The effect of antioxidants on the microstructure of tissues in the experimental aseptic inflammation focus

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Abstract. The results of studying the intramuscular administration effect of ethyl methylhydroxypyridine succinate are presented in the article, as well as administration of the Flunidzhekt drug and the new Antioxidant anti-inflammatory drug for animals on the course of aseptic inflammation in white laboratory mice. After provoking the inflammation by subcutaneous turpentine oil injection, experimental animals were administered intramuscularly with abovementioned pharmaceutical substances for five days. Abscessing soft tissues of white mice obtained from an inflammatory lesion were subjected to microscopic examination. Microscopy of substances obtained from animals of the control group, in which no therapeutic agents were used, established that the pathological process proceeded against the background of significant leukocyte infiltration of the affected areas. In contrast, development signs of regenerative processes were observed on microsections made from the tissues of mice that were injected with ethylmethylhydroxypyridine succinate. The onset of those signs was predicted by the increase in the proliferation of fibroblasts with a decrease in leukocyte infiltration. After using the “Flunidzhekt” drug, it was noted to be highly effective in suppressing the intensity of the inflammatory process. But it was also noted that the most optimal microscopic picture was observed in pathological tissues obtained from animals that used the Antioxidant anti-inflammatory drug for animals. The use of ethyl methylhydroxypyridine succinate has a positive effect on the course of the inflammatory process, helping to reduce pathological changes in tissues in the outbreak and accelerating the healing process. Moreover, the use of the Antioxidant anti-inflammatory drug for animals helps accelerate the regeneration of the outbreak and reduces the severity of its pathogenetic effects.

1. Introduction
At the present time, many diseases of farm animals causing enormous economic damage to livestock occur against the background of the inflammatory reaction development. The inflammatory process often has a tendency to complications and the development of concomitant pathology, which significantly reduces the possibilities and economic feasibility of further productive exploitation of the animal. Diseases having an inflammatory process in their pathogenesis are one of the most common causes of death, forced slaughter and culling of farm animals, they also negatively affect the quality of products of animal origin.

The development and implementation of new approaches for prevention and treatment of inflammatory diseases in animals is an urgent problem of biological and veterinary science. The
development of new substances aimed at rationally preventing the development of an inflammatory reaction, as well as helping to optimize its course both at the clinical and physiological levels, and their widespread introduction into the production process of animal products will increase their safety and usefulness.

It has been established that oxidative stress plays a decisive role in the development and course of inflammation and thereby contributes to the pathophysiology of a number of debilitating diseases [1, 2, 3, 4, 5, 6]. The formation of reactive oxygen and reactive nitrogen intermediates is considered the main factor leading to the dysregulation of the inflammatory response [7]. It has been established that free radicals exert their effect at different stages of the inflammatory process by activating nuclear factors that cause the synthesis of cytokines. They contribute to the synthesis of inflammatory mediators and adhesion molecules and exert their toxic effects at the site of inflammation, reacting with various cellular components, causing loss of function and death of cells [8, 9, 10]. There is also data on the negative consequences of redox metabolism violation for the immunoprotection of the body, which can dramatically affect the inflammation course. In particular, redox changes alter the proteome of T cells in a quantitative and qualitative way, and post-translational modifications of surface and cytoplasmic proteins can affect the function of T cells due to an increase in the number of reactive particles [11].

Oxidative stress occurs due to imbalance of prooxidants and antioxidants. The depletion of the antioxidant system functionality is one of the causes of oxidative stress, leading to an avalanche production of free radicals. The organs and tissues with intense metabolic and energy requirements are subject to the most severe oxidative damage [12]. There is considerable evidence of oxidative damage not only to cells, but also to extracellular materials. The extracellular matrix may be more susceptible to oxidative stress compared to cells because it has fewer defense mechanisms. Under the influence of oxidative stress, the matrix can undergo a pathological change in the structure, which will definitely affect the physiology of the cell [13]. The oxidative nature of the extracellular environment is significantly different from the nature of the intracellular compartment. The redox potential of the cytosolic branch of the intracellular medium limits the formation of disulfide bonds, while the oxidative extracellular medium contains proteins rich in disulfide bonds [14].

The reason for the development of oxidative stress is the launch of uncontrolled chain reactions with the formation of free radicals, which are chemically active substances that differ from other compounds in that they have unpaired electrons in their outer orbitals. They are able to destroy cellular components, and the accumulation of data attests to the fact that they can contribute to the development of various diseases, including inflammatory ones [15, 16].

There is data, according to which oxidative stress can be associated with the induction of cell death by stimulating apoptosis and / or necrosis, and increased cell death rate contributes to the formation of a necrotic nucleus, which is a sign of progressive, unstable damage [17].

A huge amount of indirect evidence indicates that free radicals derived from oxygen, especially superoxide and hydroxyl radicals (and hydrogen peroxide to a lesser extent), are mediators of inflammation and / or destruction of tissues in inflammatory disorders [18].

