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Histopathological Study of the Efficacy of Alpha Lipoic Acid and H_2O_2 on Early Stage of Fracture Healing in Rabbits

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ABSTRACT

In most animals, fractures are not compatible due to a short period of fixation because of animal movement, unlike humans, so we need to reduce or accelerate some stages of fracture healing. The objective of the present study is to determine the effectiveness of using alpha lipoic acid and hydrogen peroxide in improving bone regeneration process after the middle section of rabbit`s right femur has been gaped with a drill. Under strict aseptic condition, the middle section of right femur of twelve male rabbits has been gaped with a drill (size 1 mm) after retracting the surrounding soft tissue. The experimental animals were divided according to the treatment into four groups (3 for each group), control, H2O2 treated group, alpha lipoic acid treated group, and H2O2 – alpha lipoic acid treated group. The experimental animals were euthanized one week after surgery for tissue collection and histopathological evaluation was performed. Histopathological study of the collected bone samples showed that alpha lipoic acid stimulates fracture healing in its early stage and using alpha lipoic acid alone revealed that the healing process was improved better than other treated groups. We concluded from the present study that alpha lipoic acid has the ability to accelerate and enhance bone fracture regeneration and improve its quality in the early stage of the fracture healing.

Keywords: Alpha lipoic acid, fracture, H2O2, rabbit.

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INTRODUCTION

Fracture healing is a persistent problem in animal orthopedics. Usually bone fractures are caused by falls from heights, traffic accidents, and the movement of animals cannot be restricted for a long time, etc. Therefore, bone healing must be accelerated following the conventional surgical fracture treatment, and new medical treatments were advanced for the purpose of avoiding some complications such as vascular necrosis, infections, osteomyelitis, and bone non-union or false union (Aydin *et al.* 2014).

After fracture occurrence, there will be a rupture of blood vessels of the periosteum, which result in haematoma and necrosis of the bone fracture site and this site will be invaded by polymorphonuclear leucocytes, macrophages, and mononuclear cells (to remove the necrotic tissue from the broken ends of the bone). The hematoma is organized by newly formed vessels, and fibroblasts result in a fibrous tissue. Throughout that reparative stage, the chondrocytes are active and woven bone has been produced from the endochondral ossification with the formation of a fibrocartilaginous callus. The osteoblasts have been progressively differentiated and the bridging mineralized callus has been externally and internally created over fracture (Ulstrup, 2008).

In the gap healing the site of the fracture, it's mainly filled with the lamellar bone, which is oriented perpendicularly to long axis, thereby, it requires the secondary osteoid re-construction. The main structure of the bones will be replaced afterwards gradually with the longitudinal re-vascularized osteons that carry osteo-progenitor cells, differentiating to osteoblasts and producing lamellar bone on the gap surface (Marsell and Einhorn, 2011) Revascularization of the fracture is dependent upon the fracture stability and gap conditions (Claes *et al.* 2003).

A variety of the therapeutic modalities were advanced for enhancing the response of healing and filling the defects of the bone. A variety of the stem cells, growth factor types, natural grafts (i.e., allografts, autografts or xenografts) and synthetic- biologic-based tissue-engineered scaffolds can be considered as a few examples. Never-the-less, those therapeutic agents and synthetic and organic materials have a few important limitations, and yet, there aren't any well-approved modalities of treatment for meeting all expected needs (Bigham-Sadegh and Oryan, 2015).

Bone can be defined as one of the metabolically active tissues. After a fracture, the bone undergoes a continuous remodeling process through two procedures, re-sorption and formation of the bone that must be balanced for the purpose of preserving the integrity and mass of the bone. Bone resorption involves the degradation of bone minerals, with the osteoclasts and bone formation by the osteocytes and osteoblasts (Cicek and Cakmak, 2018).

Both the innate and adaptive immune processes are important at the anabolic and catabolic stages for fracture healing. In the primary inflammatory phase after an injury, specific cellular immune functions function by removing dead tissue, promoting angiogenesis, and initiating repair (Einhorn and Gerstenfeld, 2015).

Sufficient debridement and irrigation for the removal of the foreign bodies, contaminating microorganisms and non-vital tissues can be considered as the most significant ways for the prevention of the infections in the open fracture (Husodo, *et al.* 2016).

