstP, but hesperidin is largely broken down into hesperetin in the small intestine and colon. Thus, it is possible that some of the results from hesperidin studies may be transferred to HstP, but only those in which hesperidin is absorbed through the digestive tract. [231–233]. One interesting aspect would be to find out whether HstP also suppresses BACE1 expression in our cell model or whether this effect does not occur, which is why the Aβ lowering effect is absent. However, the data on HstP is relatively scarce and few have investigated the effects on Aβ levels, most studies have focused at AD and the resulting oxidative stress, where HstP has shown its potential.

In contrast to HstP, MgOr, Fol and their combination ID63 have significant effects on Aβ symptomatology. All substances were able to lower $A\beta_{1\text{-}40}$ levels. MgOr, Fol and Vit B6 as a single substance lowered $A\beta_{1\text{-}42}$ levels and the combinations even lowered sAPPα

levels. Similar results can be found in the literature. *Li et al*. found that administration of Fol at concentrations as we used (10µM) significantly decreased Aβ production in N2a-APP cells. The authors suggest that the effect is due to the stimulation and expression of DNA methyltransferases (DNMT) [234]. A study by *Guo et al*. indicated that hyperacetylation of histones and DNMT-dependent hypomethylation mediate the activation of stress-related signaling pathways in SH-SY5Y cells, leading to increased expression of APP, PS1 and BACE1 genes and consequent overproduction of Aβ [235]. Fol could modulate the activity of DNMT to ameliorate Aβ production. In an APP/PS1 mouse model, the administration of Fol significantly reduced Aβ levels and the expression of APP and PS1 genes. Furthermore, the application of Fol significantly increased DNMT activity compared to the control diet, which again supports the hypothesis that Fol influences DNMT activity and thereby Aβ production [236]. These results are confirmed by an HT-22 cell model in which Fol administration increased DNMT activity. Moreover, Fol significantly decreased the increased expression of APP and PS1 induced by incubation with Aβ oligomers. *Guo et al*. suggested that Fol induced methylation potential-dependent DNMT activity, which then methylated and silenced APP and PS1 [237].

Tian et al. also demonstrated in an APP/PS1 mouse model that Fol administration decreased $A\beta_{1-42}$ levels but not $A\beta_{1-40}$ levels [238]. We could not demonstrate these results because in our cell model, both levels were significantly decreased by Fol administration. In addition, *Tian et al.* Aβ wrote that protein expression of BACE1 decreased and concomitantly that of ADAM9 and ADAM10, important α-secretases of the ADAM family (disintegrin and metalloproteases), could be increased [238]. Again, we were unable to establish these results. In our case, there was decrease in sAPPβ, but a significant decreased in sAPPα levels. However, on the one hand, we tested a combination of MgOr and Fol and on the other hand, we are dealing with a different model. In addition, we suspect that the lowered sAPP levels are due to the lowered APP processing itself, which in turn lowered the sAPPα and sAPPβ levels. It must be kept in mind that sAPPα is 100 times higher than sAPPβ [239, 240]. Nevertheless, it can be concluded that in both *Tian et al.* and us Fol has an effect on Aβ production, but probably via different pathways. Incubation with MgOr decreased both $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in our studies. In contrast *Li et al.* showed in their APPswe/PS1dE9 mouse model that increasing Mg levels in mice resulted in a small plaque deposition in the brain of the mice, but no change in both Aβ monomers. However, increasing Mg levels led to decreased BACE1 expression, which

