Histpathological Anti-inflammatory Effects of Flunixin Meglumine and Ketoprofen on Excised Rat Tendon

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Abstract

Objective- This research was conducted to study the effect of flunixin meglumine and ketoprofen on the healing of excisional wounding in the tendon of rats.

Design- Experimental study.

Animals- Twenty rats were equally divided into four groups of control, placebo (excised tendon receiving saline solution), flunixin and ketoprofen.

Procedures- Right Achill complex of all groups underwent full thickness tenotomy. All the rats, except control group, received normal saline, flunixin meglumine or ketoprofen, respectively after operation for 7 days. After euthanasia of all animals on the day 15, the Achill complex was dissected free and prepared for histopathologic study. Neovascularization, edema and inflammatory cell infiltration, fibrin layer formation as well as fibroblast and fibrocyte counts were considered for the evaluation of healing process. Neovascularization, edema and inflammatory cell infiltration were scored from 0 to 3.

Results- Results showed no significant change in number of fibroblasts between the groups. Reduced angiogenesis in both treatment groups of non-steroidal anti-inflammatory drugs (NSAIDs) was observed.

Conclusion and Clinical Relevance- Our findings showed that anti inflammatory effects of ketoprofen is slightly more potent than flunixin meglumine, although differences were not statistically significant.

Key Words- Tendon, Rat, Ketoprofen, Flunixin meglumine, Healing.

Introduction

Tendons are fibrous connective tissues that connect skeletal muscles to the bones they move. Furthermore, tendons may be able to withstand tension and prevent trauma to muscle by absorbing the forces transmitted through them.[1] Tendon injuries are frequently encountered in both general and orthopedic clinical practice. These injuries are very important, disabling and typically painful.[2,3] and often require some type of analgesic in their management.[4] Tendon injuries are common conditions for which non-steroidal anti-inflammatory drugs (NSAIDs) are usually used to treat the swelling and pain. NSAIDs decrease fever, pain and prevent inflammation, therefore commonly are used in musculoskeletal trauma and orthopedic surgery to reduce inflammatory response and pain. They have been reported usually to affect bone metabolism, fracture healing and cause ectopic new bone formation in the soft tissues around the hip joint.[5,6] Studies have demonstrated improvement of healing after the usage of NSAIDs.[7,8] without any adverse effects[9,10] in different animal species. NSAIDs have been reported to decrease strength of the healing tendon and reduce diameters of the tendons.[4,11] However, other studies have indicated that negative effects of NSAIDs on tendon healing might be in the early proliferative phase. Through their adverse effects on inflammation during remodeling.[12] they might be useful in this phase.[13] However, a number of studies have reported negative effects of NSAIDs which can delay the consolidation of fractures or the incorporation of the biomaterials because they interfere with the bone remodeling, particularly by their action on the production of prostaglandins.[14,15] In addition, these drugs have been found to impair tendon healing in preclinical studies.[9]

NSAIDs such as ketoprofen and flunixin meglumine inhibit both cyclooxygenase-1 (COX-1) and COX-2 nonselectively and are widely used for the treatment of various inflammatory and noninflammatory conditions such as arthritis, cardiovascular diseases, and the

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management of post surgical and post-traumatic pain in human and animals.[16,17] The objective of this study was to investigate the effect of ketoprofen compared to flunixin meglumine (both are easily accessible, commonly used medicines in our market) on histopathological properties following experimental tendon injury in female rats.

Materials and Methods

Animals

Twenty adult female albino Wistar rats (mean body weight of 209g with a standard deviation of 20g, from animal house of the university) were randomly divided into four groups. Each group was randomly allocated five specimens. One of the groups was treated by flunixin meglumine, the second group was treated by ketoprofen and the third group was placebo, received the same volume of normal saline. Five animals were assigned as control group. The animals had access to fresh tap water and standard laboratory food (balanced rat pellet) *ad libitum*. Animals were kept in a separate polycarbonate laboratory animal cage (floor area of 1800 cm^2) with wire tops in 12 hours light/12 hours dark cycle. The room temperature range was 20-22°C and humidity was between 60 to 70%. Institutional ethical committee guidelines were observed for this experimental study.

Surgical procedure

A cocktail of ketamine 10% (75 mg/kg, alfasan, the Netherlands) and xylazine 2% (5 mg/kg, alfasan, the Netherlands) was administered intraperitoneally before surgery. Following surgical preparation, a 3 mm longitudinal skin incision was made on the lateral side of the right Achilles complex and a 3-mm-long segment of the Achilles tendon was removed from 1.5 mm proximal to the calcaneal insertion. The tendon was left unsutured and the plantaris tendon was kept intact.[9] The skin was closed with two simple interrupted sutures. The limb was not fixed and unprotected weight bearing was allowed in all rats. All procedures were performed by single surgeon at the same place and conditions.

