Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

*Article*
**Volume 13, Issue 5, 2023, 487**
https://doi.org/10.33263/BRIAC135.487

# Aging and the Liver: Mitochondrial Dysfunctions and the Impact of Humic Biological Add

**Olga Dyomshyna [1,*], Galyna Ushakova [1], Lilia Stepchenko [2]**

[1] Oles Honchar Dnipro National University, 72 Gagarin ave., Dnipro 49010, Ukraine; d.olga.1970@gmail.com (O.D.); ushakova_g@ukr.net (G.U.);
[2] Dnipro State Agrarian-Economic University, 25 Sergey Efremov str., Dnipro 49027, Ukraine; stepchenko2@gmail.com (L.M.);
* Correspondence: d.olga.1970@gmail.com; (G.U.);

Scopus Author ID 7004018560
**Received: 6.09.2022; Accepted: 19.10.2022; Published: 6.01.2023**

**Abstract**: The pro-antioxidant balance in the whole liver and its mitochondria fraction under physiologically late aging gerbils and impact with bioactive additives Humilid was studied. Thirty-six male Mongolian gerbils were randomly divided into six groups: 6-, 24-, 30-, 36-, 39-month-old, and 6-month-old animals with Humilid drinking water (5 mg/kg weight for 14 days). Obtained data show that metabolic changes with aging provoke the disbalance of the antioxidant system via the decreased activity of catalase in the liver. The sequence of events of reduction of catalase activity, accumulation of toxic peroxide metabolites, and inhibition of superoxide dismutase (SOD) activity were observed in the liver mitochondria under aging. Showed that catalase activity is higher at 24 months old among the investigated antioxidative components of the liver mitochondria. At 39-months-old of age, on the contrary, a decrease in catalase activity is accompanied by an increase in the concentration of cytochrome C. Therefore, the obtained results may indicate various compensatory reactions that are involved in the antioxidant protection of the liver of animals in critical periods of life. Furthermore, the positive preventive impact of Humilid on liver metabolism under aging was shown.

**Keywords:** aging; long-living; liver; mitochondria; oxidative stress; gerbils; Humic substances.



## 1. Introduction

The problem of aging is one that always has interested humans. However, despite the numerous ongoing studies, scientists do not have a clear answer to the question: "What is aging?" Substantial knowledge about this phenomenon has accrued for decades, which has allowed the creation of the so-called "aging theory." Mitochondrial and free radical theories are recognized as the most popular and vital [1-3]. The mitochondrial theory embodies more approaches, including that of free radicals. Mitochondria are the primary consumers of oxygen in the cell.

Nevertheless, the establishment of a significant number of active forms, including oxygen free radicals, accompanies the combined processes of redox reactions of the respiratory chain and the formation of energy in the form of ATP [4,5]. Under normal conditions, such aggressive compounds are detoxicated by the antioxidant system (AOS) components [6-8]. A decrease in AOS efficiency causes a disbalance in the energy supply processes to the cell, premature aging, and death.

Cell aging results from accumulating damaged, oxidized molecules impairing supramolecular structures. The most sensitive organelles to the excessive number of TBA-active products are mitochondria [9]. Oxidative stress provokes the aging of mitochondria, reducing their ability to carry out autophagy/mitophagy [10-13], leading to cell aging and tissue degeneration [14,15].

In contrast to the prooxidant system in the cell, evolutionary forms of protection against the excessive formation of aggressive compounds that support oxidative-reducing equilibrium are formed. The balance between peroxide oxidation, on the one hand, and the AOS, on the other, is a prerequisite for maintaining normal cell function [16]. Disbalance of the AOS function leads to the excessive accumulation of active forms of oxygen, changes in cellular metabolism and damage to cell membranes, and the appearance of oxidized proteins. Furthermore, lipid peroxidation provokes the development of mutagenic and cytotoxic compounds [17,18]. The aging process in humans and animals is accompanied, on the one hand, by changes in the generation of active forms of oxygen (AFO) in the cytoplasm. On the other, significant shifts in the AOS, in particular, decreased activity of its enzyme chain, increased activity, and the role of the individual non-enzymatic representatives of the system are the main characteristic of aging [19-21]. Various drugs with active antioxidant substances are used to prevent oxidative stress from developing with age [22]. The antioxidant properties of biologically active additives with humic acids, such as Humilid, were discussed previously [23-27].

The enantiostasis in the liver is an important condition for functioning in a highly organized organism under a physiological state. Blood protein synthesis, energy metabolism, and the elimination of toxic metabolites depend on the liver. All functions require a significant amount of energy produced in mitochondria, the effectiveness of which depends on the integrity of the external and internal membranes. Oxidizing processes, which prevail during aging, lead to a violation of all processes mates [28,29]. Nevertheless, life expectancy has increased significantly due to improved living standards and advances in technology and medicine. Every year in the world, an increasing number of long-living people are registered, but the phenomenon of longevity is not sufficiently investigated [13].

Moreover, experimental studies on aging have been described mostly in animals not older than 25 months [9,30]. The main task of our study was to establish changes in the antioxidant protection and biochemical parameters of the functional activity of the liver for long-living gerbils. Therefore, this work aimed to identify the oxidant/antioxidant balance in the liver and its mitochondria at physiologically aging gerbils and test of humic acids impact it.

## 2. Materials and Methods

### *2.1. Experimental animals.*

The experiment was carried out on 36 male gerbils (Mongolian Gerbilia, Meriones Unguiculatus, Milne-Edwards, 1867) of different ages, with an average weight of 63-83 g kept under standard vivarium conditions with a full-fledged diet of feeding. Manipulations on animals were carried out following the rules laid by the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 2010) [31] and the Law of Ukraine "On Protection of Animals from Cruel Treatment" (2006) [32].

*2.2. Study groups.*

The animals were divided into 6 experimental groups (n=6):

1 – 6-months-old animals, the age of puberty gerbils (control group, to which other groups were compared;

2 – 24 months, the maximum age at which gerbils live;

3, 4 – 30 and 36 months, every 6 months of late life;

5 – 39 months, the maximum age that was presented in experiment;

6 – 6-months-old with Humilid in drinking water (5 mg/kg weight for 14 days) animals.

*2.3 Drug*

Humilid was obtained from Khristyeva Problem Laboratory of Humic Substances, Dnipro State Agrarian and Economic University (Dnipro, Ukraine). The general characteristics of Humilid are present in Table 1.

