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THE MAIN PARAMETERS OF THE ANTIOXIDANT SYSTEM IN BLOOD OF MALE AND FEMALE RABBITS IN AGE DYNAMICS

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Abstract

Antioxidant system (AOS) is the most important unit of the general biological defense system. In assessing its state the choice of adequate, stable and sensitive indicators is important to determine the products of lipid peroxidation (LPO) and antioxidant defense enzyme complex. Antioxidant Index (AOI) is based on detecting blood and erythrocyte lipid peroxidation products such as malondialdehyde, diene conjugates of polyunsaturated fatty acids, and assessing the activity of antioxidant enzymes (i.e. superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in erythrocyte membranes. The aim of this work was to evaluate the role of AOS in the mechanism of blood oxidative homeostasis in Soviet Chinchilla rabbits (males and females) during different periods of ontogeny. Studies were performed in bioclinic on 10 rabbits. Blood was sampled from animals at the age of 60, 120 and 180 days. In the blood plasma we determined the content of diene conjugates and malondialdehyde, antioxidants and low molecular weight α-tocopherol and retinol. In hemolysates the activity of antioxidant enzymes, i.e. glutathione reductase, glutathione peroxidase, catalase, superoxide dismutase, was evaluated. For the first time for the biological evaluation of antioxidant system of rabbits a combined integrated AOI was used, which allows to reveal not only changes in the AOS of blood, but also to determine the proportion of its individual factors in the ontogenesis of rabbits. A comparison of the content of cholesterol, glucose and triglycerides in blood plasma showed the differences between male and female rabbits in lipid and energy metabolism. At the age of 180 days an increase in cholesterol, triglycerides and glucose in males was 11.6 %, 27.2 % ($P \leq 0.001$) and 12.9 % ($P \leq 0.01$), respectively, and in the females it was 14.7 %, 27.4 % ($P \leq 0.001$) and 13.0 % ($P \leq 0.05$), respectively. At the age of 120 and 180 days in the animals an insignificant reduction in all indicators was observed compared with the values obtained in the 60 day old rabbits. α-Tocopherol content in males aged 120 and 180 days increased by 14.4 and 21.9 %, respectively, and the retinol level was 8.3 and 29.2 % higher compared to the initial (day 60) values. In females there was a significant increase in the concentration of α-tocopherol by 14.9 and 18.9 %, respectively, and in retinol by 13.6 and 50.0 %, respectively, at days 120 and 180. Revealed changes in the overall and partial AOI in ontogeny indicate the initial activation of compensatory mechanism of antixidation in early ontogenesis and further decrease in a compensatory capacity of the antioxidant defense system in blood. Thus, the general index of the AOI in 4 month old rabbit males and females increased by 12-20 % and 4-12 %, respectively. A positive index value indicates the relatively high antioxidant protection despite the trend to reducing activity of the enzyme protection. In the 6 month old animals the integrated combined AOI values were negative, being typical for the state of oxidative stress. In males these changes were more evident and reliable. Usage of AOI index may be informative in assessment of the state of protective systems both in farm and laboratory animals.

Keywords: ontogeny, protection systems, rabbits, antioxidant system, AOS, blood antioxidant index, AOI.

The anti-radical defence system serves as an important link in the general biological defence chain of the body, acting at atomic and molecular levels and is usually considered as a balance between the total accumulated products of free-radical oxidation and the general activity of enzymatic and non-enzymatic antioxidant defence systems. To evaluate the state of the antioxidant system

(AOS), it is important to choose suitable, stable and sensitive indicators to determine the amount of lipid peroxidation (LPO) products induced by free radicals and a complex of the antioxidant defence enzymes. There is also an increasing understanding of the need to have integrated indices accommodating deviations of AOS indicators from reference values [1, 2].

The antioxidant index (AOI) suggested earlier [3] is based on the identification of LPO products (malondialdehyde, conjugated dienes of polyunsaturated fatty acids) in blood and erythrocytes and evaluation of the activity of structurally uniform antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in erythrocyte membranes. Total AOS is a difference between partial indices AOI1 and AOI2. AOI1 is the mean accumulated deviation from the norm of enzyme activity; AOI2 is the mean accumulated deviation from the norm of LPO product content. Using AOI revealed that the deviations of both partial indices were of similar magnitude, which made it possible to use them in the clinical practice [1, 2].

