ABSTRACT

Aim of the study: To evaluate the effect of treatment with diclofenac for 30 days on serum level of alkaline phosphatase, calcium and phosphate, bone density and its relation to healing of tooth extraction socket in rabbits.

Methods: This study was carried out on ten healthy male rabbits weighing between 1.0 – 1.5 Kg. They were divided into 2 groups: Group 1 (control) consisted of 5 rabbits treated with normal saline (0.2 ml) injection, group 2 (treatment) consisted of 5 rabbits treated with diclofenac sodium injection in dose of 30 mg/kg/day for 30 days. The animals' weights were measured at (0, 10, 20) days of treatment. After 30 days of treatment, rabbits of both groups were anesthetized by xylazine hydrochloride and ketamine hydrochloride by I.M injection at dose of 0.5 mg/Kg, 50 mg/Kg respectively. Local anesthesia was injected opposite to the right maxillary incisor, then the tooth was extracted. The surface area of extraction socket wounds was measured at 0, 3, 6, 9 days postoperatively.

All rabbits were sacrificed 10 days after surgery. Venous blood samples (3 ml) were collected from all rabbits via jugular vein during animal sacrificing. Serum Calcium, phosphate and alkaline phosphatase were determined.

Statistical analysis of data was done by SPSS program version 15. Weight of animals was analyzed by ANOVA and Duncan's Multiple Range Test. Non-parametric test was used to compare scores of healing. Mann-Whitney test was used to compare the score of extraction wound healing and T-test was used for analysis of serum Calcium, phosphate, alkaline phosphatase and number of osteocytes.

Results: Scores of extraction wound healing showed that at day 0 there was no significant differences between control and treatment groups with mean ± SD of 7.0 ± 0.0 for both groups (p = 1.00) while at 3, 6, and 9 days of healing period, mean ± SD are 3±0.0, 0±0.02, and 0±0.0 for control group while for treatment group they are 6.75 ± 0.25, 7.25 ± 0.25 and 7.5±0.28 respectively. There were significant differences between 2 groups (p= 0.01, p=0.008, p= 0.01) respectively. In comparison between scores of wound healing within one group
significant differences was found between time 1,2,3 and 4 in control group with mean ± SD of 7.0 ± 0.0, 3±0.0, 0±0.02 and 0±0 respectively, (p=0.002) while a non significant differences was found in treatment group, mean ± SD of 7.0 ± 0.0, 6.75 ± 0.25, 7.25 ±0.25 and 7.5±0.28 respectively (p=0.10).

Statistical analysis for the serum Calcium , phosphate and alkaline phosphatase showed no significant differences between control and treatment groups with mean±SD of 10.85±3.6, 4.80±2.10 and 47.83±14.45 (P-value = 0.826, 0.800, 0.720 respectively). Significant differences between 1st, 2nd and 3rd weight records in control group with mean±SD of 1.28±0.037, 1.33±0.025, 1.40±0.07 respectively (p=0.022), while in treatment group no significant difference was found with mean ±SD of 1.96±0.33, 2.00±0.49, 1.80±0.45 respectively (p=0.792).

Light microscopic examination showed that number of osteocytes was significantly higher in the control group than treatment group with mean±SD of 158.10±59.066 and 96.70±14.667 (p<0.05). The micrographs of decalcified rabbit tibia from control group showed well organized histology. The compact bone of treatment group showed disorganized lamellar architecture of bone matrix which seemed to be more porous with decreased bone marrow cells.

Conclusion: Chronic treatment with diclofenac can delay tooth extraction socket healing without significant effect on serum level of calcium , phosphate and alkaline phosphatase. Copyright © www.acascipub.com, all rights reserved.

Key words : diclofenac ,calcium ,alkaline phosphatas ,tooth extraction

INTRODUCTION

NSAIDs include some of the most frequently taken medications. These agents share a common mechanism of action, they exert qualitatively similar therapeutic and toxic effects. For the treatment of pain and inflammation that accompany various dental surgical procedures, the short-term use of NSAIDs has generally proved to be highly efficacious and safe(1).

Diclofenac is a non steroidal anti inflammatory (NSAIDS) presenting significant analgesic and anti – inflammatory activities (5). There is evidence , however , that NSAIDS may delay the repair of damaged tissues , such as skin , cartilage , fractured bone and new bone formation after tooth extraction(3,4).

