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# Intensity Of Protein Oxidative Modification's Processes In The Dynamics Of A Full Thickness Wound Healing In Pharmacotherapy By Gel With Sapropel Extract.

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**ABSTRACT**

Sapropel is a valuable raw material resource of Ukraine, the extraction of which can satisfy needs in different national economies, including health and ecology protection. The advantage of using peloid-based preparations are possibility to use them in the acute phase of the inflammatory process and absence of balneological reactions. The processes of free radical oxidation is a component of metabolic cell activity that occur constantly in living organisms. The cell membranes are composed of proteins that undergo oxidative modification, so the aim of our research was to study the intensity of these processes in the dynamics of a full-thickness excision wound healing in rats when using the gel with sapropel extract under the provisional name "Saprogel". The study of the process intensity of protein oxidative modification was performed in the dynamics of a thickness excised plane wound healing in pharmacotherapy with the with sapropel gel, carbopol-based gel and the reference sample "Pantestin-Darnitsa" (Ukraine), comparing them with indicators of intact animals and control pathology groups. In the serum of rats determined the content of the protein oxidative modification products and the level of sulfhydryl groups. It is revealed that in a full-thickness excision wound under the conditions of using the drug "Saprogel" in blood serum a decrease in the level of protein molecule oxidation and the restoration of the sulfhydryl group content, a decrease in the activation of free radical processes and an increase of antioxidant protection are detected, which provide the stabilization of cell membranes, can be due to the pronounced antioxidant properties of sapropel extract.
**Keywords**: peloids, sapropel, saprogel, protein oxidative modification, antioxidant activity.

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## INTRODUCTION

The economic potential of Ukraine in the conditions of commodity market formation is largely determined by the degree and completeness of using local raw materials, among which are organic lake sediments - sapropels. Due to their large reserves, relative simplicity of extraction, high quality properties, they are valuable natural raw materials to meet the needs of various sectors of the economy, including medicine, veterinary pharmacy and pharmacy [1].

The issue of protecting and improving the ecological status of lakes remains important [2]. Extraction of sapropel improves the ecology, hydrological status of lakes, restores their recreational capacity and simultaneously solves the problem of their conservation [1,2]. Therefore, the development of sapropel deposits and the optimization of their rational complex use have great economic and scientific importance, which makes the relevance of this study.

The advantage of using peloid-based drugs is the ability to use them in the acute phase of the inflammatory process, which is known to be unacceptable for traditional peloid therapy [3,4]. In addition, the therapeutic efficacy of drugs based on biologically active components of peloids is significantly increased due to the lack of balneo-pathological reactions, which often cause the interruption of treatment with native mud applications [5].

The main group of biologically active substances in sapropels is humic substances (HS), the content of which in sapropel is in the range - from 6.7 to 71.2% in terms of organic matter. More than half of them are composed of humic acids. [6]. Humic substances (HS) are heterogeneous, reactive organic macromolecules that play an important role in redox reactions [7].

The antioxidant activity of peat humic acids and sapropel deserves special attention because it is caused by a number of their structural features. Aromatic cores of humic acids contain a large number of carboxyl and quinoid groups, which are catalysts for redox reactions and cause antioxidant activity. [8]. Antioxidant property exhibited by humic substances and its fractions were recorded in research work carried out by Avvakumova et *al*. [9].

The basic structural units of humic acids are polycarboxylic acids, amino acids, amides, amino sugars, carbohydrates, phenoxy acids and others. Humic acids contain about 15 different types of functional groups, among which the most important are amino groups, amide, alcohol, aldehyde, carboxyl, carboxylate, ketone, methoxy, phenolic, quinone, hydroxyquinone and others [8, 10]. However, phenolic and carboxylic groups are most prevalent in humic acid structures which are responsible for their antioxidant and anti-inflammatory properties. Quinone groups are responsible for wound healing and have fungicidal / bactericidal properties [11].

Humic acids have been shown to enhance skin wound healing, oral cavity wound healing and exhibit antibacterial properties [12].

