

THE EFFECTS OF TRIMETAZIDINE AGAINST ISCHEMIA INDUCED ULCER IN RABBIT EAR MODEL

Tahssein Ali Mohammed^{1*}; Adeen A. Al-Zubaidy¹; Mukhallad Abdulkareem Ramadhan²; Rasha Kareem Khudur²

¹Department of Pharmacology. College of Medicine, Al-Nahrain University, Iraq

²Department of Phathology College of Medicine, Missan University, Iraq

Email address: tahsseinalsaadi@gmail.com

Abstract

Acute wounds, such as those created by surgery or trauma, occur suddenly and heal in a relatively predictable time. Deregulation or interruption of one or more phases of the normal healing process leads to chronic wounds. A chronic wound is a wound that fails to progress through the normal phases of healing in an orderly and timely manner. The treatment of chronic wounds poses a significant challenge for clinicians and patients in which their healing is often slow or stagnant, causing prolonged patient morbidity. The prevalence of peripheral arterial disease (PAD) is approximately 5% to 10%, whereas 15% to 20% of individuals over 70 years of age are affected; 1% to 2% of PAD patients over the age of 50 develop critical limb ischemia in which blood flow is insufficient to maintain tissue viability resulting in ischemic pain at rest, ischemic ulcers and gangrene that eventually requires limb amputation. Pentoxifylline is dimethyl xanthine-derivative used in the treatment of peripheral vascular disorders. Trimetazidine is an effective anti-ischemic agent, well-tolerated drug mainly used in angina pectoris.

Keywords: *Pentoxifylline, Trimetazidine, Rabbit*

Introduction

Skin is an organ which defends the body from external environment such as temperature, UV radiation and chemicals. The skin is composed of three layers, listing from the outside, the epidermis, the dermis, and the subcutaneous layer (Benítez JM, Montáns FJ. 2017). Wound location plays vital role in the progress of non-healing wounds; this may be due to inadequate blood flow, neuropathy, or both of them. Regular wound healing depends on the balance of promoting and inhibiting factors like cytokines, growth factors, nitric oxide; while impaired wound healing caused by an imbalance of these mediators that result from difference of inflammatory response due to infection, repeated trauma, in adequate oxygen supply or blood perfusion (Kumar V, et al. 2009), figure(1).

Pathophysiology of chronic wound (Ischemic and Hypoxic Injury)

Ischemia is a common cause of cellular injury. In comparison with hypoxia, anaerobic glycolysis continues energy production. Subsequently, anaerobic energy generation stops in tissues with ischemia after depletion of potential substrates. So that, ischemia damages tissues severely and faster than does hypoxia (Bergan JJ, et al.2006). The most significant biochemical abnormalities involved in hypoxic cells which leads to cell damage is reduced ATP generation, as a result of reduced oxygen supply leading to failure of several cellular systems including ion pumps that leading to an influx of Ca^{2+} , cell swelling, mitochondrial damage with its harmful consequences, lowering the intracellular pH by depletion of glycogen stores and increase of both ROS and lactic acid, inhibition of protein synthesis (Bergan JJ, et al.2006) figure(2). If oxygen is reestablished, all of these disorders are reversible; but if ischemia continues, irreversible injury results in which cell death either caused by necrosis or apoptosis (Laure Rittié. 2016).

Molecular progress in non-healing wound

In non-healing wounds, there is a failure of the injured tissue to progress through the expected phases of healing. Cells in the local environment of a chronic wound are subject to significant stresses, including chronic inflammation, the harmful presence of reactive oxygen species, and a

proteolytic environment secondary to bacteria, underlying ischemia, and recurrent ischemia-reperfusion cycles (Wlaschek, M. et al. 2005). All chronic wounds begin as acute wounds but fail to progress through the normal healing process and become locked in an extended inflammatory phase. Specifically, in this phase there is an excess of cytokines including IL-1 β , IL-6 and TNF- α (Falanga V. 2005). Also there are increased levels of proteases such as MMPs including MMP-2 and -9, along with correspondingly low levels of their regulators which increases the ratio of proteases relative to that of their inhibitors with high levels of elastase, plasmin, and thrombin (Ulrich, et al. 2004). These events can impair cell migration and lead to breakdown of some necessary matrix proteins and growth factors which cause deterioration of the structure of the provisional matrix and an inability of the wound cells to proliferate and migrate (Signorelli SS, et al. 2005). As the ECM is constantly being degraded, the tissue perceives that there is still injury and maintains the inflammatory cascade which continues to draw in neutrophils, macrophages, and other phagocytic cells. The massive influx of neutrophils release cytokines, reactive oxygen species, and inflammatory mediators, which injure host tissue in a continuous cycle (Mast BA, Schultz GS. 1996), figure (3).

Rabbit ear model for ischemic injury

Venous leg ulceration (VLU) is a chronic disease affecting about 1-2% of individuals (Graham ID, et al.2003). The pathogenesis of VLU is complex, but the cornerstone of disease is the inflammation in which the leukocytes interact with endothelial cells due to disruption in the expression of adhesion molecule that represent a major mechanism of the chronic vascular disease that impaired wound healing (Pukstad BS, et al.2010). Furthermore, chronic inflammation is correlated with oxidative stress in which reactive oxygen species (ROS) are involved in chronic wounds pathogenesis (Hansson GK, et al. 2002). So that, the goal of treatment of ischemic injury would have to control on the inflammatory process and suppress of oxidative stress which could ameliorate wound healing. Several models were established to study the ischemic injury (Schreml S, et al. 2010). Although animal wound healing models are imperfect

reflection of wound healing processes in human beings and its clinical challenges, these models continue to be crucial tools for the development of new strategies and approaches for therapy of the pathological conditions in clinical chronic wounds (Pandey M. 2012).

In cases where human healing occurs entirely by re-epithelialization and granulation formation without contraction, the ear wound model may be more suitable because it heals without contraction and has a vascular cartilage wound bed (Sisco M and Mustoe TA, 2003). The rabbit ear has the additional advantage of a very constant anatomy of the three major vascular pedicles. When two of these are divided, the ear is rendered reproducibly ischemic, and yet with complete survival which allows an examination of different agents during ischemic conditions with impaired healing (Sisco M and Mustoe TA, 2003).

Ischemia was induced by surgically occluding of two of the three main arteries in the rabbit ear; in which the central and rostral arteries are ligated at the base of the ear through a circumferential incision thus interrupting the entire dermal circulation, while preserving the caudal artery and all three veins (Sotomayor S., et al.2016). Several ulcers are then created down to the auricular cartilage. Since the dermis of the rabbit ear is firmly attached to the cartilage, the vascular wound bed cannot close by contraction but closes by granulation tissue formation (Sisco M and Mustoe TA.2003), figure (4). Several advantages of this model; firstly it maintains all the advantages of the open circumferential model developed by such as easy care of the animal, economic (four wounds on each ear), severe and long-lasting ischemic time. Other important advantage is the substantial simplification of the procedure in which minimal invasive model is easy to create (Sisco M and Mustoe TA.2003) and a surgical microscope or electrocautery is not necessary. However, such equipment may be used if one prefers (Lu L, Saulis AS, et al .2005). The other tissues can be cut without any trouble, also bleeding is minimal and can easily be stopped by gauze pressure and the whole procedure takes less than 1 hour by one person (Kloeters O, et al . 2007) . Secondly, in this model the wounds are easy to heal and the chance of infection

or animal loss is minimal (Reid RR, et al .2006). Because the three vascular bundles are all under surgical control, it is easy to make this model mimic various chronic wounds (Zhou YF, et al .2004). Thirdly, this model involve minimal invasive procedure, the skin inside the ear was not disrupted. However, because the small vessels deliver some blood supply to the proximal portion of the skin, perhaps these vessels can facilitate healing of the wounds (Vinay Kumar, 2013). Finally; in this model new vessel formation appears within 2 weeks in the ischemic ear, whereas new granulation tissue formation is barely detectable at day 7 after wounding (Buján J, et al. 2006).

Biomarkers involved in ischemic injury

A major advantage in the use of animal models is the ability to provide harvested samples. Wound healing investigators have used a combination of macroscopic and histological observations, biochemical measurements of wound healing markers, often in combination with analyses of cellular and immunologic responses to evaluate the progress of wound repair in which various testing methods have been used to determine the success or failure of treatment modalities (Gottrup F, et al. 2011).

A. Interleukin-6 (IL-6)

Interleukin-6 is a cytokine with a wide range of biological activities. It regulate the acute phase response and it is indicator of inflammation within body (Fuster, 2014).

B. Tumor necrosis factor alpha (TNF α)

Tumor necrosis factor alpha (TNF α) is a cell signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction.

