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DISSERTATION

THE MECHANISM OF DERMATOTROPIC ACTIONS OF NANOCRYSTALLINE CERIUM DIOXIDE

03.00.04 – Biochemistry

Submitted for obtaining a scientific degree of the candidate of biological sciences
The thesis contains results of own research. Using ideas, results and texts of other authors have a link to the corresponding resource.

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SUMMARY

Arefeh Amiri, THE MECHANISM OF DERMATOTROPIC ACTIONS OF NANOCRYSTALLINE CERIUM DIOXIDE. - Qualifying scientific work on the rights of manuscripts.

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The work is devoted to the study of dermatotropic effects of nanoceria with its pharmaceutical compositions in wound healing process on rats with full-thickness wound model.

It has been established that at all times of observation, the area of full-thickness wound under the action of the nanocrystalline cerium dioxide (Nanoceria)-based pharmacological composition was significantly less than the wound area without application of the composition. On the 6th, 9th and 20th day of experiment nanoceria-based composition treated wounds areas were by 20,1% ($p<0.05$), 37,5% ($p<0.05$) and by 34.0% ($p<0.05$) accordance decreased in comparison with control. Complete epithelialization of the wounds under the influence of the pharmacological composition occurred more quickly for 3 days (in intact group of rats it took 23.0±0.8 for complete wound closure and 20.0±0.5 for experimental group).

It has been established that a pharmacological composition containing 0.05% Nanoceria dissolved in 0.5% carbopol has a bactericidal effect on the test cultures of Staphylococcus epidermis, Staphylococcus aureus and Pseudomonas aeruginosa, and the fungistatic effect on the test culture of yeast fungi of the genus Candida.

After healing untreated full-thickness dermal wounds in homogenate of the former skin injuries of untreated wounds moisture content decreased but collagen content, smelted gelatin content and temperature of welding of a skin were increased.

After the use of Nanoceria-based pharmacological composition all of the above indicators returned to the level of intact rats that explains the healing without the formation of a rough scar at the site of the full-thickness skin wound.

We have also done the investigation on the level of endogenous intoxication by measuring the concentration of C-reactive protein and the level of Middle-mass molecules in blood serum in rats without wound treatment and in rats after wound treatment with nanoceria-based composition application. It was established that on the 3rd, 6th, 9th, 14th and 20th days after wounds modeling the concentration of C-reactive protein and the level of Middle-mass molecules in blood serum were significantly increased. Nanoceria-based composition decreased the concentration of C-reactive protein and the level of Middle-mass molecules in blood serum in blood serum of rats after injury.

According to our experiments, it was demonstrated the development of full-thickness wound is accompanied by an increase in the processes of lipid peroxidation (LPO) in the blood serum of rats. Also, we have shown the changing in the activity of antiradical enzymes (superoxide dismutase activity decreases and catalase increases), which is associated with the intensification of the formation of active forms of oxygen. It was found that in the case of full-thickness wounds under conditions of nanoceria-based composition application in experimental group of rats, the oxidative-antioxidant balance is restored in blood serum, as shown by the decrease in the products of the LPO and the normalization of antiradical enzymes activity.

In the rats with full-thickness wounds we have shown a decrease in the content of protein, non-protein and total sulfhydryl groups in blood serum, indicating that the level of free radicals increases in serum on the wounded surface area, which leads to the depletion of the level of non-protein low molecular weight thiols (cysteine, glutathione, etc.) and inhibition of the activity of thiol enzymes by blocking their sulfhydryl groups (glutathione peroxidase, glutathione transferase, glutathione reductase). Reduction of the total, protein and non-protein SH groups in this experiment (intact group) reflects the overall displacement of the redox-balance in

the pro-oxidant side. When nanoceria-based composition was applied to wounds, the restoration of sulfhydryl groups content in blood serum was observed that is evidence of antiradical property of nanoceria.

We have shown the effect of nanoceria-based composition was significant on some growth factors, such as, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), matrix metalloproteinase-2 (MMP 2), matrix metallopeptidase-9 (MMP 9) and hypoxia-inducible factor 1α (HIF 1α). Results demonstrated the positive influence of nanoceria-based composition on the abovementioned factors by having a great influence on their regulation in the healing process. The level of VEGF was well regulated in experimental group which means it was upregulated (expressed) gradually and reached its peak for accelerating the angiogenesis and then downregulated to reach the baseline level faster when compared to the control group. For NGF the same result as VEGF has been obtained, where upregulation and downregulation happened faster in experimental group which shows the wound healing acceleration by the help of Nanoceria. For MMP-2, after injury the level of this factor should increase in order to accelerate the healing process and goes back to baseline level when getting close to the complete healing. We have shown in experimental group this process happens in a faster and more regulated manner where in control group it took more time as dysregulation was seen. For MMP-9 expression and then reaching to the baseline level happened more quick in experimental group compared with control group. For HIF-1α, in control group of rats, overexpression of this factor was seen from 3rd day where it made it difficult and time consuming to reach the baseline level, while in experimental group the normal expression and downregulation was seen that makes it obvious for a faster wound healing.

Key words: full-thickness wound, nanoceria-based composition, C-reactive protein, Middle-mass molecules, collagen, antimicrobial action, lipide peroxidation, matrix metalloproteinases, growth factor, hypoxia-inducible factor 1α.

List of publications on the topic of the dissertation

1. Arefeh Amiri, Nikitina NS, Stepanova LI, Berehovyi SM, Beregova TV, Spivak M Ya. Effect of cerium dioxide nanocrystal (Nanoceria) on the concentration of some growth factors during the wound healing process in rat model. Research Journal of Pharmaceutical, Biological and Chemical Sciences (RJPBCS). March - April 2019; 10(2): 1537-1555.

2. Arefeh Amiri, Nikitina N.S., Stepanova LI., Beregova T.V., Potential impact of Cerium Dioxide Nanoparticles (Nanoceria) on the concentration of C-reactive protein and Middle-mass molecules after wound treatment. ScienceRise: Біологічні науки. 2019; 1: 14-19.

3. Arefeh Amiri, Radziminska O.V., Vereschaka V.V., Stepanova L.I., Beregova T.V. The influence of nanocrystalline cerium dioxide on physical and chemical properties of rat skin. Вісник проблем біології і медицини. 2019; Вип. 1, Том 2 (149): 108-111.

4. Arefeh Amiri, Beregova T., Nikitina N., Stepanova L. The influence of Cerium Dioxide Nanocrystal (Nanoceria) on full-thickness wound healing process. Вісник Київського національного університету імені Тараса Шевченка, Серія: Проблеми регуляції фізіологічних функцій. 2018; 1 (24): 6-11.

5. Arefeh Amiri, Beregova TV, Nikitina NS, Stepanova LI, Vovk A, Kondratiuk TO, and Spivak M Ya. Cerium Dioxide Nanocrystal (Nanoceria): A Promising Pioneer Therapeutic Agent in Wound Healing Process. Research Journal of Pharmaceutical, Biological and Chemical Sciences (RJPBCS). July–August 2018; 9(4): 859-871.

6. Arefeh A. Antimicrobial effect of Nanoceria in wound healing process / Arefeh Amiri, Beregova T., Kondratiuk T. // "Biological markers in fundamental and clinical medicine" collection of abstracts, March 2018: book of abstracts. - Prague, the Czech Republic. –p. 48-49.

7. Arefeh A.The effect of cerium Dioxide on cut-wound healing // The Bukovinian International Medical Congress (BIMCO), Book of abstracts, 2018: Chernivtsi, Ukraine. - p. 319.

8. Arefeh A. Potential impact of Cerium Dioxide nanocrystal(Nanoceria) on pro- and antioxidant system in blood serum of rats with full-thickness wounds / Arefeh Amiri, Nikitina N., Vovk A., Nagorniak E., Riazhkova D., Beregova T. // Biosciences Advance 2018 “XVI International Scientific Conference of Students & Young Scientists” book of abstracts, 2018: Kiev, Ukraine. - p. 113-114.

9. Amiri A. Correction parameters of endogenous intoxication in experimental cut-wound / Amiri A., Medvedieva N., Taburets O., Nikitina N.International scientific and practical conference «Actual questions of medicine and biology» // 30.05-1.06.17: book of abstracts, 2017: Poltava, Ukraine – p. 5-6.

CONTENT

LIST OF CONDITIONAL ABBREVIATIONS

ABD - antibacterial drug

AOS - antioxidant system

CAT - Catalase

CRP - C-reactive protein

GR - glutathione reductase

GP - glutathione peroxidase

GT - glutathione transferees

HIF 1α - hypoxia-inducible factor 1α

LPO - lipid peroxidation content

MDA - malondialdehyde

MMP - matrix metallopeptidase

MMM - middle-mass molecules

Nanoceria - nanocrystalline cerium dioxide

NGF - nerve growth factor

ROS - reactive oxygen species

SOD - superoxide dismutase

TBARS - Thiobarbituric acid reactive substances

VEGF - vascular endothelial growth factor

INTRODUCTION

Actuality of theme.

One of the actual problems of modern pharmacology is the creation of new wound healing agents with expressive anti-inflammatory, antimicrobial, reparative properties and at the same time lack of toxic effects on the body. This is due to the presence of large number of patients with wounds and skin damage that require both surgical, and local treatment. Annually millions of surgical wounds are created during routine surgical operations [World Health Organization, 2014]. The development of such drugs has become even more relevant in connection with undeclared war in the East of Ukraine, which led to an increase in the number of damages to the skin and wounds of various etiologies are accompanied with infections, and / or antioxidant skin condition and inflammation [1, 2] The consequence of this is the oxygen starvation of the skin, a violation in reparative mechanisms, with subsequent formation of keloid scars, and in in some cases, the development of gangrene [3].

Skin defects, such as ulcers, burns, surgical wounds or wounds due to injuries, cause colonization with a wider range of bacteria [4]. Microbial spectrum of pathogens of infection is characterized by growth resistance to existing medicines. At the same time treatment of patients is further complicated by the presence of factors that lead to complications and intoxication of the organism (age, presence of infections, diabetes, vascular diseases, cancer) [5]. The rates of widespread multifactorial medicines encourage the search for more effective dermatotropic agents and optimize treatment. Therefore, today, the efforts of scientists from different countries are aimed at developing new agents in medical forms for the treatment of wounds [6].

Wound healing in skin is an evolutionarily sustained, highly interconnected as well as regulated process. It takes place over the incessant yet overlapping phases of hemostasis, inflammation, proliferation, and remodeling [7, 8]. The skin is the organ most challenged by multiple extrinsic stress factors, resulting in frequent cell and

barrier damage. As such, skin has constructed a set of complex mechanisms to safeguard itself and to restitute tissue entirety when damaged, without resulting in septicemia [7]. Demographically, the number of patients enduring chronic wounds and bad healing conditions is reaching epidemic ratio and will become even more severe in both human health and economic terms [9, 10]. Penurious wound healing after trauma, surgery, acute illness, or chronic disease conditions affects millions of people worldwide each year and is the consequence of poorly regulated elements of the healthy tissue repair response, including inflammation, angiogenesis, matrix deposition, and cell recruitment [7]. The failure of many recent approaches to render specific results such as wound closure, control of fluid loss, and presenting properties such as durability, elasticity, and histocompatibility has conducted to the introduction of numerous nano-technological advances [11, 12]. As a result, nanoparticles have a capability to deliver a maintained and controlled deliverance of therapeutics that eventuates in an accelerated healing process [13, 14].

Cerium is a rare earth metal that, when incorporated with oxygen, can adopt a fluorite crystalline net structure that has an extremely reactive surface area for neutralization of radicals. In addition, nanoceria can reversibly bind oxygen and shift oxidation states (Ce3+/Ce4+) depending on the situations [15, 16]. The use of nanoceria for therapeutic goals provides several benefits over other novel antioxidant approaches. For example, the delivery of nanoencapsulated antioxidant enzymes, such as SOD or catalase, has the restriction that only one sort of reactive oxygen species can be scavenged by each enzyme, whereas, several species are involved in neurodegenerative diseases [17] and nanoceria have been shown to decline their levels [18, 19].

Because of these properties, nanoceria have been investigated in biological systems and shown to exhibit antioxidant effects in various models of disease [20]. In contrast, nanoceria exhibits both catalase and SOD- mimetic activity with SOD-mimetic catalysis overpass that of the endogenous enzyme [21-26].

Depending on the size of the nanoparticles, Nanoceria displays oxidative and

reductive properties, which is paralleled by changes in its toxicity [27]. The neuro- and radio-protective and antitumor effects of Nanoceria are described [28, 29]. Cerium salts are utilized in the treatment of burn wounds [30].

The antioxidant property of cerium oxide nanoparticles (Nanoceria) has been investigated recently; nanoceria were noticed to salvage superoxide radical [31, 32], hydrogen peroxide [33], hydroxyl radical [34] and nitric oxide radical [35]. Therefore, Nanoceria have been tested in biological systems wherein they can defend tissues against radiation induced damage [36], defend against laser- induced retinal damage [37], increase lifespan of photoreceptor cells [38], decrease spinal injury [39], reduce chronic inflammation [40] and elevate angiogenesis [41].

Such mixed valence state enables CeO2 nanoparticles (CeO2NPs) to have excellent catalytic activities and to act as free radical scavengers [42].

All this served as the basis for the creation of a Ukrainian dermatological drug based on nanocrystalline cerium dioxide and the study of mechanisms of its dermatotropic action.

Relationship of work with scientific programs, plans, themes.

The dissertation work was performed at the Laboratory of Pharmacology and Experimental Pathology of the "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv within the framework of the Research topic "Pre-clinical studies of the toxicity of melanin substance for new medicines and efficiency of dermatotropic drugs based on nanoparticles" (Number of State Registration 0116U004828, 2016 - 2017).

The topic of the dissertation is approved by the Academic Council of the Educational and Scientific Center "Institute of Biology" of Taras Shevchenko National University of Kyiv, protocol № 5 of November 14, 2016.

The purpose and tasks of the study. The aim of the work was to find out the molecular-biochemical mechanisms of the action of a Nanoceria-based pharmacological composition in the form of full-thickness wound model in rat's skin.

In accordance with the goal, we had set the following tasks:

1. To investigate the duration of healing and to determine the area of full-thickness excised plane wounds in the process of self-healing and after application of a pharmacological composition based on nanocrystalline cerium dioxide.

2. To investigate the antibacterial properties of the pharmacological nanoceria-based composition using test cultures of bacterial strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and yeast fungi of the genus *Candida albicans*.

3. To determine the physical and chemical properties of skin in rats after comlete closure of full-thickness excised plane wounds without and using a pharmacological nanoceria-based composition.

4. To determine the influence of pharmacological nanoceria-based composition on the level of C-reactive protein and the concentration of Middle-mass molecule in blood serum.

5. Determine the intensity of lipid peroxidation by the content of diene conjugates, TBA-acid products, Schiff bases in blood serum and evaluate the state of the antioxidant system by the activity of superoxide dismutase and catalase, and determine the content of SH-groups and products of oxidative modification of proteins during wound self-healing and after using a pharmacological nanoceria-based composition

6. To determine the content of growth factors (VEGF, NGF), metalloproteinases (MMP-2, MMP-9) and hypoxia-inducible factor 1α (HIF1α) in the wound bed homogenate after self-healing and after application of nanocrystalline ceric dioxide-based pharmacological composition.

*Object of research***:** biochemical mechanisms of skin injury and restoration of the full-thickness wound model.

*Subject of research***:** biochemical and physico-chemical properties of the skin in conditions of experimental full-thickness wound model, justification of correction of

these changes with the help of a Nanoceria-based pharmacological composition.

Methods of investigation. Spectrophotometric (determination of free radicals content, lipid peroxidation content, protein modification, enzymatic activity of pro- and antioxidant enzymes), fluorescence (determination of the schiff bases content), planimetric (wound area , healing rate), physical and chemical (moisture content and collagen, gelatin release, thermal welding), ELISA measurement (growth factors, MMPs, HIF 1α), turbidimetry (measurement of C-reactive protein concentration), Gabrielyan method (Middle-mass molecules concentration measurement), the methods of mathematical statistics.

Scientific novelty of the results obtained.

Scientific data on biochemical and mechanisms of development of skin lesions under the conditions of experimental full-thickness wound model in the dynamics were investigated and mechanisms of action of nanoceria-based pharmacological composition in these pathological states were established. It has been proven that a pharmacological composition consisting of 0.05% Nanoceria ($Ce O_2 O_2$), dissolved in 0.5% carbopol, has a strong bactericidal effect on *S. epidermidis*, *S. aureus*, *P. aeruginosa*, that makes the appropriate application of the drug for the treatment of infectious inflammatory processes. The degree of nanoceria influence in test cultures of microorganisms was evaluated by the presence or absence of growth inhibition zones and their size (diameter). Variants of experiments using gel containing carbopol only (without nanoceria) and options without making any drugs served as control options. In a test culture of *Candida albicans* yeasts Nanoceria produced fungistatic effect (areas of growth retardation were noted, where a decreased intensity of yeast growth was observed).

On the basis of the conducted estimation of the morphological parameters of the skin condition of rats in all terms of observation under the influence of a Nanoceria-based pharmacological composition, it was established that the area of experimental full-thickness wound was significantly smaller in each day of experiment compared

to the wounds without application of the composition.

With the use of a new pharmacological composition, wound healing in rats occurred without the formation of a gross keloid scar, which was confirmed by a decrease in the content of collagen and smelted gelatin in the skin, also, the welding temperature of wound's skin in rats with nanoceria-based composition application was lower compared to the wound's skin in rats without Nanoceria application. Nanoceria did not have any impact on the moisture content of wound's skin.

After evaluation of the effect of Nanoceria on CRP and MMM pool for wound healing process, we have seen an increased level of CRP and MMM pool in blood serum of rats and applying Nanoceria on the wound, the positive impact of this pharmacological composition on restoring these indicators to the control level has been revealed.

The experimental full-thickness wound was accompanied by the development of oxidative stress and the change in the activity of pro- / anti-oxidant system enzymes in the rat body, resulting in a rise in the blood serum of the free radical anion superoxide, hydrogen peroxide, lipid peroxidation content, and the content of protein, non-protein and total sulfhydryl groups. In this case, superoxide dismutase activity decreased, and catalase - increased against the depletion of the glutathione system of antioxidant protection. The use of a pharmacological composition with nanoceria restored oxidative stress, the content of protein, non-protein and total sulfhydryl groups and antioxidant enzyme activity to a control level.

It was shown that in the blood serum of untreated animals the expression of VEGF, NGF, MMP-2, MMP-9 and HIF 1α was dysregulated. Our experiment has demonstrated the positive influence of Nanoceria on these growth factors (VEGF, NGF, MMP-2, MMP-9 and HIF 1α) by having a great impact on their regulation in the healing process.

The practical value of the results obtained.

The obtained results supplement the idea of the pathogenesis of skin disorders and the mechanisms of action of nanocrystalline compound in the experimental full-

thickness wounds. The work contains an experimental demonstration of the clinical application of Nanoceria-based pharmacological compositions in full-thickness wounds. A new pharmacological composition for the treatment of post-cutaneous wounds, which can be used in medical practice in the treatment of patients with skin lesions, is proposed. Some provisions of dissertation work and research methods can be introduced into the educational process at the biological departments of universities and medical universities in the development of a course on the study of molecular and biochemical aspects of pathological conditions.

Conclusions

In accordance with the goal in the dissertation work the biochemical mechanisms of wound healing action are revealed:

1. Pharmacological composition containing 0.05% nanoceria dissolved in 0.5% carbopol speeds up the healing of full-thickness dermal wounds in a rat model. Healing of full-thickness dermal wounds occurred without the formation of a rough keloid scar. Also in all terms of the observation under the action of a Nanoceria-based pharmacological composition, the area of full-thickness wounds significantly reduced in comparison with the wounds without applying the composition.

2. Nanoceria-based composition has a bactericidal effect on the test cultures of Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa, and the fungistatic effect on the test culture of yeast fungi of the genus Candida which is important for the use of the composition for the prevention and treatment of infectious inflammatory processes on the wound surface.

3. After healing untreated full-thickness dermal wounds in homogenate of the former skin injuries moisture content decreased but collagen content, smelted gelatin content and temperature of welding of a skin were increased. After the use of nanoceria-based pharmacological composition all of the above-mentioned indicators

returned to the level of intact rats that explains the wound healing without the formation of a rough scar at the site of the full-thickness skin wound.

4. C-reactive protein and Middle-mass molecules concentrations in blood serum of rats with full-thickness dermal wounds on the 3rd, 6th, 9th, 14th and 20th days of the experiment were increased in comparison with intact rats. Nanoceria-based pharmacological composition had the positive impact on decrease these indicators in all terms of the observation and restored them on 14th day of experiment. It is witness that pharmacological composition diminished endogenous intoxication full-thickness dermal wounds.

5. The development of full-thickness wound is accompanied by an increase in the processes of lipid peroxidation products in the blood serum of rats and by the changing in the activity of antiradical enzymes (superoxide dismutase activity decreases and catalase increases), which is associated with the intensification of the formation of active forms of oxygen. In the case of full-thickness wounds under conditions of nanoceria-based composition application the oxidative-antioxidant balance is restored in blood serum, as shown by the decrease in the products of the LPO and the normalization of antiradical enzymes activity.

In the rats with full-thickness wounds we have shown a decrease in the content of protein, non-protein and total sulfhydryl groups in blood serum, indicating that the level of free radicals increases in blood. When nanoceria-based composition was applied to wounds, the restoration of sulfhydryl group’s content in blood serum was observed that is evidence of antiradical property of nanoceria.

Daily application of carbopol gel with nanocrystalline cerium dioxide on the wound bed restored pro- / antioxidant balance in the blood serum of rats, which was manifested in the reduction of the content of LPO products and the normalization of the activity of antioxidant enzymes, as well as restored the group to level of control. Therefore, one of the mechanisms of the dermatotropic effect of a nanoceria-based pharmacological composition is its antioxidant and antiradical action.

6. In the blood serum of untreated animals the expression of VEGF, NGF, MMP-2, MMP-9 and HIF 1α was dysregulated. The high early levels of MMP-9 appear to be associated with concomitantly elevated collagenase levels, possibly to facilitate epithelialization and degradation of denatured collagen. The prolonged elevation of MMP-2 activity is probably important for the remodelling of scar tissue. Moreover, gelatinases may serve as indicators of the progression of the wound healing process. Nanoceria-based composition represented as a great regulator for both gelatinases in wound healing process. Nanoceria-based composition has the positive influence on growth factors (VEGF, NGF) and HIF 1α by having a great impact on their regulation in the healing process.

Personal contribution of applicant.

Analysis of literature, conducting experiments, statistical processing and writing a dissertation are performed by the competitor independently. Planning for directions of research, discussion of the received results, formulation of conclusions made with participation of the scientific advisor doctor of medical sciences, Vereschaka V.V. It's been a long and at times daunting journey for the past three years. This would not have been possible without the support of many great individuals. I owe my deepest gratitude to doctor of science, Prof. Beregova T.V., doctor of science, Prof. Ostapchenko L.I., senior scientific worker, PhD, Kondratiuk T.O. The author expresses her gratitude to all the colleagues for the provided help and their participation is noted in the joint publications.

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Approbation of the results of the dissertation

Main results of dissertation work reported and discussed at the:

1. International scientific and practical conference “Actual questions of medicine and biology” (Poltava, Ukraine, 2017).

2. XVI International Scientific Conference of Students and Young Scientists “Shevchenkivska vesna: Biosciences advance” (Kyiv, Ukraine, 2018).

3. Conference “Theoretical and practical aspects of the use of biological markers in fundamental and applied medicine and biology” (Prague, the Czech Republic, 2018).

Publications.

On the theme of the dissertation, 9 scientific works were published, including 5 articles (3 articles in professional editions recommended by the Ministry of Education and Science of Ukraine, 2 articles in foreign publications, 4 publications in congresses and conferences.

Structure and volume of the dissertation

The dissertation consists of an introduction, review of literature, research methods, a section with the presentation of the results obtained, a section devoted to the analysis of the results, conclusions and the list of literature, containing 296 sources. The materials of the dissertation are presented on 137 pages of the printed text, illustrated by 3 tables and 28 figures.

SECTION 1
LITERATURE REVIEW

1.1. Biochemical and molecular mechanisms of full-thickness wounds healing

Wound healing is a complex and dynamic set of events associated with the repair of damaged and lost cellular structures and tissue layers. Lost tissue repair is a linear process where growth factors cause cell proliferation, extracellular matrix production, and proliferation of parenchymal cells. The molecular and biochemical processes occurring during healing of the wounds are divided into the following phases: the inflammation phase occurs immediately after the injury and lasts 2-3 days, the regeneration phase and the formation of granulation tissue - begins with 3-4 days; the phase of scarring and epithelization (healing) - begins on the 8th day after the injury.

The emergence and development of wound defects refers to free radical pathologies, which are accompanied by the activation of free radical reactions. During the inflammation phase, neutrophils and cytokines produce oxidants such as reactive oxygen species (ROS) or reactive forms of nitrogen. These substances act as free radicals, due to which delay the healing of wounds and cause significant damage in healthy tissue cells throughout the body. In response to oxidative stress in the wound site, antioxidants neutralize free radicals and create the necessary environment for wound healing.

A characteristic feature of the inflammatory phase is the "oxide explosion". Polymorphonuclear cells and macrophages, migrating into the wound, release a large amount of superoxide radical, which is converted into hydrogen peroxide at the expense of SOD. Superoxide is produced by NADPH oxidase, this fermental complex carries electrons from NADPH to molecular oxygen and forms a superoxide radical. It consists of proteins localized in phagosomal membranes and in plasma membranes of phagocytic cells [43, 44]. Some components of the complex are in the cytoplasm;

they move to the membrane due to phagocytosis or under the influence of soluble stimulants. The activity of NADPH-oxidase in phagocytes is controlled by small GTPase Rac2 [45].