in turn resulted to decreased sAPPβ production. The authors suggest that this is due to a restoration of the BDNF/TrkB signaling pathway [74]. Activation of BDNF/TrkB pathways result in suppression of BACE1 expression and thereby decreased Aβ production [241]. In Na2 neuroblastoma mouse cells, high doses of Mg decreased $A\beta_{1-42}$ production, but not $A\beta_{1-40}$ and a lack of Mg resulted in a significant increase in both Aβ monomers. Furthermore, incubation of different concentrations of Mg lowered sAPPβ levels and increased sAPPα levels [242]. Magnesium-l-threonate was also able to significantly reduce $A\beta_{1-42}$ levels in APP/SP1 mice [212]. The authors hypothesize that the lowering of ROS levels may have led to a concomitant lowering of Aβ levels, as excessive ROS levels can lead to abnormal production of Aβ [243]. We could not observe this phenomenon, because in our case the $A\beta_{1-42}$ levels are lowered, but the ROS levels are unaffected. It is possible that the Aβ lowering effect is not controlled by down-regulation via BACE1, since there was no reduction in sAPPβ levels. Thus, it seems that in our example another mechanism is responsible for the lowered Aβ values. This assumption applies both to the individual substances and to the combination of them. Possibly, the effect of a reduction of the general APP levels is related, which in turn would also explain the decrease of sAPPα.

Other substances we investigated on the Aβ levels were the combination of Cof, KW and CF. There was no change in Aβ levels. For the dipertenes KW and CF, there is no literature available that examines the effects of either on Aβ processing. For Cof, however, there is some literature that show different results than our experiments. *Cao et al.* showed in their study that acute but also chronic administration of caffeine reduces Aβ production in transgenic mice. Acute administration of caffeine reduced Aβ levels in the brain and intestinal fluid of old and young mice. Long-term administration in old mice caused a decrease in plasma Aβ levels and a decrease in soluble and brain Aβ deposition. Furthermore, higher plasma caffeine levels were associated with a higher decrease in $A\beta_{1-40}$ in aged mice [244]. Similar results are shown by *Arendash et al*. whereby an administration of 1.5mg caffeine, which is somewhat equivalent to the dose of 500 mg or 5 cups of coffee for humans, lowered $A\beta_{1-40}$ as well as $A\beta_{1-42}$ levels. This was achieved by significantly reducing the expression of BACE1 and PS1 in the caffeine-treated mice. The Aβ lowering effect was further confirmed in SweAPP Na2 cells, where there was a caffeine dose-dependent decrease in both Aβ monomers [102]. Not only in mice that have been given caffeine during their lifetime, but also in old mice that suffer from AD-typical limitations, there is an Aβ-reducing effect. Here not only the cognitive ability was

improved but also the Aβ deposits in the brain by over 40% reduce as well as the $A\beta_{1-40}$ and $A\beta_{1-42}$ levels. This was probably achieved by reduced BACE1 and PS expression, which were influenced by the cRAf-1/Nf_kB pathway [103]. Using SH-SY5Y cells, it was shown that methylxanthine, which includes caffeine, could significantly reduce Aβ levels. In particular, caffeine increased activity of α-secretase and decreased that of β-secretase. This was made possible by an improvement in the protein stability of ADAM10 and a reduction in the expression of BACE1 and APP. This had the effect of significantly decreasing $A\beta_{1-42}$ deposition by caffeine [97]. The study situation in humans is somewhat difficult, there are only a few studies that explicitly deal with the Aβ level. *Gardener et al.* could show that a high baseline coffee consumption over 126 months is associated with a lower Aβ accumulation [245]. Similar results were obtained by *Kim et al.*, who showed that a lifetime consumption of more than two cups of coffee per day significantly reduced Aβ positivity in non-demented elderly adults compared to those who consumed less than two cups per day [246]. Comparisons of these studies with the others from the cell or mouse models is difficult because here the beverage coffee itself was consumed and not the single substance caffeine was observed. Coffee as a beverage contains a large number of bioactive substances, many of which can have a positive effect on neuronal diseases such as AD [247]. Furthermore, only the Aβ levels as a whole were investigated without focusing on the individual monomers. Even though we did not show any effect on Aβ levels by our substitutes in our model, there seems to be a decrease in Aβ levels by caffeine. This happens in most cases via the suppression of BACE1, PS1 and/or APP expression, as well as via an activation of ADAM expression. This results in an increased α-secretase or decreased β-secretase activity and thus a reduction of Aβ monomers and the deposition of Aβ. It would be interesting to know whether the effects addressed would have been achieved by caffeine alone in our cell model, since we only studied the combination. It is possible that the effects of the individual components negate each other, as there are no studies on the effects on the Aβ values of KW and CF.