H2O2can be defined as one of the common reactive oxygen species (ROS) that are included in catalytic mechanisms, in spite of a fact that it's toxic to the cells as a result of its oxidative nature. It is not stable, and that may result in damaging the oxidizing DNA, lipidsand protein. It's utilized to prevent the local infections from bacteria as well as a clinical debridement in the joint and bone operations. The in-vivo studies, there have been reports on increased osteoclast activity levels reported due to over-production of the ROS, (i.e., the H2O2). Which is why, H2O2 is known to have the ability for the stimulation of

the formation of the osteoclast and the function by the re-sorptive cytokines (Cicek and Cakmak, 2018).

The oxidative stress has been assumed to have a relation with the decreased value of the BMD (i.e., bone mineral density), strength and stiffness, and resulted in the osteoporosis through the in-vitro studies. None-the-less, mechanisms by which the bone matrix is influenced haven't been entirely recognized yet. The H2O2 induced oxidative damages to bio-mechanical characteristics of the bones has not been yet studied thoroughly enough (Domazetovic, *et al.* 2017).

The ALA (i.e., the alpha lipoic acid) can be considered as one of the significant mitochondrial co-factors and, due to the fact that it is a free molecule, it is capable of exerting multi-level immuno-modulatory functions. It has been considered to have the ability of chelating the heavy metals, regenerating the essential antioxidants and repairing the important molecules that have been damaged with the oxidation. Its differentiated and complicated function is not capable of being simply reduced to an antioxidant anti-inflammatory and detoxifying action (Monastra *et al.* 2016).

The ALA, which is one of the natural occurring compounds and dietary supplements, was stated as one of the potent antioxidants, which is one of the strongest scavengers of the free radicals. The exact mechanisms that underlie ALA's bone-protective actions is still not fully understood. It has been focused upon ALA's anti-oxidative capacity for exerting the bone-protective impacts in vivo as well as in vitro. It has exerted the bone-protective effect through the inhibition of the generation of the ROS. Moreover, it is capable of exerting its bone-protective actions. It has resulted in the promotion of the osteoblast differentiation, maturation and mineralization as well as the inhibition of the apoptosis of the osteoblast (Fu *et al.* 2015).

The objective of this research has been aimed at the evaluation of the alpha lipoic acid and hydrogen peroxide effects upon the healing of the fracture in the rabbit model.

MATERIALS AND METHODS

Twelve local male rabbits were used in the present study, weighed (1.5-2 Kg). The animals were housed in separated cages under normal temperature conditions for 2 weeks in animal house at the Veterinary college/ University of Basrah for acclimatization.

The experimental animals have been classified to 4 groups (i.e., three for each group), control, H2O2 treated group, ALA treated group, and H2O2 –ALA group. The site of operation was prepared surgically, and surgery was done at safety general anesthesia.

After retracting the surrounding soft tissue, the middle sections of the right femur of twelve male rabbits were gaped with a drill (size 1.5 mm) **(Figure 1)**. In the control group, wihc is the first group; the wound has been irrigated by 100ml 0.90% saline. In the second group, ALA has been given orally to the animals. In the third group (hydrogen peroxide group), the wound has been entirely soaked with a solution of (3% H2O2) for two minutes and irrigated afterwards with 100ml saline. In the fourth group, rabbits were treated with alphalipoic acid and H2O2. Rabbits were euthanized at the end of one week postoperatively, in each treated fractured femur, the osseous-cartilaginous area, and the fibrous tissue in callus has been histopathologicaly assessed.