**Table 1.** General characteristics of Humilid.

| **Characteristics** | **Value for specifications U 15.7-00493675 004 2009 (in Ukraine)** | **Laboratory test** |
|---|---|---|
| General view | Viscous fluid | Viscous fluid |
| Color | Dark-brown | Dark-brown |
| Smell | Specific | Specific |
| pH | 12 | 11,4 |
| Dry matter | 11 % | 14,8 % |
| Organic substances | 9 % | 10,6 % |
| Humic acids and their salts in organic substances | 50 % | 58,4 % |
| Pathogenic microorganisms | CFU /$dm^3$<br>not presented | CFU /$dm^3$<br>not presented |

*2.3. Sampling.*

The animals were weighed and anesthetized (thiopental 60 mg/kg, Kyowa Kirin Ltd, UK) at the end of the experiment. The liver was removed, washed in saline, and used for further studies.

*2.4. Obtaining a mitochondrial fraction.*

The mitochondrial fractions of the liver (1 g) were obtained by differential centrifugation in the following buffer medium: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl; pH 7.4 at 0–3 °C; 2 мМ MgCl2 [33]. The homogenate of the liver was centrifuged at 740 g in 10 ml of medium for 5 minutes at 0-2 °C to remove the cellular fragments and isolate the nuclear fraction. The resulting supernatant was used to determine the biochemical parameters and to obtain the mitochondrial fraction. The supernatant was centrifuged at 740 g for 5 minutes at 0-2 °C. For the purification of the mitochondria, the supernatant was centrifuged twice at 9000 g for 10 min. The supernatant containing the plasma membrane, lysosomes, microsomes, and cytosol was discarded. The collected pellet, including the mitochondria, was rearranged to 10 ml in the medium for further centrifugation at 10000 g for 10 minutes. The sediment containing the mitochondria was suspended in a buffer with 0.25 M sucrose-free of EDTA and again centrifuged at 10000 g for 10 min. The procedure was repeated twice, and the dense sediment of mitochondria was thoroughly suspended in a pipette containing 0.4-0.5 ml of 0.25 M sucrose. The suspension of mitochondria was immediately used for studies.

*2.5. Enzyme assays.*

The concentration of total protein, aspartate aminotransferase activity (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2), lactate dehydrogenase (LDH, EC 1.1.1.27), γ-glutamyltransferase (GTP, EC 2.3.2.2), and alkaline phosphatase (ALP, EC 3.1.3.1) was determined with standard laboratory test kits (PZ CORMAY S.A., Lomianki, Poland) according to [34], using the manufacturer's protocol. The activity of catalase (CT, EC 1.11.1.6) was determined by the ability of hydrogen peroxide to form a stable colored complex with molybdenum salts [35] and was expressed in μcat/mg protein in all the investigated fractions. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was evaluated as the ability of the enzyme to inhibit quercetin oxidation [36] and was expressed in conventional units (c.u./mg protein). The enzyme activity that was able to induce quercetin inhibition by 50 % per 1 mg tissue protein was taken as a unit. The concentration of cytochrome C was estimated based on its ability to restore sodium dithionate [37].

*2.6. MDA concentration.*

The concentration of MDA was determined by the contents of the colored complex formed by the reaction with two molecules of thiobarbituric acid (TBA) in an acid medium [38]. The amount of MDA was expressed as μmol of TBA-active products per mg protein.

*2.7. Statistical analysis.*

Results were statistically analyzed using the one-factor dispersion analysis ANOVA. For all statistical calculations, the significance was considered as the value of $P < 0.05$. A correlation was calculated with Pearson's coefficient.

## 3. Results and Discussion

*3.1. The prooxidant-antioxidant status of the liver and its mitochondria at physiological aging.*

The main prooxidants in the cells are oxidized products, including malonic dialdehyde (MDA), formed from organic molecules damage. Table 2 shows the analyses of prooxidant-antioxidant status in the liver and its mitochondria of gerbils at different ages, indicating the increased level of prooxidant products during physiological aging.

It is known that the sexual maturity of gerbils reaches the age of 6 months, and the life expectancy mostly does not exceed 24 months. The increased concentration of MDA in 37% of the liver of 24-month-old gerbils, on 99% of 36-month-old gerbils, and in 58% of 39-month-old gerbils compared with 6-month-old animals was shown.

**Table 2**. The compounds of the pro/antioxidant system of the liver of gerbils in physiological aging, n=6.

| Parameters | Experimental group, age (months) | | | | |
|---|---|---|---|---|---|
| | 6 | 24 | 30 | 36 | 39 |
| **The liver** | | | | | |
| MDA, μmol/ mg protein | 2.19 ± 0.46 | 3.01 ± 0.17* | 2.52 ± 0.10 | 4.36 ± 0.25* | 3.47 ± 0.21* |
| Catalase, μkat/mg protein | 4.28 ± 0.47 | 3.49 ± 0.69 | 3.06 ± 0.48* | 4.04 ± 0.50 | 3.2 ± 0.56* |
| SOD, c.u./mg protein | 6.31 ± 1.40 | 9.94 ± 2.10* | 13.70 ± 1.10* | 8.13 ± 0.66 | 3.93 ± 0.41* |
| Cytochrome C, μg/g tissue | 3.69 ± 0.43 | 4.14 ± 0.32 | 3.83 ± 0.28 | 3.88 ± 0.38 | 2.53 ± 0.21 |
| **The mitochondrial fraction derived from the liver** | | | | | |
| MDA, μmol/mg protein | 6.43 ± 0.34 | 15.99 ± 2.52* | 12.04 ± 0.81* | 12.32 ± 1.76* | 26.83 ± 4.32* |

| Parameters | Experimental group, age (months) | | | | |
|---|---|---|---|---|---|
| | 6 | 24 | 30 | 36 | 39 |
| Catalase, μkat/mg protein | 7.44 ± 0,57 | 14.45 ± 2.09* | 8 86 ± 1.3 | 8.95 ± 1.78 | 7.04 ± 0.81 |
| SOD, c.u./mg protein | 12.17 ± 1.18 | 8.22 ± 1.24* | 10.8 ± 3.01 | 7.31 ± 1.79* | 4.92 ± 0.28* |
| Cytochrome C, μg/g tissue | 7.25 ± 0.87 | 6.95 ± 1.01 | 6.97 ± 1.08 | 5.57 ± 0.61* | 13.22 ± 0.13* |

**Note: In this and the next tables**: * – P < 0,05 significantly different from the 6-month-old animals

At the same time, in the mitochondrial fraction of experimental animals' livers, significant changes were observed in the direction of increased concentrations of oxidized products. The quantity of MDA increased 3-fold in 24-month-old compared to 6-month-old mature gerbils (Table 2). At later lifetimes (30-36 months), these products decreased 2-fold, though exceeding the value of 6-month-old animals, suggesting an exciting oxidative state in the mitochondria of the liver of gerbils during late aging. Usually, the average life expectancy of gerbils is 24 months. Animals over 24 months can be considered long-living. Upon examining the 39-month-old animals, an irreversible 4.5-fold increase in the concentration of oxidized products was observed compared with 6-month-old and a 2-fold increase compared to 30-36 months-old animals. The obtained results confirm the fundamental proposition that mitochondria are one of the primary sources of oxidized products. The formation of excessive amounts of oxidized metabolites in the mitochondria indicates the duality of this phenomenon: MDA can be both a consequence and a cause of processes that lead to the aging of the organism and the liver particularly (Table 2).