As with the mitochondrial studies, we were interested in what would happen if we combined all of our tested substances into one cocktail and tested it for the Aβ effect. We found that the SC significantly lowered $A\beta_{1-40}$ compared to the control. Considering the SC results, there is an additive effect on $A\beta_{1-40}$ levels. Of our tested substances, only MgOr and Fol had the potential to significantly lower Aβ levels, but in combination as SC, all substances lowered Aβ levels. Indeed, all our tested substances show the same potential in the literature. The subsequent question is, whether the effect we observed is

due to the combination of MgOr and Fol alone or do all substances reach their potential. It is possible that the additive effect results in a suppression of BACE1 and APP expression and a greater reduction in β-secretase activity than we have seen from MgOr and Fol. For this, the pathways described in the literature for the individual substances would have to be investigated. It would be necessary to see which of the substances addresses which pathway in order to examine these in the cocktail. Although most substances address the same pathway, there are subtle differences that would have to be considered in more detail. However, this is beyond the scope of this work, but offers potential for future investigations. Moreover, a possible correlation between the Aβ reduction described in the literature and the resulting reduction of ROS [8, 248] could not be shown in our experiments. Although the Aβ levels were reduced by the SC, the ROS values remained unchanged. Possibly, the combination of all substances contributed to a reduction of the Aβ levels and did not have a negative but also not beneficial effect on the ROS levels. The absence of ROS lowering effect is probably due to the large number of substances administered.

4.3 Secondary Plant Constituents and Micronutrients and their Role in Supporting Glycolytic Metabolism in Alzheimer Disease

For brain activity, glucose is the primary source of energy. Because of this, impaired glucose metabolism is thought to be associated with neurodegenerative diseases [249]. Glycolysis is used to obtain energy from glucose by breaking it down to pyruvate. While this step is anaerobic, further processing of pyruvate can be either anaerobic or aerobic. In aerobic glycolysis, pyruvate is first converted to acetyl-CoA and then further oxidized to carbon in the citrate cycle. Most energy is stored in the form of $FADH_2$ and NADH, which is used in oxidative phosphorylation to produce ATP [250]. Lactate, in turn, is the end product of anaerobic glycolysis catalyzed by lactate dehydrogenase (LDH). Lactate is considered an important bioenergetic metabolite produced in the absence of oxygen by fermentation or in the presence of oxygen by aerobic glycolysis. The latter is also known as the Warburg effect. Lactate can be used by cells as an oxidative substrate [251]. Lactate is the link between the glycolytic and aerobic metabolic pathways [250].

At the onset of AD, patients who are at high risk of developing AD usually show impairments in cerebral metabolism before they show signs on neuropsychological or imaging studies [252]. In patients with severe AD, the cerebral glucose utilization is

reduced by 45% [253]. This is also reflected in the utilization ratio, with patients with AD onset showing a 2:1 utilization ratio between glucose and alternative sources, whereas comparably aged control subjects have a 29:1 ratio, whereas young control subjects use glucose exclusively, corresponding to a 100:0 ratio [254]. The impairment cannot be attributed to loss of brain matter or electrophysiological abnormalities. Abnormalities in the brain include deficiencies in several enzyme complexes involved in mitochondrial oxidation of substrates for energy production, including the pyruvate dehydrogenase (PDH) complex. Further, it can be hypothesized that the impairment of glucose oxidation in the brain interacts with an impaired supply of oxygen and glucose to the brain [252].

