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Effect Of Cerium Dioxide Nanocrystal (Nanoceria) On The Concentration Of Some Growth Factors During The Wound Healing Process In Rat Model.

Arefeh Amiri[1]*, Nikitina NS[1], Stepanova LI[1], Berehovyi SM[1], Beregova TV[1], Spivak M Ya[2, 3]

[1]Educational Research Center "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv, Ukraine.
[2]Danylo Zabolotny Institute of Microbiology and Virology National Academy of Science of Ukraine
[3]LCL 'DIAPROF', Svitlycky Str., 35, Kyiv 04123, Ukraine

ABSTRACT

Wound healing is an evolutionarily upkept, intricate, multicellular flow that, in skin, objects at barrier amendment. This process brings in the harmonic endeavors of various cell types containing platelets, endothelial cells, fibroblasts, keratinocytes and macrophages. The infiltration, proliferation, migration, and differentiation of these cells will come to a climax in an inflammatory response, the formation of fresh and new tissue and finally wound closure. This intricate process is performed and adjusted by an equally complicated signaling pathway bringing in manifold cytokines, transcription factors, growth factors, and chemokines. The aim of this study is to emphasized on the effect of Nanoceria on biochemical mechanisms of 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) among other numerous factors that are involved in the healing process. The experimental procedure. Research was conducted on 80 white laboratory male rats weighing 200 - 250 g, which were divided into four groups(20 rats in each group): control group (without any wound), intact group (wounded animals without any dressing application), experimental group (wounded animals with Nanocria application) and carbopol group (wounded animals with carbopol application). Full-thickness wound model was performed at the back of each epilated rat using surgical scalpel and forceps by the dimension of 1 x 1 cm^2 . The animals of experimental group were treated with "Nanoceria-Gel" which contains 0.05% CeO2 (dissolved in 0.5% Carbopol) nanoparticles directly to the wound site once daily until healing for wound dressing. In the intact series wound healing happened without drug and only Carbopol while the control group was remained untreated. After complete wound closure in each group, skin samples were taken out in 3^{rd}, 6^{th} and 20^{th} days and enzyme-linked immunosorbent assay (ELISA) method was performed to measure the skin levels of VEGF, NGF, MMP-2, MMP-9 and HIF 1α. 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 experimental group the normal expression and downregulation was seen that makes it obvious for a faster wound healing. We have shown the great impact of Nanoceria on above mentioned factors that are important in wound healing process, specially for VEGF and NGF where angiogenesis and nerve growth for proper tissue remodelling are pivotal. Due to several other advantages of Nanoceria, such as Antioxidant, Antimicrobial, ROS reduction, SOD restoration, Catalase reduction properties that were investigated and has been proven in our previous studies, we consider Nanoceria as a promising drug for further investigation.

Keywords: cerium dioxide, nanocrystal, wound healing.

Corresponding author

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INTRODUCTION

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 [1]. 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 [2]. For example, throughout the inflammatory phase, fibronectin and other ECM protein fragments in the wound area act as chemo-attractants for monocytes [3] which then attach to ECM proteins. This attachment provokes phagocytosis [4] resulting in the monocytes/macrophages to latter decomposition of ECM fragments and other debris in the area [5]. Cohesion of monocytes to ECM proteins also provokes the expression of growth factors [6] that can then have an influence on cells to impress the synthesis of ECM constituents [7].

Interactions between ECM and growth factors in this dynamic reciprocal relation take various patterns (Fig. 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 [1, 8].