Further AOS investigation involves general biological evaluation of the balance between the enzymatic and non-enzymatic defence systems and the system of accumulation of free radical oxidation products. AOIs below the reference values are certain to indicate oxidative stress [4, 5]. But it is a compensatory response associated with the mean accumulated deviations above the reference values (AOI1 more than 100 %) that raises more concerns.

It is necessary to note that while clinical investigation can be based on the norm obtained for a wide group of healthy volunteers of different ages and both sexes, experiments with animals have to rely on the values established for a reference group. But in this case we mostly ignore the ontogenetic specifics of AOS development, which are important to consider, e.g., when choosing appropriate experimental models (in particular to test biological products with antioxidant qualities) or because of the issue with increased sensitivity of integrated indices.

The purpose of this work was the detailed investigation of the antioxidant system in rabbits of both sexes during different periods of ontogenesis.

Technique. The investigation involved 10 Soviet Chinchilla rabbits and was performed in the environment of a bioclinic. The infant rabbits with a live weight of 1.5 kg were selected when they were 60 days old. Based on the theory of analogues, two groups of 5 animals (males and females) were formed. In the female group there were no pregnant or lactating animals. The subject animals were kept in the same conditions in standard cages and were clinically healthy. Parameters for animals of both sexes at the age of 60 days were taken as the reference. In both groups the main ration conformed to the feeding standards developed by the V.A. Afanasiev Research Institute for Fur and Rabbit Farming [6, 7]. The animals ate formula pellets K-122 (Russia). The total nutrition value of the rations was the same for both groups.

The health of the rabbits was estimated by clinical parameters, such as rectal temperature, heart rate (HR), respiratory rate (RR), appearance, as well as by behaviour (general activity, food behaviour, defecation and the texture of faeces, grooming, social interactions, and the existence of stereotypies).

Blood was collected from the 60, 120 and 180 day old animals in sterile vials by venipuncture of the posterior auricular vein.

Samples of heparinized blood were centrifuged in a standard laboratory centrifuge Liston C 2204 Classic (Liston, LLC, Russia) at 3,000 rpm for 15 minutes. The plasma was carefully separated from the settled erythrocytes. Hemolysates were prepared by diluting the erythrocyte mixture with distilled water (1:1 vol/vol) and subsequently frozen at –10 to –15 °C.

The amount of conjugated diens in the plasma ($CD_{pl.}$) was determined using the method developed by Z. Placer [8] and modified by V.B. Gavrilov and M.I. Mishkorudnaya [9]; the concentration of malondialdehyde in the plasma ($MDA_{pl.}$) was assayed according to M. Mihara et al. [10]. The activity of the antioxidant enzymes in the hemolysates was estimated, particularly for glutathione reductase (GR) as based on the method suggested by J. Tilbotson and H. Sauberlich [11] adapted for an analyser Labsystems FP-901 (Labsystems Diagnostics Oy, Finland) [12]; for glutathioneperoxydase (GP) according to G. Mille [13] modified for an analyser FP-901 [14]; for catalase (CAT) according to N. Oshino et al. [15] modified by G.Y. Maltsev and A.V. Vasiliev [16]; for superoxidedismutase (SOD) according to the description of M. Nishikimi et al. [17]. Retinol and α-tocopherol concentrations in blood plasma were determined by high-performance liquid chromatography using extraction cartridges. An automatic open analyser Labio 200 (Mindray Medical International Ltd., China) with photometric detection (with Master Labio 200 software) was applied for biochemical analysis of the blood plasma. Reagents by BIOCON Diagnostik GmbH (Germany) were used.