C. Oxidative stress parameter (Malondialdehyde)

Malondialdehyde (MDA) is a secondary product of lipid peroxidation and is used as a parameter to determine the extent of lipid peroxidation. MDA measurements can be used to assess the levels of oxidative damage within serum tissues and other biological samples (Uchiyama M, Mihara M. 1978).

D. Matrix MetalloProteinase-9 (MMP-9)

Matrix Metalloproteinase (MMPs) are zinc endopeptidases play a pivotal role in regulating extracellular matrix (ECM) degradation and deposition that is essential for wound re-epithelialization in both acute and chronic wounds (K. Vitlianova, et al. 2015). The activeMMP-9 (gelatinase B) is expressed in several injured epithelia, including the eye, skin, gut, and lung, playing a role in wound healing and cell signaling (Castaneda FE, et al.2005).

E. Vascular endothelial growth factor-A (VEGF-A)

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic stimulators; plays a critical role in ischemic angiogenesis (Ferrara N. 2002).

F. Endothelial nitric oxide synthase (eNOS)

Biosynthesis of NO is conducted by a family of isozymes, including neuronal NOS which expressed in neurons and endothelial cells subsequently (nNOS), (eNOS), and inducible NOS (iNOS) that is not expressed in normal tissue but can be induced under pathological conditions (Anggard E. 1994). The presence of eNOS protein in the vascular endothelium of mucosa, submucosa and especially granulating tissue suggests that eNOS might play a role during ulcer healing; this is proven by the fact that angiogenesis and skin wound healing shown to be impaired in mice deficient with eNOS (Lee PC, et al. 1999).

Pentoxifylline

Pentoxifylline is a dimethyl xanthine-derivative used in the treatment of peripheral vascular disorders, that increases cyclic adenosine monophosphate (cAMP) level in the smooth muscle of blood vessels and is classified as a vasodilator agent (Machado PRL, et al. 2007). Pentoxifylline is a competitive nonselective phosphordi-esterase inhibitor (Essayan DM. 2001) that raises intracellular cAMP, activates protein kinase A (PKA), and inhibits TNF- α and leukotriene synthesis (Deree J, et al. 2008) and reduces inflammation and innate immunity (Peters-Golden M, et al. 2005).

Trimetazidine

Trimetazidine is an effective anti-ischemic agent, well-tolerated drug mainly used in angina pectoris (Onay-Besikci A, Ozkan SA. 2008). Trimetazidine acts at the mitochondrial level diminishing the beta-oxidation of fatty acids by selectively inhibiting the enzyme 3-ketoacyl coenzyme A thiolase (3-KAT), which is the final enzyme in the FFA b-oxidation pathway ; which increases the metabolic rate of glucose (Kantor PF, et al.2000). When oxygen is in low supply, the oxidative processes of FFA and glucose are disrupted paradoxically leading to an increased rate of FFA b-oxidation associated with even greater oxygen consumption while glucose metabolism decreases, which results in lactate accumulation and, in extreme cases, development of metabolic acidosis (Fantini E, et al.1994).

Aim of study

To investigate and evaluate the effects of trimetazidine against ischemic ulcer in rabbit ear model.

Methods

Forty domestic rabbits of both sex each weighing 1.5 to 2 kg were used in this study were be divided into nine groups each of ten animals .The duration of the study started in beginning of June 2017 and finished in the end of July 2018. The Experimental protocol for this study involves:

A. Induction group

Pre-operative care

The surgical procedures for the induction of ischemic wounds were based on (Sotomayor S., et al.2016) and a modified method published by (Chien S. et al. 2007). All surgical procedures were performed under sterile conditions in which the surgical areas were wiped with ethanol and the rabbits were placed on a suitable pad during the duration of the procedure. Briefly, the ventral side of ear was shaved by hair removal spray as well as the areas at the base of the ear; then anesthesia was induced with ketamine injection (50mg/kg IM) and xylazine injection (5mg/kg IM) and prophylactically treated with cefatoxime (100 mg/kg IM) before surgery to reduce the risk of systemic infections; then diluted lidocaine without

epinephrine (0.5-1%) at dose (7mg/kg) is S.C. injected y in the ear as local anesthetic agent (Jordan P, et al .2012) . In this model to induce an ischemia both the central and the rostral arteries with the central and the rostral veins were ligated at two points at the base of the ear by using silk sutures (4/0) and leaving the minor caudal artery as the sole source of blood inflow to the ear without any surgical excisional incisional wounds in ear tissues figure (4). Then several studies were done in days (3, 7, and 14) for detection the signs of ischemic in ear tissue depending on visual observation and histopathological examination.

Post operative care

Post operatively, the ischemic ear was appearing congested, cool and cyanotic with a reduced sensation distal to the injury. Ear movement was reduced but not totally eliminated because some muscles were still attached to the base of the ear. After wounding, the inflicted area was treated with 5 ml of ice cold, 10% iodide solution for 30 seconds to prevent bacterial contamination. Exudates and blood from the wound were absorbed by pressing sterile cotton swabs on the wound. After surgery, the rabbits were allowed free diet and drink and the animal received cefatoxime (100 mg/kg IM) was injected daily for 3days to prevent infection caused by *Staphylococcus aureus*, and community-acquired *Enterococcus faecium* and *Pseudomonas aeruginosa* (Gorwitz RJ. 2008). Thus, as conventional antibiotic was required followed by cefixime 100mg suspension twice daily for 5 days with paracetamol suspension to reduce possible pain and the shaved area was protected with fusidic acid cream 2% daily and the sutures were removed after 2 weeks. Then at day 30; blood samples was collected by cardiac puncture and skin biopsy was taken from the ear for tissue homogenate and histopathology preparation by using punch biopsy (10mm) that was inflicted on the anterior part of the rabbit ear with a diameter of 1 cm; 3-4 horizontal inflicted sites were prepared as shown in (Figure: 5) (Sotomayor S, et al.2016)

B. Orally administered drugs in experimental groups

Orally administered control group

Rabbits were received tap water with normal diet for 30 days then at day 30; blood samples was collected and skin biopsy was taken from the ear.

Orally administered pentoxifylline group

Rabbits were received pentoxifylline (100mg/kg/day) orally after induction of ischemia at day 16 for 15 days then at day 30 ; blood samples was collected and skin biopsy was taken from the ear.

Orally administered trimetazidine group

Rabbits were received trimetazidine (20mg/Kg/day) orally after induction of ischemia at day 16 for 15 days then at day 30; blood samples was collected and skin biopsy was taken from the ear.

Preparation of samples

After centrifugation of serum and tissue homogenates, supernatants were removed and assayed or it stored in deep freeze unless worked immediately were used for the estimation the levels of (IL-6 ,TNF- α ,MDA,MMP-9,VEGF-A and ,eNOS). Finally, ear tissue sections were prepared and stained with hematoxylin and eosin for histological evaluation.

Results

Changes in serum and tissue homogenate levels of ischemic parameters in induction group

In induction group results showed a significant elevation in serum and tissue homogenate levels of IL-6, TNF- α , MDA and MMP-9 with a significant reduction in serum and tissue homogenate levels of VEGF-A and eNOS respectively in comparison with both orally administered control group and topically administered control group. Tables (1and 2), Figures (6 to 17).

Effects of oral pentoxifylline on serum and tissue homogenate levels of ischemic parameters after induction of ischemia

Results showed a significant reduction in serum and tissue homogenate levels of IL-6, TNF- α , MDA and MMP-9 with a significant elevation in serum and tissue homogenate levels of VEGF-A and eNOS respectively in orally administered pentoxifylline group in comparison to that observed in induction group, but showed non-significant difference in

compare with orally treated control group. Tables (1 and 2), Figures (6 to 17).

Effects of oral trimetazidine on serum and tissue homogenate levels of ischemic parameters after induction of ischemia

In this group results produced a significant reduction in serum and tissue homogenate levels of IL-6, TNF- α , MDA and MMP-9 with a significant elevation in serum and tissue homogenate levels VEGF-A and eNOS respectively in comparison to that observed in induction group; But showed non-significant difference compared to orally administered control group and orally administered pentoxifylline group. Tables (1 and 2), Figures (6 to 17).

Histopathological evaluation of orally administered drugs

The mean of histopathological scores of orally administered control group was showed significant changes (Score number = 5) table (3) figure (18) in compare with induction group figure(19). While in orally administered pentoxifylline group results revealed significant changes in compare with induction group, but it's non-significant in compare to orally administered control group (Score number =3) table (3) figure (20). Also in orally administered trimetazidine group results showed significant changes in compare with induction group, but it's non-significant in compare to orally administered control group and orally administered pentoxifylline group figure (Score number =4) table (3), figure (21).

