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Research Article

Comparative Morphological Study of Diclofenac and Heparin with Diclofenac and Warfarin on Fracture Healing

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Abstract: Anticoagulants and non-steroidal anti-inflammatory drugs (NSAIDS) are frequently used concomitantly in the management of bone fractures. The present study was thus designed to evaluate the effect of combine use of these drugs on the histology and histomorphometry of bone tissues in experimental rat model of bone fractures. Animals were randomly divided into 3 groups (A, B and C) of 12 animals and submitted to diaphyseal fracture of right tibia after being anesthetized with chloroform via inhalation under aseptic conditions. Following fracture, animals in group B were administered with diclofenac and heparin, while group C were administered with diclofenac and warfarin. Group A animals served as control. Four animals were selected from each group for radiographic, histologic and histomorphometric analysis on days 7, 14 and 21 days of treatment. Radiographic assessment showed fracture lines are no longer visible at day 21 but deposit of callus is reduced in groups B and C. Histological analysis revealed intact osteocytes within lacunae, empty lacunae, and resorption cavities in all groups and presence of more immature collagen fibres in groups B and C all through the 21 days of treatment when compared to the control. Histomorphometric evaluation showed significantly increased ($p<0.05$) osteocytes count in groups B and C compared to control group, at day 7, 14 and 21. However, group C showed significant decrease ($p<0.05$) in cortical width compared to control and group B, at day 21.The study concludes that the combined use of diclofenac and anticoagulants could affect the quality of fracture healing.
Keywords: Anticoagulants, Non-steroidal anti-inflammatory drugs (NSAIDS), Diclofenac, Heparin, Warfarin, Fracture healing.

INTRODUCTION

The process of healing in a fractured bone depends on several factors related to the patient, fracture site, and treatment [1]. In contrast to healing in other soft tissue, bone fracture healing is a very remarkable process, because rather than leading to scar tissue formation, normal bone healing leads to the regeneration of the anatomy of the bone and complete return to function [2].

Administrations of different pharmacological agents have been known to have an effect on the fracture healing process. Such agents include corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDS), antibiotics and anticoagulants [3]. Among these drugs, NSAIDS and anticoagulants are commonly used in the management of fracture cases. Not only are they prescribed in daily practise, they are frequently administered concomitantly [4].

NSAIDs are often used because of their analgesic effects. They carry out their pharmacologic effect by inhibition of cyclooxygenase. Diclofenac sodium, a commonly used NSAID derived from phenylacetic acid, is indicated for the management of acute and chronic conditions. Anticoagulants on the other hand are commonly used for the prevention and treatment of deep vein thrombosis (DVT). Major orthopedic trauma is a compelling risk factor for the development of DVT. This condition has been observed to occur in 50-70% of patients submitted to acute fixation of proximal femoral fracture, multiple fracture patients, and those presenting with spinal cord trauma when no prophylactic measure is performed. The most commonly used anticoagulants are low molecular weight heparin (LMWH) and warfarin [5].

Studies have reported no difference in quantitative amount of direct or radiographically measured callus formed during NSAIDs use [6]. In another study, Muller *et al*., 2004 reported that diclofenac sodium when given orally affected the mechanical properties of bone, reduced body weight gain and reduced the coefficient of non-fractured bone [1]. A significant delay in fracture healing following administration of enoxaparin was reported by Street *et*

al. (2000). Their study found fewer proliferating cells and fewer transforming pericytes in the medullary cavity at day 7 and 14 and weaker mechanical properties at day 21 compared to the control animals [7]. Hak *et al*. (2006), however reported no deleterious effect of LMWH on fracture healing mechanical properties [8].

Regardless of the frequent use of anticoagulants as prophylaxis for DVT and NSAIDs as analgesic in the management of trauma cases, few studies have shown their combined effect during fracture healing. The present study was thus designed to evaluate the effect of combine use of these drugs on the histology and histomorphometry of bone tissues in experimental rat model of bone fractures.