Antioxidant status was estimated by the antioxidant index (AOI), which was calculated using the following equation:

$$AOI = AOI1 - AOI2 = [(GP/GP_{ref.} + GR/GR_{ref.} + SOD/SOD_{ref.} + CAT/CAT_{ref.})/4)] - [(CD_{pl.}/CD_{pl.\ ref.} + MDA_{pl.}/MDA_{pl.\ ref.})/2],$$

where AOI is the antioxidant index; AOI1 is the total for main enzymes of the AOS-system; AOI2 is the total for LPO products; GP, GR, SOD, CAT, $CD_{pl.}$ and $MDA_{pl.}$ are the activity values for glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase correspondingly, and the concentration of malondialdehyde in blood plasma (experiment and reference). AOI can be expressed both in per cents and parts. In case of the former, the reference value is 100 %, in the second case it is 0.

Obtained data were processed by methods of the standard parametric statistics using the *t*-criterion of Student.

Results. The monitoring period was optimal both for laboratory tests (60-180 days) and practical rabbit-breeding (120-180 days).

The state of the antioxidant system in rabbits was analysed by the activity of the enzymes of the erythrocyte antioxidant defence and LPO product concentration. In per cents, no significant difference between these values and the reference values for the animals of 120 and 180 days old was noticed (Table 1). Slightly unequal reduction in all the parameters was observed.

Calculated integrated indices (Table 2) gave more detailed information about the state of AOS during ontogenesis. Thus, in 4-month-old male rabbits, AOI2 was noticed to reliably decrease (by 21-23 %), while in females the changes were not so pronounced (11-1 2%). This reduction led to the relative increase in the total AOI: for 12-20 % in males and for 4-12 % in females (on average +15.8 % and +8.2 %, correspondingly). Positive AOI indicated that the antioxidant defence was in a relatively compensated state even as there was a decreasing trend in the activity of the enzyme defence.

In 6-month-old animals, AOI2 recovery was not compensated by AOI1, thus the total AOI becomes negative (–6.3 % in males and –2.1% in females), which is typical for the oxidative stress. In males, changes of the partial indices were more pronounced (up to 11 % in the confidence interval) and reliable.

Basically, the revealed trends could be equally applied to all the studied parameters, i.e. AOI informative value increased with the increase in the number of the measured similar parameters. Using more criteria in addition to the MDA content, SOD activity and some others [18, 19] significantly increases the infor-

mation value of the investigation results, with the ways the parameters are grouped becoming very important. The obtained data show that the total index (AOI) makes it possible to reliably estimate low deviations in the antioxidant status during ontogenesis.

1. The activity of the enzymes of the erythrocyte antioxidant defence and the concentration of the lipid peroxidation products in blood plasma of Soviet Chinchilla rabbits of both sexes and different ages ($M \pm m$, laboratory experiment)

Group	GP, μmol/(min · ml)	SOD, units/ml	GR, μmol/(min · ml)	CAT, thous. units/ml	$CD_{pl.}$, nmol/ml	$MDA_{pl.}$, nmol/ml
			Age 60 days			
Males (reference, $n = 5$)	20.2±0.7	1,243.0±59.0	2.2±0.2	346.0±11.0	1.6±0.1	1.1±0.3
Females (reference, $n = 5$)	18.8±1.9	1,227.0±23.0	2.3±0.1	354.0±16.0	2.0±0.2	1.4±0.1
			Age 120 days			
Males	17.6±1.2	1,198±42	2.2±0.1	333.0±22.0	1.3±0.1	0.8±0.1
Deviation from reference, %	87.1±0.3	96.4±2.1	97.8±0.2	96.2±0.4	83.0±0.2	73.7±0.3
Females	18.1±2.2	1,209.0±67.0	2.3±0.1	334.0±19.0	1.8±0.1	1.2±0.1
Deviation from reference, %	96.3±0.7	98.5±2.0	97.9±0.1	94.4±1.3	88.2±0.2	88.9±0.1
			Age 180 days			
Males	17.6±1.4	1,156.0±69.0	2.1±0.2	310.0±8.0	1.6±0.3	1.0±0.1
Deviation from reference, %	87.1±0.4	93.0±2.6	91.2±0.2	89.6±0.8	98.7±0.2	86.8±0.3
Females	17.1±1.3	1,116.0±5.0	2.1±0.1	330.0±12.0	1.9±0.2	1.3±0.1
Deviation from reference,%	90.9±0.5	90.9±1.7	90.1±0.0	93.2±1.1	93.1±0.3	93.4±0.1

Note: GP means glutathione peroxidase; GR means glutathione reductase; SOD means superoxide dismutase, CAT means catalase; $CD_{pl.}$ means conjugated dienes, $MDA_{pl.}$ means malondialdehyde.