Discussion

Several factors including hypoxia, inflammation, hyper-glycemia, venous stasis, infection and immunosuppression are associated with dys-regulation of wound healing mediators which impairs wound healing and facilitates the formation of chronic wounds (Eun BL, et al .2000). Prolonged ischemia is a common element in ulcers of many etiologies (diabetes, pressure sores, peripheral vascular disease and venous stasis) and it is a significant contributor to chronic wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation (Menke NB, et al. 2007).

The rabbit ear model has been extensively used to simulate ischemic wounds which had been validated in previous studies allowing fast and easy macroscopic and microscopic evaluations of the ischemic wounds (García-Honduvilla N, et al. 2013). Chronic wounds in animals can be created from an acute wound by inducing diabetes, mechanical pressure, ischemia, or reperfusion injury (Mustoe et al., 2006). Some notable observations have been made that point to rabbit wounds behaving similarly to human wounds. These similarities include increased scarring with delayed epithelialization and less scarring with old age, topical steroids, and collagen synthesis inhibitors (Ayman Grada, et al.2018). In this study a modified ischemic ear model was developed in which induction of ischemia was done by ligation of central and rostral arteries with the central and rostral veins to ensure complete ischemia by prevention blood flow and venous drainage without any well-established protocols for induction of acute wounds (excisional and incisional models) (DiPietro and Burns, 2003).

Effect of ischemic induction on levels of inflammatory mediators

In the current study, induction of ischemia provoked a significant notable elevation in levels of inflammatory mediators (IL-6 and TNF- α) in tissue homogenate greater than in the serum; in which after recruitment leukocytes produce a wide range of inflammatory mediators such as cytokines, ROS, and growth factors to resolve inflammation. IL-1 β , IL-6 and TNF- α play an important role in this process; this is in agreement with previous study (Steinberg JP, et al .2013) that showed ischemic wounds markedly increased expression of inflammatory cytokines, chemokines and increased inflammatory cells following tissue damage at 7-14 days post wounding that lead to increase monocyte recruitment which impaired epi-thelialization and granulation. These monocytes mature into inflammatory macrophages and perpetuate inflammation with release of TNF- α and IL-6 (DeLeo FR, et al. 2009). Also, previous study demonstrated that ischemic wounds express significantly higher levels of inflammatory cytokines such as IL-1 and IL-6 which supports the validity of this model (Gourdin MJ, et al . 2009).

Effect of ischemic induction on levels of oxidative stress

In local tissue hypoxia; the wounds exposed to ischemia-reperfusion cycles revealed greater levels of inflammation as a result of reactive oxygen species (ROS) production during periods of reperfusion. In the present study, it has been shown that ischemic injury caused harmful effects on the ear tissue which altered the oxidant /antioxidant balance that caused significant increase in the level of lipid peroxidation end product (MDA) in the tissue homogenate more than in the serum. The explanation for this elevation involved that the influx of neutrophil cells in the early inflammatory response that is essential for the clearance of bacteria and cellular debris during cutaneous wounding, this an overload of infiltrating neutrophils at the ischemic wound site is known to be adversely influence wound healing and likely to cause oxidative stress (Leor J, Rozen L, et al. 2006).

Effect of ischemic induction on levels of MMPs

The MMPs play essential role in all stages of wound healing by modifying the wound matrix, allowing for cell migration and tissue remodeling in which MMP-9 is expressed in skin injury and other injured epithelia (Castaneda FE, et al. 2005). In the present study, the results of induction group provoked high levels of MMP-9 in the tissue homogenate more than in the serum, this occur due to tissue hypoxia that increases inflammatory cytokines such as IL-6 and TNF- α that stimulate the production of neutrophil gelatinase-associated lipocalin (NGAL). The latter would be interacting with MMP-9 activity to form MMP-9/NGAL complex. This process occurs during the onset ischemia and healing phases of venous ulcers (S. de Franciscis, et al. 2013); thus yield high MMP-9 activity that caused dissolution of the basal epidermal keratinocytes hemi-desmosomes; that disrupts their contact with the basement membrane and allows their migration through the wound matrix (Mulholland B, et al. 2005). This increment in MMPs levels was in agreement with previous study that showed over expression with significantly high levels of proteases proteins during the inflammatory and proliferative phases resulting in repeated migration of polymorph nucleus (PMNs) and macrophages to the wound site that prolonged the inflammatory phase

attributed to excessive collagenolytic properties and neutrophils resulting in delayed wound healing (R. Serra, et al. 2016). Furthermore, during ischemic hypoxia, the bacterial metabolic activity would be increase due to replication and this produce high levels of MMPs that is negatively effect on the wound progression at different stages, in which MMPs degrades the ECM that is required for cell migration and causing immune cells to migrate to initiate inflammatory response (S. L. Dholakiya and K. E. Benzeroual . 2011).

Effect of ischemic induction on levels of angiogenic stimulators

In normal conditions, endothelial cells remain quiescent as 'phalanx' cells under a balance of pro- and anti-angiogenic factors. When pro-angiogenic factors dominate; the status of cells rapidly changes that caused the endothelial cells switch to angiogenic phenotypes, categorized as either migratory tip cells or proliferating stalk cells (M. Potente, et al . 2011). Tip cells respond and navigate towards the angiogenic signal and are characterized by the slow proliferation, high motility, existence of filopodia as well as the expression of protease facilitating the digestion of ECM components (H. Gerhardt, et al. 2003). Stalk cells follow a tip cell and proliferate extensively to elongate a new vessel and to extend the vascular lumen. In the present study after induction of ischemia there was a significant reduction in serum and tissue homogenate levels of both VEGF-A and eNOS; due to the combined effect of ischemia and injury that responsible for the temporary delay of expression of both VEGF-A and eNOS this was correlated with previous study that showed VEGF-A mRNA expression was scarce, and located in plasma inside the small blood vessels, among blood cell components and in the endothelial cells that are coating the inside of the blood vessels and extravasation of the angiogenic factor to the surrounding connective tissue was not observed (Sotomayor S, et al. 2016). This result was compatible with previous study that showed the fluids of non-healing wounds contain elevated levels of inflammatory cytokines such as IL-1, IL-6 and TNF- α , elevated levels of proteinases, and low levels of growth factor activity compared with fluids collected from acute healing wounds (Sotozono, et al.1999). Also previous studies showed that TNF- α

directly impairs endothelial dependent relaxation by inhibiting NO generation via a mechanism involving sphingomyelinase and ceramide activated phosphatase 2A (Smith, et al. 2006) which act as a regulator in angiogenesis via stimulate the proliferation and migration of endothelial cells, mediate VEGF-A production and provide protection for endothelial cells from apoptosis during the initial phases of angiogenesis (Frank S, et al. 2002). Moreover; TNF- α play a crucial role in recruiting leukocytes infiltration which indirectly impair vascular contractility (Frangogiannis, et al. 2002).

Effect of pentoxifylline on levels of inflammatory mediators

Control of inflammation has an important role in wound healing in which over activation of inflammatory cascade impairs this process (Rajan V, Murry RZ. 2008). In the present study, orally and/or topically administered pentoxifylline after induction of ischemia produced significant reduction in both serum and tissue homogenate levels of inflammatory mediators IL-6, TNF- α and MMP-9 due to its mechanism as a nonselective competitive PDE inhibitor (PDE-I) (Essayan DM. 2001); that raises intracellular cAMP, activates PKA, inhibits synthesis of IL-6 and TNF- α (Deree J, et al. 2008), decreases cytokine release and suppresses leukocyte hyperactivity by reducing superoxide release and neutrophil adhesion (Rasslan R, et al. 2014) and reduces inflammation and innate immunity (Peters-Golden M, et al. 2005). Consequently, these events are correlated with previous study done by (Kalay S, et al. 2013) reported that pentoxifylline suppressed caspase-3 activity and inhibited the expression of interleukins (IL-1 β , IL-6 and TNF- α mRNA) in the brain tissues after hypoxic ischemic encephalopathy in neonatal rats.

Effect of pentoxifylline on levels of oxidative stress parameter

Moreover; orally and/or topically administered pentoxifylline decrease MDA levels in both serum and tissue homogenate by reduction oxidative stress via its scavenger effect on oxygen-free radicals production (Bhat VB, Madyastha KM. 2001); this result is in agreement with previous study demonstrated a decrease in serum and tissue MDA levels after pentoxifylline administration in severe

ischemia/re-perfusion of small intestine. Thus, pentoxifylline with anti-inflammatory and antioxidant properties may improve wound healing (Lloris Carsi JM, et al. 2013).