The increase in superoxide and hydrogen peroxide concentration is short-lived, as the migration of neutrophils and macrophages decreases as a result of the AOS [46]. The ability of macrophages to produce ROS is much lower than that of neutrophils, but ROS and especially H2O2 are important to other messengers that regulate the physiological functions of macrophages [47]. Myeloperoxidase, contained in granulocytes and monocytes, catalyzes the reaction of hydrogen peroxide with chloride anions; a powerful oxidizing chlorinated acid is formed [48]. The reaction of myeloperoxidase is also a source of singlet oxygen [49].

But if the inflammatory phase is delayed in time, the concentration of ROS will exceed the antioxidant capacity of the cell, which we just see when cutting the wound without treatment, as a result, there is an oxidative stress. Oxidative stress, which is mediated by radical ROS (superoxide anion radical) and non-radical ROS (hydrogen peroxide), inhibits cell migration and proliferation and causes tissue damage, as a consequence of the lateral phase, continues in time. On the other hand, the defect of neutrophils causes complicated infections and poor healing of wounds [50]. Reducing the level of the regulatory protein of Rac2 phagocytic NADPH oxidase can be one of the causes of prolonged wound re-epithelialization.

Known members of the antioxidant system are thioredoxin-1 (Trx-1) and -2 (Trx-2), glutathione (GSH), glutathione s-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADP (H) Quinone oxidoreductase (NQO1), catalase (CAT), epoxy hydrolase, heme oxygenase-1 (HO-1), UDP-glucuronosyltransferase (UGT), and glutamylcysteine synthetase. In addition to protein ROS, cells also use non-enzymatic metabolites, which are small antioxidant molecules such as vitamin C, vitamin E, β-carotene, glutathione, coenzyme Q, bilirubin, α-tocopherol, nicotinamide adenine dinucleotide phosphate (NADP), and urate. Part of the metal ions is capable of oxidation / reduction reactions such as

transferrin and ferritin, which have an increased absorption capacity of ROS [51,52].

Oxidative stress can be estimated by studying the oxidized form of glutathione-GSSG, as well as screening of oxidative-sensitive kinases ASK-1, p38 and JNK, and transcription factors such as NF-κB and especially AP-1 [53, 54]. Due to the fact that proteins respond to oxidative stress, cells evolved to use ROS not only for signal but also for prevention of cellular pathophysiology.

Thus, the use of antioxidants during wound healing improves wound repair. Vitamin E, alpha lipoic acid, vitamin C, grape seed extract, coenzyme Q10, glutathione and lutein, by reducing the concentration of free radicals, promote the development of new tissue in the wound.

Oxygen is the main substrate required for mitochondria-dependent adenosine phosphate (ATP), which provides an increase in the amount of energy needed to restore damaged tissue when wounding.

ROS derivatives of O_2 act as secondary signaling molecules. The most important role in the ROS belongs to the superoxide anion radical ($O_2^{\bullet -}$), Hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), perhydroxyl radical ($HO_2\bullet$), and singlet molecular oxygen [55,56]. Endogenous cellular ROS arise from mitochondrial oxidative phosphorylation in the production of ATP from the endoplasmic reticulum, or from the class of oxidoreductase enzymes. The ROS, through the oxidation reactions, removes electrons from adjacent molecules, which violates their structure. Low levels of ROS induce cell cycle stop (i.e., they are cytostatic), the basic levels of ROS support normal cell functioning and hemostasis, the increase in the number of ROS induces a number of transcription factors, and the multiple induction of ROS activates pro-apoptotic proteins.

However, in addition to the positive effects of low levels of ROS on wound healing, excessive ROS formation leads to oxidative stress, which has a detrimental effect on wound healing. An increase in the ROS level was detected in vivo, which is associated with a repair disorder in chronic non-healing wounds [57]. At the molecular level, in addition to ROS-mediated transcription, which leads to secretion

of proinflammatory cytokines and induction of matrix metalloproteinases, excess ROS and reactive nitrogen species can either directly or by activation of proteolysis modify or destroy the proteins of the extracellular matrix, cause disorders of the functions of skin fibroblasts and keratinocytes [58].

Wound healing is a dynamic process, on the one hand, an excessive inflammatory process, on the other hand, does not heal injuries or chronic wounds [59, 61]. Superoxide radical anion takes direct part in the initiation of free radical processes. Its generation is the starting point for the cascade of reactions, which leads to the emergence of other ROS [62].

As already mentioned above, ROS play an important role in protecting, especially during wound healing, since phagocytic neutrophils and macrophages use their reactive and damaging properties. These cells are capable of absorbing bacteria in phagosome, which is the trigger mechanism for the cytosolic components of NADPH oxidase (p47, p67, p40 and Rac2) along with the membrane subunit (cytochrome b558). With intense absorption of O_2 , known as a respiratory burst, NADPH reduces molecular oxygen in the phagosome to $\cdot O^-_2$ or H_2O_2; it creates ROS lethal level that can destroy an infected pathogen [63]. As phagocytic macrophages and neutrophils destroy microbes in phagosomes, they release high concentrations of H_2O_2, which slows down the growth of adjacent bacteria [64].

The bacteriostatic effects of H_2O_2 in *Escherichia coli* were shown at concentrations from 25 to 50 μM, which are necessary for the clearance of bacteria. Thus, the ability of phagocytes to secrete H_2O_2 into the intracellular medium, and not only to maintain it in phagosomes, indicates the antimicrobial properties of the ROS in the wound area.

H_2O_2 acts as the primary and secondary mediator in response to wound healing, its level is regulated by the local antioxidant enzymes, SOD, and GP, which was previously shown in models of skin wound of rats [65]. The functional role of H_2O_2 is a consequence of some of its properties, it is easily synthesized, easily decomposed, present in all cell types, has a longer half-life than radical ROS, and its small,

uncharged molecule allows it to freely diffuse through membranes and tissues.

Hydrogen peroxide also stimulates the proliferation of human fibroblasts and vascular endothelial cells in a comparative series of concentrations [66]. It has been established that 500 μM H_2O_2 stimulates the production of macrophage inflammatory protein (MIP) -1α, which is a chemotactic ligand for mononuclear phagocytes, neutrophils, eosinophils, basophils and lymphocytes [67]. It has also been shown that 100 μM H_2O_2 stimulates angiogenesis through vascular endothelial growth signaling (VEGF) [68] and is chemo-active for keratinocytes; Low levels of H_2O_2 also contribute to the migration and proliferation of keratinocyte cells [69].

Investigating the wounded zebrafish dorsal fin, it has been shown that H_2O_2 released by the mechanism of double oxidase extends to the edge of the wound with decreasing concentration gradient within a few minutes after epithelial damage, and this indicates the rapid formation of leukocytes [70].

Oxygen-dependent lesions are associated with the formation of ROS leukocytes, which activate the processes of lipid peroxidation (LPO) of plasma and cell membranes. For example, in the case of purulent wounds, there is oxidative stress in the inflammation zone, that is, the functioning of the antioxidant system is disturbed. The ROS and peroxides formed at the same time increase the destruction of cells, resulting in the formation of secondary necrosis, an increase in the area of damage, cause microcirculatory disturbances.

Since the formation of the wound is defected, there is a decrease in the activity of the endogenous antioxidant system (AOS). The activity of enzymatic and non-enzymatic components of AOS (superoxide dismutase, catalase, glutathione peroxidase (GP), glutathione-S-transferase, ascorbic acid, vitamin A and glutathione) in purulent infected wounds decreases during the first 7 days and is restored only on 14-16th days [71 , 72]. The amount of LPO contents (malonic dialdehyde) remains high and decreases only on 14th day. The main non-oxygen-dependent link of secondary alteration is hydrolytic enzymes that break down the components of the interstitial fluid and the cellular elements of the connective tissue. Neutral proteases

such as collagenase, elastase, cathepsins, gelatinases, collagen, elastin and basement membranes break down. Sour proteases cause destruction of glycoproteins and proteoglycans.

48 hours after injury, the migration of monocytes from adjacent blood vessels increases and new gene expression profiles are formed, they are differentiated into macrophages that are activated by chemokine signaling, can act as cells that represent antigens and help neutrophils in phagocytosis [73].

Regeneration (Proliferation) phase. In this phase, angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction occur.[74] In angiogenesis, vascular endothelial cells form new blood vessels.[75] In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin.[74] Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' atop the wound bed, providing cover for the new tissue.[76] In wound contraction, myofibroblasts decrease the size of the wound by gripping the wound edges and contracting using a mechanism that resembles that in smooth muscle cells. When the cells' roles are close to complete, unneeded cells undergo apoptosis. [77]

Fibroblasts appear in the wound on 2-3 days after its formation and dominate among the cell populations in the first week. The early matrix is largely composed of fibronectin and hyaluronate, acting as the basis on which fibroblasts can migrate and fix. The source of these fibroblasts is derived from fibrocytes of the regional connective tissue and perivascular adventitia. Fibroblasts produce a variety of substances that are necessary for wound healing, including glycosaminoglycans (GAGs) and collagen. Proteoglycans are proteins to which polysaccharides are attached. The four major glycosaminoglycans include hyaluronic acid, chondroitin-4-sulfate, dermatite sulfate and heparin sulfate [78]. They form an amorphous gel, which plays an imp ortant role in the deposition and aggregation of collagen fibrils [79].

During fibroblast proliferation, collagen is produced. The amount of collagen is constantly increasing for 3 weeks, reaching a stable level when collagen synthesis becomes equal to collagen lysis. An increase in collagen content in the wound in the phase of fibroplasia correlates with an increase in the strength of the wound.

Synthesis of collagen begins as an intracellular process, as a result of which the monomer is initially formed, is actively secreted into the extracellular wound in the middle, where the polymerization in the collagen fibrils occurs. They then covalently form cross-links, resulting in significantly increased strength of the wound.

A signal activating collagen production is a combination of growth factors stimulated by hypoxia and products of anaerobic metabolism (lactic acid) [80]. At the 1st week after injury, the collagen synthesis activity reaches a maximum, and immature collagen fibrils become histologically visible in the wound. Collagen is a basic building material of connective tissue that forms three polypeptide chains that are twisted counterclockwise.

Normal collagen synthesis occurs intracellularly and continues in the extracellular space. Collagen content in the wound is regulated by the balance between the product and the lysis of collagen by collagenase. The activity of collagenase is controlled by many factors, including parathyroid hormones, adrenocorticosteroids and colchicine. Control of these processes opens therapeutic opportunities for intervention in the process of wound healing and prevention of pathological rumen formation.

In the phase of scarring and epithelialization there is a restructuring of granulation tissue and replacement of its mature connective cavity, and the formation of a scar. At this stage, the collagen of the third type is replaced by type I collagen, the content of fibronectin is reduced. Metalloproteinases (MMP-1,2,3,9) play an important role in this process, providing degradation of the components of the extracellular matrix [81]. The balance of MMP activity with tissue inhibitor (TIMP) determines the volume of the intercellular matrix and the probability of formation of atrophic or hypertrophic scars. The granular tissue becomes mature fibrous tissue,

poor in blood vessels, with collagen fibers and fibrocytes [82].

Matrix metalloproteinases (MMPs) exist in both acute and chronic wounds. They are crucial due to their inhibitors, in regulating extracellular matrix deterioration and deposition that is extremely important for wound re-epithelialization. The excess protease activity can result in a chronic non-healing wound. Expression and activation of MMPs in response to wounding need to happen on an appropriate time as they are essential for successful wound healing.

The MMP family consists of a group of calcium-dependent zinc-containing enzymes that are engaged in the degradation of ECM. Family members share structural (Figure 1.1) and sequence similarities, a hemopexinlike C-terminal domain and a flexible proline-rich hinge region, which acts in identification of substrates (usually ECM). Exceptions to this rule are MMP-7, MMP-23, and MMP-26, which lack the hemopexin-like domain. Some MMPs have additional insertions, which have a hand in the functional differences observed between the MMP types. MMPs can be divided into seven groups based on the substrate preference and domain organization: (1) collagenases, (2) gelatinases, (3) stromelysins, (4) matrilysins, (5) metalloelastases, (6) membrane-type MMPs (MT-MMPs), and (7) other MMPs. Table 1 sums up the diverse groups of human MMPs, their substrates, and function in cell migration [83].

Metalloproteinase activity and secretion are highly controlled and maintained. In tissues with normal condition, MMPs are expressed at basal levels, if at all. When tissue reconstruction is demanded (as in wound healing), MMPs can be quickly expressed and activated. Several different cell types express MMPs within the skin (keratinocytes, fibroblasts, endothelial cells, and inflammatory cells such as monocytes, lymphocytes, and macrophages). In response to a range of signals MMP expression can be induced, including hormones, cytokines and contact with other cell types or the ECM [84].

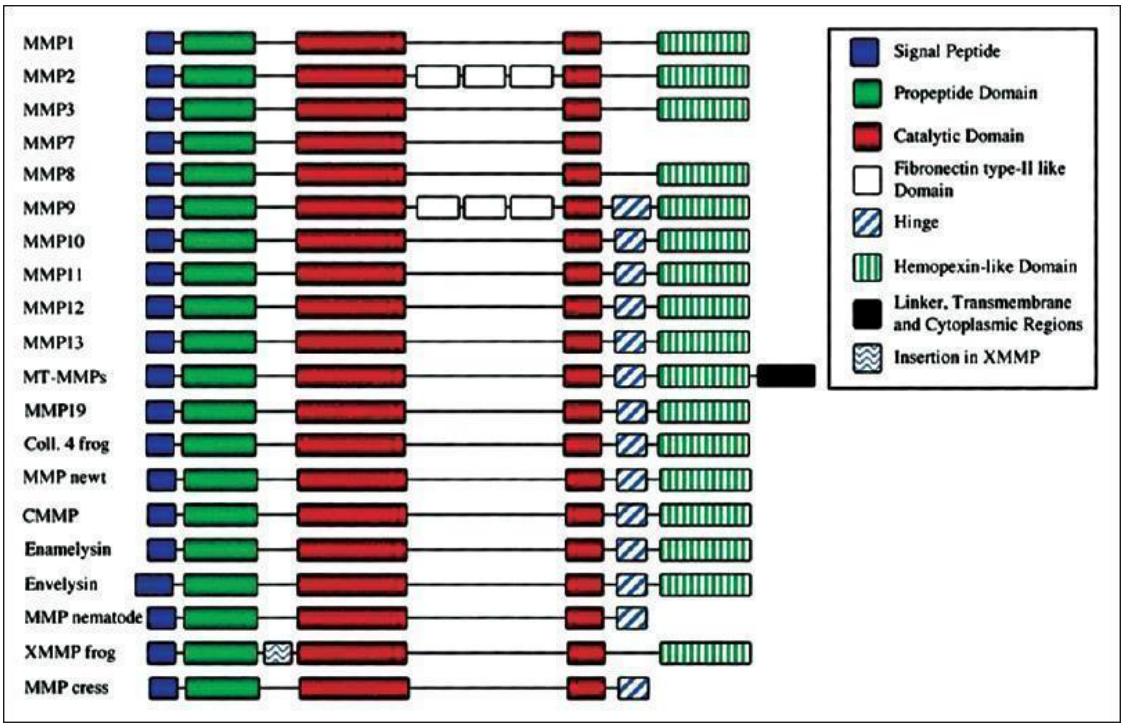

Figure 1.1. Schematics of the domain structures of the 23 representative MMPs. Catalytic domain (represented by green) has an insertion of gelatin-binding domain in MMP-2 and 9. In all other MMPs, the catalytic domain is a continuous entity [255].

A wide range of growth factors and cytokines transcriptionally take part in MMPs activation; these consist of keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor, tumor necrosis factor-a (TNF-a), transforming growth factor-b (TGF-b), as well as interleukins and interferons [85].

The basis of the mechanism of successive remodeling of the scar is the balance between collagen degradation and the synthesis of a new collagen (collagenization). Collagenase and other matrix metalloproteinases result in the degradation of collagen I and III types, in mature skin these collagen types are present in a ratio of 4: 1. The accumulation of the wound provides the organized order of the fibrils and the strength of the tissue. Over time, the number of macrophages and fibroblasts is reduced by

apoptosis [86].

Complete healing of the wound is defined as such, which leads to a complete restoration of normal anatomical structure, functions and appearance of the tissue. Certain systemic and local factors may slow down wound healing, causing a disturbance in balanced tissue repair processes, which in turn leads to chronicity of the pathological process.

1.2. Dermatotropic drugs: characteristics and application

An important stage of treatment is the local treatment of the wound bed. This method of treatment can accelerate the self-healing of the wound and increase the effectiveness of the use of other therapeutic measures [87, 88]. The treatment of skin lesions, regardless of their causes, is carried out to restore or improve the physiological functions of the skin.

Unfortunately, traditional methods of prevention and treatment of wound infection with antibiotics do not justify themselves. This is due to the high rates of evolution of the wound microflora and the rapid formation of its resistance to antibacterial drugs [89, 90].

For therapy, use of dermatotropic drugs aimed at the elimination of a specific disease, as well as increased protection against the effects of environmental factors and the prevention of loss of proteins, electrolytes and water [91].

Wound healing agents can be taken externally, systemically, or injected directly into the inflammation center.

Drugs for external use have several advantages, including: ease of use and monitoring of treatment. In addition, most of these drugs do not get into systemic blood flow and, accordingly, do not cause complications in the body. Also rarely interact with other drugs [92].

The main obstacle to the use of drugs for external use - the stratum corneum of the epidermis, namely, its overcoming the active substance - the limiting stage of

absorption [93].

The rate and extent of absorption of the drug through the skin depends on many factors. Active substances are usually mixed with bases. Bases have a significant effect on absorption, and with their correct selection they can have a positive effect on the skin. The base should be easily applied and removed, not to cause irritation and damage the appearance of the skin. The active ingredient should be well stored in the base and easily released from it [94, 95].

For the treatment of skin diseases, local pharmaceutical products are often preferred, but it is necessary to take into account the probability of developing systemic effects when applied.

Local pharmacotherapy in the general wound therapy scheme is an auxiliary, but not an alternative, role. That is, it should complement active surgical treatment, but not substitute for it.

The tasks of medical treatment of wounds can be represented as follows (Table 1.1.).

Table 1.1 Algorithm of local medical treatment of wounds used in Ukraine at different stages of wound repair

The first phase-inflammation	«Альгофін» (ХФЗ «Красная звезда», Україна); «Аргосульфан» 2% (Jelfa, Польща); «Дермазин» 1% (Lek, Словенія); «Бактробан» (Smithkline Beecham, Великобританія); «Гентаміцин» «Белмедпрепараты», Білорусь; «Київмедпрепарат», Україна); «Іруксол-моно»(Knoll, Німеччина); «Левоміцетин »(« Дарниця », Україна); «Мадекасол» (Syntex, Швейцарія); «Метилурацил» («Дарниця», Україна); «Мірамістин» («Дарниця», Україна); «Нітацид-Дарниця» («Дарниця», Україна);

The second phase-regeneration	«Метилурацил-Дарниця» («Дарниця», Україна); «Бетадин» (Egis, Угорщина); «Вундехіл» * («ЕЙМ», Україна); «Вулнузан» * (Sopharma, Болгарія); «Карбодерм-Дарниця» * («Дарниця», Україна); «Траумель С» * (Heel, Німеччина); «Репареф» («Белмедпрепарати», Білорусь);
The third phase-the remodelling of the scar	«Актовегін»* (NycomedAustria, Австрія); «Солкосерил» (Solkobasel, Швейцарія); «Бепантен» (RocheAG, Німеччина); «Мефенат» («Фармак», Україна).

As mentioned before, in each phase of the wound process it is necessary to use preparations with the corresponding types of pharmacological action and different osmotic activity [96].

Preparations for the treatment of wounds in the first phase of the wound process.

Proceeding from pathogenesis of the wound process, it is believed that pharmacological preparations taking in phase I should provide antimicrobial, dehydrating, necrolytic and analgesic action, that is, to help suppress the conditionally pathogenic microflora in order to quickly heal the wounds, while creating the conditions for the next reparation.

However, most drugs have a narrowly directed effect: only antimicrobial or dehydrating, or necrolytic, that is, they do not provide a comprehensive effect on the wound process. This is their main disadvantage.

Preparations for the treatment of wounds in the second phase of the wound process.

The negative effect of ointments in the 2nd phase of the wound process is primarily due to their high osmotic activity. The properties of these drugs, for the

treatment of the 2nd phase, they should be different from the drugs used in the first phase. In the first place, they should stimulate regenerative processes in the wound, promote the growth of granulations and accelerate epithelialization. It is also necessary that these drugs reliably protect the granular tissue from secondary infection in the wound of the defective microflora, had some anti-inflammatory effect, improved regional microcirculation and metabolic processes in tissues [97]. Frequently used various ointments with antibiotics on a fatty basis, the indifferent basis of which prevents reparative processes in the wound, and the presence of the antibiotic in the composition provides a certain antimicrobial activity, which depends on the sensitivity of the microflora to this antibiotic [98]. To enhance the effect of drugs into their composition, they introduce ingredients that are able to expand the spectrum of ointments, complementing their antimicrobial action by anti-inflammatory or stimulating effect on the wounds.

It is more advisable to take those medications that could simultaneously have antimicrobial effect and did not interfere with drainage, contributed to the growth of granulation. It is not advisable to take an ointment in the second phase on a hydrophilic basis, because due to high osmotic activity, they "dry out" the granulation.

Treatment of wounds in the 3rd phase of the wound process (remodeling and maturation).

Treatment in this phase has a similar purpose with the task of the 2nd phase: preservation of the wound from injury and stimulation of epithelialization. Recommended use of indifferent ointments and physiotherapy procedures [99].

So, summing up the results, it can be argued that there is a need for effective combination drugs that will be effective at all stages of the wound process. Also, the issue of the creation and research of new pharmacological preparations of domestic production on the basis of substances of plant and synthetic origin remains.

1.3. Nanoceria: properties and application as a dermatotropic remedy

In modern pharmacy, one of the most important directions is the search for new sources of biologically active substances, which at the same time possess both high absorption properties and biological activity. The main criteria for choosing such substances are biocompatibility and non-toxicity. The natural polyphenolic compounds correspond to these conditions. The small assortment of domestic medicinal products with dermatotropic effect on the pharmaceutical market of Ukraine, which not only prevents infection of the wound and accelerates the wound healing, but also prevents the formation of keloid scars, cause the search for and development of new drugs.

Wound healing is an innate process, which reestablishes the integrity of skin as quickly as possible. Renovation of the skin is crucial, due to the skins significance in survival through the prevention of infection, fluid loss and other vital functions [100]. Wound repair in adult skin begins with an acute inflammatory phase and ends with the formation of a permanent scar. In contrast, early gestation fetal wounds (first and second trimester) heal in a near perfect fashion, rapidly and without the production of a scar [100,101]. Often there are also obstacles of scar-degenerative origin in the area of wound - hypertrophic and keloid scars; scar restrictions (contractions) of movements scar hernia and diastase on the basis of muscle atrophy, sores and rarely – tumors [101,102]. Despite a great inventory of dressings, wound treatment stays a serious problem of regenerative medicine [103]. One of the main requirements to wound dressing is establishment and preservation of the ideal conditions for potent healing.

Modern fashions in the technologies of local treatment are based mainly on the headway and evolution of cell medicine. However, because of high biological activity of prospective products, it is vital to study their effects on wound reformation. For example, the biomedical application of cerium oxide nanoparticles (nanoceria) is a focal point of research for a few years, Cerium Dioxide (Nanoceria) seems to be a promising tool, due to its magnitude to regulate redox processes in biological tissues.

Depending on the size of the nanoparticles, Nanoceria displays oxidative and reductive properties, which is paralleled by changes in its toxicity [25]. The neuro- and radio-protective effects of Nanoceria are described [26-,28]. Cerium salts are utilized in the treatment of burn wounds [29].

The antioxidant property of cerium oxide nanoparticles (Nanoceria) has been investigated recently; nanoceria were noticed to salvage superoxide radical [30,31], hydrogen peroxide [32], hydroxyl radical [33] and nitric oxide radical [34]. Therefore, Nanoceria have been tested in biological systems wherein they can defend tissues against radiation induced damage [35], defend against laser-induced retinal damage [36], increase life span of photoreceptor cells [37], decrease spinal injury [38], reduce chronic inflammation [39] and elevate angiogenesis [40].

Cerium is a rare earth element that exhibits the ability to change in the oxidation state of Ce between Ce3+ and Ce4+ depending on the oxygen partial pressure in the surrounding atmosphere [41]. Such mixed valence state enables CeO2 nanoparticles (CeO2NPs) to have excellent catalytic activities and to act as free radical scavengers [42], (Fig.1.2).

For example, hydroxyl radical which is one of the strongest oxidants and most biologically active free radicals and nitric oxide radical that is gaseous free radical that exhibits multifaceted biological effects, both beneficial and damaging.

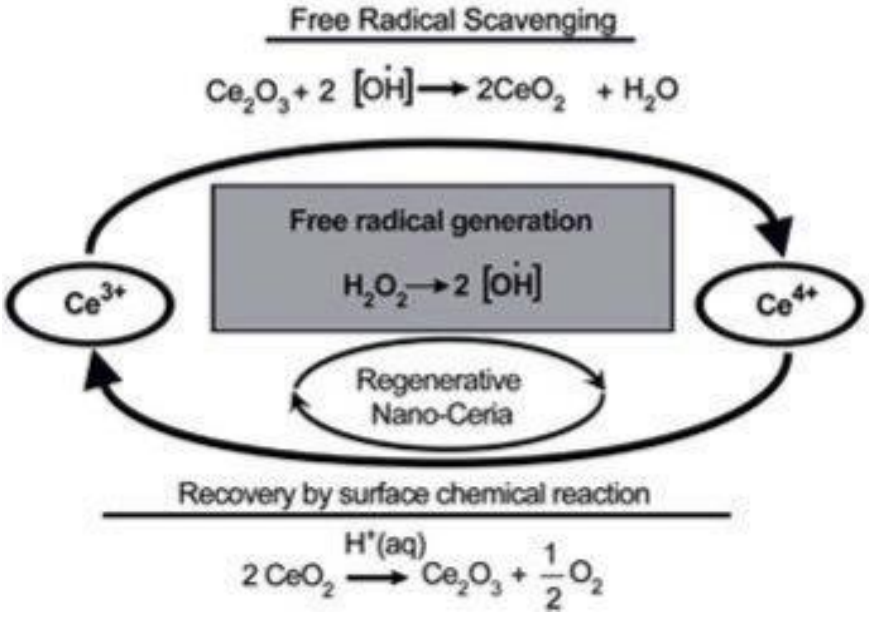

Figure 1.2. Schematic detailing the proposed regenerative properties of CeONP and probable mechanism of the CeONP free-radical scavenging property and autocatalytic behavior.53 Copyright 2007, Elsevier. [42]

Another important advantage CeO2NPs can hydrolyze phosphate ester bonds which is crucial for regulation of protein activity, for energy transfer molecules, and for the stability of DNA and RNA. Recent studies suggested that CeONP are able to hydrolyze phosphate ester bonds of many biologically relevant molecules [104, 103] Tan and colleagues [105] found that CeONP could effectively intercede the dephosphorylation of phosphopeptides.