An enzyme that can be referred simultaneously to as the high molecular antioxidant component (converts the super oxygen molecule into a less toxic peroxide of hydrogen) and the factor of prooxidant system (contributes to the excessive formation of hydrogen peroxide, which is one of the main factors of oxidative stress) is superoxide dismutase (SOD). In our experiments, increased SOD activity was detected in the liver of aged gerbils (Table 2).

The gradual increase of SOD activity on 57% in 24-month-old animals compared to the 6-month-old was shown. However, the peak activity (117%) was registered at the age of 30 months animals. The SOD activity tended to decrease until 39 months old and was just 62% referred to 6-month-old rodents. Such changes in SOD activity in the liver of gerbils could be related to the difference in the amount of substrate available to this enzyme. Perhaps, the ability of the mitochondria to absorb the oxygen molecule and its accumulation in the cytosol is reduced under aging, which, in turn, leads to the transformation of oxygen to superoxide. This assumption was confirmed by decreased activity of SOD in the mitochondrial fraction, both at the age of 24 and 30 months, compared to the 6-month-old animals.

Thus, the stable higher level of prooxidant products in the liver under physiological aging may be associated with increased permeability of the cell membrane of the hepatocytes.

The evolutionarily formed networks of protection against the excessive formation of aggressive compounds maintain an oxidation-reduction balance. The basis of the antioxidant system is macromolecular and low molecular weight compounds with high ability and sensitivity. Thus, the enzyme catalase is already active even at low concentrations of hydrogen peroxide [2,25]. The universal enzyme catalase effectively converts toxic hydrogen peroxide into water and molecular oxygen. The reacted oxygen molecule can be reused in the respiratory chain to provide the IV cytochrome oxidase complex (aa3) function. This reaction of oxygen regeneration in mitochondria is lower than in peroxisomes, where catalase activity is the highest in a cell [39]. However, it provides an additional level of oxygen in mitochondria.

The catalase activity in the liver of gerbils varies insignificantly with age (Table 2): decrease from 24 months until 30 months of age, and increases to 36 months compared to the

6-month-old gerbils. It is, presumably, associated with the activation of cellular adaptation to new conditions of existence. However, at the age of 39 months, there was a decrease in the activity of catalase noted during aging. The correlation analysis between the catalase activity in the liver of gerbils and its mitochondrial fraction showed positive relationships of high degree significance at the age of 6 months ($r = 0.75$, $P < 0.05$) and negative relationships at age 39 months ($r = -0.85$, $P < 0.01$). That is, in adulthood, the system of antioxidant protection with the participation of catalase is equally active both in the cytosol and in the mitochondria. The cytosolic protection systems of the liver of long-living animals are depleting with age. At the same time, the pro/antioxidant balance in the liver has supported by mitochondrial catalase.

Correlation analyses of the activity of catalase and the amount of MDA revealed positive relationships, with a high degree of significance in the liver mitochondrial fraction also: for 30-months-old gerbils ($r = 0.93$, $P < 0.001$), 36-months-old ($r = 0.9$, $P < 0.001$) and 39-months-old ($r = 0.7$, $P < 0.05$), that reflects an induced adaptive reaction of antioxidant system of mitochondria under aging. The positive high-level significance between the amounts of activity of catalase and SOD in the liver of 36-months-age gerbils ($r = 0.93$, $P < 0.001$) was shown with an increased level of MDA. This suggests a decrease in catalase activity that leads to the accumulation of peroxide metabolites and inhibition of SOD activity.

The lowest index of SOD activity in the mitochondria registered at the age of 39 months (a 2.5-fold decrease in activity) compared with 6-month-old animals. This fact can be explained by the reduced ability of mitochondria to use oxygen in the respiratory chain with age and reduced energy processes, as indicated by a 4-fold increase in MDA in the mitochondrial fraction of the liver of 39-months-old gerbils compared to 6-months-old animals (Table 2).

Cytochrome C has many functions as a nuclear-coded mitochondrial protein that provides vital and apoptotic cell processes [40]. The presence of this protein in the respiratory chain is decisive in the aerobic production of energy [41]. Cytochrome C also acts as cardiolipin peroxidase [42] and kinase (four sites of phosphorylation have been identified) [9,43,44]. The phosphorylated form of cytochrome C is indispensable in regulating the interconnection of the electron transport system and oxidative phosphorylation [43] and contains an excessive formation of oxidative products due to its antioxidant properties. Thus, an oxidized form of cytochrome C can convert superoxide anion to molecular oxygen more efficiently than SOD. It acts as a processor for hydrogen peroxide [45], and unlike superoxide, it is processed as a reducing agent and an oxidized form of cytochrome C. Therefore, this protein is a marker of the effectiveness of the antioxidant system of mitochondria.

In 6 to 24 months gerbils, the concentration of cytochrome C in the mitochondria decreased slightly (Table 2). However, in the liver, it increased by 12%, indicating the possible induction of apoptotic processes in old gerbils. The lowest concentration of cytochrome C in liver mitochondria was observed at the age of 36 months. That is, in adulthood, the system of antioxidant protection with the participation of catalase is equally active both in the cytosol and in the mitochondria. The cytosolic protection systems of the liver of long-living animals are depleting with age. At the same time, the pro/antioxidant balance in the liver has supported by mitochondrial catalase. However, the question of a significant 2-fold increase in the concentration of cytochrome C in the mitochondrial fraction in 39-months-age animals remains open.

Correlation analyses of the amount of cytochrome C and the SOD activity showed an established positive relationship with high-level significance ($r = 0.94$, $P < 0.001$) in 24 months old gerbils. Namely, both proteins act as functional agonists at this age by their ability to

convert the anion superoxide [45]. At 39 months of life, the correlation coefficient changed to negative ($r = -0.83$, $P < 0.01$) in the mitochondrial fraction. Consequently, at an older age, the main role in the biotransformation of anion superoxide in mitochondria lies in cytochrome C. This mechanism likely provides mitochondria with oxygen molecules under hypoxic conditions. At the same time, a negative relationship high-level of significance between the activity of catalase and the amount of cytochrome C ($r = -0.87$, $P < 0{,}001$) and ($r = -0.99$, $P < 0{,}001$) was established in the mitochondrial fraction of 24- and 39-months-old gerbils, respectively. However, it should be noted that at the age of 24 months old, an increase in catalase activity is accompanied by a decrease in the concentration of cytochrome C. At 39-months-old of age, on the contrary, a decrease in catalase activity is accompanied by an increase in the concentration of cytochrome C (Table 2). Both proteins participate in the antioxidant protection of the cell. Therefore, the obtained results may indicate various compensatory reactions that are involved in the antioxidant protection of the liver of animals in critical periods of life.