The processes of free radical oxidation are a component of the cell metabolic activity that are constantly occurring in living organisms. The main activation mechanism of free radical processes is to increase the formation of active oxygen forms (AOF). AOFs can have a distinct toxic effect on cell structures, which is mainly due to the further stimulation of the free radical oxidation processes, which leads to the development of oxidative stress, which is manifested by the accumulation of toxic products, the damage of molecules, cell membranes, tissues, the decrease in reparative processes in the wound, etc. [13,14].

The cell membranes, except lipid components, include proteins that undergo oxidative modification, so the aim of our research was to study the intensity of these processes in the dynamics of a full thickness excision wound healing in the rats when using the gel with sapropel extract under the conditional name "Saprogel".

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## MATERIALS AND METHODS

### Materials

The gel with sapropel extract (SE) under the conditional name "Saprogel", carbopol-based gel and comparator product “Pantestin-Darnitsa” gel («Darnitsa», Ukraine) were the materials of this research.

“***Saprogel”*** is gel test containing 15% aqueous SE, was prepared according to the following technology: potassium sorbate (preservative) was dissolved in purified water, the pre-weighed carbomer Ultrez 10 was added and left to swell for 30-60 minutes. During the swelling periodically the mixer was switched on and mixed at the speed of 60-90 rot/min. Gradually the SE was added and homogenized at the speed of 60-90 rot/min during 5-10 minutes until obtaining a homogeneous gel. The aqueous sapropel extract (SE) was obtained from Prybych deposit sapropel, Volyn region, Ukraine. The sapropel was treated with 0.1 N alkali solution. Cavitation was used at the temperature of (50 – 60) °C and the speed of 3000 rot/min for 60 minutes for obtaining a homogeneous mixture. The obtained extract was evaporated to 1:10 of basal volume [15,16].

The carbopol-based gel containing carbomer Ultrez 10, potassium sorbate and purified water was prepared accordance with the technology of "Saprogel", using trometamol as a neutralizing agent.

The comparator product, "Pantestin-Darnitsia" gel (“Darnitsa” Ukraine), contains such active pharmaceutical ingredients as dexpanthenol and miramistin. "Pantestin-Darnitsia" gel is used for the treatment of wounds of different localization and genesis in II-nd phase of wound healing process [17].

### Methods

Investigation of the biochemical blood parameters of experimental animals was performed in the laboratory of molecular biology and clinical biochemistry of the Institute of Animal Biology of the National Academy of Agrarian Sciences.

In the serum of rats determined the content of protein oxidative modification products and the level of sulfhydryl groups.

### Determination of the content of protein oxidative modification products

The content of protein oxidative modification (POM) products and oligopeptides was determined by the level of carbonyl derivatives, which are detected in the reaction with 2,4-dinitrophenylhydrazine [18].

### Determination of sulfhydryl group level

The level of total, protein-bound and non-protein sulfhydryl (SH) -groups was measured by the Ellman’s method [19].

### Statistical Analysis

The hypothesis testing of the normal data distribution was conducted by way of the Shapiro-Wilk test using the software package GraphPad Prism 5.04 (GraphPad Software Inc., USA). Further result calculation was performed using a two-way ANOVA with Bonferroni post hoc test. For each obtained result the arithmetic mean (M) and the standard error of the arithmetic mean (m) were determined. Difference between means was considered as statistically significant if p is less than 0.05.

## EXPERIMENTAL

Studies of the intensity of protein oxidative modification (POM) processes were performed in the dynamics of a full thickness excision wound healing in pharmacotherapy with sapropel gel, carbopol-based gel and the reference sample "Pantestin-Darnitsa" (Ukraine), comparing them with indicators of intact animals and control pathology groups.

A model of full thickness excision wounds was reproduced on pre-depilated skin in anesthetized rats. For this, the skin was excised using a surgical scalpel and forceps 1 × 1 $cm^2$ in size. The bleeding was stopped with sterile gauze swabs and 3% hydrogen peroxide solution. Treatment was started as soon as the wound was restored and fully healed [20,21].

The studies were performed on male white laboratory rats (200-250 g), n = 119. The animals were kept in the vivarium of Danylo Halytskyi National Medical University under standard conditions of temperature (21°C), light (12/12 h), humidity and diet (complete feed for laboratory animals K - 12-4, "Rizan-1", Ukraine), according to the "Standard rules for the arrangement, equipment and maintenance of experimental biological clinics".