Healing of tooth extraction socket is a complex process involving tissue repair and regeneration, it involve chemotaxis of cells into the wound and differentiation of mesenchymal cells to osteoprogenitor cells , proliferation and differentiation of bone forming cells, maturation and remodeling of bone , all these cellular events are controlled and regulated by specific signaling molecules(5,6).

The dentist may be the first to detect diseases and clinical problems affecting the bones. Chemical confirmation is dependent on the patient’s serum levels of calcium , phosphorous , and alkaline phosphatase , those biochemical markers of bone turnover are currently employed in monitoring therapies used to affect bone tissue and they are used today to reflect underlying bone remodeling process that controlled by these signaling molecules. (5,6)

Alkaline phosphatase is one of the most important Biochemical bone markers ,it is a hydrolase enzyme produced by bones in the body,it is responsible for removing phosphate group from many types of molecules by a process of dephosphorylation(9). Elevation of Alkaline phosphatase is readily observed in animals with rapid bone formation(10).

Calcium and phosphate are also very important elements of bone markers , although plasma activity of Alkaline phosphatase is considered to be more useful in distinguishing between normal and abnormal bone formation (11). The level of Calcium and phosphate should be determined whenever there is disturbance in mineral metabolism in bone tissue. Increase in total serum calcium level may be seen in osteolytic bone lesion at which calcium is liberated in the blood stream through the action of bone osteoclasts while serum phosphate level may increase with healing process of bone(10).

In this study the effects of treatment with diclofenac for 30 days on serum level of alkaline phosphatase , calcium and phosphate , bone density with it is relation to healing of tooth extraction socket in rabbit was investigated.

Materials and methods

The study was conducted at the pharmacology Lab. / College of Dentistry / University of Mosul from 20/10/2012 till 1/12/2012.
It was carried out on ten healthy male rabbits from local market weighing between 1.0 – 1.5 Kg. The animals were kept in standard animal housing condition with the room temperature of 25±2°C. They were divided into 2 groups as follows: Group 1 (control group) consist of 5 rabbits treated with daily intramuscular injection of normal saline (0.2 ml), group 2 (treatment group) consist of 5 rabbits treated with daily intramuscular injection of diclofenac sodium (veterinary and agricultural products Mfg. co.; Jordan) in dose of 30 mg /kg/day for 4 weeks. Animals’ weights were evaluated at (0,10,20) days of treatment. After 4 weeks treatment by diclofenac was stopped and rabbits were anesthetized by intramuscular administration of xylazine hydrochloride (Holland, Castenray, interchemra) and ketamine hydrochloride (Aleppo – Syria, ElSaad) at dose of 0.5 , 50 mg/Kg respectively, then few drops of local anesthesia of lidocaine 2% with adrenaline (Saint –Maur-des-Fosses Cedx ,France) was instilled at the site of right maxillary incisor. During the surgical stage of general anesthesia, right maxillary incisor have been extracted by stainless steel dental forceps after disconnection of the surrounding gingival tissue using stainless steel Tweezer, a single dose of oxytetracycline(Norbrook laboratories) in dose 15 mg /kg was given immediately after tooth extraction.

Assessment of extraction wound healing

The surface area of extraction socket wounds were measured by the same operator at 0 ,3 ,6, 9 days postoperatively. They were categorized with respect to surface area that is calculated by measuring the greatest length and the greatest width (side to side) using digital vernier (Digital Caliper/China) then multiply these two measurements (length in cm × width in cm) to obtain an estimate of surface area in cm², the scores of wound surface area were as follow: 0 = 0 , 1 = < 0.3 , 2 = 0.3-0.6 ,3 = 0.7-1.0 , 4 = 1.1-2.0 , 5 = 2.1-3.0 , 6 = 3.1- 4.0 , 7 =4.1-8.0 , 8 =8.1-12.0 ,9 =12.1-24.0 , 10 = >24.0.

A comparison of the scores measured over time and the duration of healing in days provide an indication of the improvement or deterioration in wound healing.

Biochemical assessments

All rabbits were sacrificed 10 days after surgery and Venous blood samples(3 ml) were collected from all rabbits via jugular vein during animal sacrificing, allowed to clot for 30 min at room tempreture, then centrifuged at 3000 R/min for 10 min .Serum samples were separated and stored at -20C till time of analysis.(within 5 days).