The inflammatory process and oxidative stress arising against it reduce cellular antioxidant ability and generally negatively affect the antioxidant-prooxidant balance in the organism [19,20]. The results of researches conducted by many scientists indicate that an increase in the production of reactive oxygen intermediates accompanies many inflammatory diseases in animals, such as, for example, metritis and mastitis in dairy cows. There are cases of successfully preventing these diseases and increasing the effectiveness of therapeutic measures with the appointment of antioxidant drugs [21, 22, 23, 24].

To date, there is enough information about the participation of free radicals in oncological pathology. A wide range of chronic inflammatory conditions predisposes susceptible cells to neoplastic transformation. In general, the longer the inflammation persists, the higher the risk of cancer developing. A mutated cell is sinusoidal for carcinogenesis. Inflammatory processes can induce DNA mutations in cells through oxidative stress. Inflammatory cells and cancer cells themselves
produce free radicals and soluble mediators, such as metabolites of arachidonic acid, cytokines, and chemokines, which act by further producing reactive intermediates. Reactive intermediate compounds of oxygen and nitrogen can directly oxidize DNA or can interfere with its reduction mechanisms [25].

Antioxidants are powerful free radical scavengers and serve as inhibitors of neoplastic processes. A large number of synthetic and natural antioxidants is known that have a beneficial effect on human and animal health and are used in the prevention of diseases [26]. Therefore, it is advisable to consider the possibility of pharmacological correction of antioxidant status in animals and its inclusion in complex regimens for the treatment and prevention of diseases with inflammatory syndrome. Based on this, the aim of the research was to study the effect of pharmacological correction of antioxidant status in white mice and to assess the impact of the new Antioxidant anti-inflammatory drug for animals on the microstructure of the tissues in the focus of experimental aseptic inflammation, compared to the “Flunidzhek” drug.

2. Experimental part
The experiment was conducted in the vivarium of the Veterinary Medicine Faculty of the Stavropol State Agrarian University. Male white laboratory mice were used in the experiment. They were divided into four groups of 20 animals each, taking into account the principle of analogues. Animals were kept in the same conditions, standard rations corresponding to this species were used for their feeding. The organization of experiments was carried out taking into account the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes and the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes. In all animals, aseptic inflammation was simulated by a single subcutaneous injection of 0.3 ml of purified 20% concentration turpentine oil in the region of the left femoral muscle. The first group of animals served as a control and preventive measures were not carried out. Mice from the second group for five days once daily were intramuscularly injected with ethylmethylhydroxypyridine succinate in the form of a solution with water for injection at the rate of 2.5 mg/kg for the active substance. The choice of this compound is due to the fact that it is a powerful inhibitor of lipid peroxidation processes, neutralizes free radicals, activates superoxide dismutase and glutathione peroxidase, and exhibits antihypoxant and antithrombogenic properties along with antioxidant activity, inhibits platelet aggregation, prevents tissue hemolysis and reduces hemolysis thromboplastin [27]. Animals from the third group were injected intramuscularly with the Flunidzhek drug (OOO “API-SAN”, Russia) for five days in a row once a day in the area of the right femoral muscle at the rate of 2.2 mg/kg, according to the active substance. This drug is a solution containing 83 mg of flunixinemeglumine as an active ingredient in 1 ml. In the fourth group, the Antioxidant anti-inflammatory drug for animals was similarly used (patent RU 2686462 dated 26.04.2019), developed at the Department of Therapy and Pharmacology of Stavropol State Agrarian University. This drug consists of a mixture containing 70 mg of flunixinemeglumine in 1 ml, as well as 150 mg of ethylmethylhydroxyprypidine succinate, 70 mg of ascorbic acid and 50 mg of polyvinylpyrrolidone in the form of an aqueous solution at the rate of 13.6 mg/kg body weight for the active substance.

Six days after the start of the experiment, all animals were sacrificed under mild ether anesthesia by decapitation and the left pelvic limb was dissected. Material of the left extremity tissues of white mice was taken for histological examination. The material was fixed in a 10% buffered formalin solution. Tissue sections 1×0.3 cm were made. Then the material was placed in a pure 10% formalin solution for two hours. The material was stored in six portions of isopropyl alcohol in increasing concentrations for one hour and placed in an environment “Histomix” (“BioVitrum”, Russia) for three hours at t = 62C in a thermostat. The material treated in such way was poured into histological cassettes. Using a microtome, paraffin sections were prepared with a thickness of 0.5 microns and glued to histological glasses. Histological sections were colored with hematoxylin and eosin for review purposes. Digital images were taken from each preparation (in .jpg format, 2592×1944 pixels in a 24-bit palette) of the
selected fields of view at a magnification of 4x/0.65, 5x/0.12, 10x/0.25, 20x/0.45 on a digital microscope Axio Lab.A1 ("CarlZeiss", Germany).