Two substances play a crucial role in glucose and energy metabolism, lactate and pyruvate. LDH-produced lactate seems to have a higher position within energy metabolism. It is assumed that lactate serves as a metabolite to conserve glucose in neurons. At the same time, lactate serves to promote neuronal energy production, modulate neuronal excitability and facilitate memory formation [255, 256]. However, elevated lactate levels are also associated with negative consequences. Elevated levels have been found in the brains of aged mice [257, 258] and in AD patients [259]. Furthermore, there is evidence from meta-analysis that pyruvate levels are lowered on the other side [260]. Mitochondrial dysfunction leads to a change from aerobic respiration to glycolytic metabolism in the brain of mice. This causes a change in LDH gene expression and an increase of lactate in the brain [258].

In our studies, HstP did not change the lactate values compared to the control, but slightly increased the pyruvate values. Possibly, the MD present in our cells could be attenuated by HstP, which leads to a change in glucose processing. This leads to an increase in pyruvate values, which could possibly result in an increase in ATP values at the end. The MD results to a change in the LDH-A to LDH-B ratio and thus to increased lactate and decreased pyruvate values [258, 257]. It would be interesting to investigate in further experiments whether similar changes occur in our cell model or whether HstP has an influence on these changes.

In contrast to HstP, the combination of Fol, MgOr and also Vit B6 significantly lowered the lactate values, but had no significant effect on the pyruvate values. It is assumed that both substances have an effect on glycolysis, particularly in relation to the Warburg effect. Not only cancer cells or tumor cells try to make a change from oxidative phosphorylation to anaerobic glycolysis, the so-called Warburg effect, but also nerve cells can undergo this change, especially if they are affected by Aβ [261–263]. To counteract the harmful

effect by Aβ, neurons seem to increase the expression of LDHA and PDK1, which makes them more resistant to the neurotoxic effect of Aβ [263, 264]. Inhibition of LDHA and PDK1 expression leads to a decrease in resistance and increased susceptibility to Aβ toxicity [265]. This effect was also observed in our experiments, where incubation with Fol and MgOr resulted in increased expression of LDHA and PDK1 compared to the control. A switch from oxidative phosphorylation to more glycolysis via the Warburg effect leads to an increase in lactate levels, which is produced when glucose is metabolized [266]. However, we were not able to determine this effect, since the lactate values were lowered. There is no literature dealing with the effects of MgOr and Fol on glycolysis and the Warburg effect, respectively, in the AD cell model, so we hypothesize our own. The incubation with both substances had the advantage that the Aβ levels were significantly reduced, which enabled the cells to decrease the energy production from anaerobic glycolysis and to focus more on oxidative phosphorylation. This can be observed on the one hand in the slightly increased respirometry data and on the other hand in the significantly decreased lactate values. Thus, the cells no longer have to protect themselves from Aβ toxicity with the Warburg effect. Nevertheless, the expression of LDHA and PDK1 remained elevated, possibly to maintain protection for some time.

As well as Fol and MgOr, Cof, KW and CF or their combination KCC had effects on glycolytic metabolism. There were no significant changes in lactate values, but a significant increase in pyruvate values due to KW and KCC. Regarding the ratio of lactate to pyruvate, all but CF were able to significantly reduce the ratio in favor of pyruvate compared to the control. Ensuring pyruvate generation is a high priority, as pyruvate can not only be oxidized to lactate in a low-oxygen environment, but also generally converted to glucose (gluconeogenesis), alanine (alanine transaminase), or oxaloacetate (pyruvate carboxylase) when cellular demand requires it. In general, neurons have an increased pyruvate demand due to a high energy turnover [267, 268]. Significant deviations in pyruvate concentration, as documented in the experiments, indicate a relevant influence on pyruvate metabolism [269]. Pyruvate can act as a scavenger, protecting neurons from oxidative damage. In several studies, increased pyruvate levels significantly attenuated the damaging effect of the stressor, H_2O_2 [270–272]. Furthermore, it is assumed that pyruvate in particular also protects mitochondria from oxidative stress [270, 273] and could thus counteract an MD. In addition, pyruvate can induce an anti-inflammatory effect by down-regulating the Nf-$_k$B pathway, as well as a variety of pro-inflammatory proteins [274, 275]. The protection against ROS could not be demonstrated in our