Cerium exists in two oxidation states i.e. +3 and +4, which provides it to represent two different oxide forms CeO2 (Ce4+) or Ce2O3 (Ce3+) depending on the environment [104], (Figure 1.3). The nanoparticle form of cerium oxide has been found to be highly superior to cerium oxide. The reduction/oxidation switch is responsible for observed antioxidant properties of nanoceria by giving rise to a number of oxygen vacancies on the surface of nanoparticles [105].

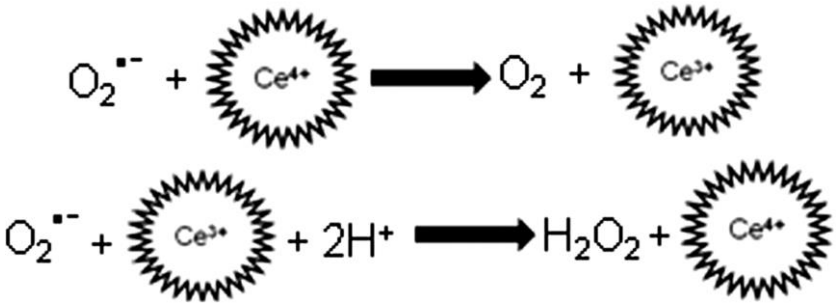

Figure 1.3. Redox cycles between Ce3 and Ce4 oxidation states. [104]

Attendance of these oxygen vacancies makes nanoceria an intense antioxidant for therapeutic applications. It has superoxide dismutase (SOD), Figure 1.3, [106] and catalase mimetic activity, Figure 1.4, [107] and also has capacity to salvage nitric oxide radicals [108]:

$$Ce^{4+} + NO \rightarrow \left[Ce^{4+}NO \leftrightarrow Ce^{3+}NO^{+}\right].$$

Nanoceria has earned attention as catalytic antioxidant in biological model systems. Due to its unbeatable ROS scavenging properties is considered as one of the important candidates for treatment of conditions involving oxidative stress. Apart from this, Nanoceria also has its impact in cancer therapy by enforcing cytotoxicity [109] and sensitizing the cancer cells to radiation therapy without modifying normal healthy cells [110]. In addition, it also has anti-angiogenic property [111].

The management of full-thickness wounds is a common part of dermatologic practice. The healing of these wounds is a complicated, interconnected series of events that eventually lead to both a structurally and functionally decent result. The initiation of this process is indicated by a hemorrhage to the tissues, whether due to an unintended trauma, such as abrasions, excoriations, blisters, burns, hypothermic injuries, ischemia, or numerous other etiologies, or a defect that has been planned, such as a surgical incision or piercing. The sequence that follows leads to the repair

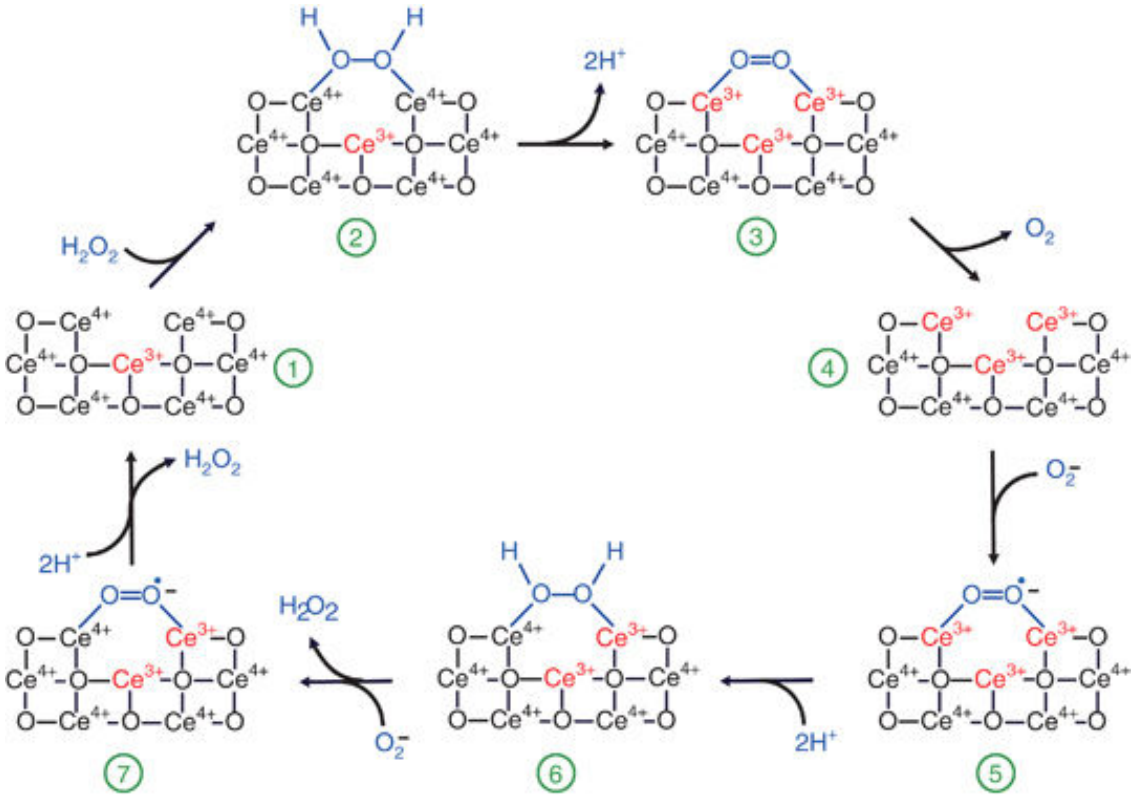

Figure 1.4. A model of the reaction mechanism for the dismutation of superoxide by cerium oxide nanoparticle (CeONP). [31] Copyright 2011, Royal Society of Chemistry.

and restoration of the site in question. Generally, there are 4 stages seen in the healing of any wound: hemostasis, inflammation, proliferation or granulation, and matrix formation or remodeling [112, 113]. A comprehension of this process permits the clinician to optimize wound healing. These stages are not presently chronologically exclusive, but rather a dynamic and integrated coordination of specific processes.

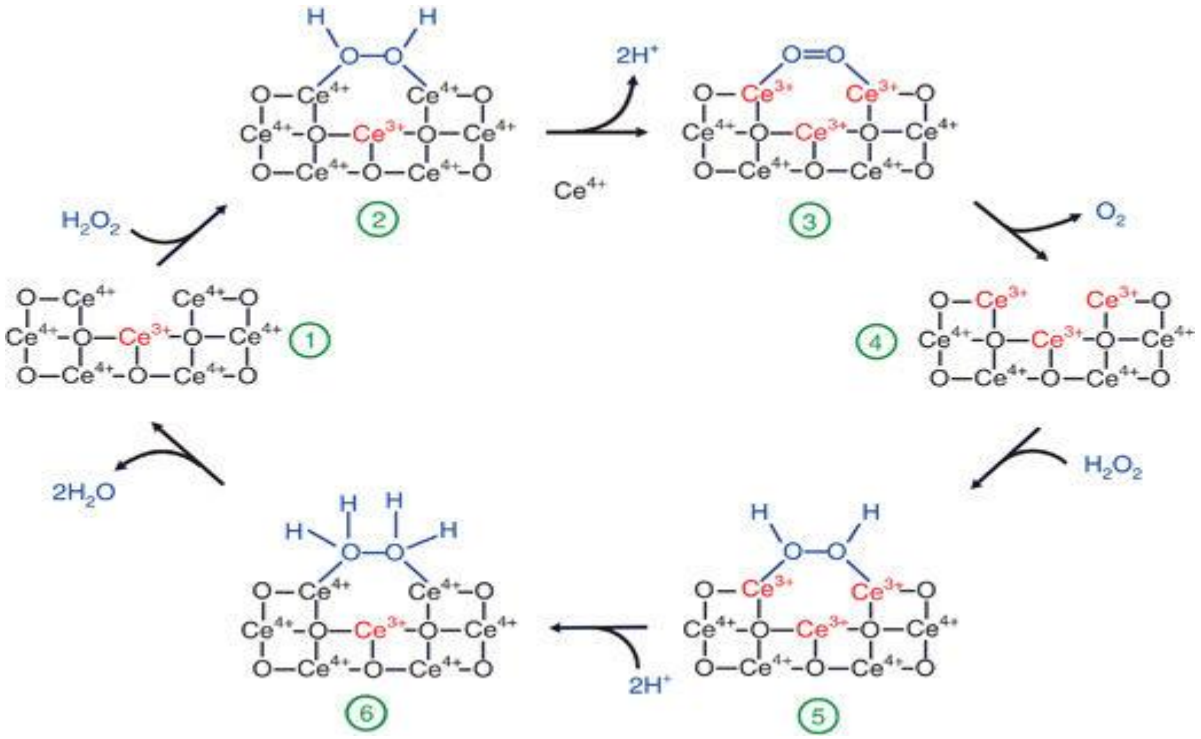

Figure 1.4. A model of the reaction mechanism for the complete dismutation of hydrogen peroxide by cerium oxide nanoparticle (CeONP).Copyright 2011, Royal Society of Chemistry [31].

These stages can be allowed to proceed ongoing, as in healing by secondary intention, or influenced by primary closure with sutures, staples, or adhesives; application of medications or topical products; or placement of various traditional or synthetic dressings. As healing proceeds, tensile strength of the wound ameliorates. In human cases, Wounds have minimal strength during the first week or so of healing, approximately 30% to 50% in 4 to 6 weeks and 60% at around 6 months.7 Tensile strengths slowly increases after this point but usually only reaches a maximum of 80% that of normal, undisrupted skin. The duration and percentages noted are general guidelines for the time frame and performance expected based on prior studies. Variation does occur due to the type, location, and size of the defect. Clearly, the less invasive, more superficial insults will conclude the entire process more rapidly [114, 115].

To take into account above mentioned data we propose to use for improving wound healing new pharmacological composition which consists of gel carbopol with nanocrystalline dioxide cerium.

Thus, the aim of the work was to find out the biochemical mechanisms of the dermatotropic action of a nanoceria-based pharmacological composition in the form of full-thickness wound model in rat’s skin.

SECTION 2
MATERIALS AND METHODS OF THE STUDY

2.1. Object of study

The studies were carried out on white laboratory rats, males weighing 200-250 g, n = 220. The animals were kept in the vivarium by the Taras Shevchenko National University of Kyiv, Taras Shevchenko educational and scientific center at the standard conditions of temperature (21 ° C), illumination (12/12 h), humidity and diet (a complete feed for laboratory animals K-12 -4, "Risan-1", Ukraine), according to "Standard rules for the ordering, equipment and maintenance of experimental biological clinics (vivarium)".

The experiments were carried out in accordance with the ethical principles adopted by the First National Congress of Ukraine on Bioethics, international agreements, and national legislation in this area.

2.2. Scheme of experiments

Before the onset of experiment, the rats were kept in quarantine, at the end of which the laboratory animals were examined and weighed. Animals were individually noticed by drawing inscriptions on the ear canals. All pain procedures and operations were performed under general anesthesia with thiopental sodium (Thiopental sodium, BiochemieGmbH / Austria) at a dosage of 60 mg / kg of animal mass. The animals were kept in individual cells under standard vivarium conditions throughout the experiment.

The experiment was conducted on IV groups of rats:

I - Intact group of rats (rats without any wounds);

II - Control group of rats with full-thickness wound model (healing was done without treatment (without Nanoceria application));

III - Carbopol group of rats with full-thickness wound model, the affected area was treated with 0.5% solution of carbopol ((universal solvent for giving them gel-like consistency (" Carbopol 980");

IV- Experimental group of rats with full-thickness wound model, which were treated with Nanoceria (0.05% concentration, dissolved in 0.5% carbopol).

Every day, starting from the first day after wound design and before healing, on the wound area of animals of the 3rd and 4th groups, the drugs were applied.

Methods for clinical and visual, biochemical, histological examination of wounds were used for the comprehensive assessment of the wound process. Clinical-visual and planimetric measurements were measured daily.

The material for biochemical studies was taken at 3, 6, 9, 14 and on the day of complete healing of the wound after starting treatment. Animals were extracted from the experiment by decapitation.

2.3. Model of full-thickness wounds

Full-thickness wounds were reconstructed on pre-depilated skin, in narcotic rats. For this, the skin was cut using a surgical scalpel and a pinch, measuring 1 × 1 см^2. The bleeding was stopped using sterile gauze swabs and 3% hydrogen peroxide solution. Treatment began immediately after the wound is design and until complete healing [116].

Skin`s wounds we were photographed with a digital camera Nikon-D3100. The images were transferred to a computer, then we calibrated and measured the square of skin`s injuries by means of programs ImageJ (NIH, USA). The obtained results expressed in percents from initial square [117].

2.4. Preparation of Nanoceria-based pharmacological composition

The wound dressings were put together by electrospinning. The film comprising 0.05% CeO2 (dissolved in 0.5% Carbopol) nanoparticles was elected as the optimal

dressing for the in vivo study on full- thickness excisional wounds of rats. A peerless feature of these nanocrystals is that they can be applied multiple times: over weeks, cerium (IV) rich particles leisurely turn over to their initial cerium (III) content. In approximately all cases, the particles subsist colloidally firm (e.g. non-aggregated) and could be applied multiple times. An in vivo study represents Nanoceria evidence in mouse tissues with no pathogenicity. Taken together, it is suggested that cerium oxide nanoparticles are well sustained in mice and are agglutinated into cellular tissues. The study illustrated that after 2 weeks, the wounds treated with the CeO2 nanoparticle- containing dressing attained a remarkable closure to nearly 100%. Our results delivered evidence supporting the feasible applicability of CeO2 nanoparticle-containing wound dressing for a favored wound treatment as it hastens complete wound closure and diminishes wound area in comparison with non-treated animals.

2.5. Blood serum collection

The serum of mammalian blood was obtained from 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 rod used to carefully separate the blood clot from the test tube walls to speed up the production of serum and centrifuged for 40 minutes. at 2000 g. The resulting supernatant (serum) was quickly separated from the formed blood elements, transferred the Eppendorf and frozen at -20 ° C until further use.

2.6. Obtaining homogenate of animal skin

After decapitation of the animals, the wound with a healthy skin adjacent to it was carved with a scalpel, and then placed in an Eppendorf by adding a physiological solution and kept frozen. In subsequent studies, the skin was homogenized in 0.9% NaCl solution in the cold. The homogenate was filtered through four layers of nylon mesh and used for biochemical studies.

2.7. Determination of physico-chemical indices of the skin

The temperature of welding of the skin was determined using a device PTZ-1 (USSR). A piece of skin 3X5 см fixed using the hooks of the apparatus, poured water and slowly heated. Under the influence of the thermal factor, the skin began to decrease linearly, which led to the tension of the cord and the commissioning of the timer indicator. The temperature of the welding was determined by the mercury thermometer, and it corresponded to the beginning of the movement of the indicator arrow [118]. The determination of the total nitrogen content was carried out using the Kjeldahl method [119].

In the study of functional properties of connective tissue, the output of collagen gelatin was determined by hydrothermal welding of the skin [119]. To determine the percentage of moisture in the tissue, skin samples were weighed on analytical scales and dried to constant weight at 80 ° C in a drying cabinet SNOL 3,5.3.5.3.5. / I1.

2.8. Determination of protein concentration by the Lowry method

The method is based on the formation of colored products of aromatic amino acids with a Folin reagent in conjunction with a biuret reaction to peptide bonds [120].

2.9. Determination of lipid peroxidation products

The content of diene conjugates was determined by the spectrophotometric method in the heptane-isopropanol extract, and the schiff bases by the fluorimetric method [121, 122]. The content of TBARS was determined by reaction with thiobarbituric acid (TBARS assay). Assay of TBARS measures malondialdehyde (MDA) present in the sample, as well as malondialdehyde generated from lipid hydroperoxides by the hydrolytic conditions of the reaction. MDA is one of several

low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products. However, only certain lipid peroxidation products generate MDA, and MDA is neither the sole end product of fatty peroxide formation and decomposition, nor a substance generated exclusively through lipid peroxidation. These and other considerations from the extensive literature on MDA, TBA reactivity, and oxidative lipid degradation support the conclusion that MDA determination and the TBA test can offer, at best, a narrow and somewhat empirical window on the complex process of lipid peroxidation. Use of MDA analysis and/or the TBA test and interpretation of sample MDA content and TBA test response in studies of lipid peroxidation require caution, discretion, and (especially in biological systems) correlative data from other indices of fatty peroxide formation and decomposition [123].

2.10. Determination of superoxide dismutase and catalase activity

The method is based on the ability of superoxide dismutase to compete with nitrosin tetrazolium (NST) for superoxide anions formed as a result of aerobic interaction of the reduced form of Nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (FMS). As a result of this reaction, NST is restored with the formation of hydrazine tetrazolium. In the presence of SOD, the percentage of recovery of NST decreases. The activity of the enzyme was determined by the calibration curve [125].

The principle of the method is that the catalase destroys the substrate H_2O_2, the undamaged part of hydrogen peroxide, when in contact with molybdenum salts, forms a stable colored complex. The activity was determined in nMol of H_2O_2 per minute per 1 mg of protein [126].

2.11. Determination of the level of sulfhydryl groups

The level of total, protein-bound and non-protein sulfhydryl (SH) -groups was measured by the Ellman's method [132].

2.12. Determination of growth factor, MMPs and HIF concentration

The growth factors levels were determined by performing ELISA. Enzyme-linked immunosorbent assay method is a criterion for antigens quantitation. The protocol commences with a captured antibody, particular for a protein of interest, coated onto the wells of microplates. In our experiment the blood level of VEGF, NGF, MMP-2, MMP-9 and HIF 1a were estimated performing ELISA [133].

Skin samples were immobilized onto 96-well plate and incubated with corresponding specific primary antibodies (Santa Cruz, USA). After that secondary antibodies conjugated with horseradish peroxidase (Bio- Rad, USA) were added. To enable colorimetric detection, reaction with the substrate o'phenylenediamine/hydrogen peroxide (Sigma, USA) was performed and the absorbance of each well was read at 422 nm ("Synergy", BioTek, USA). Values were expressed as optical density / mg of protein. Total proteins were determined by Bradford's method [134].

2.13. Investigation of the antibacterial properties of Nanoceria

To study antibacterial properties of "Nanoceria" the method of application of the drug (1 g) to the surface of the nutrient medium was used (NA - Nutrientagar, manufacturer Sigma-Aldrich, Spain). Previously surface environment was covered with suspension of test microorganisms according to the recommendations (on the recommendations, "Determination of the sensitivity of microorganisms to antibiotics" Ukraine Ministry of Health, Order number 167 of 05.04.2007). Number of colonies forming units (CFU) was determined by densitometer «Vitek-2»

(«BioMerieux» (France)). CFU load in microorganism suspensions amounted to 1,5× 10^6 10^6 CFU/ml (which corresponds to 0,5 McFarland) for bacteria, 1-5× 10^8 10^8 CFU/ml for yeasts. Culture collections of bacteria *Staphylococcus aureus* ATCC 25923, *S. epidermidis* 509/5, *Pseudomonas aeruginosa* ATCC 27853 and yeasts *Candida albicans* were used as test culture. We investigated the effect of nanoceria on these bacterial cultures of microorganisms.

2.14. Determination of C-reactive protein and Middle-mass molecule concentration

Level of Middle-mass molecules was carried out by Gabrielyan with modification [135] method where it is based on the precipitation of macromolecular peptides and proteins of biological fluids using trichloroacetic acid and quantitatively in the centrifugation obtained by the supernatant of medium-molecular peptides by absorption in a monochrome light stream at a wavelength of 254 nm.Serum and plasma of blood were collected and in the centrifuge tubes 1 ml of serum and 0.5 ml of trichloroacetic acid (100g/L), stirred and centrifuged for 30 min at 1500 rev./min. 0.5 ml of supernatant was taken and transferred to a test tube of 4.5 ml of distilled water. The contents of the test tube were mixed and a spectrophotometric measurement at λ 254 nm was performed. against the distillate, expressed in the mind. unit × mg of protein-1.

Determination of CRP concentration was carried out by Turbidimetry. The concentration of C-reactive protein was determined by the kinetic method analysis at fixed time according to standard (reaction time - 120 s, delay time - 3 s) [Turbidimetry in laboratory practice / VV Dolgov [and others]. - M.:Reafarm - 2007 - 176 pp.]. Quantitatively the concentration of C-reactive protein in the serum were evaluated by immunoturbidimetry using a CRP latex reagent. Latex particles, covered with specific antibodies to CRP, agglutinate when mixing with samples containing CRP. Agglutination causes a change Absorption at λ = 540 nm depending on the content of

CRP in the sample. Concentration of C-reactive protein was determined by concentration calibrator of the formula:

C CRP = ((A 0 -A 1) sample / (A 0 -A 1) caliber.) × C caliber.,

where A 0 - absorption after 3 s, A 1 - absorption after 120 s, C - concentration. The concentration of C-reactive protein in serum was expressed in mg/l.

2.15. Statistical analysis of results

Statistical analysis of data was carried out by the "Statistica 8.0" software package. The type of in- group data distribution was verified via the Shapiro-Wilk test. As data were distributed normally ($p > 0,05$), two-way ANOVA was conducted to determine the significance of difference between means, with Bonferroni posttest. Difference between means was judged as statistically significant if $p \leq 0,05$. Mean and standard deviation (SD) were calculated for each group.

SECTION 3
RESULTS OF RESEARCH AND THEIR DISCUSSION

MORPHOFUNCTIONAL STATE OF RATS SKIN AT DIFFERENT STAGES OF WOUND HEALING PROCESS AND APPLICATION OF A NEW PHARMACOLOGICAL COMPOSITION BASED ON NANOCERIA

3.1. Biocidal effect of the pharmacological composition on the culture of microorganisms

Resistance of infectious agents to antibacterial drugs (ABD) leads to an increase in the timing of treatment of patients, increases mortality and increases the duration of epidemics. Economically, the growth of antibiotic resistance in bacteria leads to a significant increase in the cost of therapy. The problem of antibiotic resistance of microorganisms is recognized as global and currently one of the strategic tasks around the world is the containment of the development and spread of antibiotic resistant microorganisms. Therefore, conducting laboratory studies to determine the sensitivity of microorganisms - the agents of human infectious diseases to ADP becomes more and more important [136].

With modern wound healing, an integrated approach based on the phases of the wound process is important. Therefore, in order to stimulate healing of wounds it is advisable to use such drugs that would simulate the properties of the intercellular substance of the connective tissue and would correspond to the pathogenesis of the wound process. The surface of wounds is an optimal medium for the development of a complex of pathogenic microorganisms (among which the species of the genus *Staphylococcus aureus* prevail (42.7%), *Pseudomonas aeruginosa* (10.3%) is commonly found in the gram-negative microflora, and wounds can also be infected with *Candida albicans* yeast microscopic fungi [137].

Microbial contamination substantially changes the course of the wound process. Together with mechanical damage to tissues, products of bacterial life can slow down the primary healing phases, increase osmotic pressure and acidosis in the tissues, disturb the microcirculation, which leads to the development of secondary necrosis [138].

The mixture made by us, containing nanoceria and carbopol, is a new biologically active pharmacological composition, based on Cerium a rare earth element.

Studying biocidal effect of nanoceria-based composition, it was revealed on test cultures of bacteria Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa nanoceria-based composition inhibited their growth. In a test culture of Candida nanoceria-based composition produced fungistatic effect (areas of growth retardation were noted, where a decreased intensity of yeast growth was observed) (fig. 3.1). Nanoceria-based composition has a strong bactericidal effect on S. epidermidis, S. aureus, P. aeruginosa, and fungistatic effect on C.albicans that makes the appropriate application of the drug for the treatment of infectious inflammatory processes. The degree of nanoceria-based composition influence in test cultures of microorganisms was evaluated by the presence or absence of growth inhibition zones and their size (diameter). Variants of experiments using gel containing carbopol only (without nanoceria) and options without making any drugs served as control options.