Determination of Serum Calcium

The level of serum calcium was determined by Moorehead and Briggs derived CPC (O-Cresol ptalein Complexone) method using kit manufactured by BIOLABO SA ,France In alkaline solution -Cresol ptalein Complexone react with calcium in serum to form a dark red colored complex which absorbance measured at 570 nm by spectrophotometer that is proportional to the amount of calcium in serum.

Determination of Serum alkaline phosphatase

Colorimetric determination of alkaline phosphatase activity was done by using alkaline phosphatase kit manufactured by bioMéreux® sa ,France according to the following reaction:

Phosphate + phenol + phosphate

The liberated phenol is measured in the presence of 4-aminooantipyrine and potassium ferricyanide. The presence of sodium arsenate in the reagent stopped the enzymatic reaction. The color intensity is measured by spectrophotometer at wave length 510nm.

Determination of Serum phosphate

The Phosphate Colorimetric Assay Kit manufactured by BioVision ,USA was used. The assay utilizes a proprietary formulation of malachite green and ammonium molybdate which forms a chromogenic complex with phosphate ion giving an intense absorption band around 650 nm.

Histopathological assessment
Also, right tibial bones from each rabbit were isolated and immersed in 10% formalin for 48 hr harvested and subjected to conventional decalcification by 10% nitric acid embedding in paraffin, longitudinal sectioning (4µm) stained by H&E staining and examined by light microscope (type XSZ-510 pro way).

Histological analysis of new bone formation, histological examination of fibrous tissues, bone trabeculae, blood vessels and bone cells types were assessed. For bone cell number, osteocytes were counted for control and treatment groups. Counting was performed in three randomly selected sites of each section at 40× magnification.

Statistical analysis

SPSS program version 15 was used to analyze the obtained data. Weight of animals was analysed by ANOVA and Duncan's Multiple Range Test. Non-parametric test was used to compare scores of healing. Mann-Whitney test is used to compare the score of extraction wound healing. Statistical analysis for the serum Calcium, phosphate, alkaline phosphatase and number of osteocytes in both groups were done by using T-test.

Results

A comparison of the scores of extraction wound healing between control and treatment groups at 0, 3, 6 and 9 days of healing period showed that at day 0 there was no significant differences between control and treatment groups with mean±SD of 7±0.0 for both groups (p=1.00) while at 3, 6 and 9 days of healing period there were significant differences between 2 groups with mean±SD are 3±0.0, 0±0.02, and 0±0.0 for control group while for treatment group they are 6.75±0.25, 7.25±0.25 and 7.5±0.28, (p=0.01, p=0.008, p=0.01) respectively. Table (1)

In comparison between scores of wound healing within one group, significant differences was found between time 1, 2, 3 and 4 in control group with mean±SD of 7.0±0.0, 3±0.0, 0±0.02 and 0±0.0 respectively, (p=0.002) while a non significant differences was found in treatment group, mean±SD of 7.0±0.0, 6.75±0.25, 7.25±0.25 and 7.5±0.28 respectively (p=0.10). (Figure 1)

Statistical analysis for the serum level of Calcium, phosphate and alkaline phosphatase showed no significant differences between control and treatment groups with mean±SD of 10.85±3.6, 4.80±2.10 and 47.83±14.45 and P-value = 0.826, 0.800, 0.720 respectively (Table 2).

Significant differences between 1st, 2nd and 3rd weight records in control group with mean±SD of 1.28±0.037, 1.33±0.025, 1.40±0.07 respectively (p=0.022) while in treatment group no significant difference was found with mean±SD of 1.96±0.33, 2.00±0.49, 1.80±0.45 respectively (p=0.792). (Figure 2)

Light microscopic examination of the decalcified sections showed that number of osteocytes was significantly higher in the control group than treatment group with mean±SD of 158.10±59.066 and 96.70±14.667 (p<0.05). (Table 3).