MATERIALS AND METHOD

Animal management

Thirty six male Wistar rats weighing between 150g to 200g were used. Animals were housed in clean plastic cages and provided with food and water *ad libitum* throughout the experimental period. All animals were handled in accordance with the guidelines for animal research as detailed in the NIH Guidelines for the care and use of laboratory Animals (NIH Publication, 2011) and experimental protocol were approved by local institutional research and ethics committee.

Fracture Procedures

Animals were randomly divided into 3 groups (A, B and C) of 12 animals. All animas were submitted to diaphyseal fracture of right tibia after being anesthetized with chloroform via inhalation under aseptic conditions. Animals were then allowed to move freely without any immobilization [1].

Drug administration

Following fracture, animals in group B were administered with diclofenac and heparin, while group C were administered with diclofenac and warfarin. Group A animals served as control. Diclofenac was administered intramuscularly on alternate thigh muscle at 5mg/kg/day. Heparin was administered subcutaneously at 0.5mg/kg/day and warfarin was administered orally at 0.005mg/kg/day. Drug administration commenced 12 hours following fracture was continued daily for a period of 21 days. Four animals were selected from each group for radiographic, histologic and histomorphometric analysis on days 7, 14 and 21 of treatment.

Radiologic evaluation

Standardized radiographs (Faxitron, Wheeling, IL USA) were performed at the time of sacrifice, using constant settings with the animal anesthetized and positioned prone with both hind limbs fully abducted. Fracture union was evaluated by two, blinded, independent observers. Fracture union was defined as the presence of bridging callus along opposite cortices [8].

Histological and Histomorphometric analysis

Following radiographic evaluation, animals were sacrificed, and right tibia dissected out. Tibia bones were immediately fixed in 10% formal saline for at least 24 hours. Fixed tibia tissues were then subjected to decalcification using 10% EDTA (pH 7.4) for 7 days. Following decalcification, tibia tissues were processed for routine paraffin wax embedding. Sections of 5 um thick were cut and stained using routine Haematoxylin and Eosin (H&E) procedure for general tissue histology and Van Geison staining procedure for collagen fibres.

Stained sections were observed under Leica DM750 digital research microscope. Photomicrographs were taken via attached ICC50 digital camera from 3 non-overlapping areas of stained sections. These were then imported onto Image J software (NIH sponsored public domain image analysis software) for histomorphometric analysis which included osteocytes cell count and cortical width measurement.

Statistical analysis

Data obtained from histomorphometric count and measurement were analysed using One-way ANOVA followed by Students-Newman-Keuls (SNK) tests for multiple comparison. Graph Pad Prsim 5 (GraphPad Inc., USA) software was package use for statistical analysis. Significant difference was set at $p<0.05$.

RESULTS

Radiographic analysis

X-Ray photos of rat tibia after 7days of treatment showed fracture lines that were clearly visible with no sign of callus formation. After 14 days however minimal deposition of callus formation in all groups was observed. Bridging callus was more in control and group B rats as compared with group C and group D. After 21 days of treatment fracture lineswere no longer visible but more deposits of callus was observed in control as compared with group B and C (Fig. 1).

Observe fracture lines with no callus formation (arrows) at Day 7 in all groups. At Day 14, bridging of fracture line with minimal callus formation is observed. At Day 21, fracture lines are no longer visible but more deposit of callus is observed in control compared to treated groups.

Histological analysis

H&E staining showed intact osteocytes within lacunae, empty lacunae, and resorption cavities in all groups (Fig. 2). Van Gieson staining technique differentiates between mature and immature collagen fibres (callus). Mature collagen fibres stains deep red while immature fibres stains pale orange. The colour intensity of the deep red was observed in the control

group through the 21 days of treatment. However groups B and C had more immature collagen fibres all through the 21 days of treatment when compared to the control (Fig. 3).