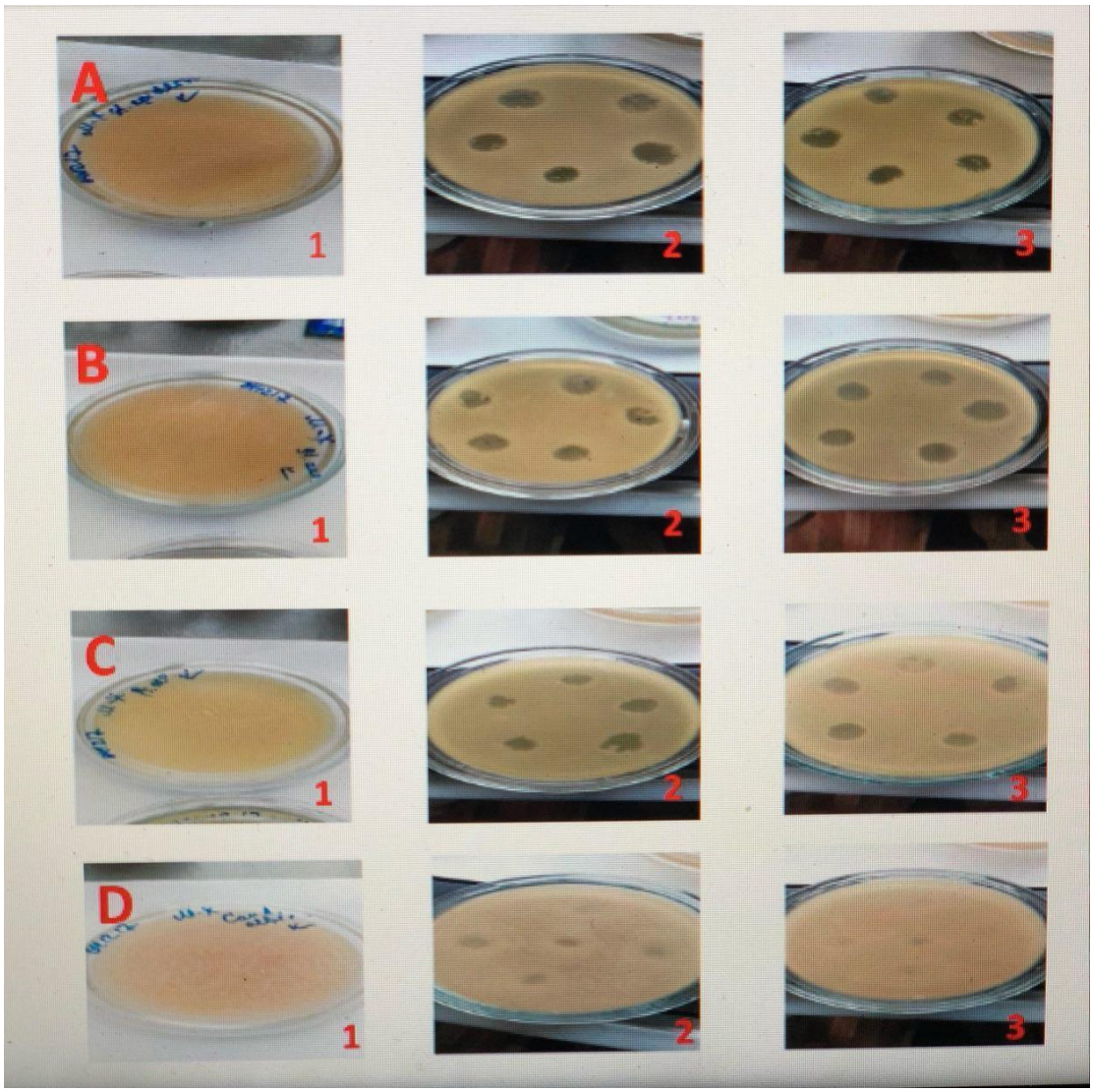

Figure 3.1: Bactericidal action of nanoceria on the bacteria *Staphylococcus epidermidis* (A), *Staphylococcus aureus* (B), *Pseudomonas aeruginosa* (C), *Candida albicans yeasts* (D). Ingredients: 0.05% nanoceria, 0.5 % carbopol. A, B, C: 1- control, 2.3 - growth inhibition zones in the places of application of Nanoceria (back side of the Petri dish). The density of the slurry $1{,}5\times 10^{8}\,10^{8}$ CFU / ml.24 hours of cultivation, D: 1 - Control, 2 - 24 hours of cultivation, 3 - 72 hours of cultivation, inhibition area of growth over grew (back side of Petri dishes).The density of the slurry $1\text{-}5\times 10^{6}\,10^{6}$ CFU/ml.

3.2. Visualization of the surface of skin wounds at various stages of the wound process

With modern wound healing, an integrated approach is considered, taking into account the phases of the wound process, as well as morphological, physiological, biochemical and molecular, which can occur in tissues. Therefore, in order to stimulate healing of wounds it is advisable to use such drugs that would simulate the properties of the intercellular substance of the connective tissue and would correspond to the pathogenesis of the wound process.

The evaluation of the wound healing effect of the pharmacological composition under study was carried out by analyzing the activity of contraction of the surface of the wound on 3rd, 6th, 9, 14th, and 20th days.

In experimental groups, each day, the pharmacological composition was applied to the affected sites using a metal spatula, which flamed before each use. Also every 24 hours. Visually assessed the general condition of animals, the condition of the affected surface and determined the size of the wound surface.

To assess the condition of the wound surface, the terms of purifying the wound from purulent-necrotic masses, the time of appearance of granulation and the beginning of marginal epithelization, as well as the terms of full epithelization of the wound surface, were studied.

The obtained data reflecting the dynamics of healing of wounds in different experimental conditions and a gradual reduction of the wound area was revealed, but the time of complete healing was different in different experimental groups.

In experimental animals, the third day was dominated by post-traumatic inflammation, the edges of the wound were swollen, swollen, and the wound was covered with a thick crust of brown color, the bottom is hyperemic. These signs were most pronounced in the animals of the control group (fig. 3.2).

Significant changes in wound condition and healing are recorded in the follow-up period. On day 3 in the control group, the signs of the inflammation process were

observed, the edges were edematous, spindle-shaped, there was a slight allocation of pus, the wound was covered with a crust. The condition of the animals treated with the pharmacological composition varied significantly with less hyperemia and edema, and the wounds were covered with a thin cortical layer. In this group, for the 3rd day, signs of inflammation were not detected.

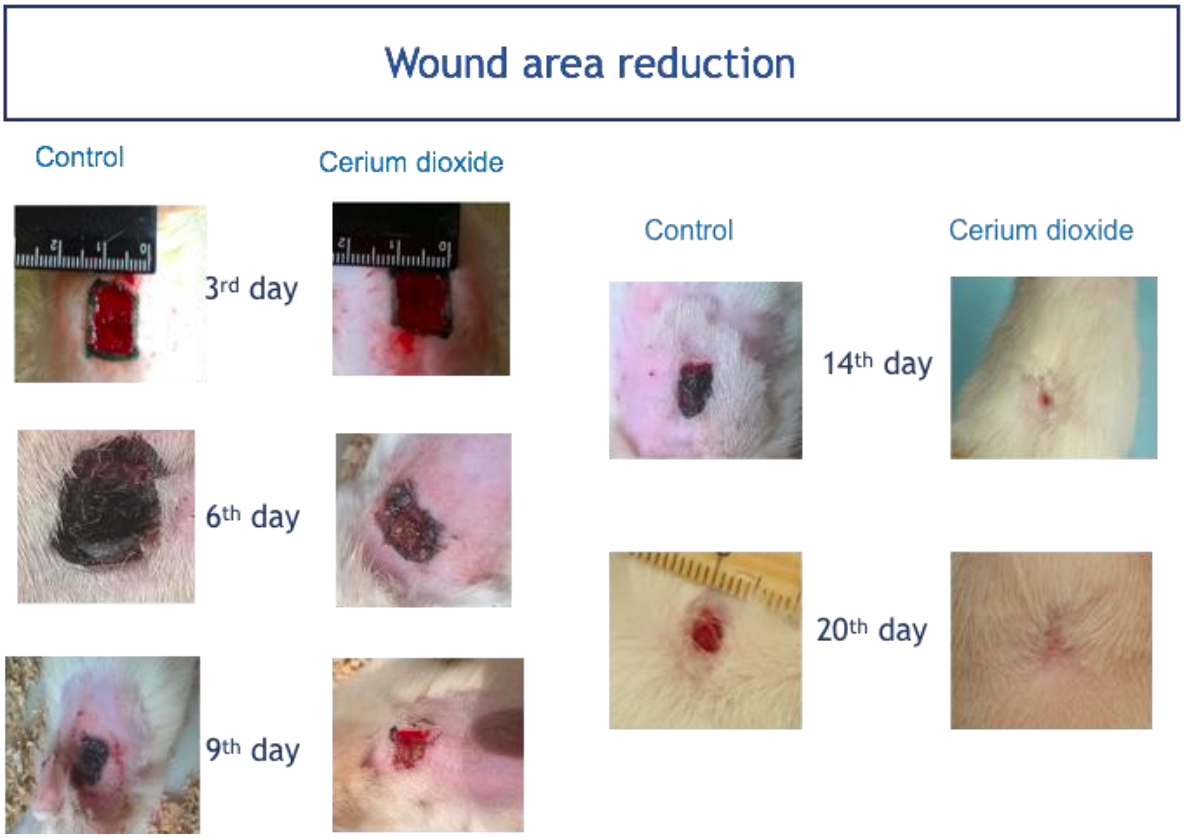

Figure 3.2. The appearance of the full-thickness surface comparing control and experimental groups:
control group – rats with full-thickness skin wounds without treatment;
cerium dioxide – experimental group of rats with full-thickness skin wounds with nanoceria-based composition treatment.

On the 6th day, the area of wounds in animals treated with a nanoceria-based composition decreased, the edges of the wound went adjacent to the bottom, on which granulation tissues began to develop. In the animal, which were not receiving

nanoceria-based composition, the rates of healing of wounds were significantly lower.

Further wound healing in all groups was characterized by the development of granulation tissue, which was covered by the epithelium from the edges. Significant dominance of these processes was observed in the experimental group of animals, which were receiving pharmacological composition.

In the model of full-thickness skin wound it was shown that complete wound closure in the intact group of animals occurred on the 23.0±0.8 day (fig. 3.2, 3.3). In the experimental group the time of full repair decreased by 13.0% ($p<0.05$) compared to the intact and was equal to 20,0±0,5 day.

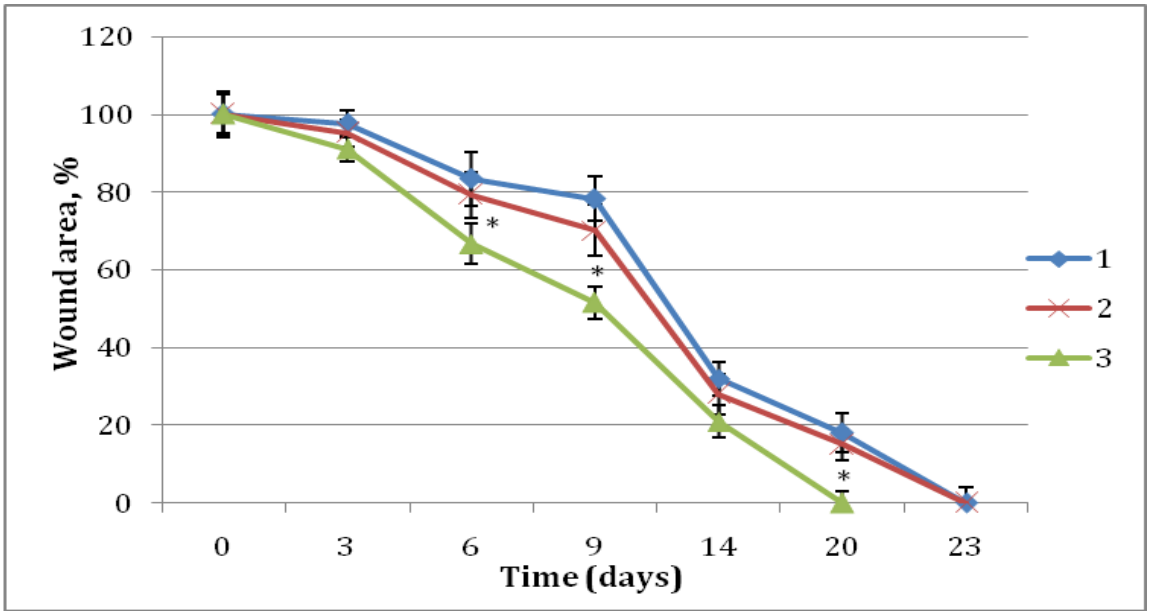

Figure 3.3. The surface of full-thickness wound area with Nanoceria action (% of the original size), M±m (n=8 in each group of animals)

1 – control group without treatment;

2 – carbopol group – the rats treated with carbopol;

3 – Experimental group – the rats treated with nanoceria-based composition.

** - p<0,05 compared with control group*

On the 6th, 9th and 20th day of experiment nanoceria-based composition treated wounds areas were by 20,1% ($p<0.05$), 37,5% ($p<0.05$) and by 34.0% ($p<0.05$) accordance decreased in comparison with control. In other time periods the rate of healing was approximately the same. in each group of animals). On the 9th and 14th day of the experiment in the above-mentioned group healing was more intense compared with the control group.

Chigurupati et al. have shown that a simple topical application of water soluble Nanoceria accelerates the healing of full-thickness dermal wounds in a mice model. In particular engineered nanoceria formulation enhances the proliferation and migration of fibroblasts, keratinocytes and VECs cells which further accelerate the wound healing process. Moreover, Nanoceria reduce oxidative stress in wounded region and protect regenerative tissue. This study suggests the therapeutic potential for topical treatment of wounds [140].

Based on Davan Revathy et al. it was proven that cerium oxide nanoparticles due to their dual oxidation state could enhance wound healing activity when compared to standard drug by scavenging ROS from the site of injury and protecting the native tissue thereby helping in producing high amount of collagen and hydroxyproline which are markers for effective wound healing. Histopathology showed no signs of inflammatory responses indicating less toxicity of these nanoparticles. Future study will include identification of other molecular mechanisms for wound healing, determining appropriate concentration of nanoparticles and formulation [141].

3.3. Physico-chemical properties of the skin during the experimental full-thickness wound model

According to the previous studies, application in the local treatment of the composition has made it possible to significantly reduce the infection of the wound

surface, that is, the composition has pronounced antibacterial and anti-inflammatory properties [139].

It was established that moisture content in the skin of animals of intact group (without wounds) was 60,3± 5,6% (table 3.1). In the skin of animals after healing of untreated wounds moisture content decreased by 39,8% ($p<0,01$). Wound treatment with carbopol gel or nanoceria-based composition did not affect moisture content in the skin after healing compared to the animals with untreated wounds.

Collagen content in the skin of animals of intact group was 35,8 ±3,3% (table 3.1). In the skin of animals after healing of untreated wounds collagen content increased by 59,8% ($p<0,05$) in comparison with the animals of intact group that confirms the formation of a scar in the place of the former full-thickness skin wound. Wound treatment with carbopol gel didn't effect on collagen content compared to the animals with untreated wounds. The use of nanoceria-based composition in the treatment of the skin wound has led to a decrease in collagen content to the level of intact animals without wounds. These findings confirm wound healing by the action of nanoceria-based composition without the formation of a rough scar at the site of the full-thickness skin wound.

Smelted gelatin content in the skin of animals of intact group was 2,1±0,2% (table 3.1). In the skin of animals after healing of untreated wounds smelted gelatin content increased by 95,2% ($p<0,001$). Wound treatment with carbopol gel didn't influence on smelted gelatin content compared to the animals with untreated wounds. But wound treatment with nanoceria-based composition has led to diminishing of smelted gelatin content to the level of intact animals without wounds.

Temperature of welding of a skin in animals of the intact group was 60,3±5,6° C (table 3.1). In rats after healing of untreated skin wounds temperature of welding of a skin increased by 41,1% ($p<0,05$). In the skin of animals after wound treatment with carbopol gel temperature of welding of a skin didn't change in comparison with animals after healing of untreated skin wounds. Wound treatment with nanoceria-

based composition has led to decrease of temperature of welding of a skin to the level of control animals without wounds.

Table 3.1.

The moisture content, collagen content, smelted gelatin content and temperature of welding of the rat's skin in different groups.

Animal group	Moisture content, %	Collagen content, %	Smelted gelatin content, %	Temperature of welding of a skin, °C
Intact (without wounds (I group))	60,3±5,6	35,8 ±3,3	2,1 ± 0,2	60,3±5,6
Control (untreated (II group))	36,3±3,4**	57,2±5,3*	4,1±0,4***	85,1±7,9*
carbopol+wound (III group)	35,1 ±3,3**	47,2±4,4*	3,2±0,3*	79,1±7,3*
Wound+ nanoceria-based composition (IV group)	40,4±3,7*	39,1±3,6#	2,6±0,2##	65,5± 6,1#

*- p<0,05, ** - p<0,01, *** - p<0,001 in comparison with intact group;

- p<0,05, ## - p<0,01 in comparison with control group.

The characteristic changes are related to the fact that when injuring the skin, the wound healing begins with acute inflammatory phase and ends with the formation of a scar. When scarring, collagen accumulates in response to tissue damage. The

formation of the scar, ultimately, is the result of excessive accumulation of extracellular matrix (EM). Although remodeling of the scars occurs months or years after the initial injury, complete restoration of normal EM architecture is never achieved. Thus, wound healing is a fibroproliferative response, which leads to incomplete regeneration of the affected tissue and excessive production of the mesenteric collagen structure, scar tissue. With the use of a new pharmacological formulation, healing occurred without scarring of tissue, which is confirmed by our research (the percentage of collagen in the skin was less than in untreated animals in the control group).

There are no sufficient data to compare our results with other scientists as Nanoceria is a newly synthesized nanomaterial and has a long way to go on further investigations.

SECTION 4

EVALUATION OF C-REACTIVE PROTEIN AND MIDDLE-MASS MOLECULES CONCENTRATION DURING WOUND HEALING

4.1. Determination of C-reactive protein concentration in blood serum during wound healing

C-reactive protein, hereinafter CRP, is and acute inflammatory protein that increases upto 1,000-fold at sites of infection or inflammation. It is present as a homopentameric protein, called native CRP (nCRP), which can detach to produce five discrete monomers, called monomeric CRP (mCRP), at the site of inflammation and infection. CRP is mainly synthesized in liver but also by lymphocytes, macrophages, smooth muscle cells, endothelial cells and adipocytes. When the tissue is injured, CRP level increases significantly in response to inflammation and infection (fig. 4.1) [142].

CRP mainly activates the C1q molecule in the complement pathway resulting in the pathogen opsonization. It can also initiate cell-mediated pathways by complement activation as well as attaching to IgG Fc receptors [143].

Once it attaches to Fc receptors, it induces pro-inflammatory cytokines releasement [144]. The ability of CRP to recognize self and foreign molecules according to their pattern recognition, makes it a unique complement activator as other activators can’t attain it due to their distinct antigenic epitopes’ recognition [145].

As mentioned before, CRP is an inflammatory marker and during bacterial infection its level inclines [142].

Kingsley and Jones demonstrated that at the site of infection, CRP level increases in response to monocytic mediators like IL-1 and IL-6 and it has a stable decay rate [146].

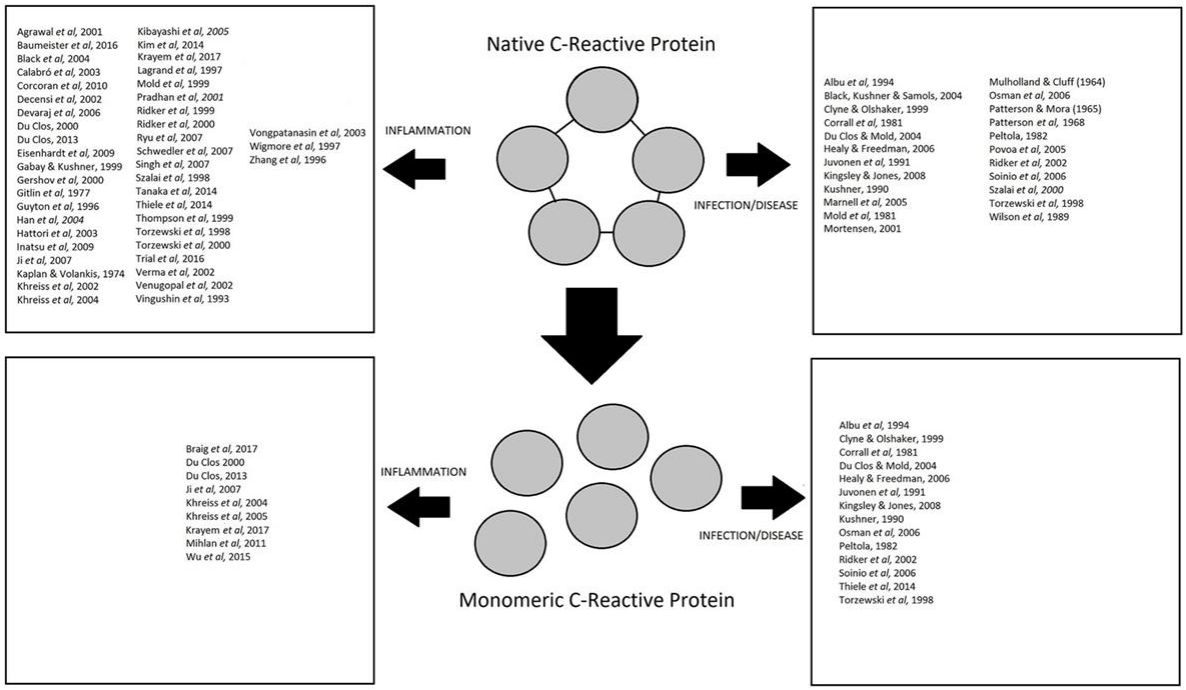

Figure 4.1: Different role of nCRP and mCRP in response to disease, infection and inflammation. [142]

In Mold et al. investigation on mice treated with 200 μg CRP, it was proven that CRP protects them against infection caused by gram-positive *Streptococcus pneumoniae* by attaching to their cell wall and leading complement pathway activation [147].

In the rats of control group we have shown an increase in the concentration of CRP in blood serum on the 3rd, 6th, 9th, 14th and 20th days of the experiment in 8,3 ($p<0,001$), 9,6 ($p<0,001$), 4,1 ($p<0,001$), 3,5 ($p<0,001$) and 1,9 ($p<0,001$) times consequently in comparison with the rats of intact group (fig. 4.2). In the rats with full-thickness wounds which were treated with nanoceria-based composition the concentration of CRP in blood serum was the same as in control group. But on the 6th, 9th and 14th days of experiment it was increased only in 5,7 ($p<0,001$), 3,1 ($p<0,001$) and 2,8 ($p<0,001$) times consequently in comparison with the rats of intact group. And on the 20th day of experiment we have shown the restoration of

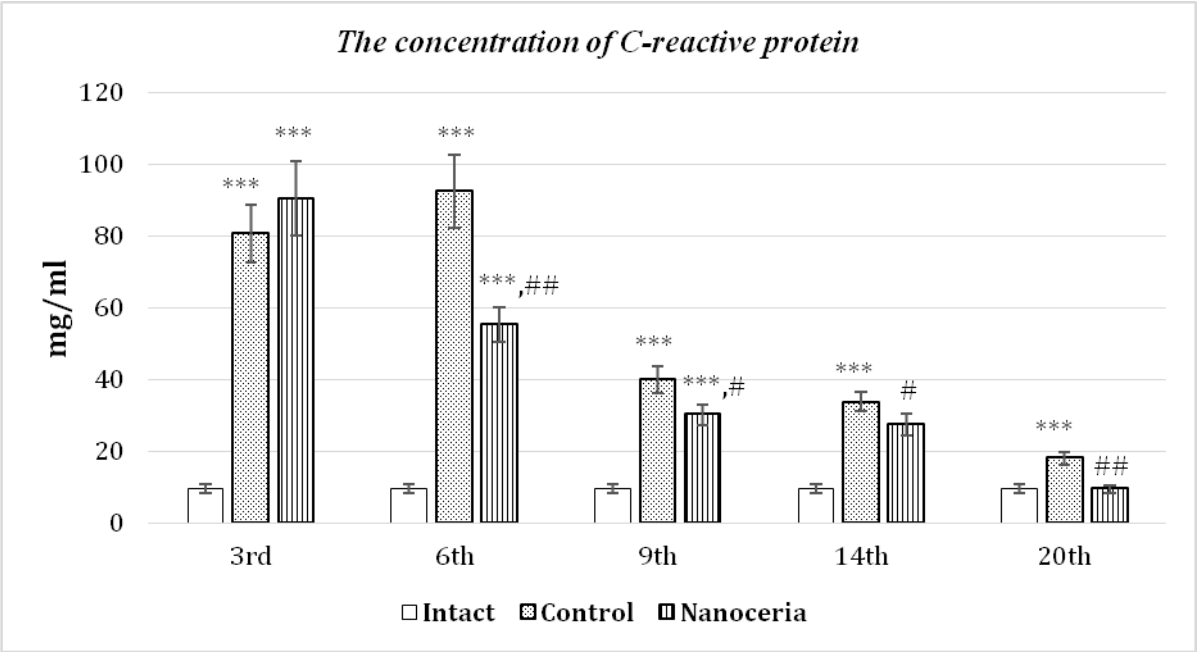

Figure 4.2. The concentration of C-reactive protein in blood serum mg / ml, M±SD:

3^{rd}, 6^{th}, 9^{th}, 14^{th}, 20^{th} – the days after wounds modeling;

** - p < 0,05, ** - p < 0,01, *** - p < 0,001 compared to the intact group of animals;*

 # - p < 0,05, ## - p < 0,01, ### - p < 0,001 compared to the control group of animals.

this indicator to the level of intact control that correlates with full re-epithelialization.

4.2. Determination of Middle-mass molecule's level in blood serum during wound healing

Middle-mass molecules (MMM) are considered as one of the suitable clinical indicators determining the development pathological process [148].

MMM is a type of combinations with the molecular mass upto 5,000Da and is divided into two major groups- average molecular mass substances and oligopeptides [149, 150].

When the metabolism alters to a reverse reaction, a big amount of metabolites and metabolic-waste products accumulate in blood remarkably high concentrations of a variety of biological substances, bacterial toxins, organs and tissues destruction products, protein and lipid hydroperoxides,etc. This pool of substances in blood is dispensed between the erythrocytes and plasma, and defines the concept of intoxication from the biochemistry point of view [151, 152].

Consequences of these substances accumulations in the blood causes tissue lysis and toxic product increment. Hence, MMM pool is the basic biochemical marker measuring the pathological protein metabolism level [153].

Current study demonstrates elevated level of endogenous intoxication markers (MMM) in blood serum in the control group of rats where nanoceria-based composition was not applied on the wounds (fig. 4.3). On the 3^{rd}, 6^{th}, 9^{th}, 14^{th} and 20^{th} days of the experiment the level of MMM in blood serum was increased in 8,0 ($p < 0,001$), 8,8 ($p<0,001$), 5,9 ($p < 0,001$), 4,8 ($p<0,001$)and 2,9 ($p<0,01$) times consequently in comparison with rats of intact group.