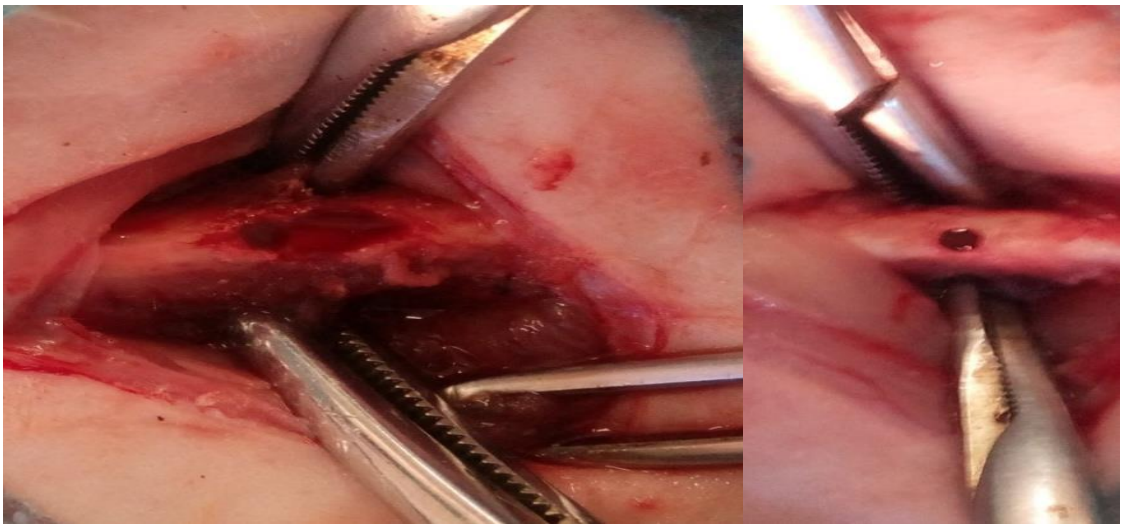

Figure 1: Middle section of femur bone was gaped with a drill (size 1 mm)

RESULTS

In the control group, the histopathological sections showed the early stage of soft callus formation and narrowing of the fractured edges **(Figure 2)**, while in H202 treated group, showed a spicule of newly formed woven bone that has filled the gap between the ends of the fracture and a few numbers of hypertrophic chondrocytes trapped in calcified matrix. In addition, there is some remaining chondrocytes, as well as presence of fibrous callus and fibrosis **(Figure 3)**. In the ALA treated group, the section of a fracture callus illustrates nicely the haphazard disposition of osteocytes in the woven bone, this mature lamellar bone, organized layers of osteocytes is noted, also there are also areas of woven bone remodeled to the lamellar bone, as well as infiltration of macrophages **(Figure 4)**. In the last group (H2O2 and lipoic acid treated group) showed formation of immature bone show, where the soft callus formation at a lower rate than the lipoic acid group and disposition of osteoblast in the woven bone is obvious. The fracture

callus shows the presence of a large number of immature chondrocytes, also remodeling of some woven bone to a lamellar bone is noted **(Figure 5).**