The micrographs of decalcified rabbit tibia from control group showed well organized histology. The section of compact bone showed bone matrix in the form of lamellae with collagen fibers, perforated by the lacunae which housed osteocytes in them. (Figure 3)

After 30 days of drug treatment, the compact bone of treatment group showed disorganized lamellar architecture of bone matrix which seemed to be more porous along with decreased bone marrow cells in central marrow cavity together with areas of necrotic bone and osteoclast cells. Osteocyte number seemed to have been lessened after one month treatment with diclofenac sodium. Tibial bone section in treatment group also showed osteoclast, necrotic bone and bone marrow of reduced size. (Figure 4 and 5)

Discussion

This study was designed to investigate the effect of treatment with diclofenac sodium for 30 days on serum levels of calcium, phosphate and alkaline phosphatase and its role in bone formation and healing of tooth extraction socket in rabbits.

In this study rabbit was chosen as an animal model since its one of the most commonly used animals for medical research and also due to the similarities between rabbit and human in the bone mineral density. Diclofenac is a phenylacetic acid derivative that is potent non selective cyclooxygenase inhibitor, the rate limiting enzyme in PG synthesis. PG have been shown to regulate many functions throughout the body including bone healing. Although the exact mechanism for diclofenac induced socket healing inhibition is unknown, the predominant theory suppose that NSAIDs, by inhibiting PG synthesis, interfere with cell signaling during the inflammatory phase leading to an uncoordinated healing response. Endogenous PGE and
PGF increase locally in the first 7 days of fracture suggesting a signaling function by PG in the early healing period\(^{(18)}\). Inhibition of angiogenesis is another suggested mechanism since that PG is stimulator of angiogenesis and it will be inhibited by diclofenac.\(^{(19,20)}\). This study was shown that treatment of rabbit with diclofenac for 4 weeks in a dose of 30 mg/kg/day which was chosen to obtain sufficient concentration of drug over a large part of the day from only single injection\(^{(21)}\) can cause delayed healing of tooth extraction socket as shown by clinical observation and measurements of the socket wound after tooth extraction for this animal. This result was in accordance with other studies stated that short term therapy with diclofenac can cause decreased bone formation during socket healing\(^{(22)}\), the inhibitory effect of diclofenac on tooth extraction wound healing in rabbit has been attributed to probable interference with inflammatory response. Considering that the presence of optimal inflammation is essential for proper wound healing. Soon after extraction the mineralized socket wall is exposed to bacterial colonization while the body attempts to form a fibrin clot. The fibrin clot becomes filled with inflammatory cells programmed to prevent wound infection. Bone is resorbed in the presence of inflammatory cells.\(^{(23)}\). By inhibiting PG synthesis, diclofenac interfere with this inflammatory phase of healing process, leading to an uncoordinated response or could be due to angiogenesis inhibition at site of healing\(^{(18,19,20)}\). NSAIDs, by decreasing the level of PGE2 may bring about a reduction of vascular endothelial growth factor (VEGF) which important growth factor in osseous repair. Callus maturation in vivo was also impaired after long term application of diclofenac which corresponds to effect of NSAID on osteoblast proliferation\(^{(24,25)}\).

The state of skeleton can be evaluated by a variety of techniques including biochemical markers of bone remodeling which provide a non invasive and more rapid responding mean for direct information about bone density\(^{(26)}\). According to the results of this study, a non significant differences were found between control and treatment groups in relation to serum level of calcium, phosphate and alkaline phosphatase, this was in agreement with studies considering that the unfavorable effect of NSAIDs was limited to the initial stages of cellular differentiation without altering the serum level of calcium, phosphate and alkaline phosphatase\(^{(27,28,29)}\). Evans and Butcher performed a study using cells obtained from human trabecular bone at which culture of osteoblasts with indomethacin resulted in a reduction of osteoblast number without significant change in alkaline phosphatase activity\(^{(30)}\).

In this study, delayed wound extraction socket healing without significant change in serum level of bone markers could be related to dose and/or duration of treatment with diclofenac\(^{(31,32)}\). In disagreement with results of this study, Daluiski et al reported that effect of celecoxib, a non steroidal anti-inflammatory drug, on human osteoprogenitor cells showed a decrease of alkaline phosphatase activity with decreased bone formation\(^{(33)}\).