The experiments were conducted in accordance with ethical principles adopted by the First National Congress of Ukraine on Bioethics [22], international agreements, national legislation in this field [23-26]. The animals were treated humanely throughout the study period adhering to the guideline for use and care of animals in declaration of Helsinki (National Research Council, 2011).

The experiment design and study protocol were approved by the Animal Ethics Committee of the Danylo Halytsky Lviv National Medical University, protocol No.3 December 10, 2019.

Before the experiment, the rats were quarantined, after which the laboratory animals were examined and weighed. Animals were individually noticed by bringing on cuts on the ear lobes. All pain procedures and surgeries were performed under general anesthesia with thiopental sodium (Thiopental sodium, Biochemie GmbH / Austria) at a dosage of 60 mg / kg animal weight. Animals were kept in individual cages under standard vivarium conditions throughout the whole experiment.

Animals were divided into 5 groups:

I. intact group of rats, in which the physiological level of the studied parameters was measured (n = 7);
II. group of rats, in which a full thickness excision wound was simulated, healing occurred independently, without treatment (n = 28);
III. group of rats, in which a full thickness excision wound was simulated, the affected area was treated with a carbopol-based gel (n = 28);
IV. experimental group of animals with experimental full thickness excision wounds, which the developed "Saprogel" was applied on the affected area (n = 28);
V. experimental group of animals with experimental full thickness excision wounds, which the comparator product “Pantestin-Darnitsa” gel was applied on the affected area (n = 28).

Daily, starting from 1-st day after wound modeling and until complete healing, the test drugs were applied to the wound surface of animals in groups 3, 4 and 5.

From II – V groups of rats on the $3^{rd}$, $7^{th}$, $20^{th}$ day of the wound process were selected 7 animals for the study of intensity of protein oxidative modification’s processes in serum. There were 7 animals left in these groups. The material for hematological studies was taken on the 3rd, 7th and 20th days,

From II – V groups of rats on the $3^{rd}$, $7^{th}$, $20^{th}$ day after the start of treatment were selected 7 animals and the material for hematological studies was taken. Study of intensity of protein oxidative modification’s processes in serum, reflecting the process of wound healing at different stages of inflammation. They were extracted from the experiment by decapitation on the day of the complete scarring of the wound bed [21].

**Blood serum collection**

The serum of experimental rat blood was obtained from the whole blood. Blood was left at 37 ° C for 4 hours to remove fibrinogen and concomitant proteins. At the next stage, a clean dry glass stick used to carefully separate the blood clot from the test tube walls to speed up the production of serum and centrifuged at 2000 rpm for 40 minutes. The supernatant (serum) was quickly separated from the formed blood elements, transferred the Eppendorf and frozen at -20 ° C until further use [21,27].

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## RESULTS AND DISCUSSION

Dynamics of lipid peroxide oxidation (LPO), protein peroxide oxidation (PPO) and antioxidant system (AOS) level indicators shows an increase or decrease in inflammatory reaction [28]. According to the literature, under oxidative stress condition, active oxygen forms (AOF) damage all biological structures [29].

In particular, under conditions of AOF excessive generation, the processes of uncontrolled protein modification develop, causing protein fragmentation, their denaturation, and formation of primary amino acid radicals, which subsequently interact with neighboring amino acid residues and this generally creates a rather complicated picture of the damaging effect of AOF on protein macromolecules. All this leads to loss of biological activity by proteins and impaired metabolic processes, including regenerative processes [30].