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

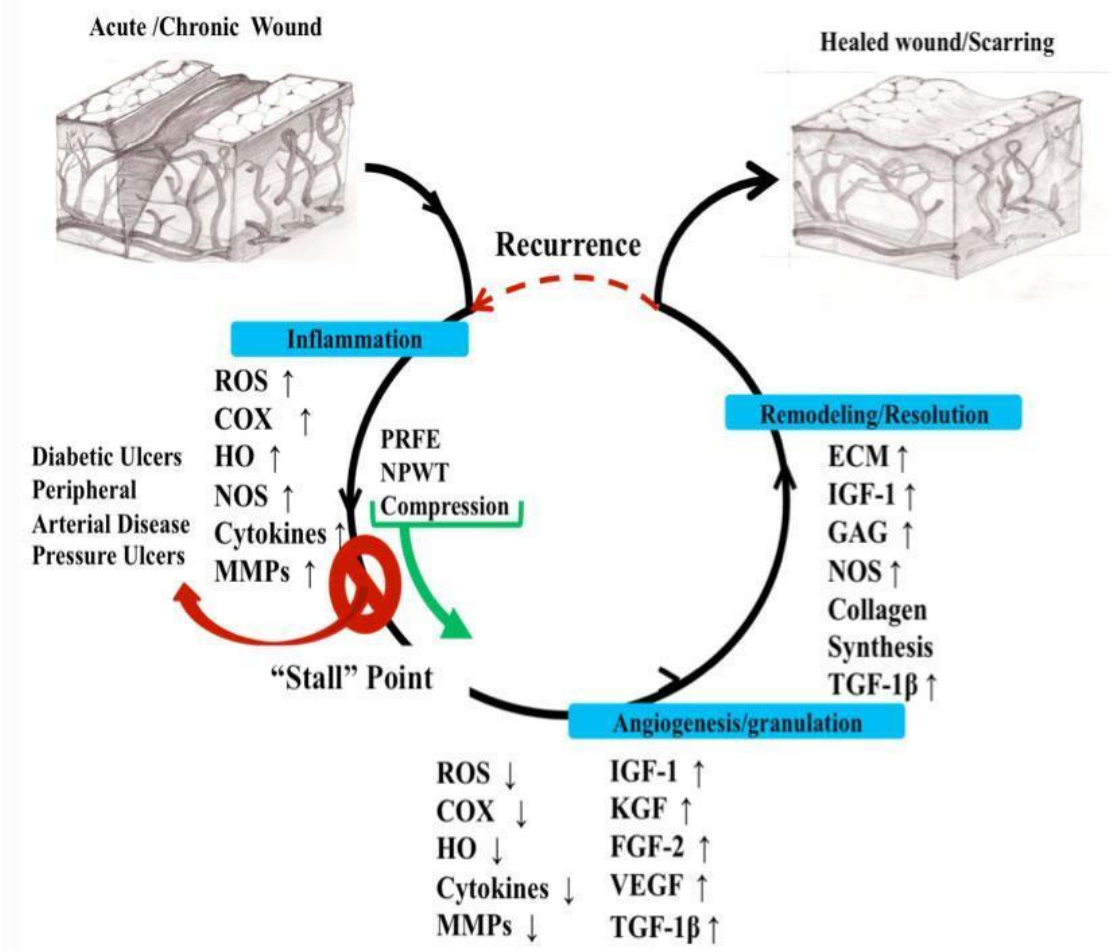

Figure 1: A cyclic model for wound healing.

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A GENERAL REVIEW OF THE ECM

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 [2,9]. Incorporation of ECM mainly happens in response to cytokines, growth factors and mechanical signals affected via cell surface receptors [10]. 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 [11]. 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 [12]. Recent studies have shown that matrix alterations can take place before actual trauma that emerge to make individuals prone to chronic repair processes [13,14].

Deterioration and alteration of the ECM by proteases, exclusively matrix metalloproteases (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 [15]. MMPs also play a fundamental role in releasing growth factors and splitting ECM proteins to disclose areas that can actuate growth factor receptors [16]. 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 [16]. 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 [17]. 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 [18]. 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 [19, 20].

The ECM come together with the whole cellular microenvironment to define cellular phenotype and performance [8]. ECM cooperate 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.

Growth factors association with wound healing process

Vascular Endothelial Growth factors (VEGF)

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 microvasculature [21]. Antibodies that block α_1 and α_2 integrin subunits significantly prevent VEGF-induced angiogenesis without needing to affect the vasculature that already exist [21]. 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 that antibodies against this integrin block angiogenesis induced by FGF and TNF-α without impacting on blood vessels that already exist [22]. Moreover, a 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 [23]. 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 [24] and in oncology, where integrin inhibitors are engaged to prevent tumor angiogenesis [25].

VEGF family members consist of: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor [26]. VEGF-A is a product of keratinocytes, neutrophils, fibroblast smooth muscle cells, endothelial cells, macrophages and platelets [27, 28, 29]. It binds to the tyrosine kinase surface receptors Flt-1 (VEGF receptor-1) and KDR (VEGF receptor–2 [VEGFR-2]) [30, 31] localized to the endothelial surface of blood vessels [32, 33]. These receptors have different roles. KDR is a significant intermediate for proliferation of endothelial cells in vitro and chemotaxis [34]. It is also responsible for persuading endothelial cell differentiation. In

contrast, Flt-1 is necessary for blood vessels organization [35, 36]. Flt-1 may also be involved in intermediating vascular permeability [37] MMP expression in vascular smooth muscle cells, [38] and the induction of anti-apoptotic proteins [39].

VEGF-A plays a significant role in wound healing because it upgrades the early events in angiogenesis, particularly endothelial cell migration [48, 40] and proliferation [241, 42] as seen in several in vitro studies. VEGF-A secretion and transcription along with the VEGFR are enhanced in the acute wound [43, 44]. Activated platelets liberate VEGF-A upon injury [45, 46]. Moreover, during wound healing macrophages release VEGF-A [47] as well as releasing TNF-α, which induces VEGF-A expression in fibroblasts and keratinocytes [43]

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. [49] VEGF-B participates also in various types of neurons. It is important for the protection of neurons in the retina [50] and the cerebral cortex during stroke [51] and of moto-neurons during motor neuron diseases such as amyotrophic lateral sclerosis [52].

