**Effects of lipoperoxidation and mitochondrial state on milk yield of dairy cows under technological stress**

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# ABSTRACT

# Evaluation of the physiological state of cattle is of crucial importance in the creation of healthy herds of highly productive dairy cows. The objective of this current work was to determinate the effect oxidative parameters on mitochondrial state in the blood, milk yield and milk composition of dairy cows under technological stress.

# The study was conducted on the black-and-white breed healthy herds. Regrouping, changing of service personnel, carrying out veterinary and sanitary manipulations were considered as technological stress factors. The concentration of cortisol in the blood serum was studied by immunological method. The concentrations of malonic dialdehyde (MDA), diene conjugates (DC), Schiff bases (SB), reduced glutathione and catalase activity were measured spectrophotometrically. The mitochondrial state was estimated by laser interference microscopy. Milk yield, the protein and lipid composition of cow milk were studied using an ultrasound analyzer.

# Technological stress caused an increase in oxidative processes, a decrease in the antioxidant activity of blood and milk at the initial stages of registration (1-7 days). The concentration of reduced glutathione remained reduced for 30 days after technological stress. These processes were accompanied by a decrease in mitochondrial refractoriness and disintegration. The indicator of milk yield decreased and was not restored to the values of intact animals by 30 days after technological stress, protein and lipid composition decreased either. Thus, a decrease in the quantity and quality of milk under technological stress may be mediated by the development of oxidative stress, the trigger of which may be mitochondria.

**Key Words:** stress; cows; hematological parameters; free-radical oxidation, mitochondria.

**INTRODUCTION**

Industrial technologies are widely used in modern agricultural enterprise among other things increase the impact of adverse environmental factors that cause animals to stress conditions. New equipment, noise exposure, table size, method of maintenance, change of care personnel are the main factors of technological stress for cattle (Breuer *et al.*, 2003; Gupta *et al*., 2007; Hernandez *et al*., 2014).

The severity of the stress reaction depends on the duration and the factors causing it but either way the regulatory mechanisms of the body are strained in the animal, a violation of physiological, behavioral and metabolic parameters has been shown (Mandal *et al*., 2011; Chikkagoudara *et al*., 2022). The sympathoadrenal and hypothalamus-pituitary-adrenal (HPA) and the sympathetic-adrenal-medullary (SAM) axes are crucial in the implementation of the action of stress factors (Bagath *et al*., 2019). Catecholamines are presumed to have an inflammatory effect whereas cortisol causes a decrease in the immune system of animals (Ibrahim *et al*., 2023).

It has been shown that stress worsens the immune response, causes immunosuppressive effects (Chen *et al*., 2018). In this regard, the consequences of stress are a decrease in susceptibility to infections (Akinmoladun *et al*., 2021). The effect any stress factors is associated with the activation of free radical processes and depletion of the antioxidant system. The effect of any stress factors could associate to the activation of free radical processes and depletion of the antioxidant system ([Chauhan](https://pubmed.ncbi.nlm.nih.gov/?term=Chauhan+SS&cauthor_id=24894002) *et al*., 2014). Additionally the acid base status is changed. Due to their wide wide-ranging impact, the development of acidosis and increased oxidative stress can lead to a deterioration of the physiological state of animals and a tangible diminution of milk yield (Semsirmboon *et al*., 2023; Raghunandan *et al.*, 2022).

Consequently, the comprehension of the stress reaction mechanisms and the analysis of more accurate stress indicators give advance opportunities to eliminate damaging factors, avoid animal diseases and increase milk yield.

Therefore, this research aim was to evaluate the relationship of oxidative parameters and the mitochondria state in the blood serum, milk yield and milk quality under technological stress.

**MATERIALS AND METHODS**

**Experimental animals and design**

This study was carried out in the conditions of the industrial complex of the Nizhny Novgorod region, where the studies were carried out on a clinically healthy dairy population of highly productive Holstein cows of the Black-and-White breed of the 2nd lactation (n=20). The conditions for feeding and keeping animals were of the same type. The animals were fed in full accordance with the norms of the Russian Academy of Agricultural Sciences, and the animals were kept tethered in standard barns throughout the year. As stress factors, regrouping, changing service personnel, and conducting veterinary and sanitary manipulations were employed in this study. The research was carried out in winter season.