*3.2. The mitochondrial and cytosolic aminotransferases in the liver in physiological aging.*

Aspartate aminotransferase enzyme plays a critical role in the metabolic activity of mitochondria [2,25,46,47]. It ensures the operation of such essential pathways in the liver as the malate-aspartate shuttle mechanism, the urea cycle, and the carbonaceous crack of aspartic acid oxaloacetate is an important substrate of the tricarboxylic acid cycle. There are two aspartate aminotransferase isoforms: mitochondrial (m-AST) and cytosolic (c-AST). However, in standard terms, the highest activity is characteristic of the m-AST [2]. Physiological aging caused changes in the main markers of liver status, including mitochondrial aspartate aminotransferase and alanine aminotransferase (Figure 1).

Significant changes in the activity of m-AST were established with aging (Figure 1). The activity of m-AST at the age of 24 months, which is a turning point in the life of gerbils, decreased 2-fold compared to 6-month-old animals. This fact may indicate an increase in the permeability of the mitochondrial membrane. At 30 months, the regeneration of the regularity of the activity of m-AST increased 1.5-fold compared with the group of 6-month-old animals, indicating the stimulation of the functional activity of mitochondria.

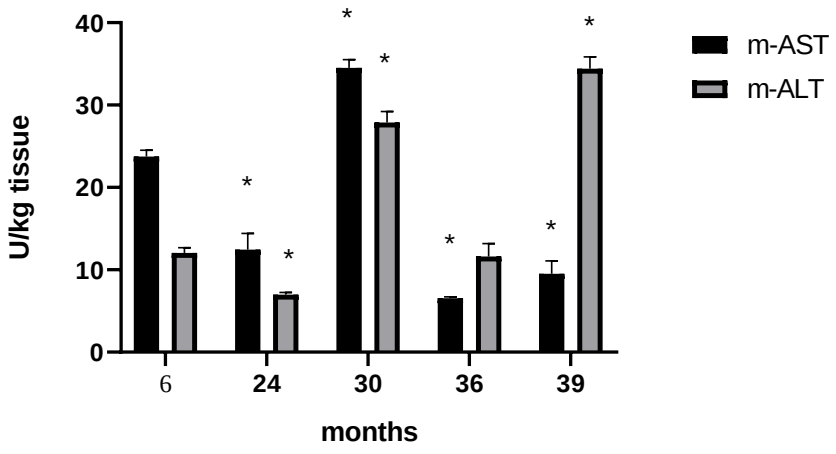


**Figure 1.** The activity of aspartate aminotransferase and alanine aminotransferase in the mitochondrial fractions derived from the liver of gerbils in aging. Values are represented as mean ± SEM (n=6); * – $P < 0.05$ vs. 6-month-old animals.

Also, such changes in the activity of m-AST for 6 months, from the age of 24 months to 30 months of life, indicate the adaptive processes occurring in the liver of gerbils. During the next 9 months of the life of these animals, there was a decrease in the activity of m-AST. Thus, we can conclude that 24 months and 36 months are the critical and vulnerable ages for the liver mitochondria of gerbils.

There are two isoforms, c-ALT, and m-ALT, for alanine aminotransferase also. However, the highest activity was detected for the c-ALT isoform, unlike aspartate aminotransferase.

The activity of m-ALT had a different tendency from its counterpart (Figure 1). At the age of 24 months, there was a significant 2-fold decrease. During the next 6 months of life, there was twice the increase compared with 6-month-old animals. At 36 months, the activity level reached that of 6-month-old animals. However, at 39 months, the activity of m-ALT increased by almost 3-fold compared to 6-month-old animals. Consequently, the peaks of m-ALT activity at the age of 30 and 39 months may indicate an increase in substrate concentration.

In the experiment, a wave-like effect of changes in the values of the De Ritis ratio in the cytosolic fraction of the liver was established. Thus, in 6-month-old animals, it was 0.67; in 24-month-old animals, it was 2.3; 30-month-old - 0.82; 36-month-old - 3.85; and in the oldest animals, 39-month-old - 2.25. Rodents are able to live up to 24 months of age in nature. That is, for most individuals of the population, this is a critical age in which important indicators of homeostasis change. In animals that lived to the age of 30 months, the De Ritis ratio reached the norm. That is, took place that stabilized nitrogen metabolism in the liver with AST and ALT involved. This phenomenon was observed against the background of a proportional decrease in the activity of both enzymes, which is associated with the slowing down of synthetic processes in aging animals. There is a significant predominance of AST activity at the age of 36 and 39 months. Maybe a compensatory mechanism that ensures nitrogen exchange in aging cells. And also the intensification of the removal of the products of the destruction of nitrogen-containing compounds.

*3.3. The lactate dehydrogenase, γ-glutamyltransferase, and alkaline phosphatase activity in the aging liver.*

Catabolic and anabolic processes of carbon exchange simultaneously occur in the cells with the enzyme lactate dehydrogenase (LDH), which defines the direction of these processes in an aerobic or anaerobic way. The accumulation of one of the LDH products, lactate, leads to acidosis of the cell. LDH activity is determined as an independent prognostic biomarker for different diseases and high physical activity [48]. The physiological aging is caused by changes in the activity of lactate dehydrogenase, γ-glutamyltransferase, and alkaline phosphatase of the liver (Table 3).

Our results showed (Table 3) a 2.5-fold prevalence of lactate dehydrogenase-related activity of pyruvate (LDHpyruvate) over lactate dehydrogenase-related activity of lactate (LDHlactate) in the liver of 6 months old gerbils. The significant transformations of activity belong to LDHpyruvate with liver aging. Thus, upon reaching 24 months, LDHpyruvate activity is increased 1.5-fold, while at the same time, LDHlactate activity remains at the level of 6-month-old animals. This fact indicates a decreased intensity of gluconeogenesis and increased lactate content in the liver at this age. One of the regulators of lactate dehydrogenase activity is the ratio of NAD+/NADH.