VEGF-B applies its effects via the FLT1 receptor [53]. VEGF-B has also been shown to control endothelial uptake and transport of fatty acids in heart and skeletal muscle [54, 55].

VEGF-C is up-regulated during wound healing and its firstly released by macrophages and is important during the inflammatory stage of wound healing [55]. VEGF-C acts mainly through the VEGF receptor-3 (VEGFR3), which is expressed in monocytes/macrophages, lymphatic endothelium and fenestrated endothelia [55, 56]. The main role of VEGF-C is in lymphangiogenesis, 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 [57]. Shortly thenceforth, it was shown to be a particular growth factor for lymphatic vessels in a diversity of models [58, 59]. 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 [60] or its secondary receptor VEGFR-2. In addition, VEGF-C plays an important role in neural formation [61] and blood pressure adjustment [62].

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 lymphangiogenesis, angiogenesis and endothelial cell growth. This secreted protein undergoes a complex proteolytic maturation, generating multiple processed forms that bind and activate VEGFR-2 and VEGFR-3 receptors. The function and structure of this protein resembles to 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.

Nerve Growth Factor (NGF)

Since first described by Levi-Montalcini and Hamburger in 1953, NGF has been well known to encourage the neural differentiation and survival of both basal forebrain cholinergic neurons and peripheral sensory neurons [63]. 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 [64]. Within hours after axonal damage, mRNA levels of NGF and its receptors temporarily elevate [65], 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 [66]. 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

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cells driven from degenerating nerves [67]. NGF also induces sphingomyelin hydrolysis, which is correspondent with the expression levels of p75NTR [68]. 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.

Matrix Metalloproteinases (MMPs) association with wound healing process

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 (Fig. 2) 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 [71].

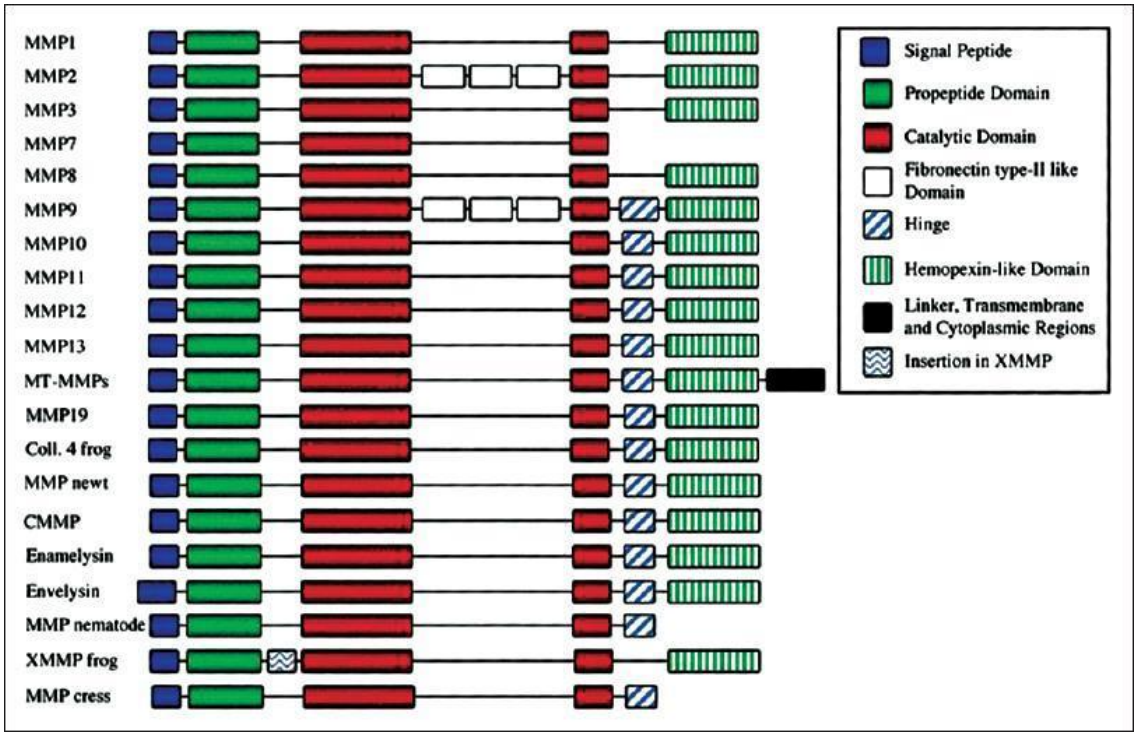

Figure 2: Schematics of the domain structures of the 23 representative MMPs. Catalytic domain (represented by green) has an insertion of gelatin-binding domain in MMP-2 and 9. In all other MMPs, the catalytic domain is a continuous entity

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 [69].