Effect of pentoxifylline on levels of angiogenic stimulators

In this study, orally and/or topically administered pentoxifylline increased the levels of both VEGF-A and e-NOS in both serum and tissue homogenate by improving red blood cell deformability, erythrocyte flexibility allowing easier vascularization which enhancing tissue oxygenation that improves blood flow in the vessels; these changes are in agreement with previous study that showed pentoxifylline improved microcirculation and oxygen delivery, especially in the ischemic states which it increases fibrinolysis and suppresses leukocyte hyperactivity by reducing superoxide release and neutrophil adhesion (Rasslan R, et al. 2014) in which due to its fibrinolytic effect it decreases blood viscosity that improve the blood flow (Lloris Carsi JM, et al. 2013).

Effect of trimetazidine on levels of inflammatory mediators

In the present study rabbits received orally and/or topically trimetazidine after induction of ischemia produce a significant reduction in the serum and tissue homogenate levels of IL-6, TNF- α and MMP-9; these results due to the effect of trimetazidine in preventing inflammatory cell infiltration. This result was in agreement with previous study that revealed trimetazidine caused a significant decrease in the levels of pro-inflammatory markers through preventing the inflammatory cell infiltration (Baumert H., et al. 2004). Other study demonstrated that trimetazidine was shown to suppress the elevation of inflammatory markers such as C-reactive protein (CRP), TNF- α and IL-6 levels during coronary interventions (Martins GF, et al. 2012) in which reactive oxygen species mediated damage enhances the release of pro-inflammatory mediators such as CRP, TNF- α , IL-1, IL-6 and IL-8 from macrophages in both inflammation and ischemia. Also these results are consistent with those of previous studies that showing trimetazidine treatment attenuates acute inflammatory responses and oxidative stress in

experimental cardiac remodeling (Zhou, et al. 2012) and pancreatitis (Tanoglu, et al. 2015).

Effect of trimetazidine on levels of oxidative stress

In this study trimetazidine reduce both serum and tissue homogenate levels of malondialdehyde (MDA) significantly by decreased mitochondrial respiration during ischemia, decreased oxygen consumption and reduced ROS production thus allowing for the conservation of a healthy mitochondrial membrane potential (P. Monteiro, et al. 2004) and also trimetazidine has anti-apoptotic effect through preservation of mitochondrial integrity (J. Kuzmicic, et al.2014); this result is in agreement with previous study that shown trimetazidine may inhibit inflammation progression by limiting ROS, maintaining cellular ATP level and protecting tissue from free radicals (J.Chen, et al.2016) and also trimetazidine prevents excessive release of free radicals, which are particularly toxic to phospholipid membranes and are responsible for both the fall in intracellular ATP concentration and the extracellular leakage of K⁺ during cellular ischemia (P. Monteiro, et al.2004). Moreover, trimetazidine acting as a potent anti-oxidant which decreased the level of malondialdehyde (the end product of lipid peroxidation) and increased the activity of superoxide dismutase and glutathione peroxidase (major endogenous antioxidant enzyme systems responsible for limiting intracellular accumulation of oxygen radicals during normal aerobic metabolism) (Iskesen I, et al. 2006).

Effect of trimetazidine on levels of angiogenic stimulators

Also in this study orally and/or topically administered trimetazidine after induction of ischemia produced a significant elevation in both serum and tissue homogenate levels of VEGF-A and eNOS; these changes due to trimetazidine improves endothelial function by increasing nitric oxide production due to up-regulation of eNOS (Q. Wu, et al. 2013) and inhibiting cell apoptosis (Y. Ruixing, et al. 2007) which improved microcirculation and endothelial cell damage (Alpaslan Tanoglua, et al. 2015). These results were been in agreement with previous evidenced that showed trimetazidine promotes the induction of cytoprotective factors such as hypoxia inducible factor-1 α (HIF-1 α) and 5-

AMP-activated protein kinase (AMPK) during ischemia (Ben Mosbah I, et al. 2007). The activation of these factors is responsible for the regulation of relevant pathophysiological processes during ischemic injury such as the ER stress, autophagy and mitochondrial damage (Nishida K, et al. 2009). While activation of AMPK up-regulate eNOS activity which enhanced NO generation during ischemic injury (Peralta C, et al. 2001) and stabilization of HIF-1 α after ischemia (Zhang WH, et al. 2006) in which accumulation of HIF-1 α triggers an increase in expression of cytoprotective genes involved in mitochondrial function, cell survival, and resistance to oxidative stress (Dawn B, Bollir. 2005).

Conclusion

1- The results of this study suggested that orally administered trimetazidine (20mg/kg/day) resulted in a significant improvement in ischemic tissue damage in rabbit ear model were associated with decreasing inflammatory biomarkers, reducing oxidative tissue damage with increasing the angiogenic activities through stimulating of growth factors and increasing endothelial cells re-epithailization which proved to be highly beneficial in treatment of skin injury in this model.

2- From all of above results that was previously discussed; we conclude that trimetazidine had noticeable effects against ischemic injury in rabbit ear model in which there is a significant difference in changing the levels of biochemical ischemic parameters in both serum and tissue homogenate, in addition to histopathological findings that refer tadalafil produce a significant viable repairing.

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Table 1. Effects of orally administered drugs on the serum levels of ischemic parameters after induction of ischemic injury (a : p < 0.05 significant in compare with control group, b : p < 0.05 significant in compare with induction group, c : p > 0.05 Non-significant in compare with pentoxifylline group, NS : p > 0.05 Non- Significant to control group).

GROUP SERUM	Control	Induction	Pentoxifylline	Trimetazidine
IL-6 (ng/L)	220.14±16.1	361.42±18.7 a	235.54±25.4 NS,b	251.19±23.4 NS,b,c
TNF-a (ng/L)	55.78±6.4	110.60±10.3 a	63.48±3.3 NS,b	72.63±6.2 NS,b,c
MDA (nmol/ml)	15.09±0.8	24.90±0.8 a	17.93±0.7 NS,b	18.24±0.8 NS,b,c
MMP-9 (ng/ml)	2.78±0.3	5.76±0.5 a	2.89±0.3 NS,b	3.60±0.5 NS,b,c
VEGF-A (pg/ml)	530.20±31.9	319.54±14.7 a	543.74±43.4 NS,b	547.83±46.0 NS,b,c
eNOS (pg/ml)	555.52±51.5	299.48±26.4 a	563.22±32.1 NS,b	614.59±45.4 NS,b,c

Table2. Effects of orally administered drugs on the tissue homogenate levels of ischemic parameters after induction of ischemic injury (a : p < 0.05 significant in compare with control group, b : p < 0.05 significant in compare with induction group, c : p > 0.05 Non-significant in compare with pentoxifylline group, NS : p > 0.05 Non- Significant to control group).

Group Tissue homogenate	Control	Induction	Pentoxifylline	Trimetazidine
IL-6 (ng/L)	509.99±34.5	748.94±43.6 a	524.44±27.5 NS,b	563.46±60.3 NS,b,c
TNF-a (ng/L)	88.30±3.2	137.64±7.4 a	91.83±3.2 NS,b	95.23±3.9 NS,b,c
MDA(nmol/ml)	20.77±0.6	28.52±0.4 a	22.71±1.1 NS,b	23.06±0.9 NS,b,c
MMP-9(ng/ml)	10.16±0.6	16.54±1.4 a	12.64±0.8 NS,b	11.13±0.7 NS,b,c
VEGF-A (pg/ml)	635.33±35.4	476.08±18.6 a	652.08±39.4 NS,b	696.15±45.7 NS,b,c
eNOS (pg/ml)	662.72±33.6	489.08±21.5 a	675.37±31.2 NS,b	690.99±36.8 NS,b,c

Table 3. Histopathological scoring system for administered groups after induction of ischemia (Altavilla D, et al. 2005 and Galeano M, et al. 2006)

Group	Re-epithelialization	Granulation tissue	Inflammatory cells	Angiogenesis	Score No.
Induction	Absence of epithelial proliferation $\geq 70\%$ of the tissue	Immature and inflammatory tissue in $\geq 70\%$ of the tissue	13-15 cells/field	Absence of angiogenesis, presence of congestion with edema	0
Orally control	Absence of epithelial proliferation with normal epidermal and dermal layers	Normal epithelial thickness with normal skin appendages	1-3 cells/field	NO	5
Orally pentoxifylline	Marked degree $\geq 60\%$	Thick layer $\geq 65\%$	6-7 cells/field	5-6 vessels/site	3
Orally trimetazidine	Marked degree $\geq 85\%$	Complete regeneration of basal layer $\geq 80\%$	1-4 cells/field	7-8 vessels/site	4

Figure 1. Structure of human skin (Laure Guenin-Mace, et al. 2014): The different tissues and cell layers composing this organ are depicted, together with the physiological mechanisms and molecular species varying during skin ulceration and healing.