**Table 3.** The activity of lactate dehydrogenase, γ-glutamyltransferase, and alkaline phosphatase in the aging liver of gerbils, n=6.

| Parameters | Experimental group, age (months) | | | | |
|---|---|---|---|---|---|
| | 6 | 24 | 30 | 36 | 39 |
| **The liver** | | | | | |
| $LDH_{lactate}$, U/kg tissue | 121.43 ± 0.44 | 121.07 ± 0.38 | 112.14 ± 0.15 | 189.63 ± 0.16* | 152.43 ± 0.66* |
| $LDH_{pyruvate}$, U/kg tissue | 259.83 ± 6.88 | 328.20 ± 9.23* | 303.59 ± 7.67* | 269.85 ± 5.26 | 226.09 ± 5.77 |
| GTP, IU/ kg tissue | 22.58 ± 1.92 | 22.77 ± 2.36 | 25.19 ± 1.59 | 28.56 ± 0.33 | 27.41 ± 1.86 |
| ALP, IU/ kg tissue | 89.6 ± 2.27 | 87.31 ± 1.56 | 117.86 ± 1.05* | 128.66 ± 0.22* | 153.47 ± 1.23* |
| **The mitochondrial fraction derived from the liver** | | | | | |
| $LDH_{pyruvate}$, U/kg tissue | 6.35 ± 0.44 | 2.18 ± 0.38* | 1.51 ± 0.15* | 1.46 ± 0.16* | 5.41 ± 0.66 |

The activity of LDH in the liver is directed towards the formation of pyruvate by an increase in the concentration of NADH. The main source of NADH in the liver is the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. Therefore, the activation of LDHpyruvate indicates the reduction of glycolysis effectiveness, in particular, its second stage.

The gradual reduction of LDHpyruvate to the level observed in 6-month-old animals was shown at the age of 39 months in the liver of gerbils. These changes indicate the direction of metabolic processes occurring with age.

A mechanism for reducing the activity of enzymes by binding the soluble fraction to subcellular structures is known. The binding pathway for LDH is the fixation on the mitochondrial membrane [48]. Our experiments showed a significant 3-fold reduction of LDHpyruvate activity in the mitochondrial fraction from 24-month-old animals, maintained at that level up to the 36-month-old (Table 3). The comparison with the data on the activity of LDHpyruvate in the liver occurs an age-related decrease in the ability of the mitochondria to regulate the concentration of free and bound forms of enzymes. At 39 months, the activity of LDHpyruvate increased 2.5-fold compared to animals aged 24-36 months but was still lower than in 6-month-old rodents. It may indicate a decrease in the ability of mitochondria to convert pyruvate and its transformation either to lactate, with the participation of LDH or alanine with involvement of ALT (Fig. 1), whose activity was elevated in the mitochondrial fraction of the liver of gerbils at this age.

Prime biochemical parameters of the state of liver tissue include γ-glutamyltransferase (GTP) and alkaline phosphatase (ALP). Both enzymes characterize the functional state of the hepatobiliary system [49,50]. The highest concentration of enzymes is observed in hepatocytes located in the area of the liver triad. Therefore, any changes in the activity of GTP and ALP may indicate changes in the ability of the liver to form and excrete bile. It is known that ALP activity increases during hepatocarcinogenesis [51]. Previous studies observed a significant increase in ALP activity in the blood during lung carcinogenesis. During metastases, the level of alkaline phosphatase can increase earlier than acid phosphatase.

A slight fluctuation of GTP activity in the liver and an increase of 1.3-fold in the blood of 30-months-old and 2-folds of 36-39-months-old compared to 6-months-old animals was observed (Table 3). Correlation analysis of the activity of this enzyme in the liver and blood showed a negative relationship high degree of significance ($r = –0.88$, $P < 0.01$). Most likely, the unpredictable changes in GTP activity in the liver of the older gerbils are associated with an increase in the permeability of the hepatocyte membrane, especially those located in the hepatobiliary triad, increasing its activity in the blood.

At the same time, the tendency of ALP activity to significantly change with age was observed (Table 3). Thus, increased alkaline phosphatase activity was observed in the liver of

24-month-old gerbils and older, and by the age of 39 months, approximately 2-fold increases were recorded in the animals compared to 6-month-old gerbils. Such changes may suggest the degeneration of the liver with age, damage to the oxidative-reducing balance in the liver, and decreased ability to carry out excretory processes.

*3.4. Impact of Humilid on the prooxidant-antioxidant status of the liver.*

Humilid is a nutritious, biologically active additive that contains humic substances. It is a highly heterogeneous group of acidic macromolecules (molecular weight 1000 to more than 300000) that bear no physical resemblance to the organic compounds of living organisms. Humic materials are believed to form through the oxidative degradation of organic tissues to relatively recalcitrant monomers, followed by polymerizing these monomeric substances into high molecular weight compounds [52]. Soil organic matter and both humic and fulvic acids have been shown to have a high affinity for oxidized products. Humic acids and their salts in organic substances were represented as 50-58% in the applicated Humilid (see table 1). Long-term studies of the properties of Humilid have proven its powerful biological effect on both plant and animal organisms [2,23,25,53]. The impact of Humilid on the main indicators of liver pro-oxidation and the high molecular weight components of the superoxide dismutase and catalase antioxidant system was shown (Table 4).

**Table 4.** Low and high molecular weight components of the pro/antioxidant system of the liver of 6-month-old gerbils under the effect of Humilid, n=6.

| **Parameters** | **Experimental group 6-month-old** | |
|---|---|---|
| | **Control** | **Humilid** |
| **The liver** | | |
| MDA, μmol/ mg protein | 2.19 ± 0.46 | 1.89 ± 0.29 |
| Catalase, μkat/mg protein | 4.28 ± 0.47 | 7.99 ± 0.67 * |
| SOD, c.u./mg protein | 6.31 ± 1.40 | 7.13 ± 0.49 |
| Cytochrome C, μg/g tissue | 3.69 ± 0.43 | 3.56 ± 0.39 |
| **The mitochondrial fraction derived from the liver** | | |
| MDA, μmol/mg protein | 6.43 ± 0.34 | 4.08 ± 0.79 * |
| Catalase, μkat/mg protein | 6.49 ± 0,11 | 7.37 ± 0.47 |
| SOD, c.u./mg protein | 12.17 ± 1.18 | 11.84 ± 1.29 |
| Cytochrome C, μg/g tissue | 7.25 ± 0.87 | 9.38 ± 1.09 * |

The activation of protective mechanisms in the liver of adult animals under Humilid application was established due to the stimulation of catalase by 86%. Moreover, the increased cytochrome C concentration in the mitochondrial fraction derived from the liver by almost 30% was also noted under the Humilid effect. This fact may indicate the stimulation of the respiratory chain with the simultaneous intensification of the cell's energy supply under the impact of Humilid.