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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 [70].

Table 1: Selected substrates of different matrix metalloproteinases (MMPs). Interstitial collagenases cleave preferentially different interstitial collagen subtypes. Stromelysins digest basement membrane proteins as substrates, whereas gelatinases process primarily cleaved matrix proteins into smaller fragments.

MMP	Name of class	Enzyme	EC No.	Substrate	Chromosome location (human)
MMP-1	Collagenases	Collagenase-1	EC 3.4.24.7	Collagens (I-III, VII, VIII, and X), gelatin, aggrecan, L-selectin, IL-1β, proteoglycans, entactin, ovostatin, MMP-2, and MMP-9	11q22-q23
MMP-8	Collagenases	Collagenase-2/ neutrophil collagenase	EC 3.4.24.34	Collagens (I-III, V, VII, VIII, and X), gelatin, aggrecan, and fibronectin	11q21-q22
MMP-13	Collagenases	Collagenase-3	13 EC 3.4.24.B4 (preliminary BRENDA-supplied EC number)	Collagens (I-IV, IX, X, and XIV), gelatin, plasminogen, aggrecan, perlecan, fibronectin, osteonectin, and MMP-9	11q22.3
MMP-18	Collagenases	Collagenase-4		Type I collagen	
MMP-2	Gelatinases	Gelatinase-A	EC 3.4.24. 24	Gelatin, collagen IV-VI and X, elastin, and fibronectin	16q13
MMP-9	Gelatinases	Gelatinase-A	EC 3.4.24.35	Collagens (IV, V, VII, X, and XIV), gelatin, entactin, aggrecan, elastin, fibronectin, osteonectin, plasminogen, MBP, and IL-1β	
MMP-3	Stromelysins	Stromelysin-1	EC 3.4.24.17	Collagens (III-V and IX), gelatin, aggrecan, perlecan, decorin, laminin, elastin, casein, osteonectin, ovostatin, entactin, plasminogen, MBP, IL-1β, MMP-2/TIMP-2, MMP-7, MMP-8, MMP-9, and MMP-13	11q23
MMP-10	Stromelysins	Stromelysin-2	EC 3.4.24.22	Collagens (III-V), gelatin, casein, aggrecan, elastin, MMP-1, and MMP-8	11q22.3-q23
MMP-11	Stromelysins	Stromelysin-3		Unknown (casein)	22q11.2
MMP-17	Stromelysins	Homology tostromelysin-2 (51.6%)			
MMP-7	Matrilysins	Matrilysin (PUMP)	EC 3.4.24.23	Collagens (IV, X), gelatin, aggrecan, decorin, fibronectin, laminin, elastin, casein, transferrin, plasminogen, MBP, β4-integrin, MMP-1, MMP-2, MMP-9, and MMP-9/TIMP-1	11q21-q22
MMP-26	Matrilysins	Matrilysin-2		Collagen IV, fibronectin, fibrinogen, gelatin, α (1)-proteinase inhibitor	11p15
MMP-14	MT-MMP	MT1-MMP (membrane type)	EC 3.4.24.80	Collagens (I-III), gelatin, casein, fibronectin, laminin, vitronectin, laminin, vitronectin, entactin, proteoglycans, MMP-2, and MMP-13	14q11-q12
MMP-15	MT-MMP	MT2-MMP		Fibronectin, entactin, laminin, aggrecan, perlecan, and MMP-2	16q13-q21
MMP-16	MT-MMP	MT3-MMP		Collagen III, gelatin, casein, fibronectin, and MMP-2	8q21
MMP-17	MT-MMP	MT4-MMP			12q24.3
MMP-24	MT-MMP	MT5-MMP		Fibronectin, but not collagen type I or laminin	20q11.2
MMP-25	MT-MMP	MT6-MMP		Progelatinase A	16p13.3
MMP-12	Other enzymes	Macrophage metalloelastase	EC 3.4.24.65	Collagen IV, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, MBP, fibrinogen, fibrin, and plasminogen	11q22.2-q22.3
MMP-19	Other enzymes	RASI-1		Type I collagen	12q14
MMP-20	Other enzymes	Enamelysin		Amelogenin, aggrecan, COMP	11q22.3
MMP-21	Other enzymes	MMP identified on chromosome 1			
MMP-22	Other enzymes	MMP identified on chromosome 1			11q24
MMP-23	Other enzymes	From human ovary cDNA			1p36.3
MMP-28	Other enzymes	Epilysin			17q11.2
MMP-29		Unnamed			

MT-MMP=Membrane type-matrix metalloproteinase, MBP=Myelin basic protein, IL=Interleukin, COMP=Cartilage oligomeric matrix protein

Amongst all the MMPs family, in this article we are going to focus on MMP-2 (gelatinase A) and MMP-9 (gelatinase B) as the major regulators during the wound healing process. MMP-1, MMP-3, and MMP-9 are the major chemokine regulators during wound healing, degrading chemokines by proteolysis to eliminate them wholly or to generate receptor antagonists (reviewed by Gill and Parks19)[72]. The loss of ECM during wound healing provokes the prompt expression of MMP-1 in basal keratinocytes at the migrating epithelial front in wounds [73]. MMP-1 (also known as interstitial collagenase and fibroblast collagenase) expression is

governed by type I collagen attachment to a_2b_1 integrin. The MMP-1 expression is induced when cells are in contact with type I collagen promoting migration [74].