Drug administration

The animals in the flunixin group received flunixin meglumine (Meganix, 5%, Erfan Darou, Iran) 2.5 mg/kg body weight subcutaneously once daily for 7 days; the first dose was injected immediately before surgery. The animals in the ketoprofen group received ketoprofen (Keptofen, 10%, Razak Laboratories, Iran) 5 mg/kg body weight and each animal in the placebo group received 0.1 m*l* of normal saline subcutaneously once daily. The rats of control group received no medication. No prophylactic antibiotic was administered before and after aseptic surgery to avoid probable interaction with healing process.

Histopathology

All animals were euthanized with a thiopental (Thiopental Sodium, Rotexmedica, Germany) overdose intraperitoneally 15 days after surgery. The healing tendons were dissected free and released from the calf muscles. The plantaris tendon was removed. The specimens were fixed in formalin and convey to laboratory. Microscopic slides were made from tendon tissues and stained with Hematoxylin & Eosin and Masson's trichrom. The pathologist was blinded about the research groups.

For histological scoring, each stained section was examined and evaluated semiquantitatively by a modified numbering score according to Ehrlich-Hunt numerical scale.[18,19] The samples were graded on a score of 0 to 3 for neovascularization, edema and inflammatory cell infiltration. The scores were given as (0) No evidence, (1) Slight or occasional evidence (<25% of the fields contained evidence of any finding), (2) Moderate occurrence (<50% of the fields contained evidence of any finding), and (3) Severe occurrence (>50% of the fields contained evidence of any finding). Fibrin layer was scored as being present (1) or absent (0). The total scores of the histological sections were analyzed with Kruskal-Wallis and Mann-Whitney U tests using SPSS-19 software.

Fibroblast and fibrocyte number were counted in all sections using a point counting lens and then volume of each zona (V_{ref}) was estimated with the usage of Cavalieri's formula. Total number of cells was calculated with the following formula:

$$N = \frac{(V_{ref} \cdot \Sigma \bar{Q})}{h \Sigma P}$$

where "ΣQ" is the total of nuclei that were counted in all of the dissectors; "h" is height of the dissector and "ΣP" is sum of frame associated points hitting reference space. In the cavalier's method dissector is part of the section that has been studied.

A minimum of 3100 points were counted. With M42 lens (that has a checker board diagram with 100 cross points) 3100 points were counted (it means 31 microscopic fields with the M42 lens). The minimum number of points to be counted has been estimated with relative standard error (RSE)

$$RSE = \sqrt{\frac{(1 - V_v)}{(n)}}$$

where "n" is number of points to be applied to a particular structure and "V_V" is volume density of the tissue that is Volume$_{ref}$ / percentage of occupied points of the checker board diagram. The data of cell number was analyzed using one way ANOVA and Tukey as post hoc. P<0.05 was considered as significant. In this

study SPSS statistical analysis software -version 19-IBM Inc- was used.

Results

The skin wounds of all rats were healed after 15 days. No wound infection was observed in the animals.

In all groups, mononuclear cells and especially lymphocytes were the only inflammatory cells present in sections (Figure 1 and Figure 2). Histopathologically, wound healing was incomplete in tendons of all cases and remodeling was yet in progress. Collagen fibers were thinner than normal and bundles were not well organized dimensionally (Figure 1 and Figure 3). Some fibers and cells had even whirls pattern. Connective tissue density of cellular and vascular elements was more than a normal tendon (Figure 1 and Figure 4). All the cells and fibers were remodeling from vertical to parallel aspect of the cut surface. There was an intensive nuclear polymorphism in some parts among fibroblasts.
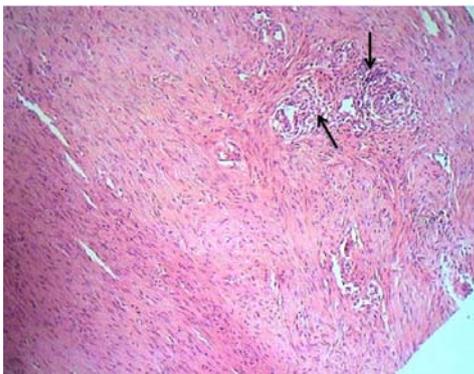
Histopathologic scores of the groups – expressed as mean ± SEM – are available in Table 1.

The number of fibroblasts and fibrocytes among the groups did not show any significant differences (P >0.05).