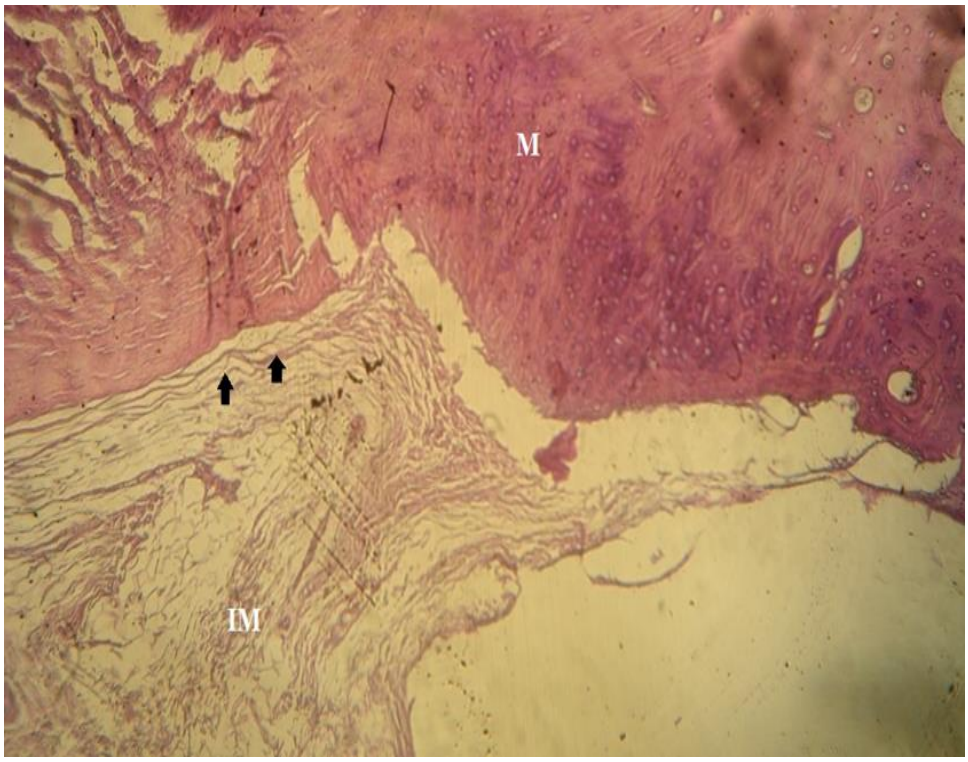

Figure 2: Bone of the control group, the histopathology showed the mature bone (M) in above side of the slide. The immature bone (IM) showed the early stage of soft callus formation (black arrows) H&E stain 100X.

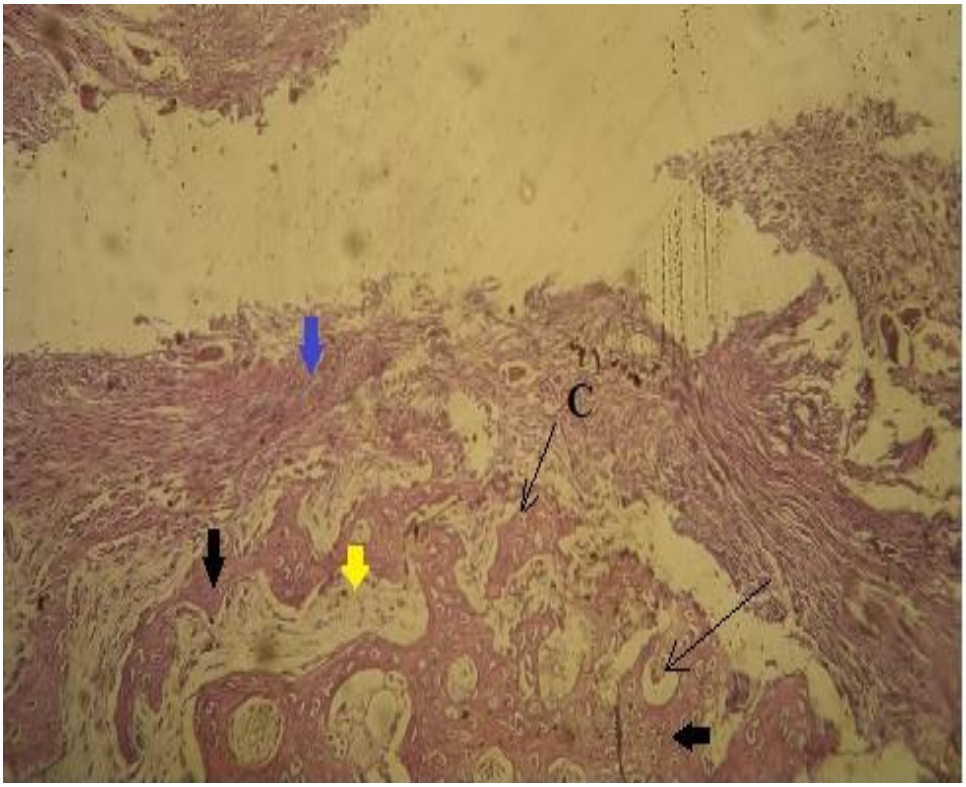

Figure 3: Bone of H202 group, histopathological section of fracture callus shows spicules of newly formed woven bone (black arrows) that has filled the gap between the ends of the fracture and few numbers of hypertrophic chondrocytes (C) trapped in calcified matrix. Some remnant chondrocyte (thin black arrow), as well as presence of fibrous callus (yellow arrow) and fibrosis (blue arrow) H&E stain 100X.