For maintained MMP-1 expression, cross talk between a_2b_1 integrin and the EGF receptor is needed [75]. At day 1, the MMP-1 expression reaches to its peak after wounding occurs in migrating basal keratinocytes at the wound edge followed by a gradual reduction until re-epithelialization is completed. MMP-1 downregulation seems to be quite substantial for normal tissue remodeling as there are high levels of MMP-1 in chronic non-healing wounds.

The presence of active MMP-2 and MMP-9 in wound fluids initially identified a role for these MMPs in wound healing [76].

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 [77, 78] MMP-9 plays an important role in keratinocyte migration; it is expressed at the leading edges 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 [79]. Hypoxia, a feature of chronic wounds, elevates keratinocyte migration and MMP-9 activity [80, 81]. In MMP-9- deficient mice, MMP-9 has also been shown to inhibit cell proliferation [81].

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) [83, 84].

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 [86-88]. 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 [89-91].

Gelatinases (MMP-2 and MMP-9) cleave other collagen types (IV, V, VII, and X), elastin, basement membranes, and denatured collagen [92]. 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 [93].

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 [94].

In a wound excision/gel zymogram study of various extracellular matrix components, Arumugam et al [95] 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 [96] 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 MMP9 was not only primarily expressed during inflammation, but perhaps it also played a role later in healing and was secreted by keratinocytes. Essentially, MMP9 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 [97] 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 [98]. 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 [99].

Hypoxia-Inducible factor 1α

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 [100]. 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 [101, 102].

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 an fundamental regulator of migration, energy metabolism, bactericidal activity and aggregation in inflammatory cells [103].

Functional deactivation of HIF-1a eventuates in significantly reduced adhesion,invasiveness and motility in isolated macrophages [104].Moreover, it has been shown that HIF-1a expression plays a critical role in increasing the differentiation of myeloid cells into macrophages and monocytes [105].

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 [106]. Angiogenesis is controlled by a balance between inhibitory and stimulatory growth factors and by physiological stresses such as changes in oxygen levels [107]. 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) [108].

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

MATERIALS AND METHODS

1. Preparation of Nanoceria solution

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. Animal Model

Research was administered on white laboratory male rats weighing 200 - 250 g, which were divided into four groups: control group (without any wound), intact group (wounded animals without any dressing

application), experimental group (wounded animals with nanocria application) and carbopol group (wounded animals with carbopol application). Keeping animals and experiments were conducted according to ethical principles adopted by Ukraine First National Congress on Bioethics, international agreements and national legislation in this area [85]. Before the experiment, the rats were retained in quarantine and marked. Before performing the full-thickness wound model, animals were anesthetized by sodium thiopental (BiochemieGmbH / Austria), at a dosage of 50 mg / kg. The animals of experimental group were treated with "Nanoceria-Gel" which contains 0.05% CeO2 (dissolved in 0.5% Carbopol (Carbomer Carbopol Ultrez 21, Belgium) nanoparticles for wound dressing. In the intact series wound healing happened without drug and only Carbopol while the control group was remained untreated. Before the experiment epilation was carried out on the back area after anesthetizing rats and one full-thickness wounds of 1 x 1 cm^2 was formed in the skin of each mouse using surgical scalpel and forceps ("Surgiwell" Pakistan). Mice were treated with Nanoceria solution applied directly to the wound site once daily until healing.

3. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method is a criterion for antigens quantitation. The protocols commence with a captured antibody, particular for a protein of interest, coated onto the wells of microplates. In our experiment the skin levels of VEGF, NGF, MMP-2, MMP-9 and HIF 1α were estimated by performing ELISA . [109]

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 ophenylenediamine/hydrogen peroxide (Sigma, USA) was performed and 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 [110].

Statistical analysis

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 post test. Difference between means was judged as statistically significant if $p \leq 0,05$. Mean and standard deviation (SD) were calculated for each group.

RESULT AND DISCUSSION

When the skin is wounded the cells in wound area lead to VEGF induction. Once VEGF is induced the angiogenesis starts. As seen in fig. 3, in control group of rats, VEGF level in blood serum was highly increased (upregulated) on 3rd day and then was down-regulated gradually on 6th and 20th days. In experimental group of rats, in comparison with control group, VEGF was upregulated on 6th day and then down-regulated on 20th day. VEGF level was lower on 20th (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 the angiogenesis was accelerated by the help of Nanoceria.