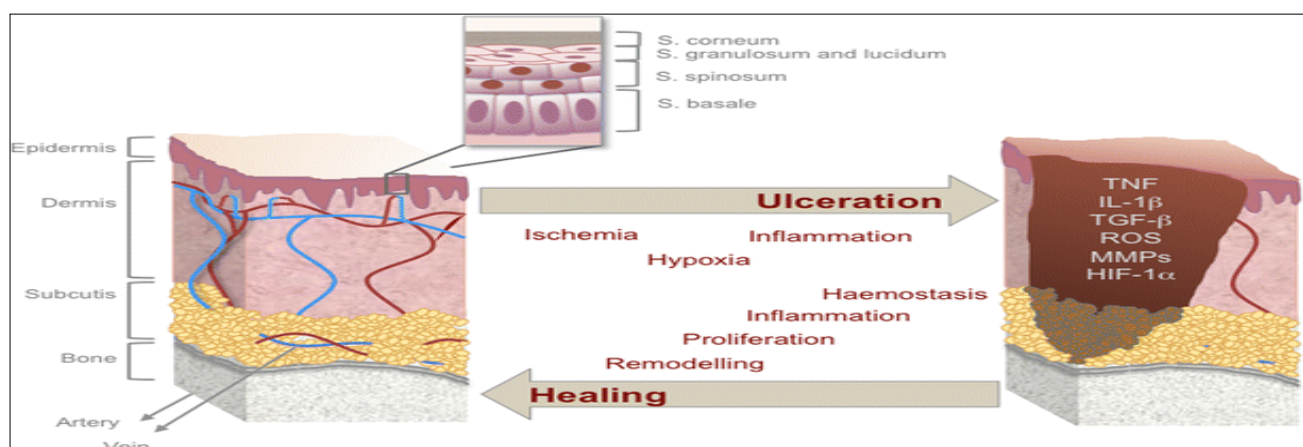


Figure 2. Functional and morphologic consequences of decreased intracellular ATP during ischemic injury (Robbins and Cotran.2010).

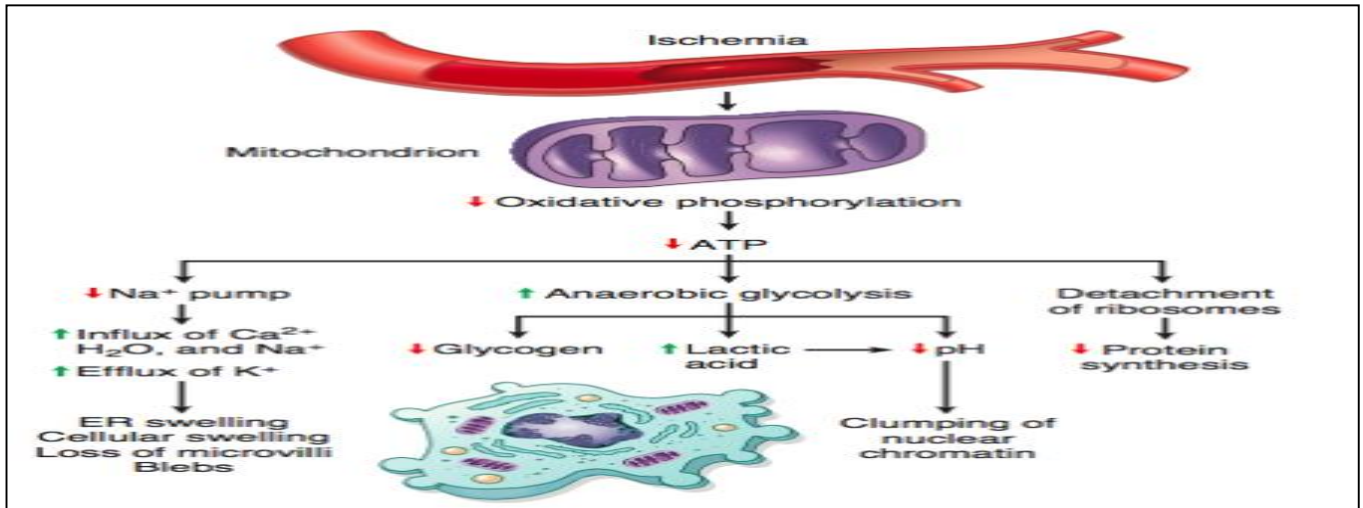


Figure 3. Hypothesis of chronic wound pathophysiology (Mast BA and Schultz GS.1996).

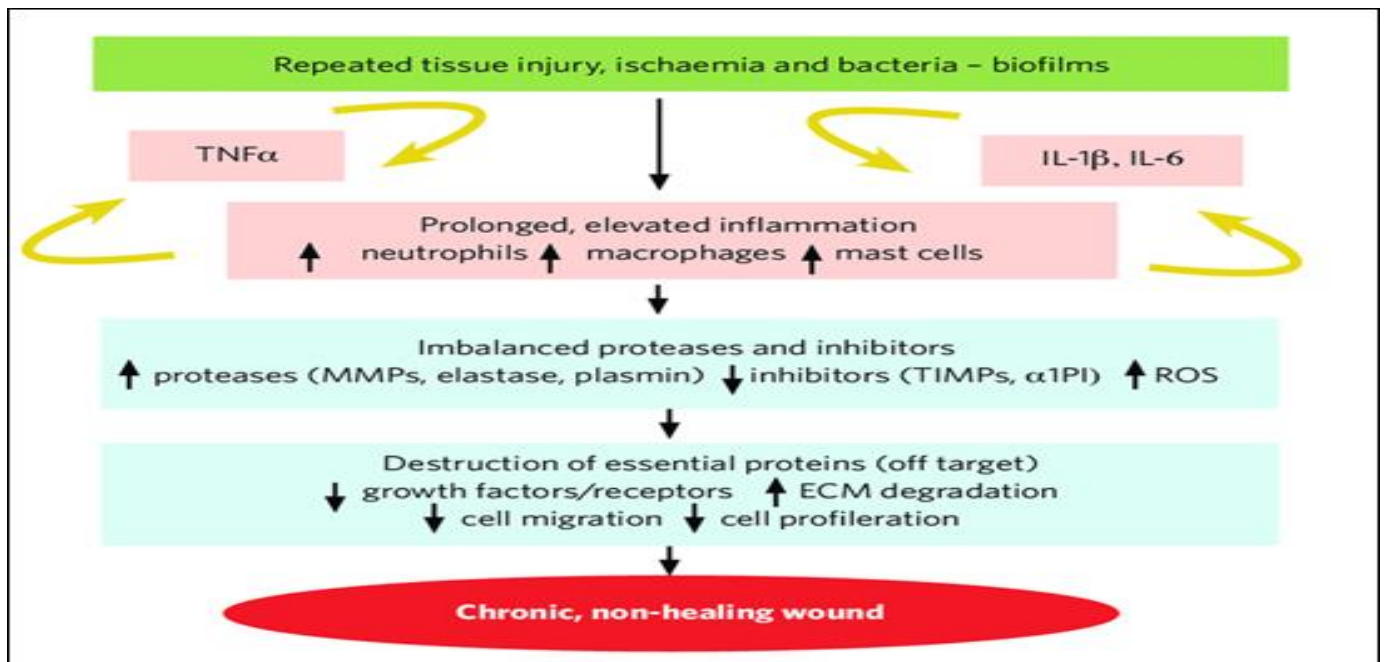


Figure 4. Ischemic rabbit ear model (The central and rostral arteries and veins are ligated, leaving the small caudal artery and vein intact) (Saulis A, Mustoe TA; 2001)

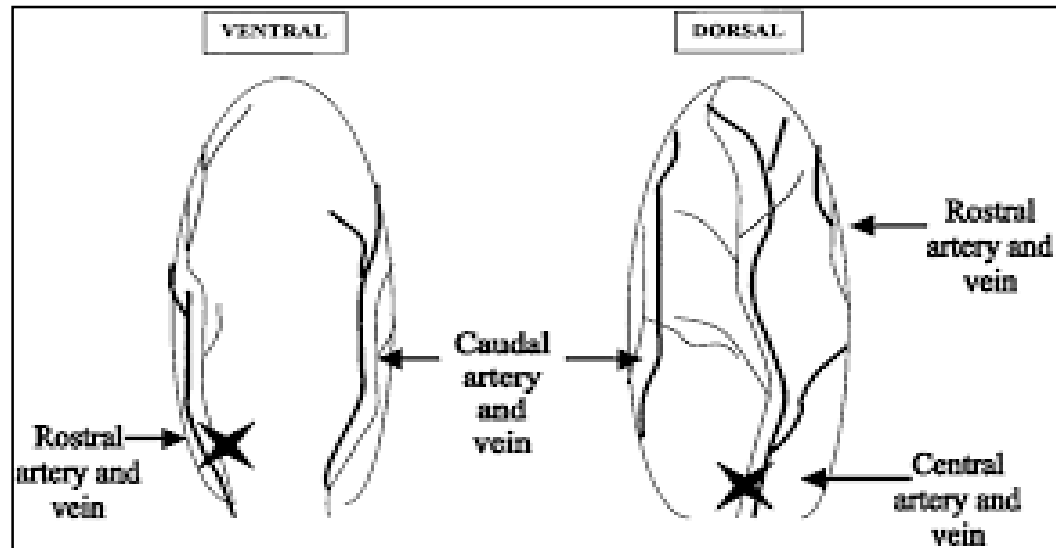


Figure 5. (A): Four circular biopsies with diameter (10 mm) done by using punch biopsy. (B): Macroscopic image shows complete removal of epidermis, dermis and cartilage.