Fig. 1: Radiographs of control (A), diclofenac plus heparin (B) and diclofenac plus warfarin (C) groups. Observe fracture lines with no callus formation (arrows) at Day 7 in all groups. At Day 14, bridging of fracture line with minimal callus formation is observed. At Day 21, fracture lines are no longer visible but more deposit of callus is observed in control compared to treated groups

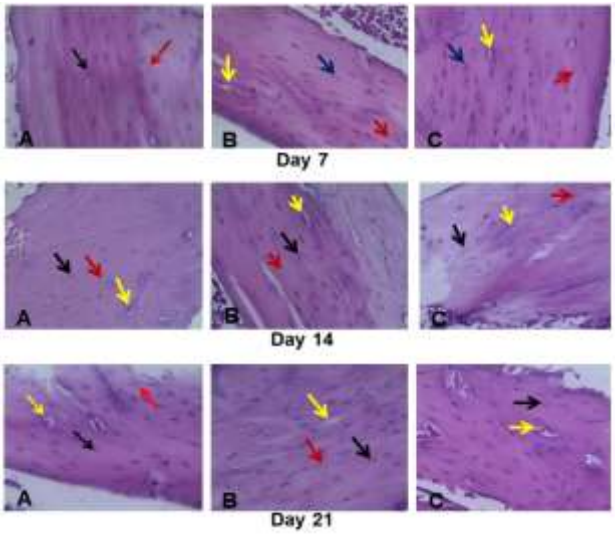

Fig. 2: Micrographs of tibia (H&E X400) of control (A), diclofenac plus heparin (B) and diclofenac plus warfarin (C) groups. Black arrows – intact osteocytes in lacunae; red arrows – empty lacunae; yellow arrows – resorption cavities

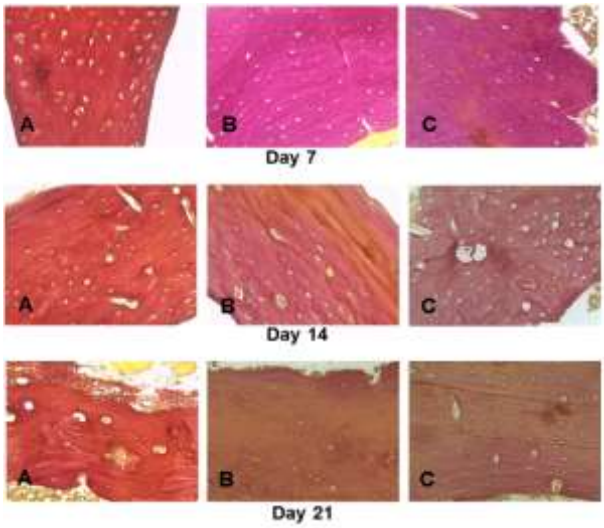

Fig. 3: Micrographs (Van Giesson X400) of control (A), diclofenac plus heparin (B) and diclofenac plus warfarin (C) groups

Histomorphometric measurements

Data analysis shows that administration of diclofenac plus heparin and diclofenac plus warfarin in groups B and C respectively, significantly increased ($p<0.05$) osteocytes count compared to control group, at day 7, 14 and 21. No significant difference was observed in oseteocytes count between groups B and C. In addition, diclofenac plus warfarin group showed significant decrease ($p<0.05$) in cortical width compared to control and diclofenac plus heparin group, at day 21 only (Fig. 4).