2. Indices characterizing antioxidant status in Soviet Chinchilla rabbits of both sexes and different ages ($M \pm m$, laboratory experiment)

Group	AOI1,%	AOI2,%	AOI = (AOI1–AOI2),%
		Age 60 days	
Males (reference, $n = 5$)	100	100	0
Females (reference, $n = 5$)	100	100	0
		Age 120 days	
Males	94.2±2.1	78.4±0.1*	+15.8±2.1
Females	96.8±2.5	88.6±0.2*	+8.2±2.5
		Age 180 days	
Males	90.2±2.8	96.5±0.4	–6.3±2.8*
Females	91.2±2.1	93.3±0.2	–2.1±2.1

Note: AOI1 is the total value for main enzymes of the antioxidant system; AOI2 is the total value for lipid peroxidation products; AOI is an integrated antioxidant index.
* Differences compared with the reference are valid for $P \leq 0.05$.
** Differences between AOI1 and AOI2 are valid for $P \leq 0.05$.

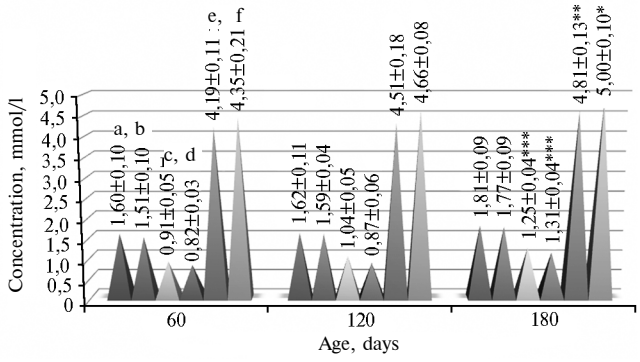
Thus, compensatory abilities of the antioxidant system were proved to increase in pubescent rabbits, with the trend of increased oxidative stress developing in older animals (this was also confirmed by the results of the analysis on concentration of retinol and α-tocopherol in blood presented below). Both for the estimation of the breeding conditions (at animal husbandry units, farms, etc.) and in experiments with animals (in particular with rabbits) as biological models, the important task is to identify subtle changes in the antioxidant status [20-22].

The energy exchange, which is one of the main metabolic functions, is the mechanism behind the adaptive abilities of rabbits in the conventional conditions of a bioclinic. But keeping the animals in cages results in enforced hypodynamia since their movement is limited. Less locomotion, in turn, impacts the supporting-motor, respiratory and other functions of the body, which re-

duces the energy expenditure.

The observed changes in the lipid and energy exchange patterns (shifts in the synthesis and decomposition ratio) can be traced, in particular, by the content of cholesterol, triglycerides and glucose in blood plasma (Fig.).

Total cholesterol and triglycerides increased in rabbits with their age. Thus, by the end of the experiment the level of cholesterol both in males and females had increased by 11.6 % and 14.7 % correspondingly compared with the reference; and the amount of triglycerides (the main energy source for the cells) had increased by 27.2 % and 27.4 % ($P \leq 0.001$). At the age of 120 days, the difference in parameters between the males and females was reliably 16.4 % (at $P \leq 0.05$), with the general advantage in the content of both studied substrates in males at all the investigation stages.



Content of cholesterol (a, b), **triglycerides** (c, d) **and glucose** (e, f) **in blood plasma of Soviet Chinchilla rabbits of both sexes and different ages:** a, c, e — males; b, d, f — females (laboratory experiment).