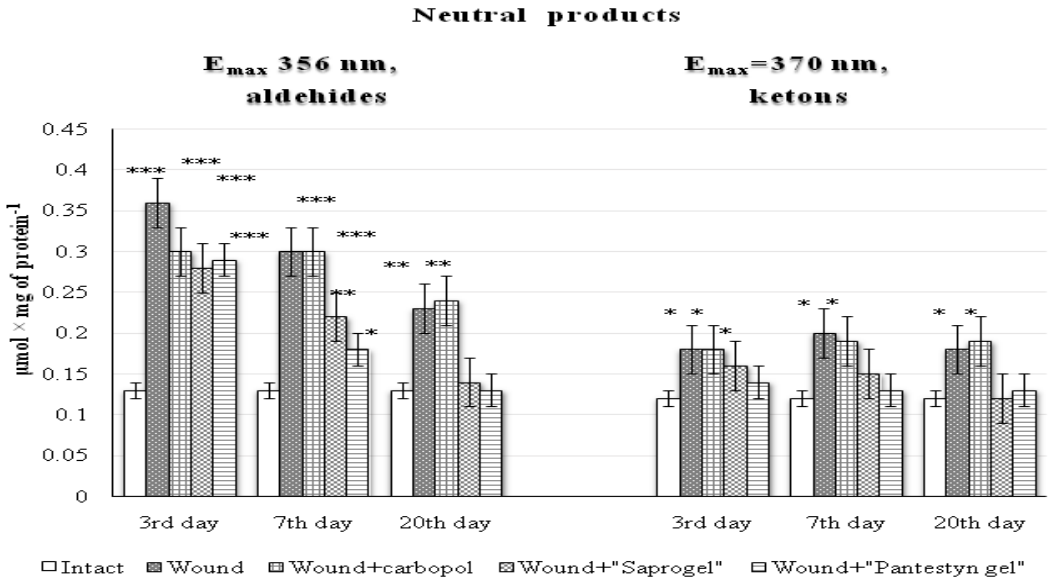
It is known that the recovery of oxidized proteins is almost non-existent. They become targets for the action of specific neutral and alkaline proteases, the activity of which depends on lots of factors [31]. In addition, it is considered that POM products are a source of free radicals which deplete the antioxidant reserves in the organism and provide the negative effect. It has been shown *in vitro* that the products of free radical oxidation of proteins lead to oxidative damage to DNA. In this case, protein peroxidation is not only the trigger mechanism of pathological processes, but also the earliest marker of oxidative stress [32].

According to the results of previous studies, treatment of full thickness excision wounds in rats with Saprogel gel reduced the healing time to 18.2 ± 1.3 days, ie by 24.5% ($p < 0.05$. Saprogel showed wound healing action in all phases of the healing process with the most pronounced effect in phase II and phase III of wound healing) [21].

The action of Saprogel in rats with full thickness excision wounds in blood serum reduces the content of LPO products and normalizes the activity of enzymes antioxidant protection of SOD and CAT, indicating that one of the mechanisms of wound healing action of sapropel is its antioxidant properties [21].

**Determination of the content of protein oxidative modification products**

**Figure 1: The content of POM products - neutral nature products - in blood serum of rats with a full thickness wound, µmol × mg protein$^{-1}$**