In contrast, where the treatment of wounds was carried out by nanoceria-based composition in experimental group, the level of MMM decreased significantly in each day of experiment: on the 3^{rd}, 6^{th} and 9^{th} days of the experiment it was increased only in 6,0 ($p<0,001$), 4,5 ($p<0,001$) and 4,2 ($p < 0,001$) times consequently. In this group of rats on the 14^{th} and 20^{th} days of the experiment the level of MMM in blood serum returned to the level of intact control.

Thus, it was established that in rats modeling of full-thickness wound of skin is accompanied by synthesis of acute inflammatory protein that shows up in increase of concentration of C-reactive protein and the level of MMM in blood serum which are indicators of endogenous intoxication and decompensation of vitally important

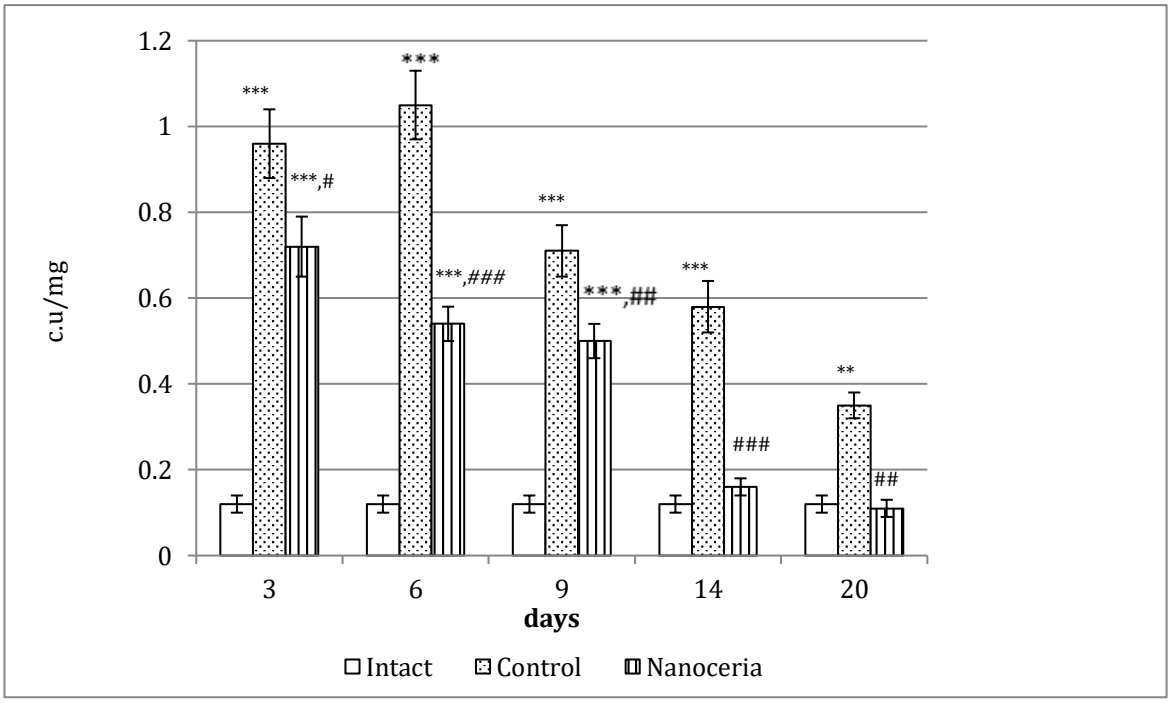

Figure 4.3. Level of Middle-mass molecules in blood, c.u/ml, M±SD.
** - p < 0,05, ** - p < 0,01, *** - p < 0,001 compared to the intact group of animals;*
 # - p < 0,05, ## - p < 0,01, ### - p < 0,001 compared to the control group of animals.

system. The use of nanoceria-based composition for the treatment of full-thickness wound of skin assists lowering of investigated parameters of inflammatory answer.

SECTION 5

PRO- / ANTIOXIDANT SYSTEM OF BLOOD SERUM OF EXPERIMENTAL FULL-THICKNESS WOUNDS AND UNDER TREATMENT BY PHARMACOLOGICAL COMPOSITION BASED ON NANOCERIA

5.1. Intensity of lipid peroxidation in full-thickness wound and in case of its correction by nanoceria-based pharmacological composition

Lipid peroxidation can be described as a process in which oxidizing agents such as free radicals attack lipids containing carbon-carbon double bond, especially polyunsaturated fatty acids (PUFAs) [154]. Lipids can also be oxidized by enzymes such as lipoxygenase, cyclooxygenase and cytochrome p 450 [155, 156]. In response to peroxide oxidation of lipid membranes and depending on specific cellular metabolic states and regenerative abilities, they can promote cell survival or cause cell death. At physiological or low lipid peroxidation rates, cells stimulate their support and survival through constitutional antioxidant defense systems or activation of signaling pathways that activate antioxidant proteins, leading to an adaptive stress response. On the contrary, with average or high lipid peroxidation (toxic conditions), the degree of oxidative damage overloads the ability to recover, and cells induce apoptosis or necrosis, programmed cell death; both processes ultimately lead to cell damage, which may contribute to the development of various pathological conditions [157].

Lipid peroxidation gives a wide range of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH), superoxide, hydroperoxide and hydroxyl, and diethyl conjugates. Among many different aldehydes that can be formed as secondary products during lipid peroxidation, malonic dialdehyde (MDA), propanol, hexanol and 4-hydroxynonenal (4-HNE)

[158]. MDA, apparently, is the mutagenic product of lipid peroxidation itself, while 4-HNE is the most toxic [159]. The final substances of LPO are fluorescent compounds of oxide copolymerization of lipids and proteins - schiff bases, gaseous products, nitrates and nitrites [160].

Our previous studies showed that application in the local treatment of Nanoceria-based pharmacological compositions significantly reduced the infection of the wound surface, healing occurred without the formation of a rough scar, that is, the composition possesses pronounced antibacterial and anti-inflammatory properties [161].

Disturbance of oxidative-antioxidant balance during skin injury is systemic, therefore it is expedient to determine these parameters in blood serum of rats.

Conjugated dienes measurement: It is generally accepted that the appearance of conjugated dienes in lipids stands for autoxidation of lipids. In fact, because of the divinylmethane structure, PUFA (polyunsaturated fatty acids) are particularly influenced by hydrogen removal by free radical attack, becoming themselves free radical intermediates. This results in the rearrangement of the double bond to conjugated dienes and, in the attendance of O2, the development of fatty acid hydro-peroxides [162]. The methods commonly used for specifying conjugated dienes are based on measuring their absorption at 233-236 nm [163 - 165]. The conjugated diene moiety is a powerful chromophore that can be indicated spectrophotometrically. When existing in fatty acids they display a characteristic absorption in the UV region [162].

The results have shown that in the blood serum of control group of animals, the content of LP products increases. Thus, the content of conjugated dienes increases: on 3^{rd} day - 1.5 ($p < 0,05$), onr 6^{th} day - 1.6 ($p < 0,05$) and on 20^{th} day the level of conjugated dienes decreased to the control level. The content of conjugated dienes in the blood serum of wounded rats under the action of carbopol had changes similar to the control group (fig 5.1). When using nanocria-based composition in experimental

group of rats, there is a decrease in the level of conjugated dienes, and on the 20th day their content was lower than the control level of 1.7 ($p < 0,05$).

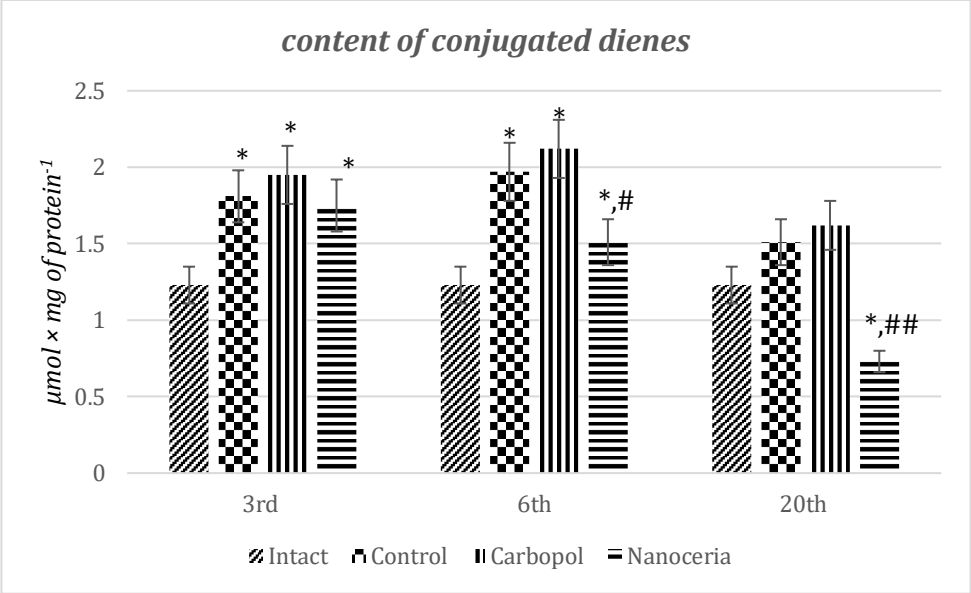

Figure 5.1: The content of conjugated dienes in blood serum of rats with full-thickness wound, µmol × mg of protein^{-1} (- $p < 0,05$ - compared to the control group of animals).*

** - $p < 0,05$, ** - $p < 0,01$, compared to the intact group of animals;*

 # - $p < 0,05$, compared to the control group of animals;

TBARS assay: Thiobarbituric acid reactive substances - TBARS - are made as a byproduct of lipid peroxidation (i.e. as degradation products of fats) which can be indicated by the TBARS assay using thiobarbituric acid as a reagent. Because reactive oxygen species (ROS) have extremely short half-lives, they are difficult to measure directly. Instead, what can be measured are several products of the damage produced by oxidative stress, such as TBARS [166]. Assay of TBARS measures malondialdehyde (MDA) existing in the sample, as well as malondialdehyde produced from lipid hydroperoxides by the hydrolytic conditions of the reaction [167]. MDA is one of several low-molecular-weight end products

formed via the decomposition of certain primary and secondary lipid peroxidation products.

Our results have shown in intact group of animals, the content of TBARS compounds increases: on 3rd day by - 2.1 ($p < 0,05$), 6th day - 3 ($p < 0,05$) and on 20th day by - 1.3 ($p < 0,05$) compared with the control group. The content of TBARS compounds in the blood serum of wounded rats under the action of carbopol was similar to that of intact group. In the blood serum of experimental group of rats, it was shown that when applying Nanocria, the level of TBARS products decreased on 3rd and 6th and 20th days. Their content was below the reference level by 1.5 ($p < 0,05$) (fig. 5.2).

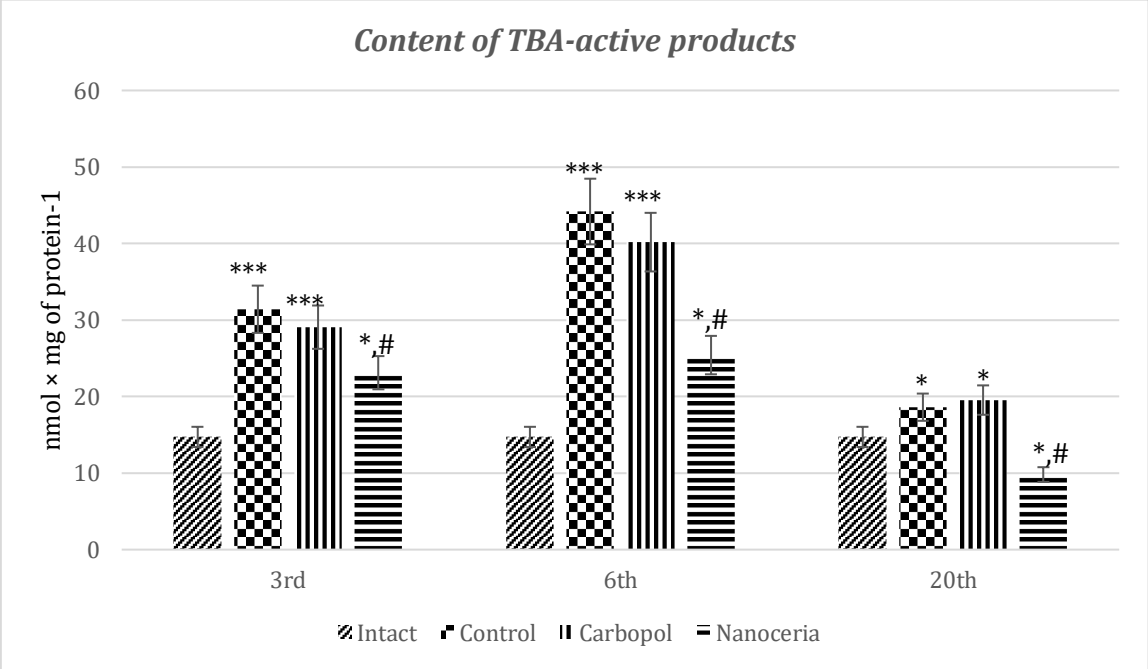

Figure 5.2: Content of TBA-active products in blood serum of rats with full-thickness, nmol × mg of protein-1, *M±SD,*

** - p < 0,05, ** - p < 0,01, compared to the intact group of animals;*

 # - p < 0,05, compared to the control group of animals;

Anisidine value (Schiff base absorption) : Schiff base is a compound that is being used as ligands to form harmonized complexes with metal ions. Conjugated Schiff

bases absorb strongly in the UV-vis region of the electromagnetic spectrum. This absorption is the basis of the anisidine value, which is a measure of oxidative deterioration for fats and oils. *p*-Anisidine condenses readily with aldehydes and ketones to form Schiff bases, which absorb at 350 nm. This colorimetric reaction is used to test for the presence of oxidation products in fats and oil [168].

We have shown in the control group of animals, the content of schiff bases increased on for 3rd day by - 1.5 ($p < 0,05$), 6th day by - 1.8 ($p < 0,05$) and on 20th day by1.3 ($p < 0,05$) compared to the intact group. Under the influence of carbopol, the level of schiff bases in the blood serum of wounded rats had similar changes of intact group. When using nanoceria, it was observed that in the blood serum of wounded rats, the level of schiff bases on 3rd, 6th and 20th days is reduced. Their content was 1.7 ($p < 0,05$) lower than the control level (fig. 5.3).

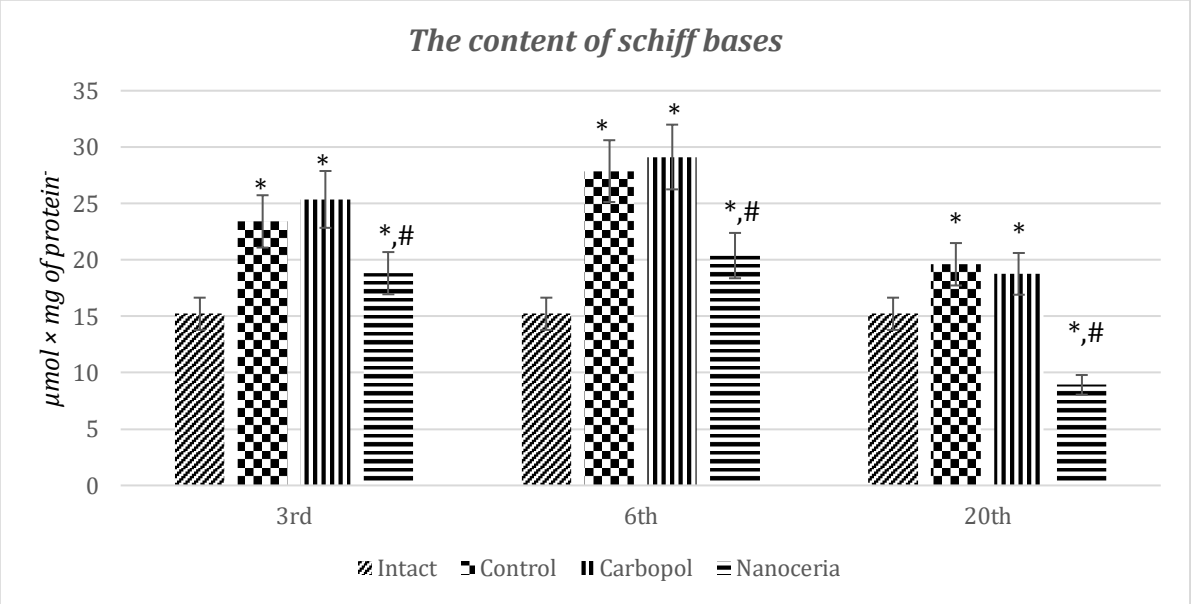

Figure 5.3: The content of schiff bases in blood serum of rats with full-thickness wounds, µmol × mg of protein^{-1}, M±SD.

** - p < 0,05, ** - p < 0,01, compared to the intact group of animals;*

 # - p < 0,05, ## - p < 0,01 compared to the control group of animals;

Thus, the data obtained by us indicate that in the blood serum at the beginning of the experiment, in response to the intensive formation of free radical compounds, the activation of lipid peroxidation was observed. The application in the local treatment of Nanoceria-based compositional compositions has led to a significant decrease in lipid peroxidation in serum, to correct the course of all phases of the wound process.

5.2. Activity of enzymes of antioxidant protection during experimental full-thickness wound healing

Oxidative stress is the root of many drastic diseases and one of its primitive features is the cellular disbalance between endogenous antioxidant defenses (free radical scavenging by small molecule antioxidants and/or redox enzymes) and ROS (e.g., superoxide radical anion, hydrogen peroxide ($□_2O_2$ $□_2O_2$); hydroxyl radical) production inside the cells [168]. The capability of nanoceria to switch between oxidation states is comparable to that of biological antioxidants. This capability imparts nanoceria with the very important biological property of radical scavenging [169]. Researchers have suggested that nanoceria possesses predominantly SOD-mimetic and CAT-mimetic activities [169, 170].

The received data have shown that in the case of a full-thickness wound in the blood serum, products of the LPO accumulate, indicating a disturbance of the balance between the intensity of free radical procedures and the work of the antioxidant system. The obtained data indicate that the cerium-based drug contributes to the effective blockage of the LPO stages, which declares itself as a steady decrease in the content of lipid peroxidation products.

Cerium is a rare earth metal that, when incorporated with oxygen, can adopt a fluorite crystalline net structure that has an extremely reactive surface area for neutralization of radicals. In addition, nanoceria can reversibly bind oxygen and shift oxidation states (Ce3+/Ce4+) depending on the situation [15]. The use of nanoceria

for therapeutic goals provides several benefits over other novel antioxidant approaches. For example, the delivery of nanoencapsulated antioxidant enzymes, such as SOD or catalase, has the restriction that only one sort of reactive oxygen species can be scavenged by each enzyme, whereas, several species are involved in neurodegenerative diseases [16] and nanoceria have been shown to decline their levels [17, 18]. Because of these properties, nanoceria have been investigated in biological systems and shown to exhibit antioxidant effects in various models of disease [19]. In contrast, nanoceria exhibits both catalase and SOD-mimetic activity with SOD-mimetic catalysis overpass that of the endogenous enzyme [20, 21]. One study showed that 5.8 μM nanoceria was equivalent to 527 U of SOD [22]. There are two categories of thoughts on the SOD-mimetic, hydrogen peroxide catalase mechanism of ceria. The first is that the Ce3+/Ce4+ ions, interact directly to both neutralize (i.e. oxidize) superoxide and overthrow peroxide. We will name this the ionic mechanism. The second school of thought is that both reactions proceed by oxygen vacancy creation and annihilation (filling), with the cerium ionic states interchanging between plus three and plus four to incorporate the oxygen vacancy population [23]. Returning now to the ionic mechanism the SOD mimetic component is believed to be facilitated by an enhancement in the Ce3+/Ce4+ ratio 110 while the catalase component is facilitated by a decline in this ratio [24].

The manifestation of negative harmful effects of free radicals and peroxide compounds is obstructed by a multi-component antioxidant system, providing binding and recombination of radicals, preventing the formation or destruction of peroxides. The system of antioxidant protection of the organism consists of two main parts: enzymatic and non-enzymatic. Antioxidant enzyme systems include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), glutathione transferase (GT), glutathione reductase (GR), which are involved in the neutralization of free radicals. SOD - an enzyme that plays a key role in the utilization of superoxide anion radicals [171, 172]. CAT catalyzes the two-electron reduction of hydrogen peroxide to water [173].

Glutathione peroxidases have five isoforms, which include cytosolic (GP-1), epithelial-specific enzyme, gastrointestinal (GP-2), plasma (GP-3) and GP-4 hydroperoxide lipids and nuclear GP [174]. Accordingly, GP is the most important antioxidant enzyme involved in preventing the harmful appetite of intracellular hydrogen peroxide. GP, uses a reduced form of glutathione as a substrate, efficiently cleaves not only hydrogen peroxide, but also organic hydroperoxides. Under certain conditions, its role in tissues with the disinfection of hydrogen peroxide is more important than catalase, since the effect of catalase is limited to peroxisomes [175]. GP has a high affinity for H_2O_2, which determines its leading role in the metabolism of hydrogen peroxide at reduced physiological concentrations of catalase. The activity of the GP increases in pathological conditions.S-transferase (GT) plays an important role in the antioxidant protection of the body play. These are antioxidant enzymes that catalyze recombined glutathione (GSH) conjugation with various toxic compounds, including xenobiotics and oxidative derivatives (e.g., lipids and DNA) [176, 177]. This reduces their toxicity and facilitates the separation from the cell. Their key role in the cell is detoxification protection of macromolecules from the adverse effects of ROS, carcinogens and chemotherapeutic agents [178]. More and more evidence suggests that the expression of GT and its functions play an important role both in the progression of the growth of cancerous tumors and acute inflammatory states [179].

Antioxidants (Catalase and SOD activities): We represented in the control group of animal the activity of enzymes in the first line of cell protection from oxidative stress changes: superoxide dismutase activity decreases and catalase activity increases with respect to the intact group of animals. Thus, superoxide dismutase activity is reduced on the 3^{rd} day - by 9.1 ($p < 0,05$), 6^{th} day - by 6.6 ($p < 0,05$) and on 20^{th} days - by 6.1 ($p < 0,05$) compared to the intact group. In this case, the catalase activity increases on 3^{rd} day - by 2.9 ($p < 0,05$), on the 6th day - by 3.3 ($p < 0,05$) and on 20^{th} day - by 1.2 ($p < 0,05$) compared with the intact group. The activity of antiradical enzymes in blood serum of rats with an experimental wound under the

influence of carbopol was similar to that of intact group. With the use of nanoceria on experimental group, restoration of superoxide dismutase activity and reduction of catalase activity to the control level are observed (fig. 5.4, 5.5).

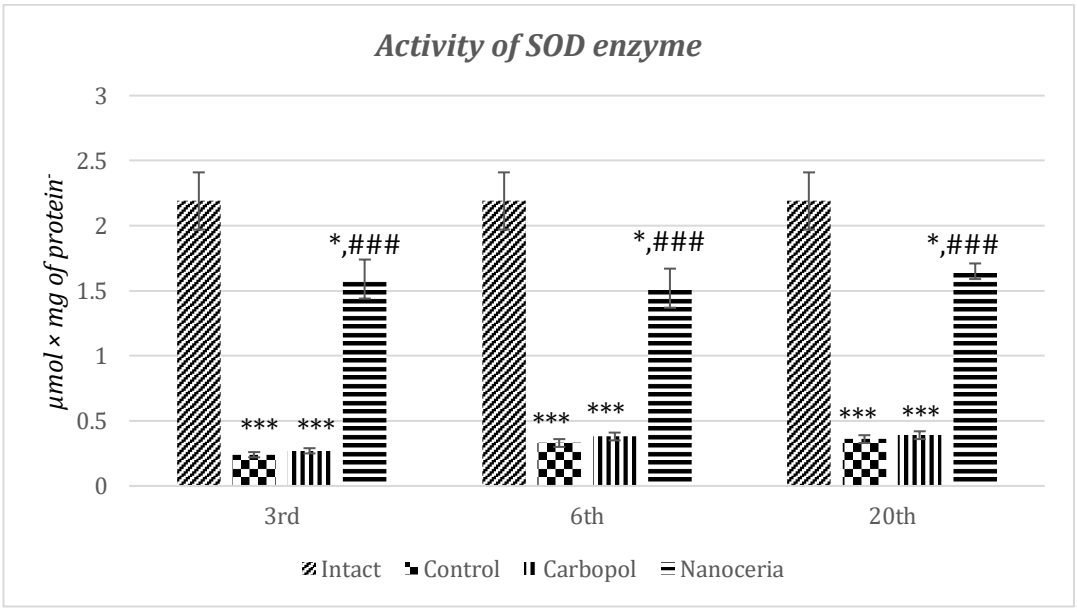

Figure 5.4: Activity of superoxide dismutase in blood serum of rats with full-thickness wounds, M±SD.