The study was carried out as per the suggestions of the European Convention for the Protection of Vertebrate Animals used for Experimental or Scientific Purposes (ETS No. 123, Strasbourg, 1986) and the Ministry of Health of the Russian Federation No. 708 N dated August 28, 2010.

During the study, blood sampling was carried out in all animals before and after 1, 3, 14, 30 days of exposing the selected stresses from the jugular vein in the morning before feeding. This dynamic made possible to analyze the role of stress in the short-term (up to 3 days) and long-term (up to 30 days) periods. Cortisol concentration, indicators of oxidative stress (concentration of MDA), diene conjugates (DC), Schiff bases, catalase activity, content of reduced glutathione of blood serum were recorded in the blood as per standard methodology as suggested in subsequent paragraphs.

**Blood research methods**

The content of cortisol in the blood serum of cows was determined using an automatic ELISA analyzer (Evolis Twin Plus, Russia) (Asuzu *et al*., 2023).

The concentration of MDA was determined by reaction with thiobarbituric acid to form a colored trimethine complex with an absorption maximum at 530 nm (Deryugina *et al*., 2019).

Catalase activity was analyzed by the decrease in peroxide in the sample (Deryugina *et al*., 2018). The measurements were carried out spectrophotometrically immediately and 20 sec after the introduction of H2O2 into the cuvette with the sample at a wavelength of 240 nm. Catalase activity (A) was calculated by the formula: A = (log E1/E2 × 120000)/Hb, where E1, E2 are the extinction of the experimental sample immediately and 20 sec after the addition of H2O2; Hb is the amount of hemoglobin in the sample. Catalase activity was expressed in mmol/gHb × min.

The concentration of reduced glutathione in the blood was studied using 5,5'-di-thio-bis(-2-nitrobenzoic) acid according to the method of Ellman (1959) using a solution of sulfosalicylic acid to precipitate protein in samples, which, in contrast to the use of metaphosphoric or trichloroacetic acids, excluded the spontaneous transition of the reduced form of glutathione to the oxidized one. The concentration of reduced glutathione was expressed in nmol/L.

The intensity of free-radical lipid oxidation in the blood was assessed by the content of molecular products of lipid peroxidation (LPO) - diene (DC) conjugates, as well as Schiff bases (SB) - by spectrophotometry (Volchegorsky, 1989) on an SF 2000 spectrophotometer (Russia). Each phase was evaluated against the corresponding control at wavelengths of 220 nm (absorption of isolated double bonds), 232 nm (absorption of diene conjugates), 400 nm (absorption of Schiff bases). The content of diene and triene conjugates and Schiff bases were estimated by relative values of E232/E220, E400/E220 and expressed in relative units.

**Methods for mitochondria isolation**

60-100 ml of venous blood was taken, mixed with 25 ml of medium containing 5% dextran 250000, 0.12 M NaCl, 10 mM EDTA, pH 7.4; and erythrocytes were precipitated for 45 min at 4 °C. The upper phase was collected and centrifuged for 10 min at 5000 g. The precipitate was suspended in hypotonic medium (10 mM Tris & HCl, pH 7.6) for 7 min; osmotic shock was stopped by adding 0.25 M sucrose. The suspension was centrifuged for 10 min at 600 g, the supernatant was stored, and the precipitate was subjected to a second osmotic shock and centrifuged again. The supernatants were combined and centrifuged for 20 min at 12,000 g to precipitate the mitochondria. The mitochondrial precipitate was suspended in medium containing 0.25 M sucrose, 2 mM EDTA, pH 7.4 (Egorova *et al*., 2011).

Structural changes in mitochondria were studied using a laser interference microscope MII-340 (Yekaterinburg, Russia) with a 30x objective (NA=0.65), λ laser=650 nm. A VS-415U CCD video camera (NPK Videoscan, Russia) with a resolution of 782x582 pixels was used to capture images. During the study biological objects were placed on a mirror substrate from which the light passing through the cell was reflected. As a result, a double phase shift of a coherent light source beam at each point of the object was recorded and an additional wave from the same source was used to form an interference image of the organelle. Images of 10 sites with monolayer arrangement of organelles in the interference channel and reflected light in each sample were obtained for the study. The state of mitochondria was assessed by recording the mean value of the optical path difference and the diameter of the phase image of mitochondria. To obtain a reliable result, the indices were calculated using at least 20 mitochondria from each sample.