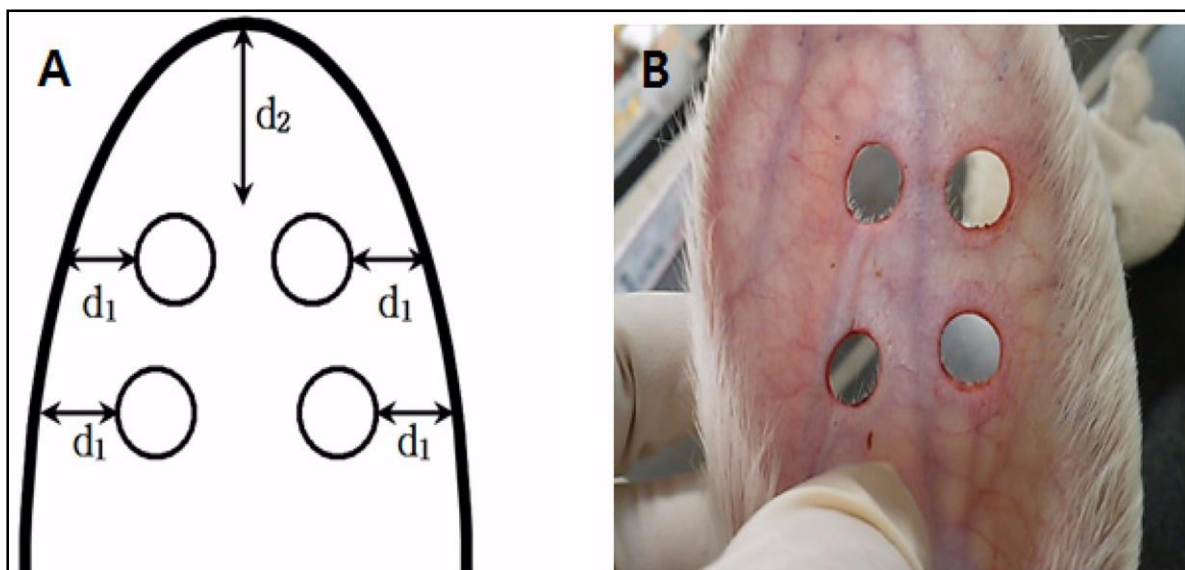


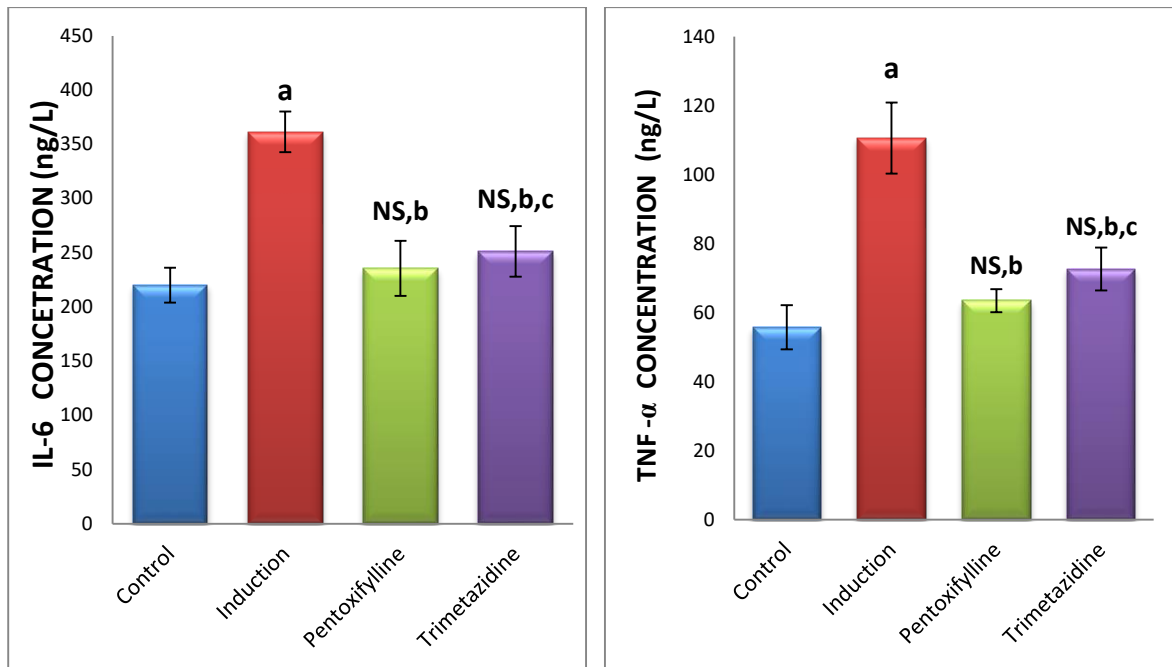
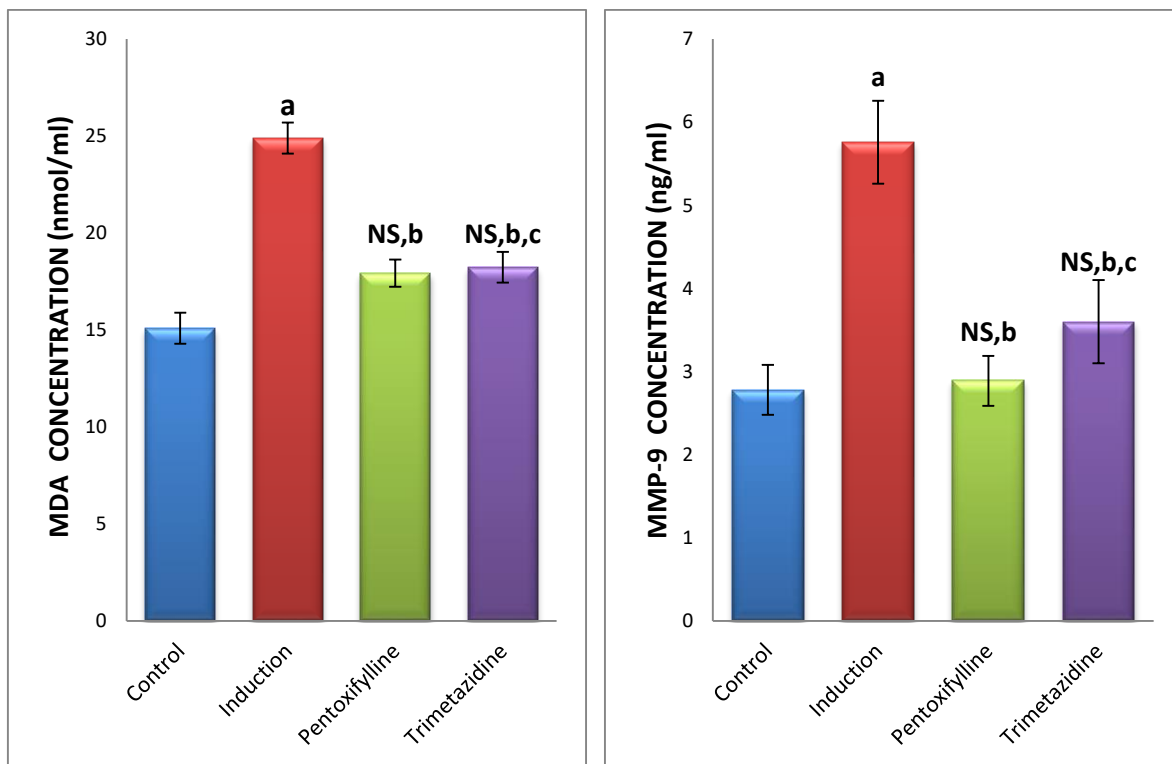
Figure 6. Effects of orally administered drugs on the serum levels of interleukin-6 (IL-6).**Figure 7.** Effects of orally administered drugs on the serum levels of tumor necrotic factor-alpha (TNF- α)**Figure 8.** Effects of orally administered drugs on the serum levels of malondialdehyde (MDA).**Figure 9.** Effects of orally administered drugs on the serum levels of matrix metalloproteinase-9 (MMP-9)