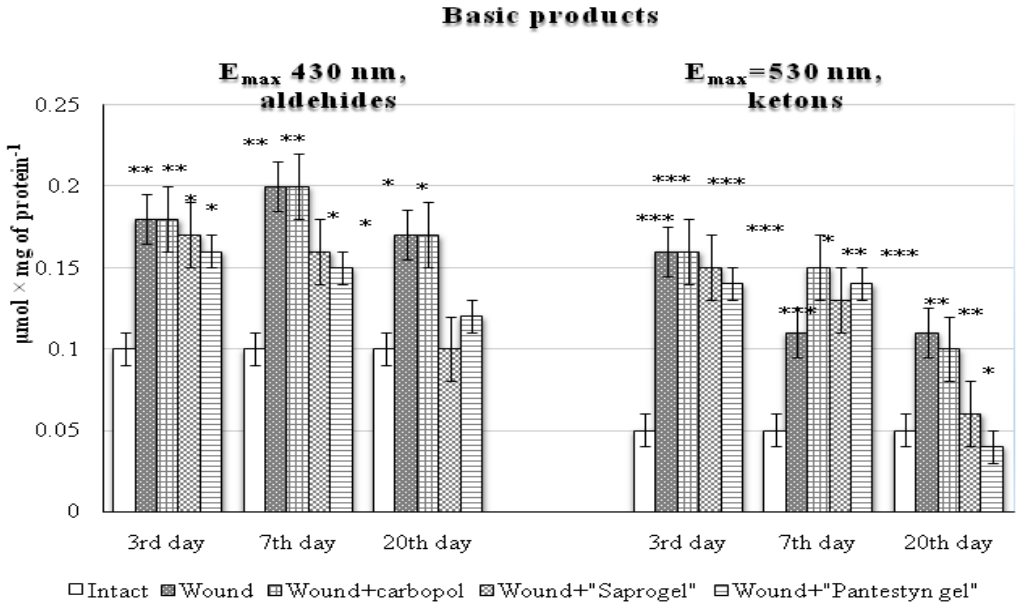

*** - p <0.05, ** - p <0.01, *** - p <0.001 compared to the intact group of animals**

In the course of the experimental studies, it was found that an increase in the level of redox proteins in blood serum is showed in the rats with full thickness excision wounds. Thus, the level of neutral aldehyde (max. absorbance at 356 nm) products is increased on the 3rd and 7th day by 2.5 and on the day 20 by 1.9 times relative to the control. Also, an increase in the content of neutral ketone ($E_{max}$ = 370 nm) products in

blood serum was detected on day 3 – by 1.3 times, on the 7th day - by 1.6 times and on the 20th day - 1.5 times compared to the control (Figure 1).

It is shown that the number of basic aldehyde (maximum absorption at 430 nm) products in a full thickness excision wound in blood serum of rats is increased on the day 3 – by 1.9 times, on the day 7 – by 2.2 times and on the day 20 – by 1.9 times relative to the control. Under the same experimental conditions, the level of basic ketone (E max = 530 nm) products is increased on the 3rd and the 7th days – by 3.5 times and on the 20th day – by 2.3 times relative to the intact group of animals (Figure 2).

**Figure 2: The content of POM products - basic nature products - in blood serum of rats with a full thickness excision wound, µmol × mg protein$^{-1}$**




*** - p <0.05, ** - p <0.01, *** - p <0.001 compared to the intact group of animals.**

In the group of rats with a full thickness wound, which was treated with carbopol, an increase in the level of red-modified proteins was observed in the serum, similar to that in the group of animals with experimental wound (Figures 1, 2).

When using "Saprogel" in blood serum there is a decrease in the content of both neutral aldehyde and ketone products, as well as the main aldehyde and ketone products of protein oxidative modification (Figures 1, 2).

**Determination of the sulfhydryl group level**

An important criterion for protein modification is the oxidation of their sulfhydryl groups, which can occur both directly and enzymatically through the involvement of hydroperoxides and lipid hydroperoxides. The rate and nature of sulfhydryl group oxidation depend on lots of factors: reagent concentration, the pH value, temperature, pKa, spatial location of SH groups in protein and their microenvironment [33].

Under these conditions, a decrease in the level of protein and non-protein sulfhydryl groups is observed in blood serum, indicating their damage by free radicals.

In animals with a full thickness excision wound the content of sulfhydryl groups is reduced in blood serum. Thus, the level of non-protein SH-groups decreased on the 3$^{rd}$ day - by 1.8 times, on the 7$^{th}$ day - by 1.9 times and on the day 20- by 1.4 times relative to control (Figure 3).