*3.5. Impact of Humilid on the aminotransferases of liver*

The mechanism of transamination is adopted due to total protein and amino acids metabolism in aging. Results of changes in the activity of aspartate and alanine aminotransferase in the liver of 6-month-old gerbils with the preventive application of Humilid were shown (Figure 2). In addition, the positive impact of Humilid in drinking water (5 mg/kg weight for 14 days) for 6-month gerbils was noted.

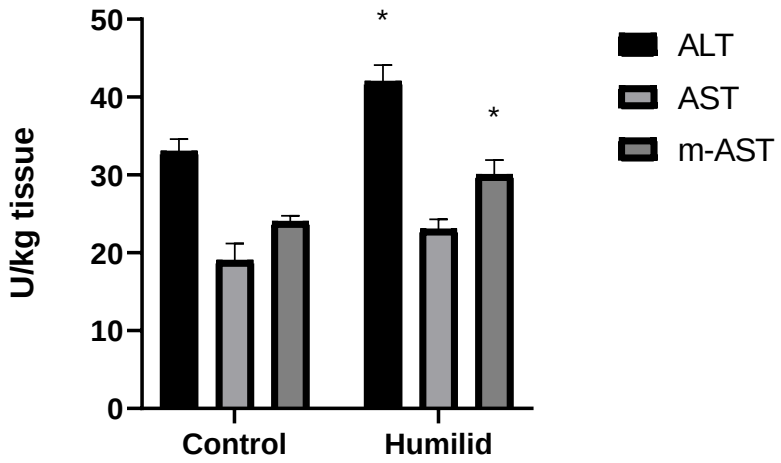


**Figure 2**. The total activity of aspartate (AST) and alanine (ALT) aminotransferases and mitochondrial aspartate (m-AST) aminotransferase in the liver of gerbils. Values are represented as mean ± SEM (n=6); * – P < 0.05 vs. 6-month-old animals.

The activity of ALT increased by 37% under the stabile activity of AST after the influence of Humilid to 6-month-old gerbils was registered (Figure 2). The activation of the glucose-alanine cycle and transamination reactions occur under the action of humic substances in the liver. Increased activity of AST in mitochondria was shown at 22% under Humilid application, while AST activity in the liver did not change. These facts may indicate the regulatory activity of humic substances to cell behavior previously described [2,23,25] and note the integrity of the mitochondrial membrane under the Humilid effect. The absence of toxicity of humic substances to mitochondria was evidenced by the stability of the cytochrome C concentration in the liver (Table 4).

## 4. Conclusions

The obtained results conclude that inhibiting the protective antioxidant system induces the aging liver. That leads to a decrease in liver function. The chief antioxidant enzymes of the liver in long-living animals are mitochondrial catalase, and cytochrome C. Preventive support by Humilid can save the stability of the antioxidant system and prolong the function of the liver in the adult.

## Funding

This research received no external funding.

## Acknowledgments

The authors would like to thank Khristyeva Problem Laboratory of Humic Substances, Dnipro State Agrarian, and Economic University.

## Conflicts of Interest

The authors declare that they have no competing interests.

## References


1. Li, D.; Li, L.; Li, P.; Li, Y.; Chen, X. Apoptosis of HeLa cells induced by a new targeting photosensitizer-based PDT via a mitochondrial pathway and ER stress. *OncoTargets and therapy* **2015**, *8*, 703–711, https://doi.org/10.2147/OTT.S76370
2. Dyomshina, O.O.; Ushakova, G.O.; Stepchenko, L.M. The effect of biologically active feed additives of humilid substances on the antioxidant system in liver mitochondria of gerbils. *Regulatory Mechanisms in Biosystems* **2017**, *8*, https://doi.org/10.15421/021729.
3. Teng, H.; Hong, Y.; Cao, J.; Li, H.; Tian, F.; Sun, J.; Wen, K.; Han, G.; Whelchel, A.; Zhang, X.; Li, X.; Dong, L. Senescence markerprotein30 protects lens epithelial cells against oxidative damage by restoring mitochondrial function, *Bioengineered* **2022**, *13*, 12955-12971, https://doi.org/10.1080/21655979.2022.2079270.
4. Lee, Y. H.; Park, J. Y.; Lee, H.; Song, E. S.; Kuk, M. U.; Joo, J.; Oh, S., Kwon, H. W.; Park, J. T.; Park, S. C. Targeting Mitochondrial Metabolism as a Strategy to Treat Senescence. *Cells* **2021**, *10*, 3003, https://doi.org/10.3390/cells10113003.
5. Zhang, C.; Zhao, Y.; Yu, M.; Qin, J.; Ye, B.; Wang, Q. (). Mitochondrial Dysfunction and Chronic Liver Disease. *Current issues in molecular biology* **2022**, *44*, 3156–3165, https://doi.org/10.3390/cimb44070218.
6. Chimienti, G.; Orlando, A.; Russo, F.; D'Attoma, B.; Aragno, M.; Aimaretti, E.; Lezza, A.; Pesce, V. The Mitochondrial Trigger in an Animal Model of Nonalcoholic Fatty Liver Disease. *Genes* **2021**, *12*, 1439, https://doi.org/10.3390/genes12091439.
7. Napolitano, G.; Fasciolo, G.; Venditti, P. Mitochondrial Management of Reactive Oxygen Species. *Antioxidants (Basel, Switzerland)* **2021**, *10*, 1824, https://doi.org/10.3390/antiox10111824.
8. Villanueva-Paz, M.; Morán, L.; López-Alcántara, N.; Freixo, C.; Andrade, R. J.; Lucena, M. I.; Cubero, F. J. Oxidative Stress in Drug-Induced Liver Injury (DILI): From Mechanisms to Biomarkers for Use in Clinical Practice. *Antioxidants (Basel, Switzerland)* **2021**, *10*, 390, https://doi.org/10.3390/antiox10030390.
9. Zhao, L.; Zou, X.; Feng, Z.; Luo, C.; Liu, J.; Li, H.; Chang, L.; Wang, H.; Li, Y.; Long, J.; Gao, F.; Liu, J. Evidence for association of mitochondrial metabolism alteration with lipid accumulation in aging rats. *Experimental Gerontology* **2014**, *56*, 3-12, https://doi.org/10.1016/j.exger.2014.02.001.
10. Martinez-Lopez, N.; Athonvarangkul, D.; Singh, R. Autophagy and Aging. In: Atzmon, PhD G., Eds. Longevity Genes. Advances in Experimental Medicine and Biology; Publisher: Springer, New York, **2015,** *847*, https://doi.org/10.1007/978-1-4939-2404-2_3.
11. Ghosh-Choudhary, S. K.; Liu, J.; Finkel, T. The role of mitochondria in cellular senescence. *FASEB journal : Official publication of the Federation* of *American Societies for Experimental Biology* **2021**, *35*, e21991, https://doi.org/10.1096/fj.202101462R.
12. Maduro, A. T.; Luís, C.; Soares, R. Ageing, cellular senescence and the impact of diet: an overview. *Porto biomedical journal* **2021**, *6*, e120, https://doi.org/10.1097/j.pbj.0000000000000120.
13. Amorim, J. A.; Coppotelli, G.; Rolo, A. P.; Palmeira, C. M.; Ross, J. M.; Sinclair, D. A. Mitochondrial and metabolic dysfunction in ageing and age-related diseases. Nature reviews. *Endocrinology* **2022**, *18*, 243–258, https://doi.org/10.1038/s41574-021-00626-7.
14. Gündüz, G.; Fişkin, K. (). Aging and cancer: molecular facts and awareness for Turkey. *Turkish Journal of Biology* **2014**, *38*, 708-719, https://doi.org/10.3906/biy-1405-9.
15. Ayers, M.; Horslen, S. P.; Gómez, A. M.; Squires, J. E. Mitochondrial Hepatopathy. *Clinics in liver disease* **2022**, *26*, 421–438, https://doi.org/10.1016/j.cld.2022.03.006.
16. Beharry, K.D.; Cai, C.L.; Henry, M.M.; Chowdhury, S.; Valencia, G.B.; Aranda, J.V. Co-Enzyme Q10 and n-3 polyunsaturated fatty acid supplementation reverse intermittent hypoxia-induced growth restriction and improved antioxidant profiles in neonatal rats. *Antioxidants* **2017**, *6*, 1-18, https://doi.org/10.3390/antiox6040103.
17. Ekoue, D.N.; Diamond, A.M. It takes 2 antioxidants to tango: the interaction between manganese superoxide dismutase and glutathione peroxidase-1. *Turkish Journal of Biology* **2014**, *38*, 748-753, https://doi.org/10.3906/biy-1406-55.
18. Gong, Z.; Tas, E.; Yakar, S.; Muzumdar, R. Hepatic lipid metabolism and non-alcoholic fatty liver disease in aging. *Molecular and Cellular Endocrinology* **2017**, *455*, 115–130, https://doi.org/10.1016/j.mce.2016.12.022.
19. Bonomini, F.; Rodella, L.F.; Rezzani, R. Metabolic syndrome, aging and involvement of oxidative stress. *Aging and disease* **2015**, *6*, 109–120, https://doi.org/10.14336/AD.2014.0305.