Bone healing process is either direct (primary) or indirect (secondary) process. There are 3 phases of bone healing: inflammatory, reparative and remodeling phase\(^{(18)}\). The histological analysis demonstrated that osteocytes density was significantly reduced in the treatment group and these changes in osteocyte density correlated with changes in osteoblasts and osteoclasts activity. Depletion of osteocytes could be an important factor contributing to bone loss in this model since they may adversely affect intercellular communication between osteoblasts and osteoclasts\(^{(34)}\). Histology of decalcified bone revealed increased amount of cartilage and thinning of trabeculae after diclofenac administration. All these deviations hinted at the decreased rate of bone formation from cartilage after drug treatment. In an agreement with these results, Shalini Chouhan and Sushma Sharma(2012). They concluded that diclofenac treatment over a continuous period of 30 days seems to be a matter of clinical concern being an interfering agent with osseous metabolism that affected osteoblastogenesis, osteoclastogenesis and collagen fibers organization\(^{(35)}\).

The presence of necrotic bone indicated loss of bone strength. Similar results were documented in earlier work having lesser amount of trabeculae in diclofenac treated rat bones undergoing repair as compared to control and calcitonin treated bone\(^{(36)}\). Histological observations of the present study were supported by previous works where diclofenac and tenoxicam resulted in numerous chondrocytes and immature bone formation during distraction osteogenesis study in rabbit tibiae\(^{(37)}\).

Although increasing evidence from animal studies suggests that non steroidal anti-inflammatory drugs suppresses early wound healing in vivo studies involving human subjects have not provided convincing evidence to substantiate this concern\(^{(38)}\).

**Conclusion**

In summary, treatment with of rabbit with diclofenac for 30 days at dose of 30 mg/kg/day can delay tooth extraction socket healing without significant decrease in serum level of calcium, phosphate and alkaline phosphatase, however it is necessary to determine if the analgesic effect of diclofenac is beneficial in treating pain after tooth extraction and other surgical procedure of bone involvement pain, since it is clear that the
function of cyclooxygenase is critical for bone regeneration. So, defining the risk–beneficial ratio of NSAIDs use is very important.

References


[34] Zarrinkalam MR , Atkins GJ .(2012): Changes in osteocyte density correspond with changes in an osteoporotic sheep model . Osteoporos Int ; 23: 1329-1336


**Table (1):** A comparison of the scores of extraction wound healing between control and treatment groups at 4 different times of healing.

<table>
<thead>
<tr>
<th>Days of healing</th>
<th>Control group Mean ±SD</th>
<th>Treatment group Mean ±SD</th>
<th>Mann-Whitney U</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7 ± 0.0</td>
<td>7 ± 0.0</td>
<td>8.000</td>
<td>1.000</td>
</tr>
<tr>
<td>3</td>
<td>3 ± 0.0</td>
<td>6.75 ± 0.25</td>
<td>0.000</td>
<td>0.011*</td>
</tr>
<tr>
<td>6</td>
<td>0 ± 0.2</td>
<td>7.25 ± 0.25</td>
<td>0.000</td>
<td>0.008*</td>
</tr>
<tr>
<td>9</td>
<td>0 ± 0.0</td>
<td>7.5 ± 0.28</td>
<td>0.000</td>
<td>0.013*</td>
</tr>
</tbody>
</table>

* significant at p<0.0

**Table (2):** Mean ±SD of biochemical bone markers for Control and treatment groups.

<table>
<thead>
<tr>
<th>Biochemical markers</th>
<th>Control group Mean ±SD</th>
<th>Treatment group Mean ±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium</td>
<td>10.85±3.6</td>
<td>10.325±2.7</td>
<td>0.826</td>
</tr>
<tr>
<td>phosphate</td>
<td>4.80±2.10</td>
<td>5.175±1.8</td>
<td>0.800</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>47.83±14.45</td>
<td>43.10±20.58</td>
<td>0.720</td>
</tr>
</tbody>
</table>

* significant at p<0.05
**Figure (1):** A comparison of the mean scores of extraction wound healing between 4 different times of healing period for both control and treatment groups.

**Figure (2):** A comparison of the animal weights between control and treatment groups at 0, 3, 6 and 9 days of study period.
Figure (3) : Light micrograph of normal tibial bone in control group showing osteocytes (OS) and blood vessels (BV). H&E. [X-2].

Figure (4) : Light micrograph of tibial bone in treatment group showing osteoclast (OC), necrotic bone (NB) and bone marrow (BM). H&E. [X-2].
Figure (5): Light micrograph of tibial bone in treatment group showing osteoclast (OC), necrotic bone (NB), and bone marrow (BM) with reduced size. H&E. [X-2].