** - p < 0,05, ** - p < 0,01, *** - p < 0,001 compared to the intact group of animals; # - p < 0,05, compared to the control group of animals;*

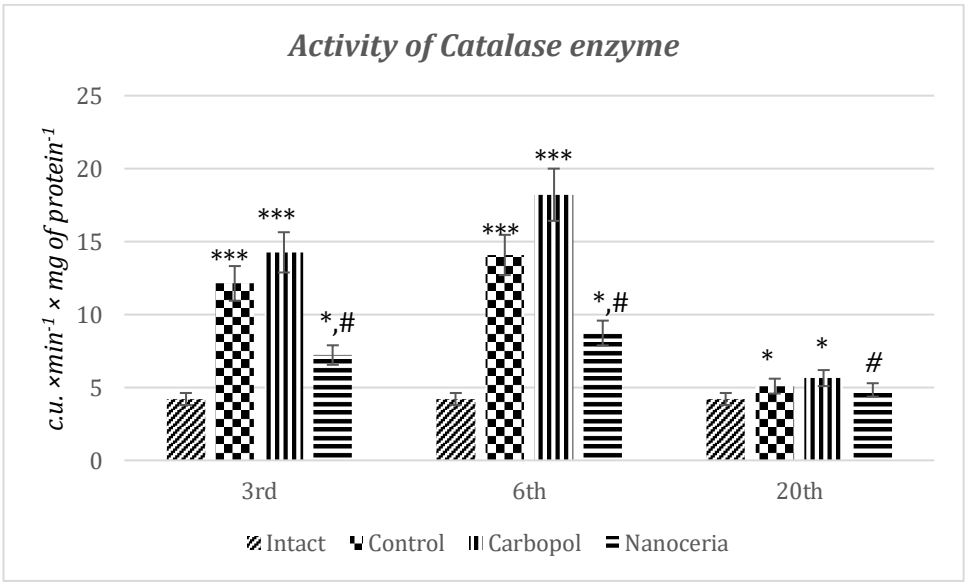

Figure 5.5: Activity of catalase enzyme in blood serum of rats with full-thickness wounds, c.u. ×min^{-1} × mg of protein^{-1}, M±SD.

** - p < 0,05, ** - p < 0,01, *** - p < 0,001 compared to the intact group of animals; # - p < 0,05, compared to the control group of animals;*

Measurement of SH-group content (or Glutathione assay): For glutathione (GSH) content assessment in blood serum, 5, 5′-Dithiobis (2-nitrobenzoic acid) (DTNB) was used as the indicator. Cells were analyzed with the spectrophotometric method [180]. A modest report of spectrophotometric method was carried out for the routine attendant determination of sulfhydryl groups in PB-SH, NP-SH, and T-SH fractions. These spectrophotometric procedures are based on the method of Ellman [181, 182], who reported that 5, 5'-dithiobis- (2, -nitrobenzoic acid) is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro-mercaptobenzoic acid anion has an intense yellow color and can be used to measure SH groups. The reaction is presented on figure 5.6.

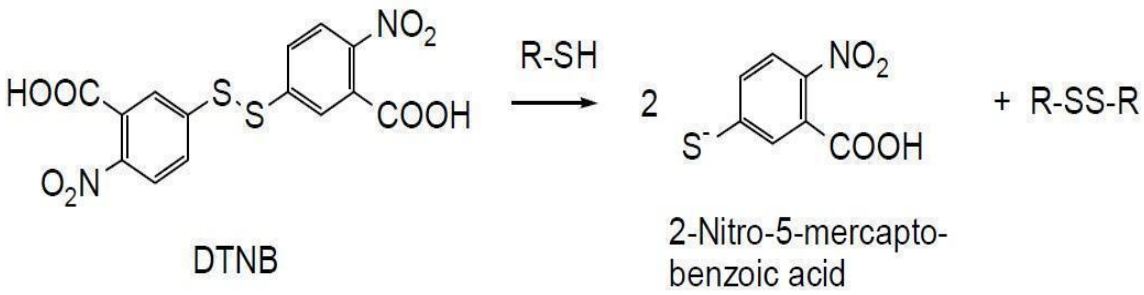

Figure 5.6: Reaction of Ellman's Reagent with sulfhydryls. [181]

Ellman's reagent is used for the modification of free thiols in proteins. It quickly forms a disulfide bond with the thiol and liberates a thiolate ion which is colored (yellow). The maximal absorbance of this thiolate is at 412 nm. Its presence can be plotted against a standard curve for characterization of the total amount of free thiols in proteins. The participation of three types of GSH (Glutathione peroxidase (GSH-

Px), glutathione S-transferase (GSH-Tr) and glutathione reductase (GSSG-Rx)) in the detoxification of reactive species of oxygen and in the inactivation of electrophiles such as carcinogenic-epoxide metabolites has been well established. Both types of processes can occur enzymatically. Particularly, GSH-Px assists the breakdown of H2O2 and other organic hydro-peroxides whereas GSH-Tr is responsible for the deactivation of electrophiles [183, 184]. The non-protein low molecular weight sulfhydryl groups, known as thiols, such as cysteine and glutathione play considerable functions in cells. Glutathione represented many impressive roles such as antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune responses, and regulation of leukotriene and prostaglandin metabolism [185] (fig. 5.7).

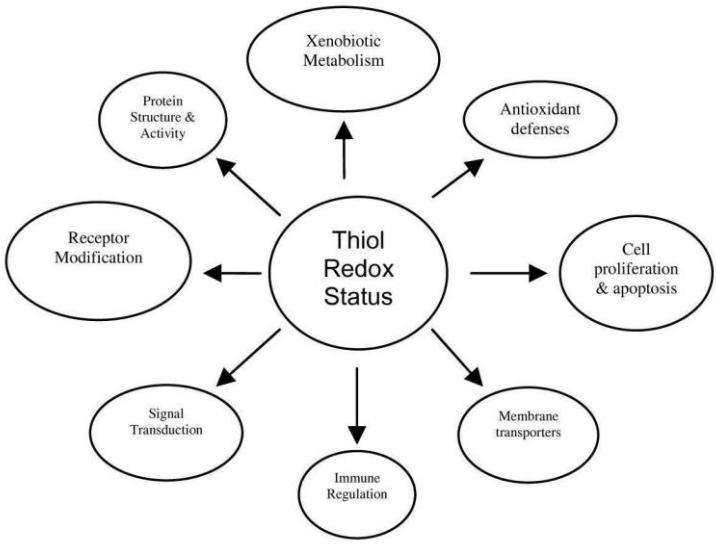

Figure 5.7: Thiol redox status and its many accompanying roles [24].

On the other hand, cysteine a sulfur containing amino acid has been offered as a nucleophile (i.e. the reactive center of an enzyme). Cysteine's ability to happen in up to 10 different sulfur oxidation states in vivo directs to a range of cysteine modifications in peptides and proteins. Each of these cysteine modifications

represents its own particular chemical and biochemical characteristics such as stability, redox-behavior, metal-binding, acidity, nucleophilicity, and catalytic activity. This inimitable reactivity of cysteine is reflected by the innumerable functions that cysteine complies in vivo, including structural stabilization, catalysis, redox-activity, and metal-binding (fig.5.8) [186].

We have shown a decrease in the level of protein and non-protein sulfhydryl groups is observed, indicating that they are damaged by free radicals. According to our results in the blood serum of control group of rats, the content of sulfhydryl groups is reduced (fig. 5.9). Thus, the level of non-protein SH-groups is reduced on 3rd day - 1.8 ($p < 0,05$), 6th day 1.9 ($p < 0,05$) and by 20th day by 1.4 ($p < 0,05$) compared to the intact group. In this case, the content of protein SH groups is reduced on 3rd day by 1.3 ($p < 0,05$), 6th day - 1.6 ($p < 0,05$) and 20th day - 1.4 ($p < 0,05$) compared to the intact group (fig. 5.10). Similar changes in the level of sulfhydryl groups have been found in the blood serum when investigating the effects of carbopol.

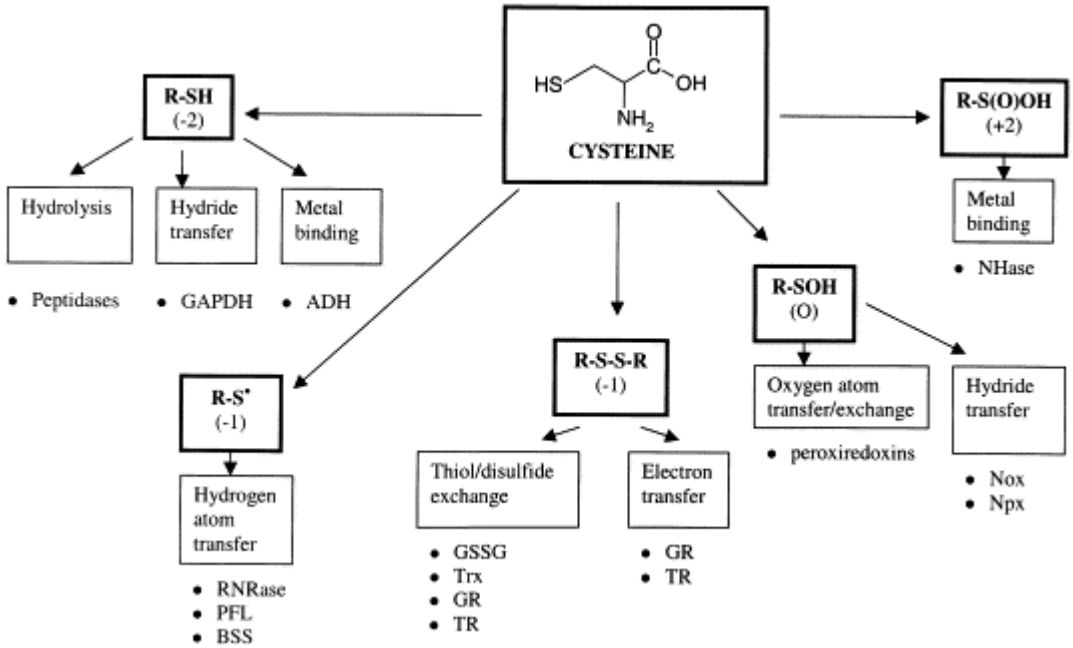

Figure 5.8: Oxidation states, properties, reactivity, and occurrences of different cysteine modifications in vivo. Enzymes discussed in the text are shown as examples. Oxidation states of sulfur are given for R in oxidation state +1 [186].

In the experimental group of animals, the level of total-protein SH-groups is reduced on 3rd day by- 1.3 (p <0,05), 6th day - 1.2 (p <0,05) and on 20th day - 1.4 (p <0,05) compared to the intact group. It was demonstrated when nanoceria-based composition was applied to wounds, the restoration of sulfhydryl groups was observed (fig. 5.11). The obtained results indicate that the level of free radicals increases in serum on the wounded surface area, which leads to the depletion of the level of non-protein low molecular weight thiols (cysteine, glutathione, etc.) and inhibition of the activity of thiol enzymes by blocking their sulfhydryl groups (glutathione peroxidase, glutathione transferase, glutathione reductase). Reduction of the total, protein and non-protein SH groups in this experiment (intact group) reflects the overall displacement of the redox-balance in the pro-oxidant side.

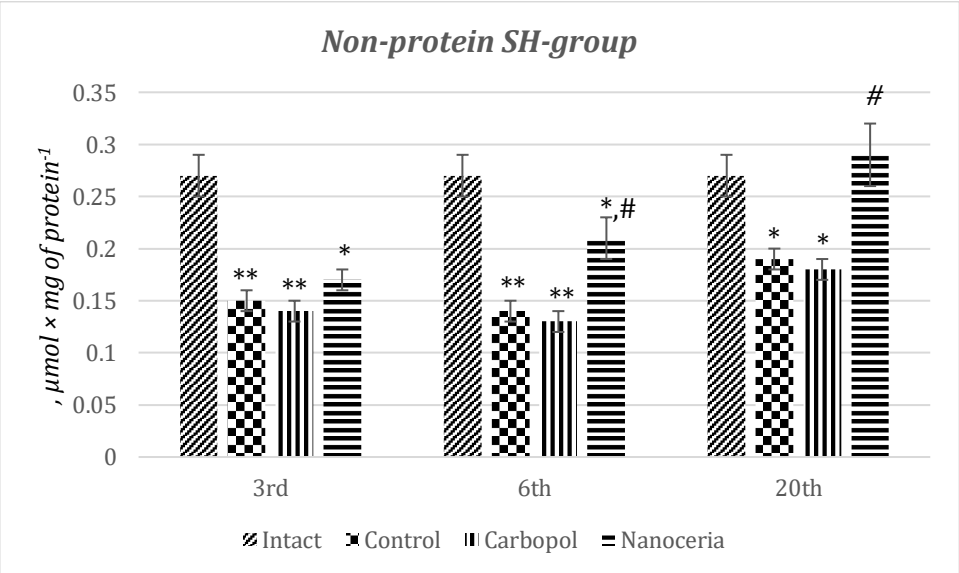

Figure 5.9: The content of non-protein sulfhydryl (SH-) groups in blood serum of rats with full-thickness, µmol × mg of protein^{-1}, M±SD.

** - p < 0,05, ** - p < 0,01, compared to the intact group of animals;*

 # - p < 0,05, compared to the control group of animals;

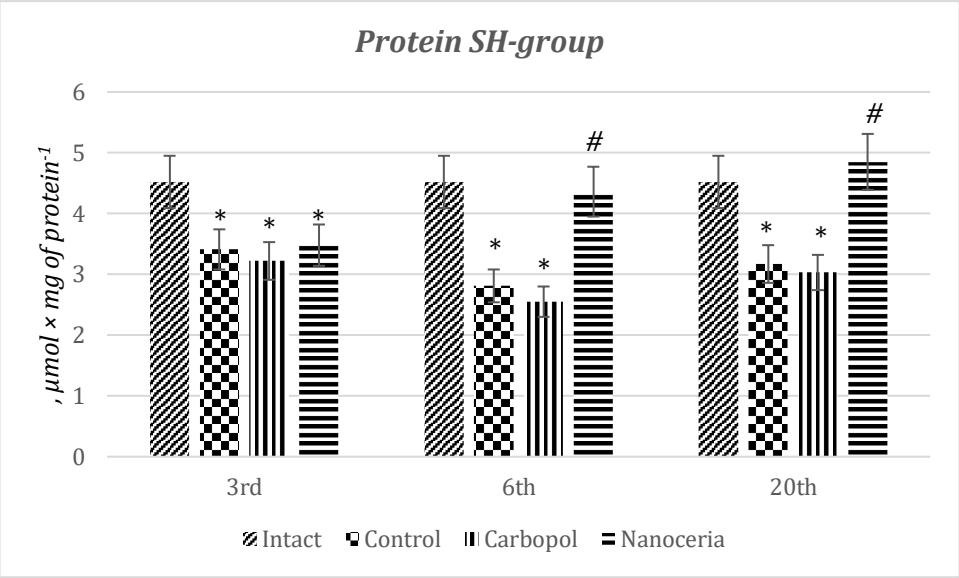

Figure 5.10: The content of protein sulfhydryl (SH-) groups in blood serum of rats with full-thickness, µmol × mg of protein^{-1}, M±SD:

** - p < 0,05, ** - p < 0,01, compared to the intact group of animals;*

 # - p < 0,05, compared to the control group of animals;

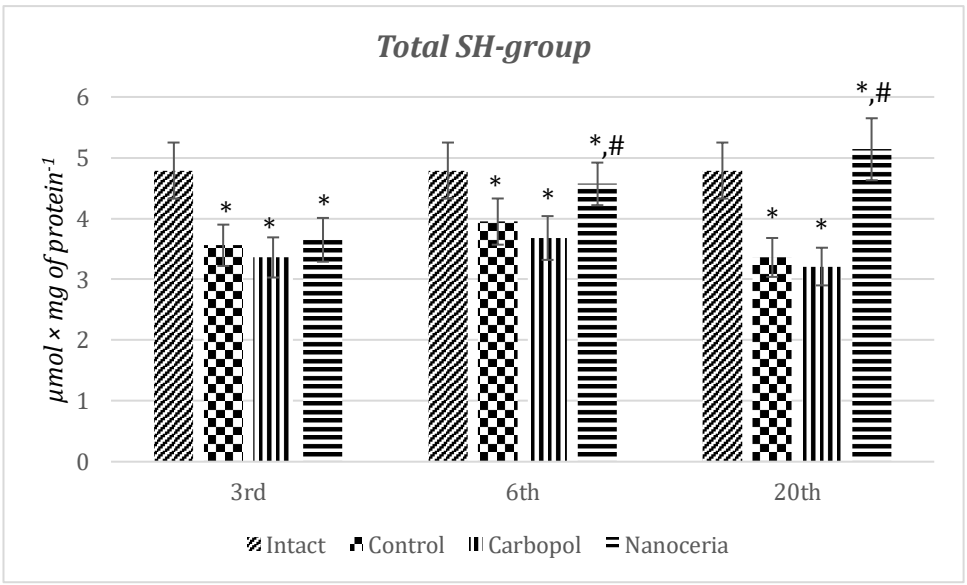

Figure 5.11: The content of total sulfhydryl (SH-) groups in blood serum of rats with full-thickness, µmol × mg of protein^{-1}, M±SD:

** - p < 0,05, compared to the intact group of animals;*

 # - p < 0,05, compared to the control group of animals;

Taking the whole topic of Pro-/Antioxidant activity of Nanoceria some authors have proven our research accurate.

Supporting our data, A.S. Karakoti et al. have shown that vacancy-engineered ceria nanoparticles exhibit unique capabilities for quenching reactive oxygen intermediates, and could find widespread use in the treatment of ROI-mediated medical disorders [187].

In addition, Talib Pirmohamed established the catalase mimetic capabilities of nanoceria and affirms that this catalytic reaction is not equivalent for all nanoceria preparations. Our findings suggest that one of the mechanisms by which nanoceria protect cells from oxidative stress may be due to their ability to act as catalase mimetics, in addition to exhibiting SOD mimetic activity [188].

Bharat Bhushan et al. suggested that nanoceria was synthesized by hydrothermal method and their enzyme mimetic activity was analysed by SOD assays. The biocompatibility and protective effect of nanoceria against H2O2 induced cytotoxicity was evaluated in vitro in fibroblast cells by MTT assay. Further, the flow cytometric and fluorescence microscopic analysis revealed the ROS scavenging potential of nanoceria in vitro. The cell cycle and mitochondrial membrane potential analysis further supports the role of nanoceria in protecting the cells against oxidative stress induced cell death. Moreover, the antioxidant potential of nanoceria was also investigated in vivo in zebrafish model. The results suggest the biocompatibility and protective role of nanoceria against the H2O2 induced embryotoxicity in zebrafish by effective removal of toxic free radicals. Thus, the present study suggests that the nanoceria and zebrafish model could be exploited as an alternative tool for future antioxidant therapy [189].

5.3. The influence of pharmacological nanoceria based composition on content of the products of oxidative modification of proteins in the serum of rats with full-thickness wounds

It was found that in rats with excisional full-thickness wounds there is an increase in the level of redox proteins of the serum (table 5.1). Thus, the level of neutral aldehyde (max. Absorption at 346 nm) products increases on 3rd and 6th days - 2.5 times and on 20th days - 1.9 times compared to the control. Also, an increase in serum content of neutral ketone (E max = 370 nm) products on 3rd day -

Table 5.1.

The content of the products of oxidative modification of proteins in the blood serum of rats with full-thickness wounds, μmol × mg protein-1

Group of animals indicator		Products neutral in nature		Products main character	
		356 nm, aldo-derivatives	370 nm, keto-derivatives	430 nm, aldo-derivatives	530 nm, keto-derivatives
Control		0,118 ± 0,012	0,123 ± 0,019	0,089 ± 0,009	0,042 ± 0,004
3 rd day	Wounded rats	0,293 ± 0,026*	0,162 ± 0,015*	0,173 ± 0,016*	0,145 ± 0,014*
	Carbopol	0,285 ± 0,027*	0,167 ± 0,016*	0,188 ± 0,018*	0,151 ± 0,015*
	Wounded rats+ Nanoceria	0,271 ± 0,025*	0,154 ± 0,014*	0,162 ± 0,016*	0,141 ± 0,014*
6th day	Wounded rats	0,293 ± 0,028*	0,194 ± 0,019*	0,195 ± 0,021*	0,149 ± 0,013*
	Carbopol	0,285 ± 0,026*	0,185 ± 0,017*	0,218 ± 0,022*	0,143 ± 0,013*

	Wounded rats+ Nanoceria	0,242 ± 0,023*	0,161 ± 0,015	0,149 ± 0,014	0,121 ± 0,011*
20th day	Wounded rats	0,221 ± 0,021*	0,183 ± 0,015*	0,166 ± 0,015*	0,098 ± 0,009#
	Carbopol	0,242 ± 0,023*	0,188 ± 0,019*	0,159 ± 0,015*	0,091 ± 0,008*
	Wounded rats+ Nanoceria	0,154 ± 0,015	0,107 ± 0,011	0,094 ± 0,009	0,064 ± 0,005*

* - p <0.05 - compared to the control group of animals

1.3 times, on the 6th day - by 1.6 times and on 20th day - 1.5 times compared to the control group.

It is shown that in the serum of excisional full-thickness wounded rats, the number of basic aldehyde (maximum absorption at 430 nm) products is increased on 3rd day by - 1.9 times, 6th day - 2.2 times, and 20th day - 1,9 times compared to control group. Under the same experimental conditions, the level of basic ketone (E max = 530 nm) products increased on 3rd and 6th days - 3.5 times and on 20th days - 2.3 times compared to the control group in the serum (table 5.1).

In the group of rats with excisional full-thickness wounds, which were treated with carbopol, a serum similar to the group of animals with an experimental wound showed an increase in the level of redox proteins (table 5.1).

When using nanoceria, there is a decrease in the content of both neutral aldehyde and ketone products in serum, as well as the main aldehyde and ketone products of oxidative modification of proteins (table 5.1).

So, we confirmed above mentioned cocnclusion about antioxidant and antiradical action of nanoceria in conditions of skin wound healing.

SECTION 6

EVALUATION OF THE CONCENTRATION OF SOME GROWTH FACTORS, MMPs AND HIF-1α DURING THE WOUND HEALING PROCESS IN EXPERIMENTAL FULL-THICKNESS WOUNDS IN RAT MODEL

6.1 Growth factor association with wound healing process

The deposition and synthesis of extracellular matrix (ECM) is a serious specification in the healing of chronic wounds, that are defined by fundamental detriment of the dermal matrix, and acute wounds. Interactions between the growth factors, cells and ECM underlay tissue procreation and recreation, consisting wound healing. These elements communicate in a continuous, bilaterally crucial dynasty of incidents that has been denoted to as dynamic reciprocal interaction [190]. Wound healing has been voluntarily segregated into the overlapping stages of inflammation, proliferation, and remodeling—each of which is determined by dynamic reciprocity among growth factors, cells, ECM [191]. For example, throughout the inflammatory phase, fibronectin and other ECM protein fragments in the wound area act as chemoattractants for monocytes [192] which then attach to ECM proteins. This attachment provokes phagocytosis [193] resulting in the monocytes/macrophages to latter decomposition of ECM fragments and other debris in the area [194]. Cohesion of monocytes to ECM proteins also provokes the expression of growth factors [195] that can then have an influence on cells to impress the synthesis of ECM constituents [196].

Interactions between ECM and growth factors in this dynamic reciprocal relation take various patterns (fig. 6.1). Some are unmediated, suchlike the direct attachment of growth factors by ECM components, and some are indirect, such as the necessity for cells to be attached to ECM in order to react to the growth factor signal.

In this article we discuss about different kinds of ECM-growth factor interactions. Here we concentrate on the connection of these interactions to wound healing [190, 196].

Before mediating particular types of ECM-growth factor interactions, we briefly discuss the compounds of the ECM and their functions in the wound healing.

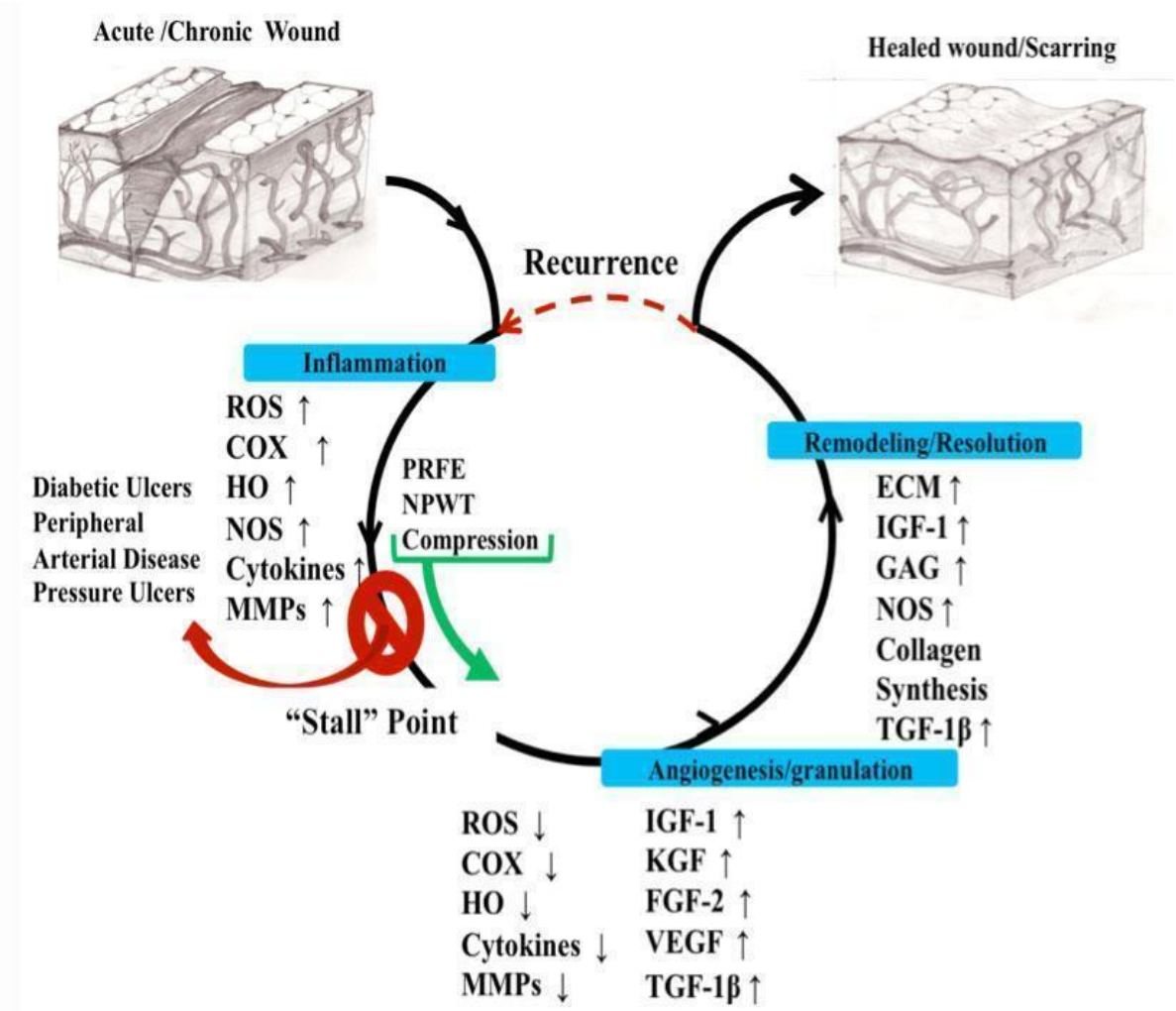

Figure 6.1: A cyclic model for wound healing. [191]

ECM is collected from compounds synthesized in the outer surface of the cells that allow functional and structural integrity to the organs and connective tissues [191,197]. Incorporation of ECM mainly happens in response to cytokines, growth factors and mechanical signals affected via cell surface receptors [198]. These cell surface receptors bring forth points of adherence that cells can utilize to perceive

mechanical interruption and to alter the deposited matrix to deliver it functionally and structurally viable [199]. The ECM can also act as a repository matrix for proteins and growth factors deposited over wounding from plasma proteins and degranulating cells detected in the blood [200]. Recent studies have shown that matrix alterations can take place before actual trauma that emerge to make individuals prone to chronic repair processes [201,202].