**Methods of studying milk and milk productivity of cows**

Milk productivity of animals was controlled by the results of control milking a month after the start of the experiment. When investigating milk productivity, we determined fat and protein content using an ultrasonic analyzer "Lactan 1-4" (Russia).

The intensity of lipoperoxidation processes in animal milk was assessed by determining primary and secondary lipoperoxidation products using a spectrophotometric method with separate registration of free-radical lipid damage products in the heptane phase of the lipid milk extract. Spectrophotometry of the lipid extract was performed at three wavelengths i.e., 220, 232, 278 nm which allowed determining the content of primary oxidation products (diene conjugates “DC”), the content of secondary oxidation products (ketodienes and conjugated trienes “CD/CT”). The final products of lipoperoxidation-Schiff bases-were determined by the method of Lvovskaya *et al*. (1991). Content of free radical lipid oxidation products was expressed in units of the oxidation index.

**Statistical analysis**

Statistical processing of the obtained data was carried out using the Statistica program, and reliability was assessed using Student's t-test.

**RESULTS AND DISCUSSION**

**Cortisol concentration analysis**

The development of a stress reaction is accompanied by an increase in the content of corticolibyrin in the blood, which increases the production of adrenocorticotropic hormone (Mormede, 2007; Deryugina *et al*., 2019).

Registration of cortisol proves the development of a stress response in cows after technological stress in our studies. It was shown that before the technological stress, the level of cortisol in the blood was within the physiological parameters characteristic of cattle and amounted to 17.68 ± 0.79 nmol/l. By day 1, there was an increase in the concentration of the cortisol hormone in the blood by 2.5 times, which corresponded to 44.77 ± 5.61 mol/l. By days 7 and 14, the cortisol level was 29.43±1.69 and 25.89, respectively. By the 30th day of the experiment, the amount of cortisol in the blood decreased, but exceeded the values obtained before the technological stress (19.32 ± 0.60 nmol/l). Percentage change in cortisol (Fig. 1).

**Lipoperoxidation and blood antioxidant system**

An integral part of the imbalance of internal homeostasis in animals under stress is a change in the concentration of free radicals and the development of oxidative stress against this background (Slimen *et al*., 2016).

Considering the dynamics of the concentration of lipid peroxidation products in blood samples obtained a day after the onset of exposure, a 2-fold increase in the level of diene conjugates was recorded with the maintenance of elevated values during 14 days of observation relative to the indicator before stress. The concentration of malondialdehyde (MDH) increased from the first day, the peak of the increase in the level of this product was found in blood samples obtained 14 days after the technological stress: by 24% relative to the initial values. A similar pattern was observed for the concentration of fluorescent Schiff bases. Studies have shown that on the 14th day, the level of Schiff bases was maximum relative to the data before stress (Table 1).

The effect of stress also affected the state of the antioxidant system in the blood of cows (Table 1). In particular, the level of catalase was below the initial level for 14 days after technological stress. The amount of reduced glutathione during the experiment was reduced by 30-50% over 30 days, and it depending on the timing of registration.

**Mitochondrial analysis**

The study of mitochondria by laser interference microscopy showed that the phase characteristics of the organelles changed under technological stress (fig. 2). It was shown that the ratio of mitochondrial phase height to phase diameter allows to calculate mitochondrial refractoriness (Yaguzhinsky *et al*., 2008). Under technological stress, the refractoriness of individual mitochondria decreased, which may be related to the inhibition of the electron transport chain. The number of disintegrated mitochondria under technological stress increased 2-fold by day 1 relative to the values of the control group. Mitochondria are the main source of reactive oxygen species (Long *et al*., 2009; Guevera *et al*., 2011) and the growth of disintegrated mitochondria with an altered refractory level can enhance the development of oxidative stress in cows, which will have a negative effect on their productivity.