3. Results
The obtained microscopic examination results of the drugs indicate that microsections made from pathological material obtained from first group mice contain tissue fragments with focal purulent inflammation. It is characterized by the formation of a cavity filled with purulent masses represented by following cellular elements: leukocytes, neutrophils (Fig. 1). This inflammation area is limited by a shaft of granulation tissue rich in capillaries, through the walls of which increased leukocyte emigration occurs. Outside, a component of connective tissue adjacent to the unchanged tissue is determined.

This updates the data on the mechanism of inflammatory damage development and confirms the data obtained by Conner E.M. and Grisham M.B. (1996), according to which, phagocytic leukocytes (e.g., neutrophils, monocytes, macrophages and eosinophils) are the most likely sources of free radicals in this case, penetrating the tissue. These reactive radicals and oxidizing agents can damage cells and tissues directly through oxidative degradation of the main cellular components, as well as indirectly injure cells by changing the protease / antiprotease balance, which usually exists in tissue interstitium [28]. There is data, according to which neutrophils secrete an excessive amount of reactive oxygen intermediates in the presence of adequate stimuli. Reactive oxygen intermediates can cause damage to cells and tissues [29].

![Figure 1](image1)

**Figure 1.** Tissue microstructure at the focus of an aseptic inflammatory process (10x/0.25 magnification).

Analysis of microsections made of pathological material obtained from second group white mice indicates the presence of regenerative processes in the focus of aseptic inflammation (Figure 2). Tissue sections with purulent inflammation were observed using the microscope. The sections had slight infiltration of the underlying tissues by leukocytes. At the same time, enhanced proliferation of fibroblasts involved in the formation of collagen fibers was noted.
Figure 2. Tissue microstructure at the focus of an aseptic inflammatory process with ethylmethylhydroxypyridine succinate treatment (magnification 10x/0.25).

The presence of tissue sections with purulent inflammation was observed in white laboratory mice from the third group after considering the microscopic picture while analyzing the microsections made from pathological material obtained from the focus of aseptic experimental inflammation. The inflammation was spreading only at the level of the formed granulation tissue, diffuse foci of leukocyte infiltration are detected in the velum of limited purulent inflammation (abscess) (Figure 3). This can be considered as signs of the formation of a demarcation line, less pronounced leukocyte infiltration than in the first group, but more pronounced than in the second group, while there was a significant number of fibroblasts along the periphery of the pathological process focus, which indicated the development of regenerative processes.

Figure 3. Tissue microstructure at the focus of an aseptic inflammatory process against the background of treating with Antioxidant anti-inflammatory drug for animals (10x/0.25 magnification).

The presence of tissue sections with purulent inflammation was observed in white laboratory mice from the fourth group after considering the microscopic picture while analyzing the microsections made from tissues obtained from the focus of aseptic experimental inflammation. The inflammation was spreading only at the level of the formed granulation tissue, diffuse foci of leukocyte infiltration are detected in the velum of limited purulent inflammation (abscess). In this case, in our opinion, the most optimal microscopic picture was observed, in which minimal leukocyte infiltration was detected, as well as the presence of fibroblast growth and the formation of connective tissue, a clearly formed demarcation line and the capsule formed by it.
We assume that the injection of ethylmethyhydroxypyridine succinate, a compound with a pronounced antioxidant effect, to animals from the second group allowed us to reduce the concentration of free radicals in the inflammation focus and thereby prevented their damaging effect on the affected tissues. When analyzing the results of studies obtained in the third and fourth groups, the conclusion was made that using the non-steroidal and anti-inflammatory “Flunidzhekt” drug based on flunixinameglumen is an effective way to optimize the inflammatory process. Also, the use of the new Antioxidant anti-inflammatory drug that we developed for animals, which additionally contains ethylmethyhydroxypyridine succinate, ascorbic acid and polyvinylpyrrolidone, allows achieving a more significant positive effect. It makes it possible to say that considering the use of antioxidant agents as an adjunct to standard pathogenetic therapy is appropriate in the case of development of local inflammatory processes in the organism of animals.

4. Conclusion

Thus, summing up the experiment conducted, it can be concluded that the normalization of antioxidant status in animals by injecting ethylmethyhydroxypyridine succinate prevents the development of oxidative stress. Therefore, it limits the excessive and uncontrolled overproduction of free radicals, which positively affects the course of the inflammatory process. Injecting the antioxidant anti-inflammatory drug complex for animals allows increasing the inhibitory effect on the inflammatory process, probably due to the content of antioxidant components in its composition in the form of ethylmethyhydroxypyridine succinate and ascorbic acid. This confirms that free radicals are direct participants of the inflammatory process and exacerbate its pathogenesis. The results of the conducted research allow recommending the study of the effectiveness of the ethylmethyhydroxypyridine succinate use, as well as the use of new Antioxidant anti-inflammatory drug for animals in complex regimens for treating diseases of farm animals occurring with the inflammatory process development.

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