Glucose concentration also increased with age. Thus, by 180 day its content had increased in males by 12.9 % ($P \leq 0.01$), and in females by 13.0 % (at $P \leq 0.05$) compared to the beginning of the experiment (see Fig.). This parameter reflects the state of aerobic energy metabolism. Glucose concentration was somewhat higher in the blood plasma of females compared with males in all investigated periods. In general, the lipid and energy exchange during ontogenesis of males and females was identical.

The non-enzymatic systems of the antioxidant defence system of the body are known to promote quick inactivation of oxygen free radicals [5, 23].

Depending on the age, the total shift in the rabbit metabolism was accompanied by changes not only in the activity of the antioxidant enzymes but also in concentrations of low-molecular antioxidants, such as α-tocopherol and retinol, in the blood plasma. Vitamins with antioxidant qualities, α-tocopherol (vitamin E) and retinol (vitamin A), are among the most important nonenzymatic antioxidants acting in the membrane and lipoprotein phases.

3. Content of vitamins E (α-tocopherol) and A (retinol) in blood plasma of Soviet Chinchilla rabbits of both sexes and different ages (*M*±*m*, laboratory experiment)

Parameter	Age, days	Group	
		males	females
α-Tocopherol, μg/ml	60	1.46±0.08	1.48±0.18
	120	1.67±0.26	1.70±0.13
	180	1.78±0.12*	1.76±0.09
Retinol, μg/ml	60	0.24±0.03	0.22±0.04
	120	0.26±0.03	0.25±0.02
	180	0.31±0.05	0.33±0.04

* Differences compared with the reference (60 days) are valid for $P \leq 0.05$.

At the age of 120 and 180 days, the concentration of α-tocopherol in males increased by 14.4 % and 21.9 % correspondingly, and the concentration of retinol was 8.3 % and 29.2 % higher compared to the initial (age 60 days) values. The same age dynamics was typical for the females too: reliable reduction in

the concentration of α-tocopherol (by 14.9 % and 18.9 %) and retinol (by 13.6 % and 50.0 %) was observed at the age of 120 and 180 days correspondingly (Table 3).

Rabbits are the quickest breeding and growing of all the farm animals. Does (female rabbits) of large breeds like the Soviet Chinchilla reach maturity and can be bred at the age of 4 months. Bucks (male rabbits) reach maturity at about the same age. Rabbits continue to grow and develop until they are 8-10 month old. The results we obtained reveal important specifics of the ontogenetic structure of the antioxidant defence in rabbits of both sexes and different ages. The main of them was the strengthening of compensatory mechanisms, including erythrocyte enzymes, in pubescent rabbits which was replaced by decompensation with the signs of oxidative stress in older rabbits.

The enzymatic link in the antioxidant defence is a chain of consecutive reactions neutralizing oxygen radicals (superoxide anion radical, singlet oxygen, hydrogen peroxide, hydroxyl radical, etc.) and the products of their reactions with organic compounds, primarily unsaturated fatty acids, forming fatty acid hydroperoxides. The main function of enzymatic antioxidant systems is to provide a long-term protection of body organs and tissues when steady-state concentrations of reactive oxygen species and free radicals increase. We should note that it is the hematopoietic growth factors responsible for new erythrocyte production that play the primary role in the long-term enzymatic defence [24, 25].

Thus, we discovered the strengthening of the antioxidant defence mechanisms involving enzymatic erythrocyte systems in pubescent rabbits (120 days) followed by decompensation and oxidative stress (180 days). In general, both in male and female rabbits the age dynamics of biochemical parameters characterizing the state of the antioxidant system (AOS) does not vary considerably. Nevertheless, the detected variations were more pronounced in males. Apparently, more conservative ontogenesis changes in females cannot be considered accidental and need further examination. Therefore, when using rabbits in biomedical experiments or while monitoring husbandry farms, sometimes it is advisable to account for the sex of the animals. It was the first time that we used the integrated antioxidant index, which makes it possible not only to detect changes in the blood AOS but also to estimate the influence of different antioxidant defence factors during ontogenesis, to determine antioxidant status in rabbits.

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