Non parametric values including neovascularization, edema, inflammation and fibrin were significantly different between all groups (P<0.05). Multiple comparisons (Mann-Whitney U Test) between each group of the study showed that there was no significant difference between Flunixin meglumine and Ketoprofen groups. Comparison of control and flunixin group showed reduction in edema, inflammation and fibrin layer in flunixin group, but they were not significant. Significant reduction in neovascularization was seen in flunixin group compared to control and placebo groups ($P< 0.05$). Ketoprofen group showed reduction in neovascularization and inflammation compared to control and placebo groups ($P< 0.05$). Flunixin and ketoprofen groups had no significant difference between the assessed parameters.

Figure 1- Inflammation with mononuclear cells predomination around vessels (arrows). Collagen fibers have different orientations and cellular population is higher than normal. Cells are composed of fibroblast, fibrocyte, leukocyte and vascular endothelial cells, Flunixin meglumine group. Hematoxylin and Eosin staining; magnification ×200.
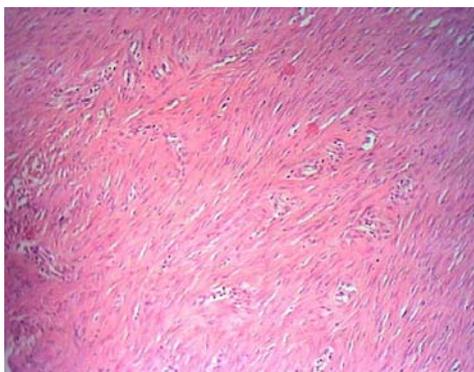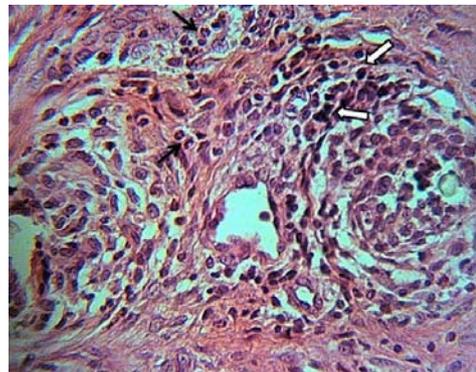

Figure 2- Tendon inflammation in higher magnitude. Although most of infiltrated leukocytes are lymphocyte (white arrows) but some neutrophils are present too(black arrows). Flunixin meglumine treated group. Hematoxylin and Eosin staining; magnification ×800.

Figure 3- Multidimensional arrangement of young collagen fibers in healing tendon. Collagen bundles do not follow a distinct pattern of orientation in this phase. Ketoprofen treated group. Hematoxylin and Eosin staining; magnification ×200.

Figure 4- Neovascularization (black arrows) and innervations (white arrow) in healing tendon. Lots of small vessels are evident around larger vessels and in parenchyma. Ketoprofen treated group. Masson's-Trichrom staining; magnification ×200.

Table 1- Semiquantitative histopathologic (inflammation and repair) evaluation of healed tendons after 15 days from excision. Edema, Inflammation and fibrin presence are indicators of inflammation. Neovascularization and cell count are indicators of repair.

Groups	Neovascularization	Edema	Inflammation	Fibrin	Cell Count $\times10^{8}$
Control	3 ± 0.0	1.2 ± 0.37	2.2 ± 0.37	1 ± 0.0	3.25 ± 0.04
Normal Saline	3 ± 0.0	1.2 ± 0.2	2.2 ± 0.37	0.8 ± 0.2	3.29 ± 0.15
Flunixin Meglumine	2 ± 0.31^{ab}	0.2 ± 0.2^{b}	1 ± 0.31	0.4 ± 0.24	3.23 ± 0.01
Ketoprofen	1.2 ± 0.2^{ab}	0.2 ± 0.2^{b}	0.6 ± 0.24^{ab}	0.2 ± 0.2	3.27 ± 0.03^{a}

Data are expressed as mean ± SEM.
[a] Statistical difference with control ($\alpha=0.05$)
[b] Statistical difference with normal saline group ($\alpha=0.05$)