20. Kim, I.H.; Kisseleva, T.; Brenner, D.A. Aging and liver disease. *Current opinion in gastroenterology* **2015**, *31*, 184–191, https://doi.org/10.1097/MOG.0000000000000176.
21. Cheng, Y.; Wang, X.; Wang, B.; Zhou, H.; Dang, S.; Shi, Y.; Hao, L.; Luo, Q.; Jin, M.; Zhou, Q.; Zhang, Y. Aging-associated oxidative stress inhibits liver progenitor cell activation in mice. *Aging* **2017**, *9*, 1359-1374, https://doi.org/10.18632/aging.101232.
22. Carrasco, C.; Rodriguez, A.B.; Pariente, J.A. Melatonin as a stabilizer of mitochondrial function: role in diseases and aging. *Turkish Journal of Biology* **2015**, *39*, 822-831, https://doi.org/10.3906/biy-1504-26.
23. Stepchenko, L.M. The value of cathepsin and its inhibitors in the regulation of metabolic processes in chicken-broilers for the action of humic substances. *The Animal Biology* **2010**, *12*, 180-187.
24. Paronik, V.; Stepchenko, L.; Diachenko, L.; Lievykh, A.; Shevtsova, A. Influence of corvitin and humilid on the oxidant-antioxidant system in rats after injectoin of adrenalin. *The Animal Biology* **2015**, *17*, 109-114, https://doi.org/10.15407/animbiol17.04.
25. Stepchenko L. M., Dyomshyna O. O., Ushakova H. O. Impact of biologically active additives of humic nature on the metabolic profile of broiler chickens, Achievements of Ukraine and the Eu In Ecology, Biology, Chemistry, Geography and Agricultural Sciences, Collective monograph, Baltija Publishing, Riga, Latvia, **2021**, *3*, Section 14, 262-288, https://doi.org/10.30525/978-9934-26-086-5-47.
26. de Lourdes Angeles, M.; Gómez-Rosales, S.; Téllez-Isaias, G. Mechanisms of Action of Humic Substances as Growth Promoters in Animals. In (Eds.), Humus and Humic Substances - Recent Advances [Working Title]. Publisher: IntechOpen, London, UK, **2022**, https://doi.org/10.5772/intechopen.105956 .
27. Wang, D.; You, Z.; Du, Y.; Zheng, D.; Jia, H.; Liu, Y. Influence of Sodium Humate on the Growth Performance, Diarrhea Incidence, Blood Parameters, and Fecal Microflora of Pre-Weaned Dairy Calves. *Animals: an Open Access Journal from MDPI* **2022**, *12*, 123, https://doi.org/10.3390/ani12010123.
28. Roger, L.; Tomas, F.; Gire, V. Mechanisms and Regulation of Cellular Senescence. *International journal of molecular sciences* **2021**, *22*, 13173, https://doi.org/10.3390/ijms222313173.
29. Miwa, S.; Kashyap, S.; Chini, E.; von Zglinicki, T. Mitochondrial dysfunction in cell senescence and aging. *The Journal of Clinical Investigation* **2022**, *132*, e158447, https://doi.org/10.1172/JCI158447.
30. Sohal, R.S.; Sanjiv, A.B.; Sohal, H. Oxidative stress and aging in the Mongolian gerbil (Meriones unguiculatus). *Mechanisms of Ageing and Development* **1995**, *81*, 15-25, https://doi.org/10.1016/0047-6374(94)01578-a.
31. Directive 2010/63/EU of the european parliament and of the council of 22 September **2010** on the protection of animals used for scientific purposes. Official Journal of the European Union L276/33. 86/609/EC. 20.10.2010.
32. Law of Ukraine "On Protection of Animals from Cruel Treatment" Vidomosti Verhovnoi Rady Ukrainy – Official Bulletin of the Verkhovna Rada of Ukraine **2006**, 21 February, 27
33. Wieckowski, M.R.; Giorgi, C.; Lebiedzinska, M.; Duszynski, J.; Pinton, P. Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nat. Protoc*. **2009**, *4*, 1582–1590, https://doi.org/10.1038/nprot.2009.151.
34. Burtis, C.A.; Ashwood, E.R. ed. Tietz Textbook of Clinical Chemistry, 3rd ed. Philadelphia, PA: Moss D. W., Henderson A.R. **1999**, 652.
35. Koroljuk, M.A.; Yvanova, L.Y.; Majorova, Y.G.; Tokareva, V.E. Method for the determination of catalase activity. *Laboratornoe Delo* **1988**, *1*, 16-19, https://pubmed.ncbi.nlm.nih.gov/2451064/.
36. Kostjuk, V.A.; Potapovych A.Y.; Kovaleva, Zh.V. A simple and sensitive method for determining the activity of superoxide dismutase, based on the oxidation reaction of quercetin. *Voprosi Medicinskoi Chimii* **1990**, *36*, 88-91, https://pubmed.ncbi.nlm.nih.gov/2363268/.
37. Choi, S.; Swanson, J.M. Interaction of cytochrome c with cardiolipin: an infrared spectroscopic study. *Biophys. Chem*. **1995**, *54*, 271–278, https://doi.org/10.1016/0301-4622(94)00151-9.
38. Andreeva, L.Y.; Kozhemjakyn, L.A.; Kyshkun, A.A. Modification of the method for the determination of lipid peroxides in the test with thiobarbituric acid. *Laboratornoe Delo* **1988**, *2*, 41-43, https://pubmed.ncbi.nlm.nih.gov/2463416/.
39. Salvi, M.; Battaglia, V.; Brunati, A.M.; Rocca, N.L.; Tibaldi, E.; Pietrangeli, P.; Marcocci, L.; Mondovì, B.; Rossi, C. A.; Toninello, A. Catalase takes part in rat liver mitochondria oxidative stress defense. *Journal of Biological Chemistry* **2007**, *282*, 24407-24415, https://doi.org/10.1074/jbc.M701589200.
40. Hüttemann, M.; Pecina, P.; Rainbolt, M.; Sanderson, T.H.; Kagan, V.E.; Samavati, L.; Doan, J.W.; Lee, I. The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian