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 [111].

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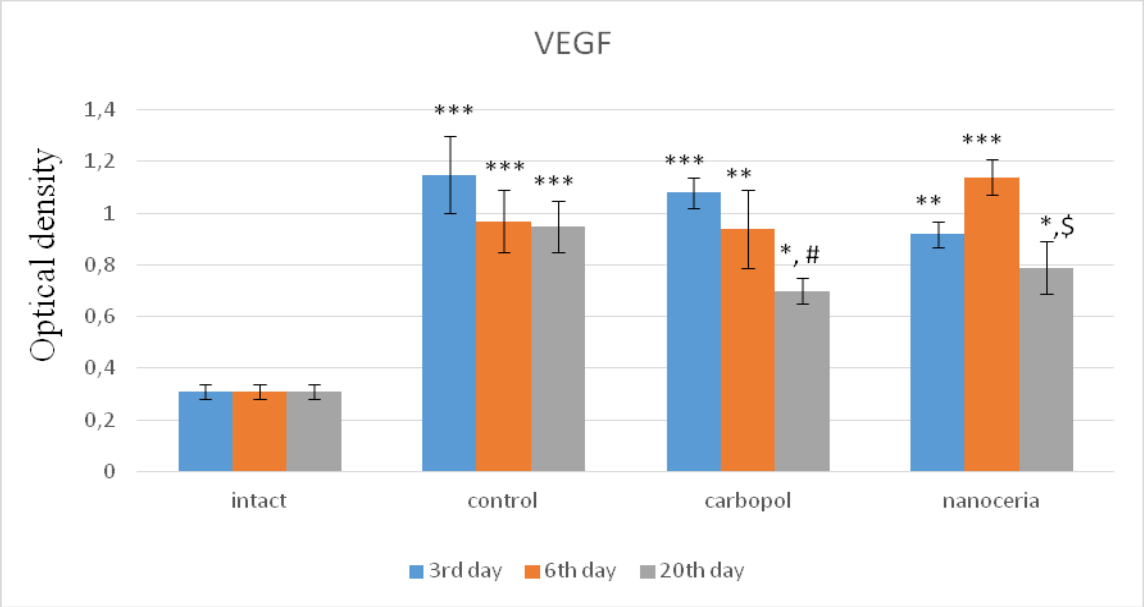

Figure 3. Effect of Nanoceria on VEGF in skin samples in rats.(*Opt. density/mg of protein*)
M±SD, n=6 in each group of animals
**** - compared to the intact group*
 # - compared to the carbopol group on 3rd day
$ - compared to the Nanoceria group on 6th day

The same results have been obtained regarding NGF.

NGF content was determined in blood serum of rats on 3rd, 6th and 20th 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 fig. 4 ,in control group of rats, NGF was highly increased on 3rd day (by 1.14±0.15 ($p<0.05$)) and then down-regulated gradually on 6th and 20th days. Comparing experimental group with control group, the NGF upregulation was more significant on 6th 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 ($p<0.05$)) on 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 [112].

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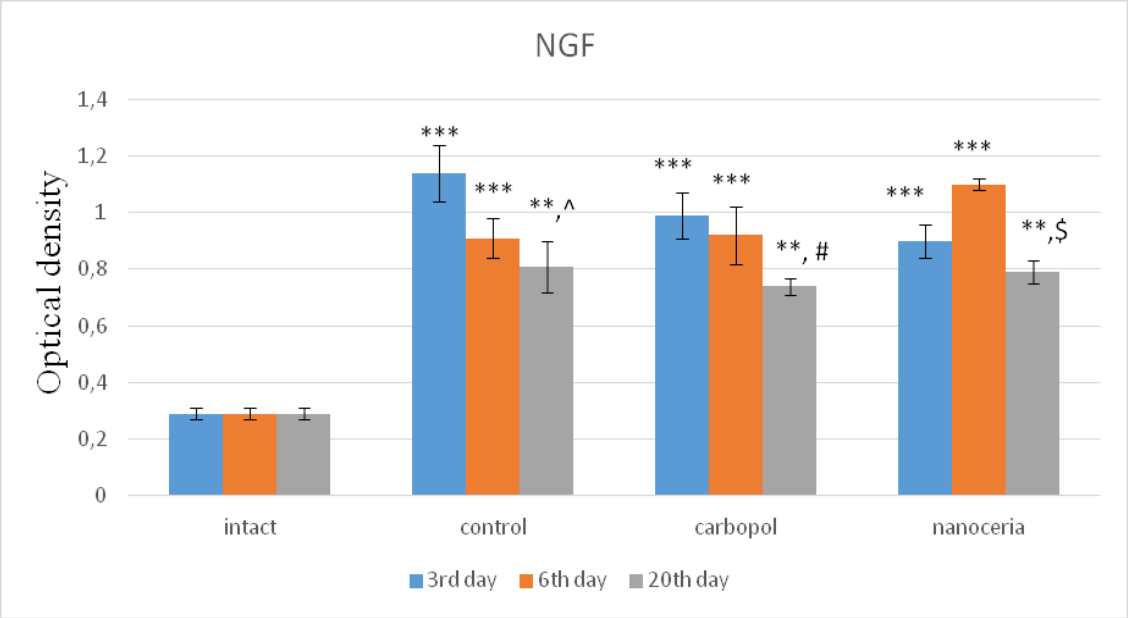