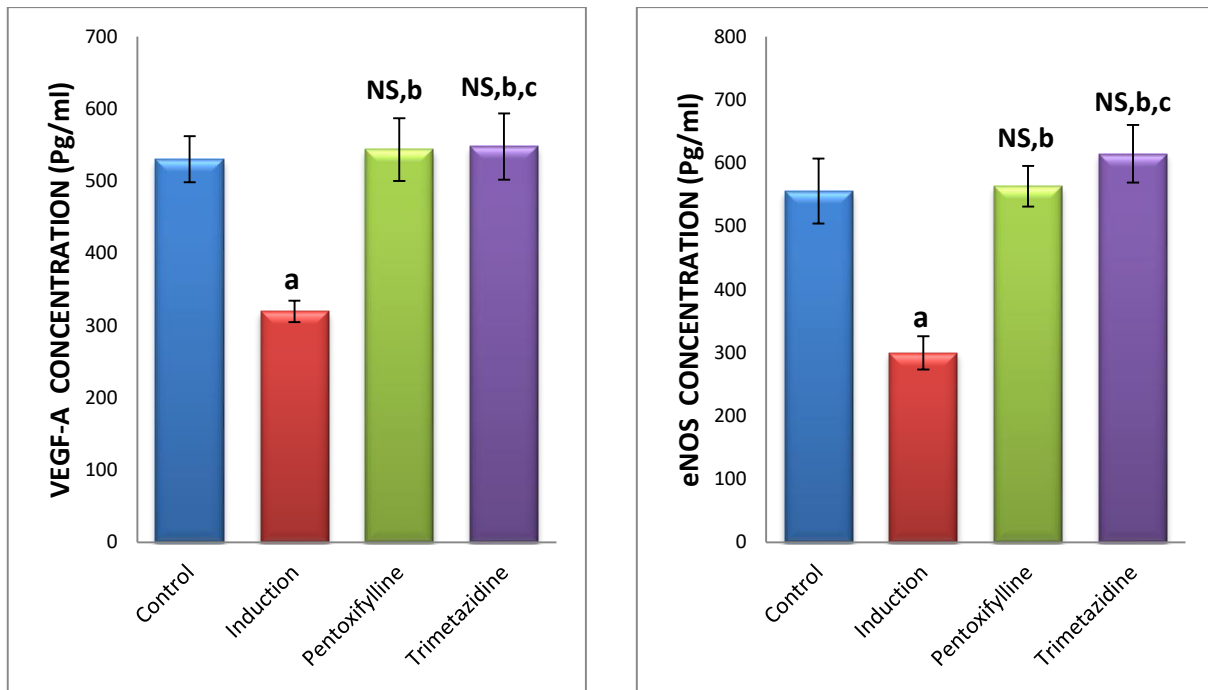
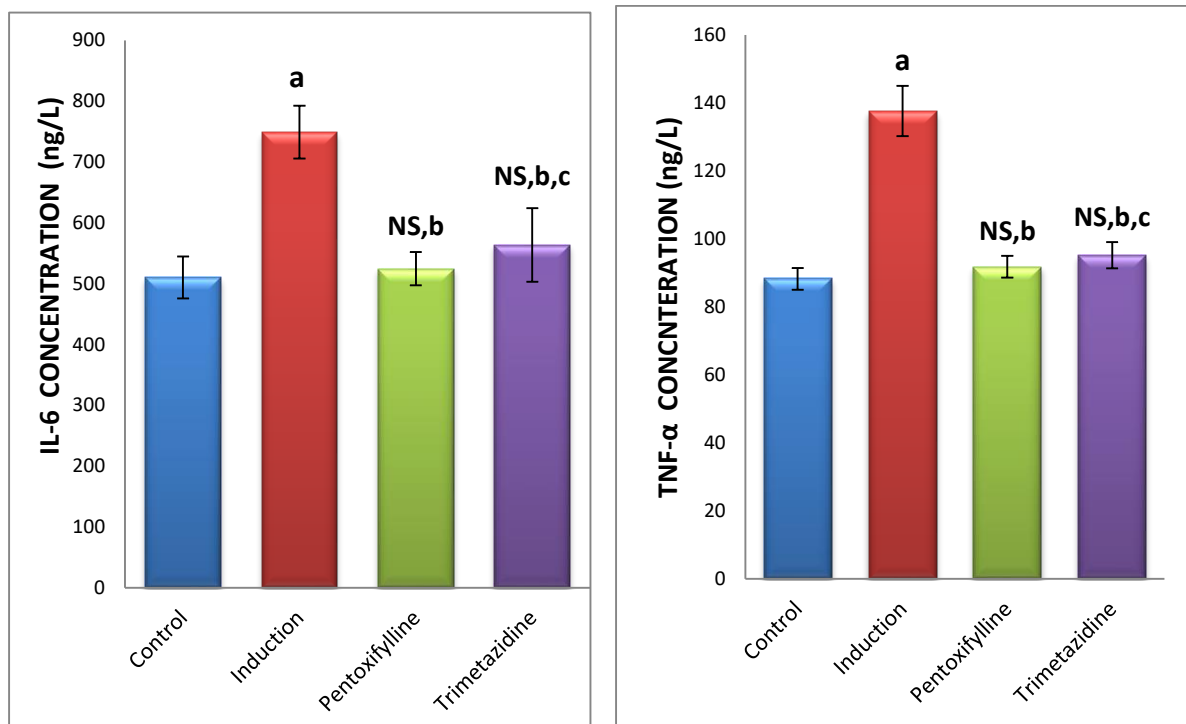
Figure 10. Effects of orally administered drugs on the serum levels of vascular endothelial growth factor –A (VEGF-A).**Figure 11.** Effects of orally administered drugs on the serum levels of endothelial nitric oxide synthase (eNOS)**Figure 12.** Effects of orally administered drugs on the tissue homogenate levels of interleukin-6 (IL-6).**Figure 13.** Effects of orally administered drugs on the tissue homogenate levels of tumor necrotic factor- α (TNF- α)

Figure 14. Effects of orally administered drugs on the tissue homogenate levels of malondialdehyde (MDA).

Figure 15. Effects of orally administered drugs on the tissue homogenate levels of matrixmetalloproteinase-9 (MMP-9)

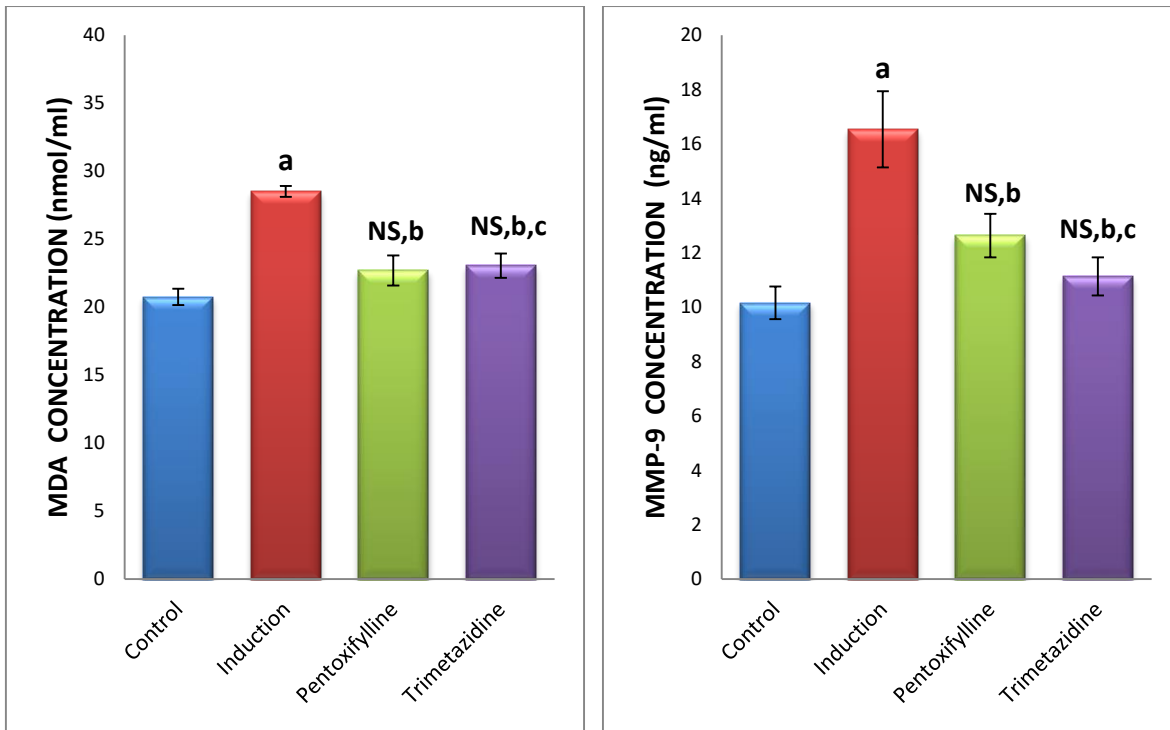


Figure 16. Effects of orally administered drugs on the tissue homogenate levels of vascular endothelial growth factor-A (VEGF-A).