Deterioration and alteration of the ECM by proteases, exclusively matrix metalloproteinases (MMPs), is a main reason of tissue remodeling, angiogenesis, leukocyte influx and re-epithelialization. MMPs also derogate angiogenic factors as well as growth factors and their receptors. Regulation of these numerous elements by MMPs defines whether angiogenesis will be stimulated or prevented [203]. MMPs also play a fundamental role in releasing growth factors and splitting ECM proteins to disclose areas that can actuate growth factor receptors [204]. Thus, MMPs participate not only in degrading and remodeling selected ECM compounds at suitable times, but also to divulge chosen bioactive ECM parts through targeted cleavage that eventually affect cellular performance [204]. MMPs are produced by keratinocytes at the wound ledge during wound healing and eventually dispatch from the basement membrane and move towards the wound bed [205]. Generation of MMPs is controlled by cellular interactions with the matrix, as displayed by the capability of human keratinocytes flourished on native type I collagen, but not denatured collagen or Matrigel, to represent high levels of MMPs [206]. These data render one more example of the ECM's adjustment of the level /pattern of cellular gene expression. Although regulated generation of proteases is crucial to typical wound healing, chronically increased levels of specific MMPs can result to matrix deterioration and are accompanied with ruinous wound healing [207, 208].

The ECM come together with the whole cellular microenvironment to define cellular phenotype and performance [196]. ECM cooperates with growth factors in various ways that eventually yield a reciprocal regulation. In the following section,

we will discuss various major kinds of correlations between growth factors and ECM, focusing on examples pertinent to wound healing and our researches.

6.1.1 The influence of Nanoceria on Vascular Endothelial Growth factors (VEGF) concentration in the skin during healing of full-thickness wounds

Angiogenesis is a process that arises during wound healing that necessitates attachment of cells to the ECM for the mean of growth factors' response. VEGF demonstrated to elevate collagen binding integrins $\alpha_1\beta_1$ and $\alpha_1\beta_2$ expression in dermal microvascular [209]. Antibodies that block α_1 and α_2 integrin subunits significantly prevent VEGF-induced angiogenesis without needing to affect the vasculature that already exist [209]. This represents that these integrins are pivotal to VEGF-induced angiogenesis. Related research has revealed that the integrin $\alpha_v\beta_3$ is not expressed in blood vessels of normal skin but expression occurred on human wound granulation tissue, and those antibodies against this integrin block angiogenesis induced by FGF and TNF-α without influencing blood vessels that already exist [210]. Moreover, an interim relation between $\alpha_v\beta_3$ expression and wound angiogenesis has been recognized, with this receptor first expressed on hypertrophied micro-vessels and later on capillary sprouts that attack the fibrin clot; antibodies against this receptor also temporarily prevent granulation tissue formation [211]. The crucial nature of integrin binding to angiogenesis is also noticed in other states like embryogenesis, where inhibition of β_1 integrins intervene with the formation of the embryonic vasculature [212] and in oncology, where integrin inhibitors are engaged to prevent tumor angiogenesis [213].

VEGF family members consist of: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor [214]. VEGF-A is a product of keratinocytes, neutrophils, fibroblast smooth muscle cells, endothelial cells, macrophages and platelets [215 - 217]. It binds to the tyrosine kinase surface receptors Flt-1 (VEGF receptor-1) and KDR (VEGF receptor–2 [VEGFR-2]) [218, 219] localized to the

endothelial surface of blood vessels [220, 221]. These receptors have different roles. KDR is a significant intermediate for proliferation of endothelial cells in vitro and chemotaxis [222]. It is also responsible for persuading endothelial cell differentiation. In contrast, Flt-1 is necessary for blood vessels organization [223, 224]. Flt-1 may also be involved in intermediating vascular permeability [225] MMP expression in vascular smooth muscle cells, [226] and the induction of anti-apoptotic proteins [227].

VEGF-A plays a significant role in wound healing because it upgrades the early events in angiogenesis, particularly endothelial cell migration [221, 228] and proliferation [229, 230] as seen in several in vitro studies. VEGF-A secretion and transcription along with the VEGFR are enhanced in the acute wound [231, 232]. Activated platelets liberate VEGF-A upon injury [233, 234]. Moreover, during wound healing macrophages release VEGF-A [235] as well as releasing TNF-α, which induces VEGF-A expression in fibroblasts and keratinocytes [236].

In comparison to VEGF-A, VEGF-B plays a less significant role in the vascular system: While VEGF-A is substantial for the generation of blood vessels, such as during development or in pathological conditions, VEGF-B seems to play a role only in the preservation of newly constructed blood vessels during pathological conditions. [237] VEGF-B participates also in various types of neurons. It is important for the protection of neurons in the retina [238] and the cerebral cortex during stroke [239] and of moto-neurons during motor neuron diseases such as amyotrophic lateral sclerosis [240].

VEGF-B applies its effects via the FLT1 receptor [241]. VEGF-B has also been shown to control endothelial uptake and transport of fatty acids in heart and skeletal muscle [242, 243].

VEGF-C is up-regulated during wound healing and its firstly released by macrophages and is important during the inflammatory stage of wound healing [243]. VEGF-C acts mainly through the VEGF receptor-3 (VEGFR3), which is expressed in monocytes/macrophages, lymphatic endothelium and fenestrated endothelial [243,

244]. The main role of VEGF-C is in lymph angiogenesis, where it primarily acts on lymphatic endothelial cells (LECs) via its receptor VEGFR-3 supporting migration, growth, survival. It was discovered in 1996 as a ligand for the orphan receptor VEGFR-3 [245]. Shortly thenceforth, it was shown to be a particular growth factor for lymphatic vessels in a diversity of models [246, 247]. However, besides its impact on lymphatic vessels, it can also encourage the blood vessels growth and adjust their permeability. The effect on blood vessels can be mediated via its primary receptor VEGFR-3 [248] or its secondary receptor VEGFR-2. In addition, VEGF-C plays an important role in neural formation [249] and blood pressure adjustment [250].

C-fos-induced growth factor (FIGF) (or vascular endothelial growth factor D, VEGF-D) is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family and is active in lymph angiogenesis, angiogenesis and endothelial cell growth. This secreted protein undergoes a complex proteolytic maturation, generating multiple processed forms which bind and activate VEGFR-2 and VEGFR-3 receptors. The function and structure of this protein resembles those of vascular endothelial growth factor C.

Placental growth factor (PLGF) is also up-regulated during wound healing which is a proangiogenic molecule. In the skin, this growth factor is expressed by keratinocytes and by endothelial cells. This growth factor implements its action by binding and activating the VEGFR-1. Like VEGF-C, PLGF plays a role during the inflammatory stage of wound healing.

When the skin is wounded the cells in wound area lead to VEGF induction. Once VEGF is induced the angiogenesis starts. As seen in figure 6.2, in control group of rats, VEGF level in blood serum was highly increased (upregulated) on 3^{rd} day and then was down-regulated gradually on 6^{th} and 20^{th} days. In experimental group of rats, in comparison with control group, VEGF was upregulated on 6^{th} day and then down-regulated on 20^{th} day. VEGF level was lower on 20^{th} (0.79 ± 0.1 ($p<0.05$)) day in experimental group compared to the control group (0.95 ± 0.1 ($p<0.05$)) which means the angiogenesis happened faster (by 0.16($p<0.05$)) and VEGF level was going

back to the baseline as in intact group of rats and angiogenesis was accelerated by the help of Nanoceria.

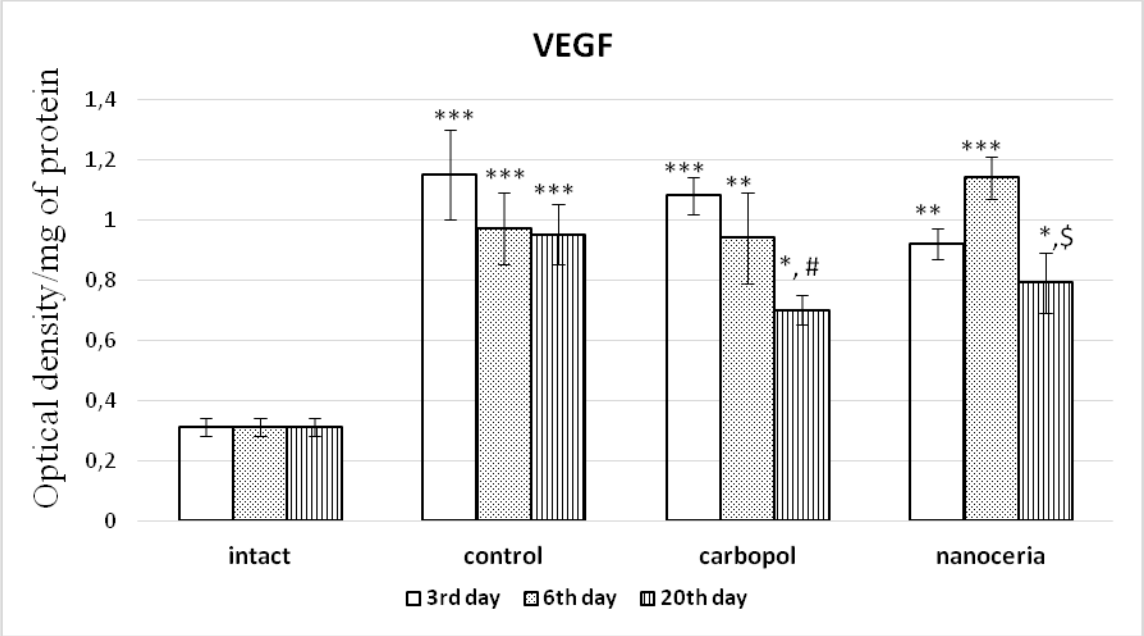

Figure 6.2. Effect of Nanoceria on VEGF in skin samples in rats, M±SD:
- p < 0,05, ** - p < 0,01, * - p < 0,001, compared to the intact group*
 # - p < 0,05 compared to the carbopol group on 3rd day
$ - p < 0,05 compared to the Nanoceria group on 6th day

Nogami et al. investigated the vascular endothelial growth factor expression in rat skin incision wound and have shown the VEGF protein expression was elevated from 1 to 7 days after injury and reached a peak at day 3. It supports VEGF as a significant factor released by inflammatory cells in the initial stage of normal wound healing [251, 252].

6.1.2 The influence of Nanoceria on Nerve Growth Factor (NGF) concentration in the skin during healing of full-thickness wounds

NGF has been well known to encourage the neural differentiation and survival of both basal forebrain cholinergic neurons and peripheral sensory neurons. Expression of two classes of cell surface receptors that are low-affinity neurotrophin receptor (p75NTR) and high-affinity TrkA receptor (TrkA) leads the target cells to respond to NGF. Within hours after axonal damage, mRNA levels of NGF and its receptors temporarily elevate [253], and represent second peak of expression at 2–3 days after injury. Schwann cells that play a crucial role in nerve regeneration in PNS produce NGF after when the nerve is damaged, which is enforced by interleukin-1 (IL-1) liberated from distal end of transected nerves [254]. In addition to their phagocytic function, macrophages engaged to the damaged site release NGF, probably in response to local IL-1 and/or tumor necrosis factor-alpha (TNF-). Moreover, during the procedure of nerve regeneration, Schwann cells react to loss of axons by dedifferentiation, proliferation, demyelination, and finally align in tubes to become a conductor for axonal extension. NGF signaling via p75NTR provokes ceramide-mediated apoptosis and differentiation in Schwann cells driven from degenerating nerves [255]. NGF also induces sphingomyelin hydrolysis, which is correspondent with the expression levels of p75NTR [256]. These detections indicate that NGF plays a part in both phenotypic elimination and regulation of dedifferentiated Schwann cells, while aiding survival and regeneration of peripheral axons during nerve repair.

The same results have been obtained regarding NGF. NGF content was determined in blood serum of rats on 3^{rd}, 6^{th} and 20^{th} days and the level of NGF in the wounded skin site was significantly increased as compared to the unwounded site (intact group). As represented in figure 6.3, in control group of rats, NGF was highly increased on 3^{rd} day (by 1.14 ± 0.15 ($p<0.05$)) and then down-regulated gradually on 6^{th} and 20^{th} days. Comparing experimental group with control group, the NGF upregulation was more significant on 6^{th} day (by 1.1 ± 0.02 ($p<0.05$)) and down-

regulated on 20th days. Level of NGF on 20th day was lower (by 0.06 ($p<0.05$)) in experimental group in comparison with control group which means the wound healing was accelerated by Nanoceria and reaching the baseline level faster in experimental group.

Hiroshi Matsuda et al. investigated the role of NGF in cutaneous wound healing and have shown that low levels of NGF were detected at uninjured control skin sites isolated on various days after wounding, ranging from 0.81 to 1.7 ng/g. In contrast, at the wounded sites, NGF reached a maximal level of 7.8 ng/g 1 d later, and then its levels were gradually decreased but were higher than those at uninjured control skin sites during the period of 14 d.

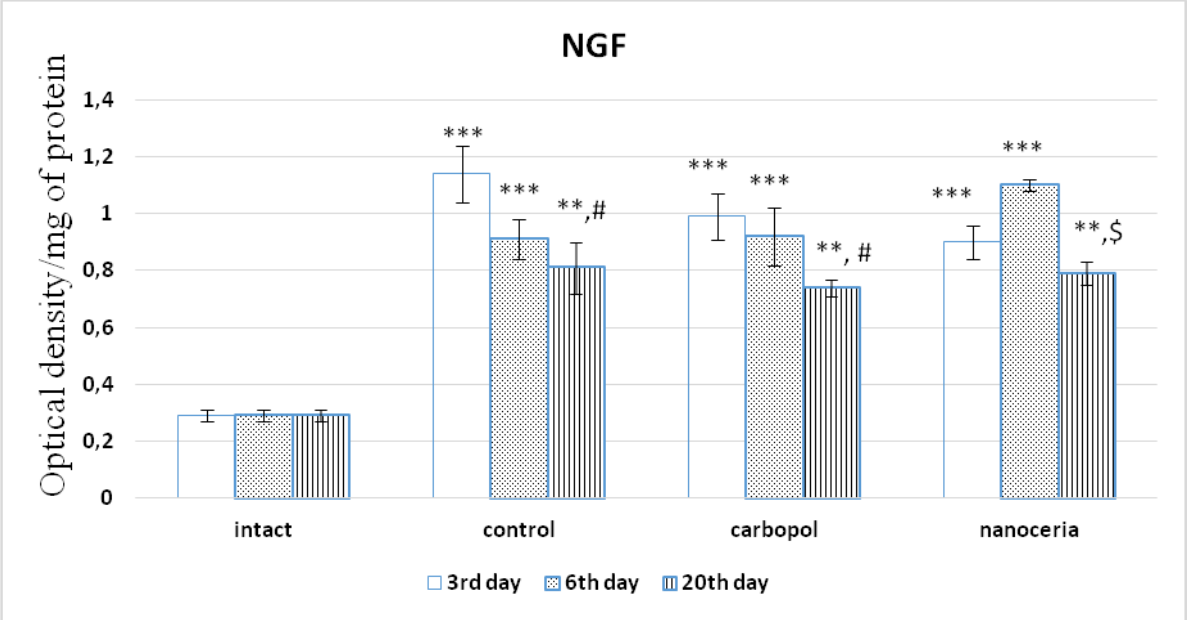

Figure 6.3. Effect of Nanoceria on NGF in skin samples in rats

M±SD, n=6 in each group of animals

- p < 0,05, ** - p < 0,01, * - p < 0,001, compared to the intact group*

 # - p < 0,05 compared to the carbopol group on 3rd day

$ - p < 0,05 compared to the Nanoceria group on 6th day

6.2. The influence of Nanoceria on Matrix metalloproteinase-2 and Matrix metalloproteinase-9 concentration in the skin during healing of full-thickness wounds

Amongst all the MMPs family, in our work we are going to focus on MMP-2 (gelatinase A) and MMP-9 (gelatinase B) as the major regulators during the wound healing process. Realy, the presence of active MMP-2 and MMP-9 in wound fluids initially identified a role for these MMPs in wound healing [257].

Metalloproteinase-9 or gelatinase B (GELB), is expressed in various damaged epithelia, including the skin, eye, gut, and lung, being involved in cell signaling and wound healing and [258, 259] MMP-9 plays an important role in keratinocyte migration; it is expressed at the leading edge of migrating keratinocytes during wound closure.

MMP-9 knockout (KO) mice show a delayed wound closure highlighting the significance of MMP-9 in wound healing [260]. Hypoxia, a feature of chronic wounds, elevates keratinocyte migration and MMP-9 activity [261, 262]. In MMP-9-deficient mice, MMP-9 has also been shown to inhibit cell proliferation [263].

Angiogenesis is a crucial process during wound healing. Both MMP-2 and MMP-9 have a hand in angiogenesis regulation during wound healing through the activation of proangiogenic cytokines, including TNF-a and VEGF, and by generating antiangiogenic peptides (e.g., endostatin from type XVII collagen, expressed in the basement membrane) [264, 265].

Levels of gelatinases are different during each stage of wound repair. Many studies have shown basal levels of gelatinase-A (MMP-2) in non-injured skin. Prolonged periods of increased MMP-2 expression occur following injury [266-268]. Pro-MMP-2 levels detected in fibroblasts appear similar in acute and chronic dermal wounds. Activated MMP-2 protein, however, is higher in chronic wounds [269-271].

Gelatinases (MMP-2 and MMP-9) cleave other collagen types (IV, V, VII, and X), elastin, basement membranes, and denatured collagen [272]. The gelatinases may

also act synergistically with the collagenase family by further degrading types I, II, and III after they have been cleaved from the triple helix [273].

MMP-2 and MMP-9 are secreted by different cells. MMP-2 is secreted by fibroblasts, and the molecularly larger MMP-9 is produced predominantly by leukocytes and perhaps also by keratinocytes [274, 275].

In a wound excision/gel zymogram study of various extracellular matrix components, Arumugam et al [276] observed that MMP-2 and MMP-9 levels persisted even after wound closure, suggesting that these matrix metalloproteinases probably play an important role in matrix (and possibly scar) remodeling. Furthermore, Salo et al [263] serially evaluated acute experimental wounds in the oral mucosa, demonstrating that MMP-2 remained stable during wound healing, while MMP-9 peaked between days 2 and 4. They hypothesized that MMP-9 was not only primarily expressed during inflammation, but perhaps it also played a role later in healing and was secreted by keratinocytes. Essentially, MMP-9 could participate in several key areas of wound healing, namely detaching anchored keratinocytes from the basement membrane and remodeling of the extracellular matrix, potentially enabling more efficient cellular migration. In contrast, Makela et al [277] evaluated wounded cell cultures and found that keratinocytes continued to grow and migrate when heterocyclic carbonate-derived compounds inhibited MMP-9. When MMP-2 was inhibited by tetracycline analogs, there was a drastic reduction in the rate of keratinocyte growth. These authors hypothesized that MMP-2 plays a key role in detachment and promotion of keratinocyte migration along the extracellular matrix.

In a recent study, the two cloned and sequenced 72- and 92-kDa gelatinases. MMP-2 and MMP-9. were examined using substrate gel chromatography before and after making partial- and full-thickness trephine wotinds in the rabbit cornea [278]. MMP-2, but not MMP-9 was detected in uninjured cornea. The authors noted different activities of the two MMPs as a function of time after wounding, with MMP-9 showing early peak levels, and disappearing after 2 - 4 weeks, depending on wound type, whereas MMP-2 levels remained elevated at all-time points. Higher levels of

MMP-9 after initial wounding are consistent with inflammatory cells as the major source of MMP-9 and fibroblasts as the major source of MMP-2 [279].

MMP-2 was indicated in uninjured skin. After wounding, the MMP-2 activity enhanced above that of normal skin, and stayed elevated at a justly stable level during the whole experimental course (fig. 6.5). It has been suggested that MMP-2 plays a surveillance role in the skin, i.e. it preserves collagen homeostasis in the tissues. The activity of MMP-2 may then indicate the number of fibroblasts in the wounds. The durability of MMP-2 represents that this matrix metalloproteinase also has a hand in the remodeling process.

As seen in figure 6.4, MMP-2 is having a regulated gradual incline in experimental group while having a dysregulated expression in control group. Moreover, the level of MMP-2 in experimental group on 20^{th} day was lower by 0.68 ($p<0.05$) compared to the control group of rats. This result demonstrates the great impact of Nanoceria by affecting on MMP-2 downregulation and reaching to the baseline level faster.

Jessica A, et al. have done an investigation on scarless wound healing in athymic nude mice and demonstrated when qRT-PCR analyses were performed on skin samples of mice collected on Day 7 post-injury, MMP-2 (4-fold; $p<0.01$) was up-regulated in nude post-injured skin relative to uninjured nude skin. In comparison, MMP-2 expression in control/wild-type post-injured skin samples were only up-regulated 1.5-fold relative to uninjured controls [280].

The initial eruption of gelatinase B activity anon after injury is believed to be unleashed by post-injury inflammation, this MMP-9 activity is thought to take part in inflammatory cell recruitment and migration to the wound site.

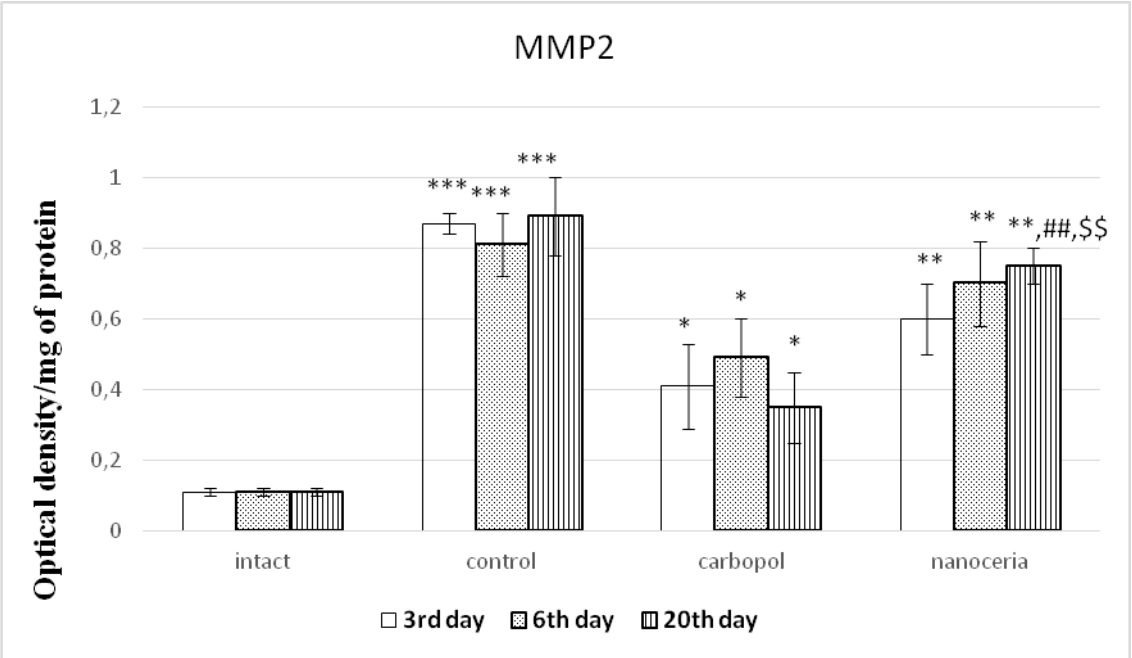

Figure 6.4. Effect of Nanoceria on MMP-2 in skin samples in rats

M±SD, n=6 in each group of animals;

- p < 0,05, ** - p < 0,01, * - p < 0,001, compared to the intact group*

 # - p < 0,05, ## - p < 0,01, compared to the carbopol group on 3rd day

$ - p < 0,05, $$ - p < 0,01 compared to the Nanoceria group on 6th day

During normal wound healing (control group) the MMP-9 expression level is higher than experimental group and then gradually starts decreasing after the initial burst. It is thought that the high level of MMP-9 (and other members of MMPs family) in the granulation tissues of chronic pressure ulcers and elevated amounts in wound fluid from chronic leg ulcers contributes to the chronicity of this wounds [281]. On the contrary, low levels of MMPs have been correlated with excessive scar formation characterized by superfluous tissue repair, with increased collagen production and reduced collagen breakdown [282].

As our results demonstrated (fig. 6.5), the level of MMP-9 in experimental group is lower in 3^{rd},6^{th} and 20^{th} days than in control group which means the downregulation of MMP-9 to the baseline level happened faster that implements the meaning of accelerated wound repair by applying Nanoceria.

In the same investigation of Jessica A et al. (in aforementioned MMP-2 result section), it was shown that when qRT-PCR analyses were performed on skin samples of mice collected on Day 7 post-injury, MMP-9 (3.5-fold; $p<0.01$) was up-regulated in nude post-injured skin relative to uninjured nude skin. In comparison, MMP-9 expression in control/wild-type post-injured skin samples were only up-regulated 1.5-fold relative to uninjured controls [280].