Discussion

Numerous studies have indicated that NSAIDs might have negative effects on tendon healing. Fibroblasts play a critical role in wound healing by synthesizing the extracellular matrix and collagen. Any change in fibroblast count may alter structural integrity and so physical properties of connective tissues. It has been mentioned that prostaglandins play an important role in proliferation of fibroblasts and wound repair.[20] NSAIDs inhibit prostaglandin synthesis and therefore may interfere with fibroblast cell growth.[21] However, our results showed no significant change in number of fibroblasts and fibrocytes between experimental groups. It's to be noted that we left the rats with no external support after operation. It means that they could bear weight and move freely in their cages. Some research stated that prostaglandin levels increase with repetitive motion.[5] Motion and prostaglandin release can cause increase in DNA synthesis. DNA synthesis is necessary for cell division of fibroblasts and healing of the injurious tendon.[22] We couldn't find any marked negative effect in tendon healing with the usage of mentioned NSAIDs after this period of study (15 days). Angiogenesis and formation of new capillary blood vessels is an essential component of the healing process since it assures delivery of oxygen and nutrients to the healing site.[23] Although some researchers have reported no effect of NSAIDs on neovascularization following full thickness incisional skin wounding in mice, others have shown that NSAIDs inhibit angiogenesis via direct effects on vascular endothelial cells.[24,25] On the other hand traditional NSAIDs may delay wound healing via reducing the synthesis of prostaglandin. They inhibit both COX-1 and COX-2 non-selectively, which are important in the regulation of angiogenesis.[26]

In our study, we observed reduced angiogenesis in both treatment groups of NSAIDs, compared to control and placebo groups, on postoperative day 15. Erpek et al. (2006) studied the effects of flunixin meglumine, diclofenac sodium and metamizole sodium on experimental excisional skin wound healing in rats. They showed that angiogenesis in the granulation tissue of the NSAID-treated groups was less pronounced, compared to controls postwounding.[19] Their findings are consistent with ours.

NSAIDs decrease pain, swelling, stiffness and inflammation. In current study, a reduction in edema was observed in both experimental groups in comparison to placebo. Inflammatory cell infiltration showed a significant decrease in ketoprofen group compared to placebo and control groups. Although we observed a decrease in inflammatory landmarks in flunixin group, this finding was not statistically significant.

Although in this research just pathologic study was done but we think that anti inflammatory effects of ketoprofen may be slightly more potent than flunixin meglumine because ketoprofen decreased most evaluated parameters more than flunixin meglumine, although there was not any statistically significant difference between the two groups (Table 1).

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work.

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اثرات ضدالتهابی فلونیکسین مگلومین و کتوپروفن بر ترمیم تاندون بریده شده رت، مطالعه هیستوپاتولوژیک

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هدف- مطالعه اثرات فلونیکسین مگلومین و کتوپروفن بر التیام تاندون بریده شده رت

طرح- مطالعه تجربی

حیوانات– بیست سر رت ماده بالغ نژاد ویستار

روش‌کار- بیسـت سر رت بالغ به طور تصادفی به چهار گروه کنترل، دارونما، فلونیکسین مگلـومین و کتـوپروفن تقسـیم شـدند. تانـدون آشیل پای راست تمامی رت‌ها تنوتومی شد. تمامی حیوانات، بجز گروه کنترل، بعد از جراحـی تـا هفـت روز بـه ترتیـب سـالین نرمـال، فلونیکسین مگلومین (۲/۵ میلی‌گرم بر کیلوگرم) یا کتوپروفن (۵ میلی‌گرم بر کیلوگرم) دریافت نمودند. پس از یوتانایز نمـودن رت‌هـا در روز ۱۵ بعد از عمل، تاندون‌های التیام یافته جدا شده و برای مطالعات هیستوپاتولوژیک آماده شدند. تراکم رگزایی، ادم و نفوذ سلول‌های التهابی، تشکیل لایه فیبرینی و تعداد فیبروبلاست و فیبروسیت برای ارزیابی پیشرفت روند التیام مورد مطالعه قرار گرفتند.

نتایج- نتایج نشان داد که تغییر معنی‌داری در تعداد فیبروبلاست‌ها میان گروه‌های آزمایش وجود نداشت. کاهش رگزایی در هر دو گروه درمان با ضدالتهاب‌ها مشهود بود. تفاوت پارامترهای اندازه‌گیری شده بین کتوپروفن و فلونیکسین مگلومین معنی‌دار نبود ولی کتوپروفن باعث تغییر بیشتری در پارامترهای اندازه‌گیری شده گردید.

نتیجه‌گیری و کاربرد بالینی- از نتایج این تحقیق می‌توان چنین نتیجه‌گیری کرد که کتوپروفن می‌تواند جهت کنتـرل التهـاب بعـد از جراحی‌های تاندون رت، گزینه مناسب‌تری نسبت به فلونیکسین مگلومین باشد.

کلمات کلیدی- ضدالتهاب غیراستروئیدی، تاندون، رت، کتوپروفن، فلونیکسین مگلومین.