***Figure 4: Effect of Nanoceria on NGF in skin samples in rats.*(Opt. density/mg of protein*)**
M±SD, n=6 in each group of animals
**** - compared to the intact group*
 # - compared to the carbopol group on 3rd day
$ - compared to the Nanoceria group on 6th day

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. 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 fig. 5, 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 20th day was lower by 0.68 ($p<0.05$) compared to the control group of rats. This results demonstrate 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 was 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 [113].

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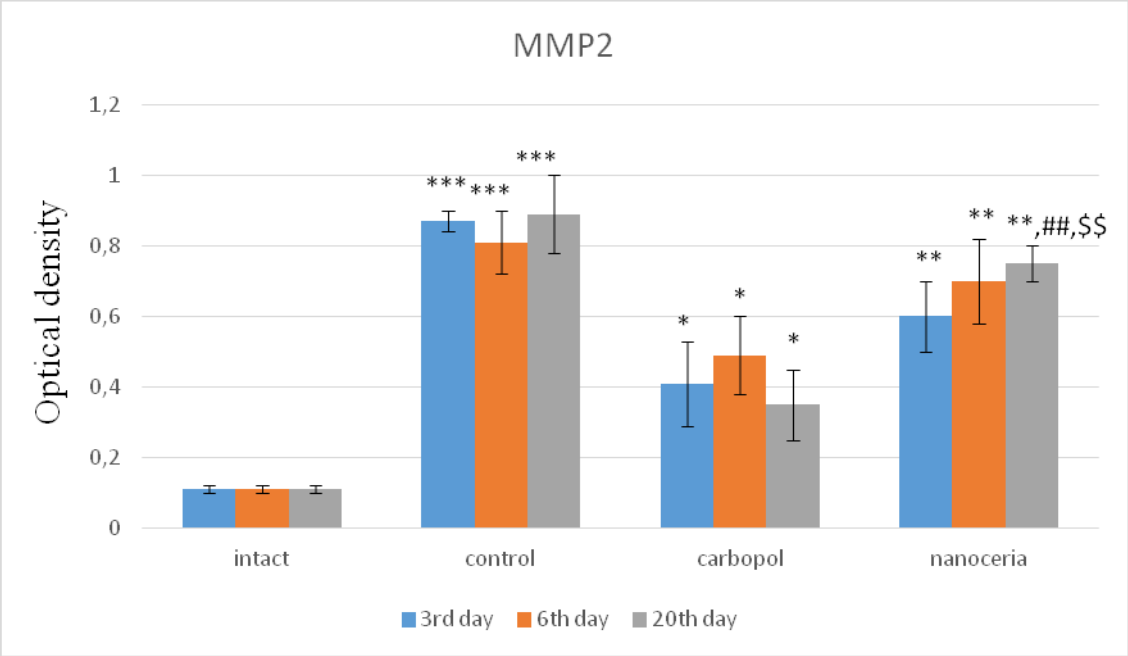

Figure 5: Effect of Nanoceria on MMP-2 in skin samples in rats.(*Opt. density/mg of protein*)
M±SD, n=6 in each group of animals
**** - compared to the intact group*
 # - compared to the carbopol group on 3rd day
$ - compared to the Nanoceria group on 6th day

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.

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 [114]. 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 [115].

As our results demonstrated, the level of MMP-9 in experimental group is lower in 3rd,6th and 20th 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 was 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 [113].