cell: From respiration to apoptosis. *Mitochondrion* **2011**, *11*, 369–381, https://pubmed.ncbi.nlm.nih.gov/21296189/.
41. Li, K.; Li, Y.; Shelton, J.M.; Richardson, J.A.; Spencer, E.; Chen, Z. J.; Wang, X.; Williams, R. S. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* **2000**, *101*, 389-399. https://doi.org/10.1016/s0092-8674(00)80849-1.
42. Kagan, V.E.; Bayir, H.A.; Belikova, N.A.; Kapralov, O.; Tyurina, Y.Y.; Tyurin, V. A.; Jiang, J.; Stoyanovsky, D. A.; Wipf, P.; Kochanek, P. M.; Greenberger, J. S.; Pitt, B.; Shvedova, A. A.; Borisenko, G. Cytochrome c/cardiolipin relations in mitochondria: a kiss of death. *Free Radical Biology and Medicine* **2009**, *46*, 1439-1453, https://doi.org/10.1016/j.freeradbiomed.2009.03.004.
43. Lee, I.; Salomon, A.R.; Yu, K.; Doan, J.W.; Grossman, L.I.; Hüttemann, M. New prospects for an old enzyme: mammalian cytochrome c is tyrosine-phosphorylated in vivo. *Biochemistry* **2006**, *45*, 9121-9128, https://doi.org/10.1021/bi060585v.
44. Yu, D.M.; Jung, S.H.; An, H.T.; Lee, S.; Hong, J.; Park, J. S.; Lee, H.; Lee, H.; Bahn, M. S.; Lee, H. C.; Han, N. K.; Ko, J.; Lee, J. S.; Ko, Y. G. Caveolin-1 deficiency induces premature senescence with mitochondrial dysfunction. *Aging Cell* **2017**, *16*, 773-784, https://doi.org/10.1111/acel.12606.
45. Wang, Z.B.; Li, M.; Zhao, Y.; Xu, J.X. Cytochrome C is a hydrogen peroxide scavenger in mitochondria. *Protein and peptide letters* **2003**, *10*, 247-253, https://doi.org/10.2174/0929866033479013.
46. Toney, M.D. Aspartate aminotransferase: an old dog teaches new tricks. *Archive Biochemistry Biophysics* **2014**, *544*, 119-127, https://doi.org/10.1016/j.abb.2013.10.002.
47. Birsoy, K.; Wang, T.; Chen, W.W.; Freinkman, E.; Abu-Remaileh, M.; Sabatini, D. M. An Essential role of the mitochondrial electron transport chain in cell proliferation is to enable aspartate synthesis. *Cell* **2015**, *162*, 540-551, https://doi.org/10.1016/j.cell.2015.07.016.
48. Elustondo, P.A.; White, A.E.; Hughes, M.E.; Brebner, K.; Pavlov, E.; Kane, D. A. Physical and functional association of lactate dehydrogenase (LDH) with skeletal muscle mitochondria. *The Journal of biological chemistry* **2013**, *288*, 25309–25317. https://doi.org/10.1074/jbc.M113.476648.
49. Whitfield, J.B. Gamma glutamyl transferase. *Critical Reviews in Clin Lab Sci* **2001**, *38*, 263-355, https://doi.org/10.1080/20014091084227.
50. Orman, M.A.; Ierapetritou, M.G.; Androulakis, I.P.; Berthiaume, F. Effect of fasting on the metabolic response of liver to experimental burn injury. *PLoS ONE* **2013**, *8*, e54825, https://doi.org/10.1371/journal.pone.0054825.
51. Espíndola, K.; Ferreira, R.G.; Narvaez, L.; Silva Rosario, A.; da Silva, A.; Silva, A.; Vieira, A.; Monteiro, M. C. Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma. *Frontiers in oncology* **2019**, *9*, 541, https://doi.org/10.3389/fonc.2019.00541.
52. Hayes, M.H.B. Evolution of concepts of environmental natural nonliving organic matter. http://dx.doi.org/10.1002/9780470494950.ch1. In: Senesi, N.; Xing, B.; Huang, P.M. (Eds) IUPAC Series On Biophysico-Chemical Process In Environmental Systems. Biophysico-chemical process involving natural nonliving organic matter in environmental systems. John Wiley & Sons, New York, **2009**; 2, 1–39, http://dx.doi.org/10.1002/9780470494950.ch1. .
53. Buchko, O.M. Free radical processes in the pig lets for the actions of humic additives. *Anim. Biol* **2013**, *15*, 27-33, https://doi.org/10.15407/animbiol15.01.027.