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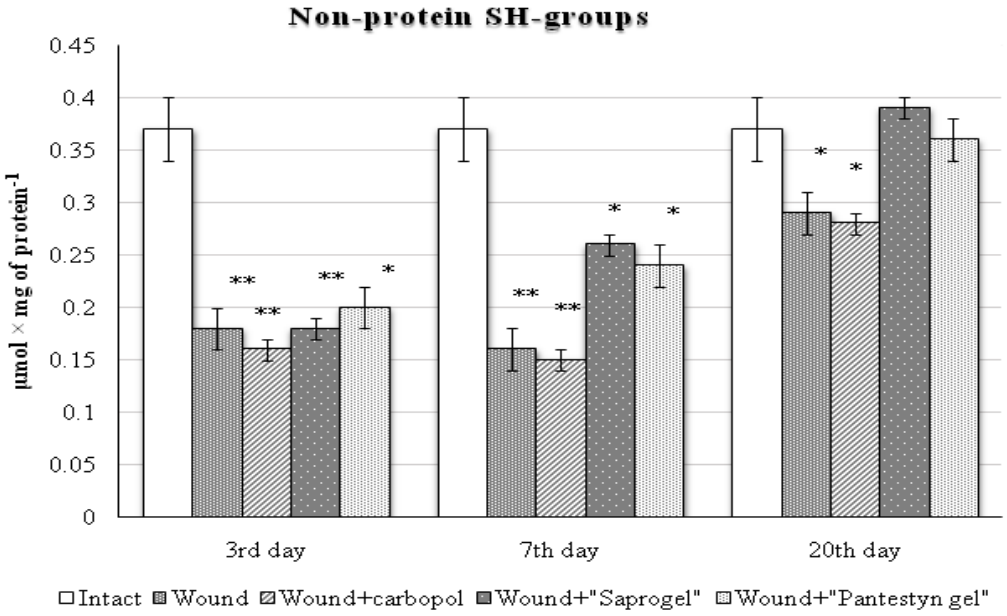
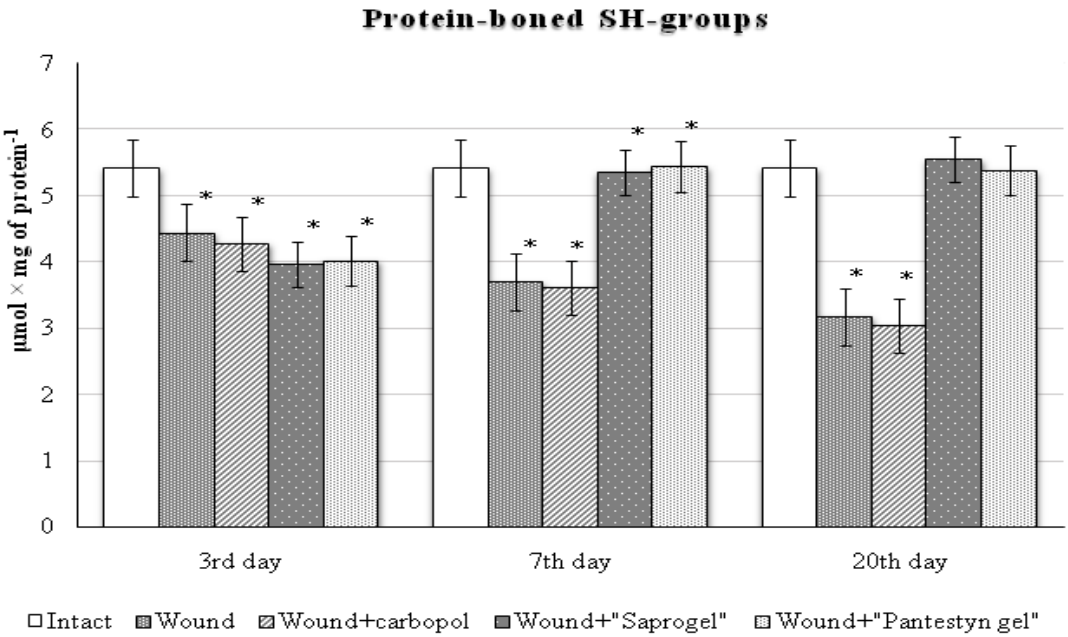
**Figure 3: The content of non-protein sulfhydryl (SH-) groups in blood serum of rats with full-thickness wound, µmol × mg of protein$^{-1}$, M± m; n=7**




*** - p <0.05, ** - p <0.01, *** - p <0.001 compared to the intact group of animals**

The content of protein-bound SH-groups decreased on day 3 - by 1.3 times, on day 6 - by 1.6 times and on day 20 - by 1.4 times compared to the intact group (Fig. 4).

**Figure 4: The content of protein-bound sulfhydryl (SH-) groups in blood serum of rats with full-thickness excision wound, µmol × mg of protein$^{-1}$, M±m, n=7**




*** - p <0.05, ** - p <0.01, *** - p <0.001 compared to the intact group of animals**

In the conditions of the experimental wound, the level of total SH-groups decreased on day 3 – by 1.3 times, on day 7- by 1.2 times and on day 20- by 1.4 times relative to the control (Figure 5).