**Milk analysis**

The analysis of milk productivity in cows at day 1 after technological stress showed a decrease in milk productivity by 32% relative to the values of cows without technological stress and preservation of the reduced milk productivity indicator at day 30 of the study. Under technological stress, the amount of protein significantly decreased by 13% at the day 1 after technological stress and recovered by day 30. Mass fraction of fat tended to decrease. An increase in lipoperoxidation products in milk was recorded under technological stress. The amount of diene conjugates and Kettani and related trienes increased significantly by day 1 of technological stress while at day 30 the indices tended to decrease (Table 2).

Results of the study suggested that the level of cortisol after 30 days of technological stress reached to the initial values but after this also it remained above the normal limit. Glucocorticoids are known to function as checkpoints for energy homeostasis and mediate many of the effects associated with stress on metabolism. A high level of cortisol, suppressing the immune system of the animal, and also increases the incidence of diseases (Fomichev *et al*., 2012).

# Additionally, in most pathologies of an infectious and non-infectious nature, the activity of lipid peroxidation enlarges leading to pronounced changes in the physicochemical properties of lipids. Lipid interactions modulate the function, folding, structure and organization of membrane proteins (Hammerschmid *et al*., 2013). Violation of lipid and protein components with increased lipid peroxidation leads to cellular dysfunction. A detrimental impact of lipid peroxidation is manifested by a violation of lipid and protein components of the membranes and leads to cellular dysfunction (Villalón-García *et al*., 2023).

# Furthermore, a decrease in the content of SH-groups and an increase in the concentration of SS-groups is noted in a state of oxidative stress of any etiology.

# Thiol compounds, due to their ability to quickly but reversibly oxidize, are the most sensitive to adverse effects of a very different nature and intensity. Among the multiple antioxidant mechanisms that have to prevent cell damage, the essential place is occupied by the control of thiol–disulfide exchange, and glutathione is the key component which is involved in the processes of cellular redox regulation (Asanuma et al., 2021). Glutathione (γ-glutamyl-cysteinylglycine) is a thiol-tripeptide which exists in two interconvertible forms, reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduced glutathione serves in the intracellular space as the main sulfhydryl buffer to maintain the reduced state of cysteine residues in all proteins. According to its chemical properties, glutathione is able to independently participate in detoxification processes, reacting with both hydrogen peroxide and organic peroxides (Kuhn *et al*., 2017; Ighodaro *et al*., 2018; Bayır *et al*., 2020). The results of the study demonstrated that the content of the GSH was reduced after technological stress. The reduced level of glutathione remained on the 30th day of the study, which indicates a decrease in the adaptive capacity of the body and a decrease in its resistance to oxidative stress.

Oxidation of fatty acid residues in membrane phospholipids leads among the primary mechanisms of cell damage under oxidative stress. The main substrates for free-radical reactions are the double bonds of unsaturated fatty acids in phospholipids (Gaschler *et al*., 2017). Mitochondrial membranes are particularly sensitive to reactive oxygen species (ROS), since cardiolipin, localized in the inner mitochondrial membrane, in most animals contains four linoleic acid residues (Schenkel *et al*., 2014). Under technological stress, the functional activity of mitochondria decreases, which is caused by the disruption of the antioxidant system and the formation of non-selective mitochondrial pores. The opening of these pores leads to irreversible disruption of mitochondrial functions (Bernardi *et al*., 2015). At the same time, the enzyme glutathione peroxidase (GPx) is required to maintain the levels of ROS in mitochondria (Shimura *et al*., 2022).

Consequently, the decrease in glutathione revealed during the study may negatively affect to the efficiency of mitochondria.

Meanwhile, mitochondrial dysfunction can be considered as a trigger of oxidative stress in cows. The separation of respiration and phosphorylation process in mitochondria leads to a superoxide anion radical generation by the respiratory chain (Skulachev *et al*., 2012).

Thus, under the action of technological stress it is necessary to consider its intensity so that a vicious circle does not develop: increasing free-radical oxidation, damage to mitochondria, increasing oxidative stress.