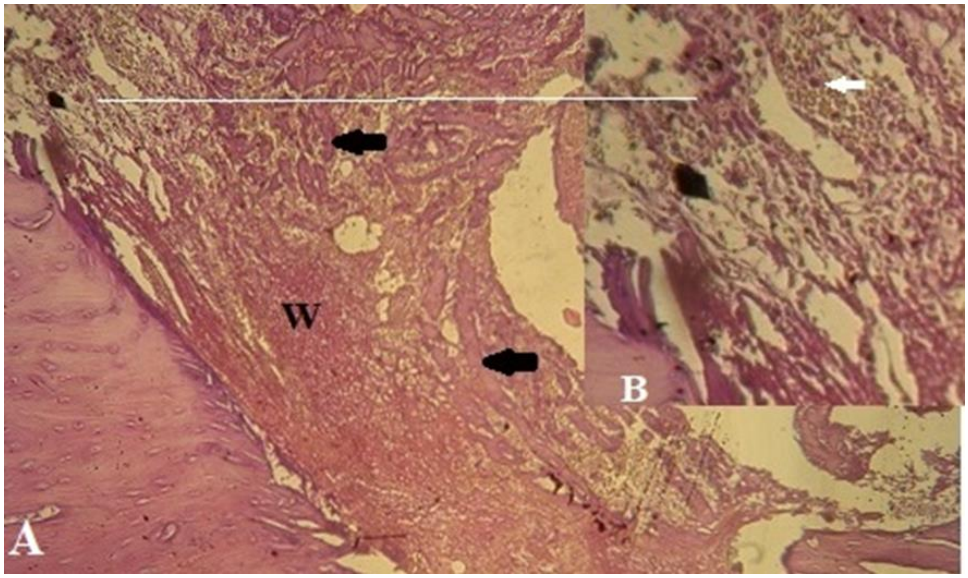

Figure 4: A-Bone of Lipoic acid group showed histopathology of fracture callus showed a nicely the haphazard disposition of osteocytes in the woven bone (W). This mature, lamellar bone, organized layers of osteocytes (black arrows) (and matrix) would be apparent, also, there are also areas of woven bone remodeled to the lamellar bone (L). H& E stains, 100X. B- There is Infiltration of macrophages (white arrow) H& E stains 400 X.

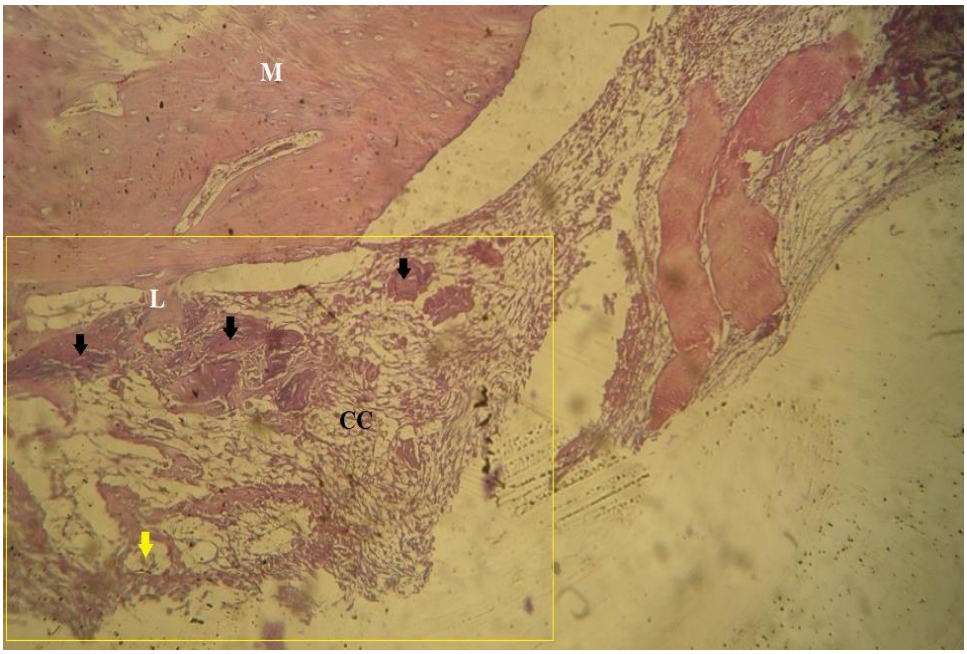

Figure 5: Bone of alpha lipoic acid and H2O2 group showed formation of immature bone (yellow box), where the initial stage of a fracture callus formation and disposition of osteoblast (black arrows) in the woven bone is obvious. The fracture callus shows the presence of a large number of immature chondrocytes (CC), also remodeling of some woven bone to a lamellar bone (L) is noted. The above side of the section showed the mature bone (M). H&E X100.