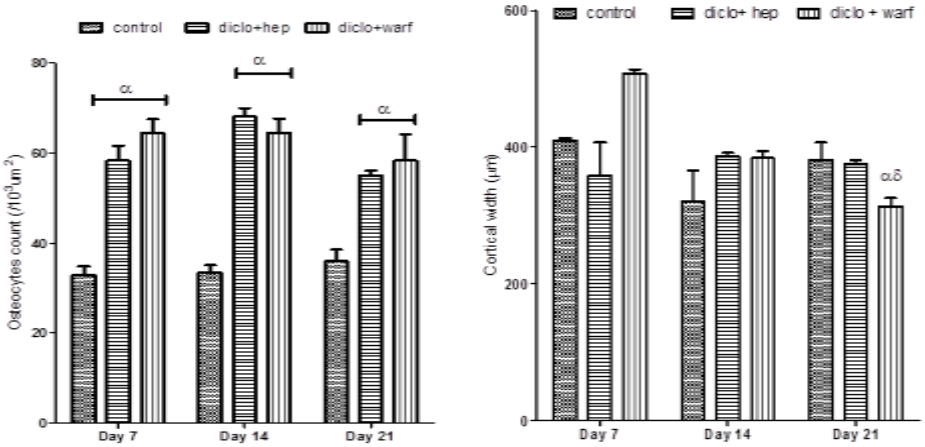

Fig. 4: Osteocytes count and cortical width of rats in control, diclofenac plus heparin and diclofenac plus warfarin groups. *α*, and δ denote p<0.05 compared to control and between group B and C respectively.

DISCUSSION

In the current study, we found that administration of heparin and diclofenac as well as warfarin and diclofenac resulted in increased number of osteocytes count at week 1, 2 and 3when compared with the control. Increased osteocytes number is associated with increase in activity of osteoclast, subsequently increasing bone resorption [9]. This loss of bone substance is thus likely to slow down rate of fracture healing and may account for the increased presence of immature fibres observed in bone tissue of treated animals.

Studies by Avioli *et al*. in 1975 and Matzsch *et al*. in 1990 identified long term use of heparin to be a risk factor for the development of osteoporosis in humans [11, 12]. Their finding was supported by Chowdhury *et al*. in 1992, who showed that low doses of standard heparin directly stimulates bone resorption by increasing the number of differentiated osteoclasts and by enhancing the activity of individual osteoclast [13]. One study by Nishiyama *et al*., (1997) comparing the effects of heparin and LMWH (Dalteparin) after 8 days of injection, observed that rats treated with standard heparin showed a significant reduction in osteoid surface and mineral apposition rates and seven of eight rats suffered spontaneous femoral fracture [14]. When compared with the rats treated with LMWH, they observed minimal decrease in bone indices and no fractures. These finding is supported by this study were we observe decrease cortical thickness in animals treated with diclofenac and anticoagulants when compared with the control. However this decrease was more marked in diclofenac warfarin group than in diclofenac heparin group. Decrease in cortical width has been said to lead to cortical porosity resulting in increased fragility of bone [15, 16]. In this study radiographic evidence showed reduced callus formation in anticoagulants and diclofenac treated animals at the end of the 3rd week. However no reduction in callus formation was observed in control group. This is consistent with studies done by Hak*et al*. (2006) [8].

The use of anticoagulant is associated with surgical site heamatoma formation. The early use of LMWH in patients with fractures may lead to larger fracture site hematoma [7, 10]. It is generally accepted that fracture site hematoma could be beneficial in fracture healing. A study by Grundnes and Reikera (1993) showed that evacuation of this hematoma could be deleterious on fracture healing [10]. However Street *et al*. in 2000 showed that though hematoma could be beneficial, high concentration of potassium in fracture site hematoma is cytotoxic to endothelial cells and osteoblasts [7]. Therefore increased fracture site heamatoma volume may have deleterious effect on fracture healing. Hak *et al*. in 2006 reported the presence of heamatoma formation in short term administered LMWH in animals [8]. In this study, we observed no hematoma formation at fracture site in diclofenac heparin administered animals. However, the presence of hematoma was observed in animals receiving diclofenac and warfarin which persisted for the period of 3 weeks.

CONCLUSION

In conclusion the combined use of diclofenac and anticoagulants could affect the quality of fracture healing, hence the study recommends that concomitant use of diclofenac and anticoagulants should be applied with caution.

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