**CONCLUSIONS**

The study shows that technological stress significantly affects the processes of lipid oxidation in blood serum and milk, which is accompanied by a decrease in milk productivity. The most pronounced changes were registered on the 1st day after the action of technological stress. The revealed disorders of oxidative processes may be mediated by mitochondrial disintegration. It is shown that the index of antioxidant system i.e., reduced glutathione was not restored to the initial values, which was combined with a decrease in milk productivity on day 30 of registration.

Effects on mitochondrial energetics can significantly increase the efficacy of therapeutic drugs. However, the mechanisms of regulation of these processes are not fully understood. The answer to the question to what extent cell energy modulation will contribute to the adaptation of the organism to stress is extremely important for the development of an effective direction of prevention and therapy.

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**Author contributions.** Deryugina A.V. designing the experiment and planning, budget allo-cation, project management, supervision, critically reviewed the manuscript. Ivashchenko M.N. esigning, planning, and execution of the experiment, data collection, data analysis, writing a draft paper, and editing. Metelin V.B. designing the experiment and planning, supervision, critically reviewed the manuscript. Danilova D.A. designing the experiment and planning, supervision, critically reviewed the manuscript. Polozova A.V. designing the experiment, data analysis, editing the manuscript. Talamanova M.N. budget allocation and project management, writing a draft paper, and editing. All authors read and approved the final manuscript.

**Conflict of interest.** The authors declare that the presented research was conducted in the absence of commercial or financial relationships that could be interpreted as a potential conflict of interest.

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**Table 1**

**The level of peroxidation products and indicators of the antioxidant defense system in the blood of cows**

|  |  |  |
| --- | --- | --- |
| Indicator | Before stress | After technological stress |
| 1 | 3 | 14 | 30 |
| DC, units opt. sq. / mg lipid | 0.34 ± 0.02 | 0.70 ± 0.01 \* | 0.73 ± 0.03 \* | 0.62 ± 0.02 \* | 0.39 ± 0.02 |
| MDA, µmol/l | 1.45 ± 0.03 | 1.71 ± 0.01 \* | 1.74 ± 0.02 \* | 1.96 ± 0.04 \* | 1.39 ± 0.02\* |
| Schiff bases, rel. units/ml serum | 0.33 ± 0.02 | 0.34 ± 0.02 | 0.40 ± 0.01\* | 0,58 ± 0.02 \* | 0.34 ± 0.04 |
| Catalase, μM H2O2 / l min 103 | 18.87 ± 1.29 | 15.43 ± 1,55 \* | 14.45 ± 1.53 \* | 15.13 ± 1.27 \* | 17.88 ± 0.73 |
| Glutathione reduced, mmol/l | 0.25 ± 0.02 | 0.14 ± 0.01 \* | 0.12 ± 0.01 \* | 0.18 ± 0.01\* | 0.19 ± 0.03\* |

Note: \* - statistically significant differences in relation to indicators before technological stress (p < 0.05)

**Table 2**

**The effect of low-intensity laser radiation on dairy productivity of cows and content of lipoperoxidation products of cow's milk**

|  |  |  |
| --- | --- | --- |
| Indicator | before stress | after stress, hours |
| 1 | 30 |
| Milk productivity, kg | 44.9±2.20 | 30.1±2.17\* | 32.3±2.13\* |
| Mass fraction of fat, % | 5.13±0.56 | 4.47±0.7 | 4.52±0.70 |
| Mass fraction of protein, % | 3.20±0.08 | 2.82±0.18\* | 3.06±0.24 |
| Diene conjugates (c.u.) | 0.94±0.16 | 1.32±0.17\* | 1.29±0.21 |
| Kettani and related trienes (c.u.)  | 0.085±0.01 | 0.103±0.012\* | 0.104±0.014 |
| Schiff base (c.u.)  | 0.015±0.001 | 0.023±0.01 | 0.022±0.013 |

Note: \* - statistically significant differences in relation to indicators before technological stress (p < 0.05)

Fig. 1. Dynamics of blood cortisol concentration after technological stress

Note: 100% - the level of the indicator before technological stress, \* - statistically significant differences in relation to the indicators before technological stress (p < 0.05)

 

Fig. 2. Phase portraits of mitochondria obtained by interference microscopy