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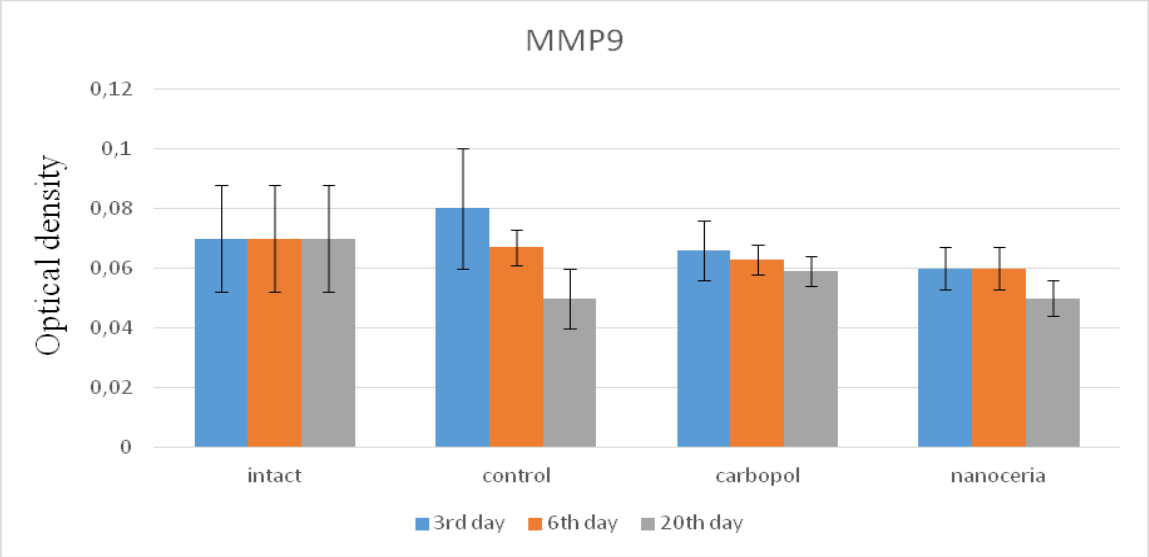

***Figure 6: Effect of Nanoceria on MMP-9 in skin samples in rats.*(Opt. density/mg of protein*)**

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 [113].

As shown in fig. 7 overexpression of HIF-1α on 3rd day (reached its peak) is observed in control group of rats then it was being downregulated gradually 3rd day onward. In other hand, HIF-1α expression level was lower in experimental group and reached its peak on 6th day and downregulated gradually afterwards approaching the baseline level faster compared to the control group. Rate of HIF-1α on 20th 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.

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[116].

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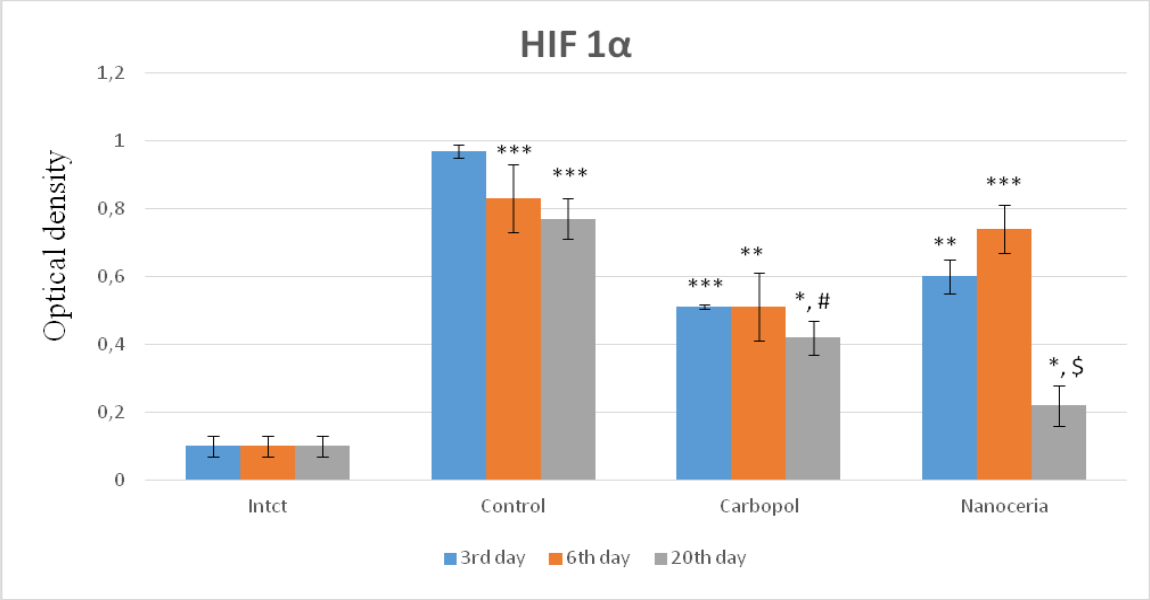

Figure 7: Effect of Nanoceria on HIF-1(in skin samples in rats.
M±SD, n=6 in each group of animals
**** - compared to the intact group*
 # - compared to the carbopol group on 3rd day
$ - compared to the Nanoceria group on 6th day

CONCLUSION

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.

Furthermore, revealing that the Nanoceria has a significant impact on VEGF and hence aiding faster angiogenesis as well as on NGF accelerating nerves growth at the wound site concludes the potentiality of this dermatotropic drug for further investigations.

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