Figure 17. Effects of orally administered drugs on the tissue homogenate levels of endothelial nitric oxide synthase (eNOS)

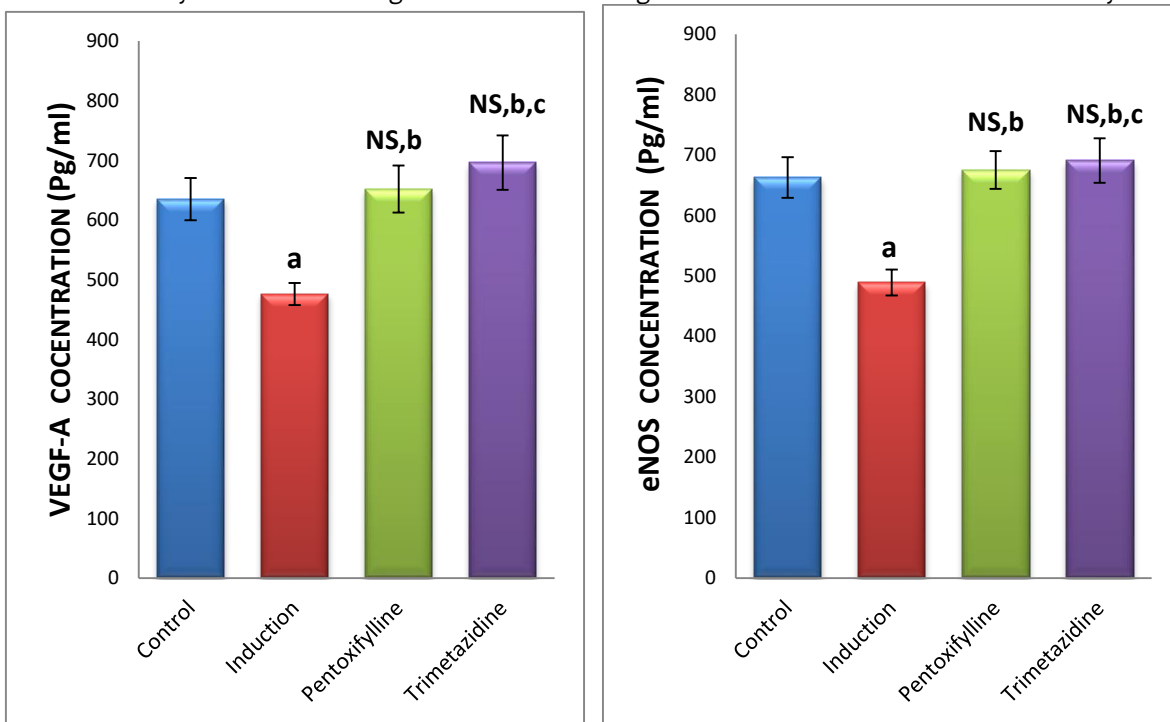


Figure 18. Section of skin of orally administered control group reveals the normal appearance of component of appendage (green arrow) (H&E - 40X).

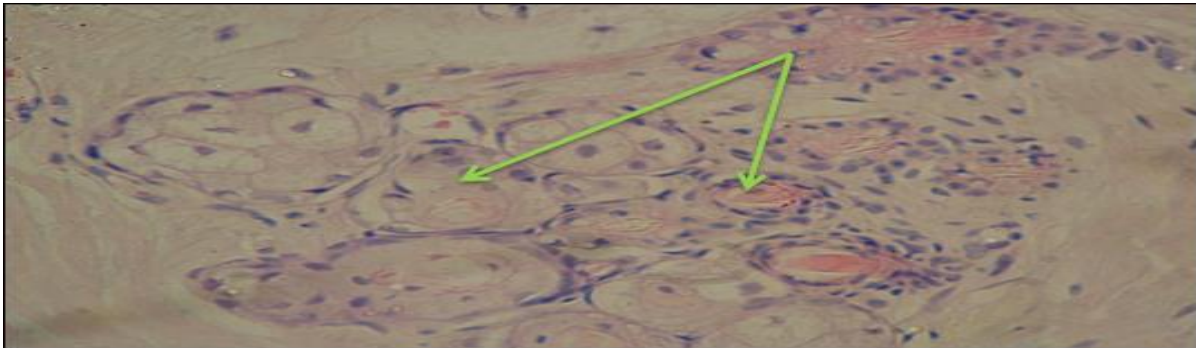


Figure 19. Skin sections induction group at 14 day interval shows marked features of ischemia which include marked inflammatory infiltration of mixed acute and chronic type (red arrow) with edema (blue arrow) and destruction of sebaceous and hair follicles (green arrow). (40X H&E)

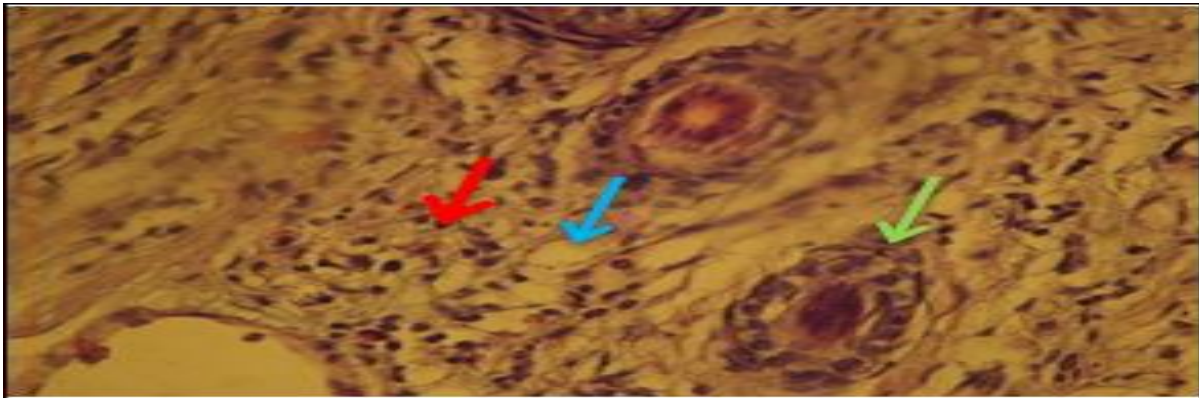


Figure 20. Skin section of orally administered pentoxifylline group show marked inflammatory infiltration of both acute (green arrow) and chronic (blue arrow) in the dermal layer (H&E - 40X).

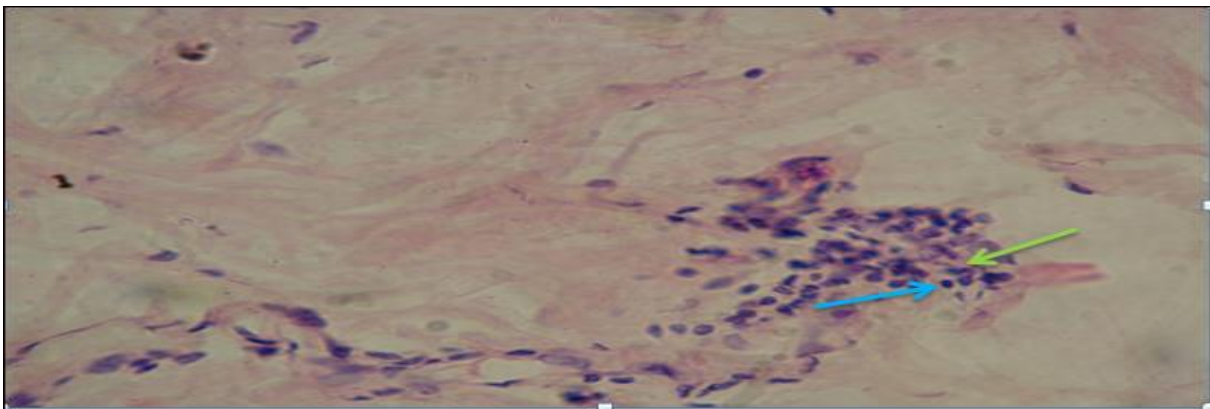


Figure 21. Skin section of orally administered trimetazidine group reveal marked angiogenesis (green arrow) and chronic inflammatory infiltration (blue arrow) (H&E - 40X).