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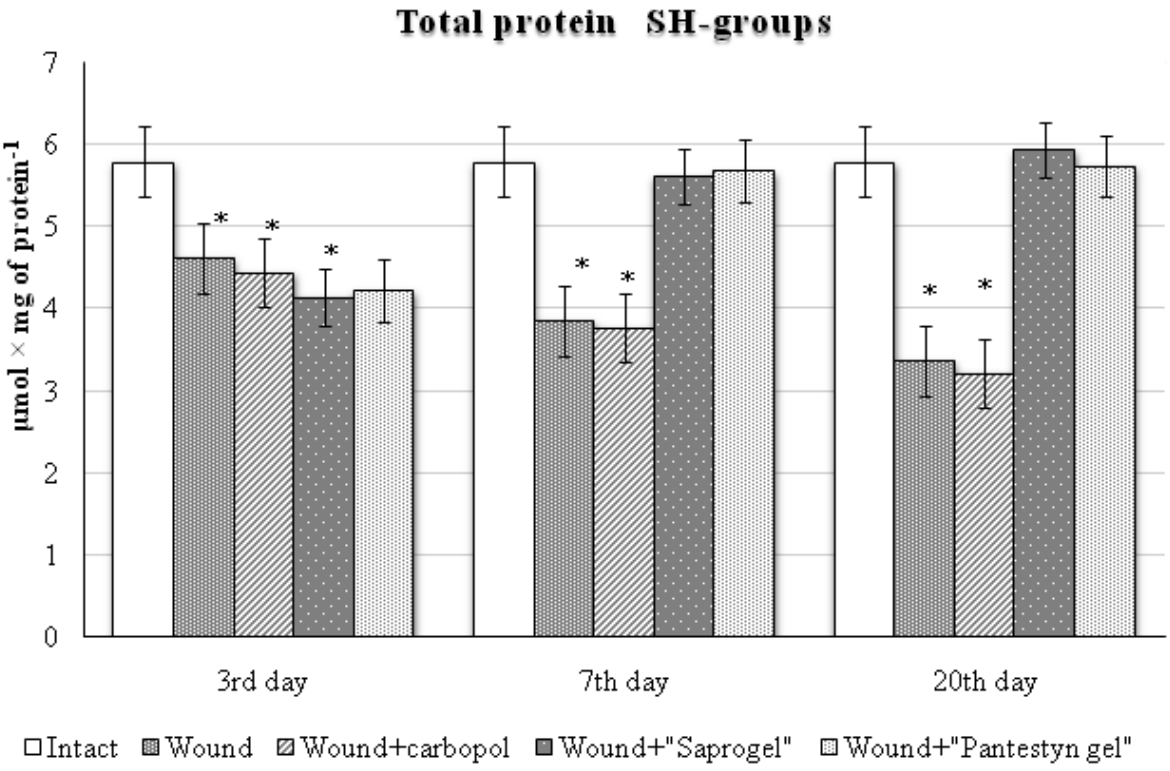
**Figure 5 : The content of total protein sulfhydryl (SH-) groups in blood serum of rats with full-thickness wound, µmol × mg of protein$^{-1}$, M±m, n=7**




*** - p <0.05, ** - p <0.01, *** - p <0.001 compared to the intact group of animals.**

Similar changes in the level of sulfhydryl groups were found in blood serum in the study of carbopol effects. When using «Saprogel» in blood serum, the content of sulfhydryl groups is restored (Figures 3-5).

The obtained results show that the level of free radicals increases in a full thickness excision wound in blood serum, which leads to the depletion of the level of non-protein low molecular thiols (cysteine, glutathione, etc.) and inhibition of the activity of thiol enzymes by blocking their sulfhydra groups. The decrease in total, protein and non-protein SH groups under experimental full thickness excision wounds reflects the overall shift of the redox balance to the prooxidant side.

The observed decrease in the content of sulfhydryl groups in blood serum of the rats under the conditions of wound modeling indicates the accumulation of covalent bonds with the participation of cysteine and methionine, as well as the oxidation of SH groups.

## CONCLUSIONS

Detected decrease in the level of protein oxidation molecules in blood serum when using "Saprogel" on the basis of sapropel extract in the rats with an experimental model of a full thickness excision wound, may be associated with pronounced antioxidant, antibacterial, anti-inflammatory and regenerative properties of sapropel extract.

It is revealed that with a full thickness excision wound under the conditions of using the drug "Saprogel", the content of sulfhydryl groups is restored in blood serum, the activation of free radical processes is reduced and the antioxidant protection is increased, providing stabilization of cell membranes.

## ACKNOWLEGMENTS

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