experiments, as the ROS levels were not altered by the incubations. Therefore, the increased ATP levels cannot be explained by this effect. Additionally, the increase in pyruvate did not lead to an increased OXPHOS, as there was no change in the respirometry data. Thus, the increase in ATP can be explained by an increased glycolysis. This is supported by the lactate to pyruvate ratio (L/P), as studies have shown that an increase in L/P is associated with an increase in $NADH/NAD^+$. Therefore, in our experiments, a decreased ratio of $NADH/NAD^+$ corresponding to L/P should be present [250]. A shift in this ratio could affect glycolysis, as the regeneration and maintenance of NAD^+ is dependent on glycerol-3-phosphate dehydrogenase. Therefore, a decrease in the $NADH/NAD^+$ ratio could lead to an increase in glycolysis [250, 276]. This could explain the ATP increase and increased pyruvate level shown in our study. However, this hypothesis would still need to be supported by sufficient experiments investigating the $NADH/NAD^+$ ratio.

As a final point, the cocktail was examined for the effects of glycolysis. Here, there was an increase in lactate levels due to incubation, with a simultaneous decrease in pyruvate levels and an L/P in favor of lactate. These values indicate that the SH-SY5Y cells undergo increased anaerobic glycolysis, which explains the lower pyruvate and increased lactate values. The SC, like MgOr and Fol, decreased Aβ levels but did not have the same effects as MgOr and Fol on lactate and pyruvate levels. Furthermore the Warburg effect seems to be inhibited by SC, although more lactate is produced which rather indicates that the cells perform more anaerobic glycolysis. However, there is no decrease in OXPHOS, which would indicate increased energy production due to the Warburg effect. The increased anaerobic glycolysis can possibly be explained by the altered L/P ratio, which can lead to an increased $NADH/NAD^+$ ratio. This, in turn, would suggest an increase in anaerobic glycolysis. Incubation with SC leads to increased OXPHOS, which together with increased anaerobic glycolysis could explain the increased ATP levels. It is possible that ATP gain from the increased OXPHOS is not sufficient, so additional ATP must be generated via anaerobic glycolysis, which would explain the increase in both metabolic pathways and altered lactate level. This could be due to the administration of the different solvents, as the ATP in the control are considerably lower than in the medium control. The SC seems to compensate this effect to a certain extent. Which is why the cells upregulate both OXPHOS and anaerobic glycolysis to provide sufficient ATP as compensation. The decreased pyruvate levels due to the SC could be a result of both of these increased metabolic rates as it results in more pyruvate being consumed.

5. Summary/Zusammenfassung

5.1 Summary

One advance that mankind has achieved over time as a result of medical and technical progress is increasing life expectancy. However, this is also accompanied by an increase in age-related diseases. One of these is Alzheimer's disease (AD), which is associated with the loss of physical and cognitive abilities as well as personality changes. To date, there is no cure for AD. Since symptoms do not appear until later in life, it is important to find a way to delay symptomatology as much as possible in advance and to take preventive or supportive measures.

AD is characterized, among other things, by changes in three major areas, including mitochondrial dysfunction (MD) with a decrease in ATP and a change in the respiratory chain. Impaired amyloid-beta (Aβ) processing and alterations in glycolytic metabolism.
It has been shown that physical activity, as well as nutrition, can prevent certain diseases, including AD. With this in mind, this work investigated the effects of hesperetin (HstP) from orange peel, magnesium orotate (MgOr) and folic acid, as well as the constituents caffeine, kahweol, and cafestol found in coffee, on the early symptoms of AD.