DISCUSSION

Usually using of animal's models to study fracture consolidation is of great benefits for understanding the bone healing process in human, and to answer questions involved to the most efficient method to treat human beings. In the current study, we use α-lipoic acid and hydrogen peroxide for treatment of iatrogenic gap fracture induced in rabbit femur for investigating the effects of these materials on the fracture healing.

We found oral administration of α-lipoic acid as antioxidant and 2 minutes irrigation of induced femoral bony gap with hydrogen peroxide showed acceleration of bone healing. This healing characterized by formation of immature bone with soft callus at the margins of the gap in one week postoperatively, as compared with control and treated groups. These results are because of anti-inflammatory antioxidant prosperities of α-lipoic acid, which suppress the destructive effects of the oxidant free radicals and enhancing the healing of the fracture, and the biochemical local effect of hydrogen peroxide that influence biological behavior in addition with its topical antiseptic property. Oxidative stress can impair bone healing by modulate the process of osteoblasts differentiation, and ROS can increase osteoblast apoptosis (Mody *et al.*2001).

When the body is expose to more stress, reactive oxygen species production is exaggerated (Gerber *etal.* 2002) making the endogenous enzymatic and the non-enzymatic antioxidant substance unable to control ROS overload causing cells damages (Bhatia *etal*. 2003) and health problem (Steer *etal* 2002). Deficiency of antioxidant complexes in the daily diet may result in developing the degenerative illnesses like neurodegenerative diseases, cardiovascular diseases, cancer, Alzheimer's disease, and various inflammatory illness (Krishnaiah *et al.* 2011; Di Matteo and Esposito, 2003; Gerber *et al*. 2002). There isn't any oxidative stress injury that can occur throughout the ischemic fracture healing period; however, it can be considerable throughout the inflammation and callus formation. Therefore, using of antioxidants could be suggested as part of the therapeutic protocol, which can be important in long bone fracture healing (Sandukji *et al*. 2011 and Turgut *et al*. 1999).

Numerous researches were focused upon the influence of medications and anti-oxidant supplementary substances on fracture healing. In a previous study, it was stated that the oral dose of grape seed proanthocyanidin extract (GSPE), a potent antioxidant, had had a positive impact upon the healing of the bone and enhanced mechanical strength of healing bone that has indicated the fact that the GSPE can be one of the effective therapeutic agents on the health of the bones and the healing of the fractures (Gurger *et al.* 2019).

Other study has been experimented on 40 female rats for the purpose of investigating the effects of the ALA that has been given orally upon osteogenesis-promoting effect of the ALA against the glucocorticoid-induced osteoporosis (GIOP). They found that LA has the ability for the prevention of the GIOP and promoting the osteogenesis through the antagonizing of the oxidative stress and suppressing the apoptosis, and it can be one of the candidates for treating GIOP (lu *et al*. 2017). Orally administering the alpha-lipoic acid can be used as one of the promising targets to improve fracture healing therapies. It found, to promote femoral fracture healing in rat at once daily dose rate 25 mg /kg after 30 day (Aydin *et al.* 2014).

In previous study on mice, it was found that Intra Peritoneal injection of alpha lipoic acid could diminish the formation of osteoclast and bone loss induced by interleukin-1 (IL-1) in parietal bone (Ha *et al.* 2006).

CONCLUSION AND RECOMMENDATION

Our study finding demonstrated using of alpha lipoic acid stimulate fracture healing in its early stage inhibiting oxidative stress effects, and using alpha lipoic acid only showed that the histopathological picture of healing process was improved (a nicely disposition of osteocytes in the woven bone during callus formation ect..) better than other treated groups. However, fracture healing is a complex and sequential event aimed to return bone to its normal function and shape. Therefore, further studies are required for investigating the effects of the ALA during the four stages of fracture healing in mature and immature animal.

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