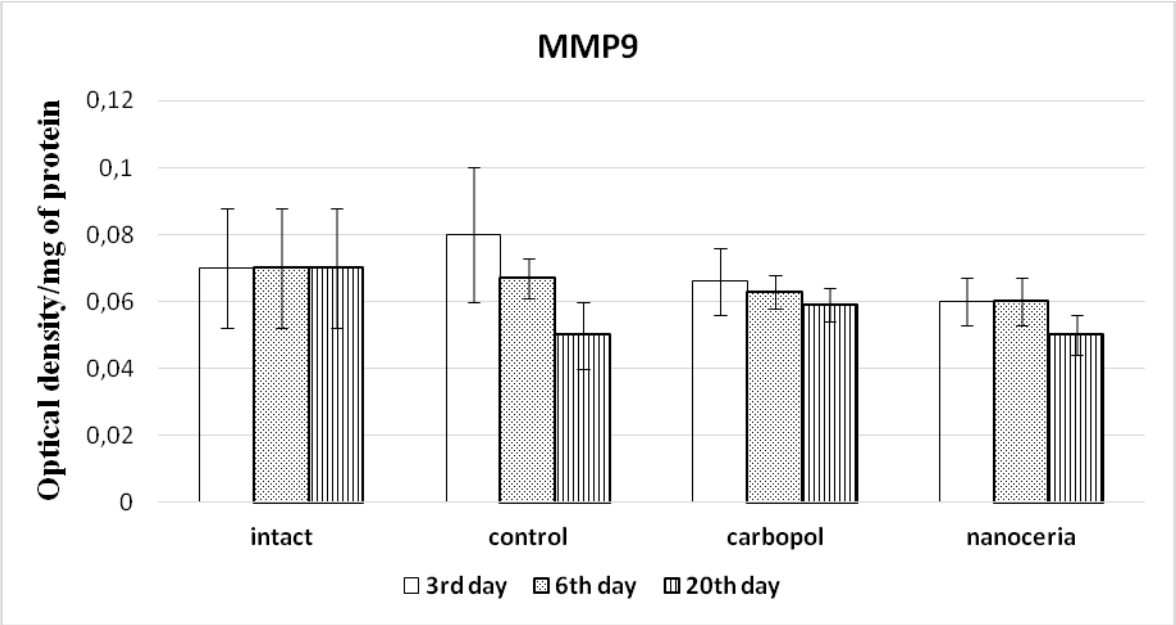

Figure 6.5. Effect of Nanoceria on MMP-9 in skin samples in rats.

Jessica A. et al. also represented that Zymographic evaluation of MMP-2 and MMP-9 enzymatic activity on Day 7 showed four white bands on Coomassie Blue-stained zymogram gels. Comparison of the sites of gelatinase activity with known molecular weights of MMPs suggests that two bands of approximately 97 and 87 kDa represent pro- and active forms of MMP-9 and bands of 72 and 66 kDa represent pro- and active forms of MMP-2. The most prominent gelatinolytic activity was represented by MMP-2 with an elevated level of its active form [280].

Jessica A, et al. have done an investigation on scarless wound healing in athymic nude mice and demonstrated when qRT-PCR analyses were performed on skin samples of mice collected on Day 7 post-injury, MMP-2 (4-fold; $p<0.01$) was up-regulated in nude post-injured skin relative to uninjured nude skin. In comparison, MMP-2 expression in control/wild-type post-injured skin samples were only up-regulated 1.5-fold relative to uninjured controls [280].

The initial eruption of gelatinase B activity anon after injury is believed to be unleashed by post-injury inflammation, this MMP-9 activity is thought to take part in inflammatory cell recruitment and migration to the wound site.

6.3. The influence of Nanoceria on Hypoxia-Inducible factor-1α concentration in the skin during healing of full-thickness wounds

Throughout the primary inflammatory process, wound areas are often hypoxic. This is because of outage of vasculature encompassing the wound, resulting in damaged oxygen delivery, and intensified by a prompt influx of inflammatory cells taking part in the healing process with elevated metabolic requirements for oxygen. These inflammatory cells preferentially gather in hypoxic sites to play a crucial role in re-epithelialization, granulation and other healing processes [283]. HIF-1, consist of a dimer of an alpha (HIF-1a) and a beta (ARNT or HIF-1b) subunit, is available in all nucleated cells of metazoan organisms. The subunits of HIF-1 attach to each other to obtain transcriptional attributes, letting it to adjust the transcriptional activity of numerous genes that elevate cell survival in hypoxic circumstances. HIF-1 is believed to be a master regulator of oxygen homeostasis, and acts chiefly under hypoxic conditions. The HIF-1a subunit is oxygen regulated and regulation of HIF-1 is then specified by the prompt post-translational degeneracy or consolidation of the HIF-1a subunit [284, 285].

In regular cutaneous wounds, HIF-1 is crucial for increment of proper angiogenic and inflammatory responses. Eventuates from investigations that engage

HIF-1a-gene-knockout mice have represented that HIF-1a is a fundamental regulator of migration, energy metabolism, bactericidal activity and aggregation in inflammatory cells [286].

Functional deactivation of HIF-1a eventuates in significantly reduced adhesion, invasiveness and motility in isolated macrophages [287].Moreover, it has been shown that HIF-1a expression plays a critical role in increasing the differentiation of myeloid cells into macrophages and monocytes [288].

HIF-1α activation is also a preliminary motive of angiogenesis, the formation of new blood vessels from pre-existing vessels, in both pathological and physiological conditions [289]. Angiogenesis is controlled by a balance between inhibitory and stimulatory growth factors and by physiological stresses such as changes in oxygen levels [290]. Hypoxia stimulates the remodeling and growth of the existing vasculature. This increases blood flow to oxygen-deprived tissues via the activation of various HIF target genes. These include vascular endothelial growth factor (VEGF), a potent angiogenic factor, as well as other angiogenic growth factors, such as angiopoietin 2 and stromal cell-derived factor 1 (SDF-1) [291].

HIF-1α regulation is pivotal for proper wound healing as both overexpression and deficiency of this factor lead to impaired wound healing.

As shown in figure 6.6 overexpression of HIF-1α on 3^{rd} day (reached its peak) is observed in control group of rats then it was being downregulated gradually 3^{rd} day onward. In other hand, HIF-1α expression level was lower in the experimental group and reached its peak on 6^{th} day and downregulated gradually afterwards approaching the baseline level faster compared to the control group. Rate of HIF-1α on 20^{th} day in experimental group was lower by 0.55 ($p<0.05$) compared to the control group of rats. This reveals the significant influence of Nanoceria on HIF-1α regulation leading to accelerated wound repair.

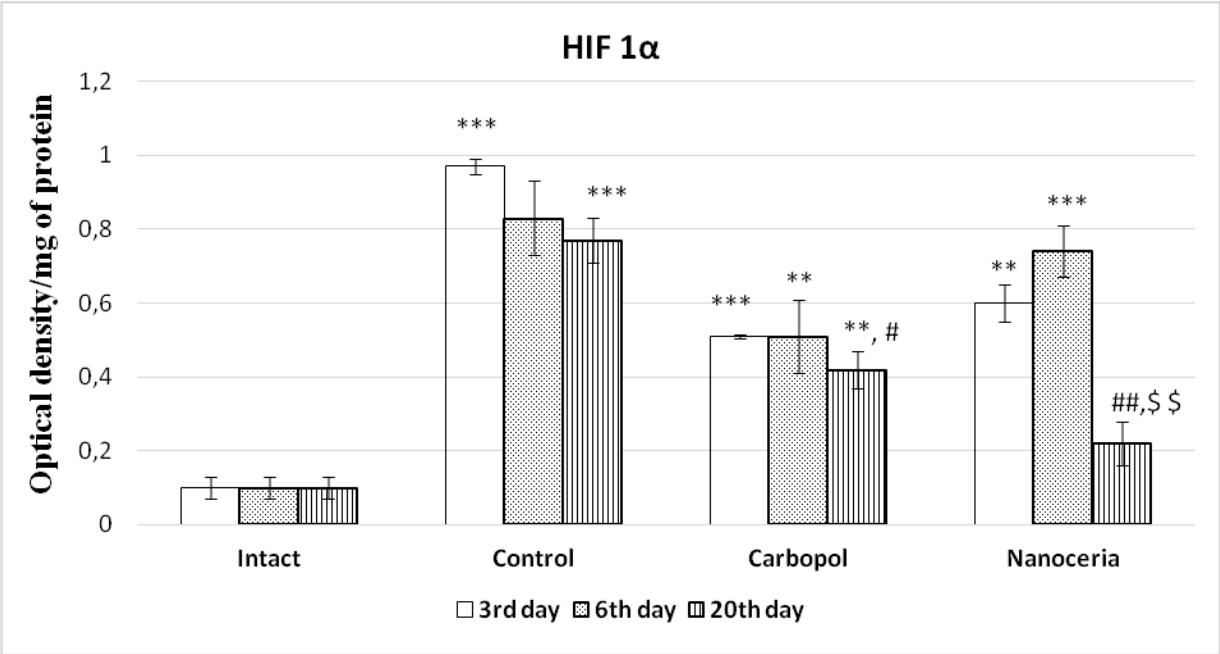

Figure 6.6. Effect of Nanoceria on HIF-1a in skin samples in rats, M±SD:
*** - p < 0,01, *** - p < 0,001, compared to the intact group*
 # - p < 0,05, ## - p < 0,01, compared to the carbopol group on 3rd day
$$ - p < 0,01 compared to the Nanoceria group on 6th day

In Kimberly A. Mace et al. investigation it was shown that in situ hybridization analysis of HIF-1α mRNA in Day 7 wound tissue from both nondiabetic and diabetic mice revealed high expression levels of HIF-1α mRNA in keratinocytes and fibroblasts of wounded skin in both groups of mice at Day 7, with no apparent difference in spatial organization. They have also demonstrated that reduction of HIF-1α protein levels is associated with the reduction of Vegf, Hmox1, and Nos2 expression in diabetic wounds and sustained expression of HIF-1α results in restored expression of HIF-1 target genes in vivo in diabetic mice[292].

Our findings suggest that gelatinases are present for an extended period of time during tissue repair. The high early levels of MMP-9 appear to be associated with concomitantly elevated collagenase levels, possibly to facilitate epithelialization. and degradation of denatured collagen. The prolonged elevation of MMP-2 activity is probably important for the remodelling of scar tissue. Moreover, gelatinases may

serve as indicators of the progression of the wound healing process. As per our results, Nanoceria represented as a great regulator for both gelatinases in wound healing process.

Taking HIF-1α into account, Nanoceria has shown to influence it greatly in order to allow it to regulate the transcriptional activity of hundreds of genes that promote cell survival in hypoxic conditions.

DISCUSSION OF OBTAINED RESULTS

At present, an increase in industrial and domestic injuries is closely linked to the negative influence of human factors on the human body as a result of wounds and civilian injuries, man-made disasters, which are accompanied by the appearance of mechanical damage, which leads to a violation of the integrity of the covering tissues - the formation of skin wounds [293,294]. Despite the wide arsenal of dermatotropic drugs used to treat the wound process, the creation of an effective domestic dermatotropic drug is an urgent problem of the present.

Our attention was attracted by cerium oxide nanoparticles (nanoceria) that is a focal point of research for a few years, Cerium Dioxide (Nanoceria) seems to be a promising tool due to its ability to regulate redox processes in biological tissues.

Depending on the size of the nanoparticles, Nanoceria dis- plays oxidative and reductive properties, which is paralleled by changes in its toxicity [11]. The neuro- and radio-protective effects of Nanoceria are described [12-14]. Cerium salts are utilized in the treatment of burn wounds [15].

Cerium is a rare earth element that exhibits the ability to change in the oxidation state of Ce between Ce3+ and Ce4+ depending on the oxygen partial pressure in the surrounding atmosphere [179]. Such mixed valence state enables CeO2 nanoparticles (CeO2NPs) to have excellent catalytic activities and to act as free radical scavengers [180]. For example, hydroxyl radical which is one of the strongest oxidants and most biologically active free radicals and nitric oxide radical that is gaseous free radical that exhibits multifaceted biological effects, both beneficial and damaging.

The antioxidant property of cerium oxide nanoparticles (Nanoceria) has been investigated recently; nanoceria were noticed to salvage superoxide radical [16, 17], hydrogen peroxide [18], hydroxyl radical [19] and nitric oxide radical [20]. Therefore, Nanoceria have been tested in biological systems wherein they can defend tissues against radiation induced damage [21], defend against laser- induced retinal damage [22], increase lifespan of photoreceptor cells [23], decrease spinal injury [24],

reduce chronic inflammation and elevate angiogenesis.

To study the dermatotropic properties of Nanoceria, we have modeled the experimental full-thickness wounds on rats.

The mixture of Nanoceria and carbopol, made by us, is a new biologically active pharmacological composition based on 0.05% CeO2 (dissolved in 0.5% Carbopol) nanoparticles was elected as the optimal dressing for the in vivo study on full-thickness excisional wounds of rats. A peerless feature of these nanocrystals is that they can be applied multiple times: over weeks, cerium (IV) rich particles leisurely turn over to their initial cerium (III) content. In approximately all cases, the particles subsist colloidally firm (e.g. non-aggregated) and could be applied multiple times. An in vivo study represents Nanoceria evidence in mouse tissues with no pathogenicity. Taken together, it is suggested that cerium oxide nanoparticles are well sustained in mice and are agglutinated into cellular tissues. The study illustrated that after 2 weeks, the wounds treated with the CeO2 nanoparticle-containing dressing attained a remarkable closure to nearly 100%. Our results delivered evidence supporting the feasible applicability of CeO2 nanoparticle-containing wound dressing for a favored wound treatment as it hastens complete wound closure and diminishes wound area in comparison with non-treated animals.

It has been established that a pharmacological composition containing 0.05% Nanoceria dissolved in 0.5% carbopol has a bactericidal effect on the test cultures of *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and the fungistatic effect on the test culture of yeast fungi of the genus *Candida*. According to Reshma P, Ashwini K, nanoceria particles exhibited inhibition with respect to the gram-negative bacteria as they are more prone to cytotoxicity [295]. [295, 296].

At all times of observation, the wound area under the action of the Nanoceria-based pharmacological composition was significantly less than the wound area without application of the composition. The greatest healing rates of a full-thickness wound under the influence of a Nanoceria-based pharmacological composition were

observed on the 6th-9th day. Complete epithelialization of the wound under the influence of the pharmacological composition occurred more quickly for 3 days (in intact group of rats it took 23.0±0.8 for complete wound closure and 20.0±0.5 for experimental group).

Chigurupati et al. have shown that a simple topical application of water soluble Nanoceria accelerates the healing of full-thickness dermal wounds in a mice model. In particular engineered Nanoceria formulation enhances the proliferation and migration of fibroblasts, keratinocytes and VECs cells which further accelerate the wound healing process. Moreover, Nanoceria reduce oxidative stress in wounded region and protect regenerative tissue. This study suggests the therapeutic potential for topical treatment of wounds [140].

According to our experiments, it was demonstrated the development of full-thickness wound that is accompanied by an increase in the processes of lipid peroxidation in the blood serum of rats. This is manifested by enhancement in the content of LPO products: conjugated dienes, TBARS compounds and schiff bases, which are indicators of the development of oxidative stress. This changes the activity of antiradical enzymes: superoxide dismutase activity decreases and catalase increases, which is associated with the intensification of the formation of active forms of oxygen. Also, under these conditions in serum, a decrease in the level of protein and non-protein sulfhydryl groups is observed, indicating that they are damaged by free radicals. It was found that in the case of full-thickness wound under conditions of nanoceria application in experimental group of rats, the oxidative-antioxidant balance is restored in serum, as shown by the decrease in the products of the LPO and the normalization of antiradical enzymes.

Based on Davan Revathy et al. it was proven that cerium oxide nanoparticles due to their dual oxidation state could enhance wound healing activity when compared to standard drug by scavenging ROS from the site of injury and protecting the native tissue thereby helping in producing high amount of collagen and hydroxyproline which are markers for effective wound healing. Histopathology

showed no signs of inflammatory responses indicating less toxicity of these nanoparticles. Future studies will include identification of other molecular mechanisms for wound healing, determining appropriate concentration of nanoparticles and formulation [141].

Supporting our data, A.S. Karakoti et al. have shown that vacancy-engineered ceria nanoparticles exhibit unique capabilities for quenching reactive oxygen intermediates, and could find widespread use in the treatment of ROI-mediated medical disorders [87, 30].

In addition, Talib Pirmohamed established the catalase mimetic capabilities of nanoceria and affirms that this catalytic reaction is not equivalent for all nanoceria preparations. Our findings suggest that one of the mechanisms by which nanoceria protect cells from oxidative stress may be due to their ability to act as catalase mimetics, in addition to exhibiting SOD mimetic activity [188].

Bharat Bhushan et al. suggested that nanoceria was synthesized by hydrothermal method and their enzyme mimetic activity was analysed by SOD assays. The biocompatibility and protective effect of nanoceria against H2O2 induced cytotoxicity was evaluated in vitro in fibroblast cells by MTT assay. Further, the flow cytometric and fluorescence microscopic analysis revealed the ROS scavenging potential of nanoceria in vitro. The cell cycle and mitochondrial membrane potential analysis further supports the role of nanoceria in protecting the cells against oxidative stress induced cell death. Moreover, the antioxidant potential of nanoceria was also investigated in vivo in zebrafish model. The results suggest the biocompatibility and protective role of nanoceria against the H2O2 induced embryotoxicity in zebrafish by effective removal of toxic free radicals. Thus, the present study suggests that the nanoceria and zebrafish model could be exploited as an alternative tool for future antioxidant therapy[189].

We have shown the effect of Nanoceria was significant on some growth factors, such as, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), matrix metalloproteinase-2 (MMP 2), matrix metallopeptidase-9 (MMP 9) and

hypoxia-inducible factor 1α (HIF 1α). Results demonstrated the positive influence of Nanoceria on the aforementioned factors by having a great influence on their regulation in the healing process. The level of VEGF was well regulated in experimental group which means it was upregulated (expressed) gradually and reached its peak for accelerating the angiogenesis and then downregulated to reach the baseline level faster when compared to the control group. For NGF the same result as VEGF has been obtained, where upregulation and downregulation happened faster in experimental group which shows the wound healing acceleration by the help of Nanoceria. For MMP-2, after injury the level of this factor should increase in order to accelerate the healing process and goes back to baseline level when getting close to the complete healing. We have shown in experimental group this process happens in a faster and more regulated manner where in control group it took more time as dysregulation was seen. For MMP-9 expression and then reaching to the baseline level happened more quick in experimental group compared with control group. For HIF-1α, in control group of rats, overexpression of this factor was seen from 3rd day where it made it difficult and time consuming to reach the baseline level, while in the experimental group the normal expression and downregulation was seen that makes it obvious for a faster wound healing.

We have also done the investigation on the level of endogenous intoxication by measuring the concentration of C-reactive protein and Middle-mass molecules after wound treatment and the results have shown a great positive influence of these factors regulation after Nanoceria application. The experimental procedure was conducted by Gabrielyan method for Middle-mass molecules concentration measurement and turbidimetry for C-reactive protein. Our data has shown that nanoceria decreases the level of Middle-mass molecules and C-reactive protein in blood serum of rats after injury.

Another research was conducted on the effect of Nanoceria on the physical and chemical properties of skin. It was established that Nanoceria did not have any effect on the moisture content of skin after treatment but it has influenced the collagen

content by decreasing its level to the baseline level which means the wound healed without any rough scar formation.

On the other hand, Nanoceria's influence was observed on the smelted gelatin content, which was increased after healing in untreated animals, by decreasing its level to the baseline level (normalization).

Jessica A, et al. have done an investigation on scarless wound healing in athymic nude mice and demonstrated when qRT-PCR analyses were performed on skin samples of mice collected on Day 7 post-injury, MMP-2 (4-fold; $p<0.01$) was up-regulated in nude post-injured skin relative to uninjured nude skin. In comparison, MMP-2 expression in control/wild-type post-injured skin samples were only up-regulated 1.5-fold relative to uninjured controls [280].

In the same investigation of Jessica A et al. (in aforementioned MMP-2 results section), it was shown that when qRT-PCR analyses were performed on skin samples of mice collected on Day 7 post-injury, MMP-9 (3.5-fold; $p<0.01$) was up-regulated in nude post-injured skin relative to uninjured nude skin. In comparison, MMP-9 expression in control/wild-type post-injured skin samples were only up-regulated 1.5-fold relative to uninjured controls [280].

Jessica A. et al. also represented that Zymographic evaluation of MMP-2 and MMP-9 enzymatic activity on Day 7 showed four white bands on Coomassie Blue-stained zymogram gels. Comparison of the sites of gelatinase activity with known molecular weights of MMPs suggests that two bands of approximately 97 and 87 kDa represent pro- and active forms of MMP-9 and bands of 72 and 66 kDa represent pro- and active forms of MMP-2. The most prominent gelatinolytic activity was represented by MMP-2 with an elevated level of its active form [280].

Hiroshi Matsuda et al. investigated the role of NGF in cutaneous wound healing and have shown that low levels of NGF were detected at uninjured control skin sites isolated on various days after wounding, ranging from 0.81 to 1.7 ng/g. In contrast, at the wounded sites, NGF reached a maximal level of 7.8 ng/g 1 d later, and then its levels were gradually decreased but were higher than those at uninjured control skin

sites during the period of 14 d [255].

Nogami et al. investigated the vascular endothelial growth factor expression in rat skin incision wound and have shown the VEGF protein expression was elevated from 1 to 7 days after injury and reached a peak at day 3. It supports VEGF as a significant factor released by inflammatory cells in the initial stage of normal wound healing [246].

Taking HIF-1α into account, Nanoceria has shown to influence it greatly in order to allow it to regulate the transcriptional activity of hundreds of genes that promote cell survival in hypoxic conditions.

In Kimberly A. Mace et al. investigation it was shown that in situ hybridization analysis of HIF-1α mRNA in Day 7 wound tissue from both nondiabetic and diabetic mice revealed high expression levels of HIF-1α mRNA in keratinocytes and fibroblasts of wounded skin in both groups of mice at Day 7, with no apparent difference in spatial organization. They have also demonstrated that reduction of HIF-1α protein levels is associated with the reduction of Vegf, Hmox1, and Nos2 expression in diabetic wounds and sustained expression of HIF-1α results in restored expression of HIF-1 target genes in vivo in diabetic mice[292].

There are no sufficient data to compare our results with other scientists as Nanoceria is a newly synthesized nanomaterial and has a long way to go on further investigations.

CONCLUSIONS

In accordance with the goal in the dissertation work the biochemical mechanisms of wound healing action are revealed:

1. Pharmacological composition containing 0.05% nanoceria dissolved in 0.5% carbopol speeds up the healing of full-thickness dermal wounds in a rat model. Healing of full-thickness dermal wounds occurred without the formation of a rough keloid scar. Also, in all terms of the observation under the action of a Nanoceria-based pharmacological composition the area of full-thickness wounds significantly reduced in comparison with the wounds without applying the composition.
2. Nanoceria-based composition has a bactericidal effect on the test cultures of Staphylococcus epidermidis, Staphylococcus aureus and Pseudomonas aeruginosa, and the fungistatic effect on the test culture of yeast fungi of the genus Candida which is important for the use of the composition for the prevention and treatment of infectious inflammatory processes on the wound surface.
3. After healing untreated full-thickness dermal wounds in homogenate of the former skin injuries moisture content decreased but collagen content, smelted gelatin content and temperature of welding of a skin were increased. After the use of nanoceria-based pharmacological composition all of the above-mentioned indicators returned to the level of intact rats that explains the wound healing without the formation of a rough scar at the site of the full-thickness skin wound.
4. C-reactive protein and Middle-mass molecules concentrations in blood serum of rats with full-thickness dermal wounds on the 3rd, 6th, 9th, 14th and 20th days of the experiment were increased in comparison with intact rats. Nanoceria-based pharmacological composition had the positive impact on decrease these indicators in all terms of the observation and restored them on 14th day of

experiment. It is witness that pharmacological composition diminished endogenous intoxication full-thickness dermal wounds.

5. The development of full-thickness wound is accompanied by an increase in the processes of lipid peroxidation in the blood serum of rats and by the changing in the activity of antiradical enzymes (superoxide dismutase activity decreases and catalase increases), which is associated with the intensification of the formation of active forms of oxygen. In the case of full-thickness wounds under conditions of nanoceria-based composition application the oxidative-antioxidant balance is restored in blood serum, as shown by the decrease in the products of the LPO and the normalization of antiradical enzymes activity.

   In the rats with full-thickness wounds we have shown a decrease in the content of protein, non-protein and total sulfhydryl groups in blood serum, indicating that the level of free radicals increases in blood. When nanoceria-based composition was applied to wounds, the restoration of sulfhydryl groups content in blood serum was observed that is evidence of antiradical property of nanoceria.

   Daily application of carbopol gel with nanocrystalline cerium dioxide on the wound bed restored pro- / antioxidant balance in the blood serum of rats, which is manifested in the reduction of the content of LPO products and the normalization of the activity of antioxidant enzymes, as well as restored the group to level of control. Therefore, one of the mechanisms of the dermatotropic effect of a nanoceria-based pharmacological composition is its antioxidant and antiradical action.

6. In the blood serum of untreated animals, the expression of VEGF, NGF, MMP-2, MMP-9 and HIF 1α was dysregulated. The high early levels of MMP-9 appear to be associated with concomitantly elevated collagenase levels, possibly to facilitate epithelialization and degradation of denatured collagen. The prolonged elevation of MMP-2 activity is probably important for the remodelling of scar

tissue. Moreover, gelatinases may serve as indicators of the progression of the wound healing process. Nanoceria-based composition represented as a great regulator for both gelatinases in wound healing process. Nanoceria-based composition has the positive influence on growth factors (VEGF, NGF) and HIF 1α by having a great impact on their regulation in the healing process.

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