Almost all compounds were shown to significantly increase ATP levels in the SH-SY5Y-APP_{695} cell model. Specifically, HstP was able to decrease the levels of reactive oxygen species. MgOr and Fol, in particular, altered Aβ-processing and lowered the levels. Coffee constituents affected glycolytic metabolism and led to an increase in aerobic glycolysis. Each of the groups of compounds acted on a different area of AD. To check whether a combination of all substances (cocktail) addressed all areas, this cocktail was also tested for its effect. It was found that ATP levels were increased and Aβ levels were decreased. There were no effects on any other areas.

In summary, although there is no cure for AD, certain substances can positively influence the disease and have a preventive effect. This makes them promising agents that deserve further research.

5.2 Zusammenfassung

Ein Fortschritt, den die Menschheit im Laufe der Zeit durch den medizinischen und technischen Fortschritt erreicht hat, ist die steigende Lebenserwartung. Damit einher geht jedoch auch eine Zunahme altersbedingter Erkrankungen. Eine davon ist die Alzheimer-Krankheit (AD), die mit dem Verlust körperlicher und kognitiver Fähigkeiten sowie mit Persönlichkeitsveränderungen einhergeht. Bis heute gibt es keine Heilung für AD. Da die Symptome erst in späteren Lebensabschnitten auftreten, ist es wichtig, bereits im Vorfeld einen Weg zu finden, die Symptomatik so weit wie möglich hinauszuzögern und präventiv bzw. unterstützend zu wirken.

Die AD ist unter anderem durch Veränderungen in drei großen Bereichen gekennzeichnet, darunter die mitochondriale Dysfunktion (MD) mit einer Abnahme des ATP und einer Veränderung der Atmungskette. Die gestörte Amyloid-beta (Aβ)-Prozessierung und Veränderungen im glykolytischen Stoffwechsel.

Es hat sich gezeigt, dass durch körperliche Bewegung, aber auch durch die Ernährung vorbeugend gegen bestimmte Krankheiten, darunter auch AD, vorgegangen werden kann. Vor diesem Hintergrund wurde in dieser Arbeit untersucht, wie sich Hesperetin (HstP) aus Orangenschalen, Magnesium-Orotat (MgOr) und Folsäure sowie die im Kaffee enthaltenen Inhaltsstoffe Koffein, Kahweol und Cafestol auf die Frühsymptome der AD auswirken.

Es konnte gezeigt werden, dass fast alle Substanzen den ATP-Spiegel im SH-SY5Y-APP_{695} Zellmodell signifikant erhöhen. Im Einzelnen konnte HstP die Spiegel der reaktiven Sauerstoffspezies senken. MgOr und Fol veränderten insbesondere die Aβ-Prozessierung und senkten die Spiegel. Die Inhaltsstoffe des Kaffees beeinflussen den glykolytischen Stoffwechsel und führten zu einer Steigerung der aeroben Glykolyse. Jede der Substanzgruppen wirkte auf einen anderen Bereich der Alzheimer-Krankheit. Um zu überprüfen, ob eine Kombination aller Substanzen (Cocktail) alle Bereiche anspricht, wurde dieser Cocktail ebenfalls auf seine Wirkung getestet. Es zeigte sich, dass der ATP-Spiegel erhöht und der Aβ-Spiegel gesenkt wurde. Auf alle anderen Bereiche gab es keine Effekte.

Zusammenfassend lässt sich sagen, dass es zwar keine Heilung für AD gibt, aber bestimmte Substanzen die Krankheit positiv beeinflussen und vorbeugend wirken können. Dies macht sie zu viel versprechenden Wirkstoffen, die es verdienen, weiter erforscht zu werden.

6. References

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7. Publication List

First authorships

Babylon, L., Grewal, R., Stahr, P., Eckert, R. W., Keck, C. M. & Eckert, G. P. (2021). Hesperetin Nanocrystals Improve Mitochondrial Function in a Cell Model of Early Alzheimer Disease. Antioxidants, 10(7), 1003, (IF = 7.675)

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