Effect of Enrofloxacin on Histochemistry, Immunohistochemistry and Molecular Changes in Lamb Articular Cartilage

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Abstract- Enrofloxacin is a synthetic chemotherapeutic agent from the class of the fluoroquinolones that is widely used to treat bacterial infections. It is metabolized to ciprofloxacin in the body as active metabolite. Fluoroquinolones change in the articular cartilage, especially with high doses and more than two weeks use. So, due to relatively excessive use of enrofloxacin in mammals and similarity of lambs to human subjects with respect to skeletal activity cycles, this study was done to investigate the effects of enrofloxacin on some cellular and molecular changes in growing lamb articular cartilage to evaluate some possible mechanisms involved these changes. Twelve, 2-month-old male lambs divided into three groups: control group received only normal saline; therapeutic group received 5mg/kg enrofloxacin subcutaneously, daily, for 15 days and toxic group received 35 mg/kg enrofloxacin in the same manner as therapeutic group. Twenty four hours after the last dose, the animals were sacrificed, and their stifle joints were dissected. Sampling from distal femoral and proximal tibial extremities was done quickly for further histological and molecular studies. Collagen-п content was studied with avidin-biotin immunohistochemistry method in different groups. Expression of Sox9 and caspase-3 was evaluated by Real–time PCR. Immunohistochemical changes were included decreases of matrix proteoglycans, carbohydrates, and Collagen-п in the toxic group. Some of these changes were observed in the therapeutic group with less intensity in comparison to the toxic group. Enrofloxacin were significantly decreased ($P\leq0.05$). Sox9 expression in therapeutic and toxic groups compared to control group. But caspase -3 expressions in the toxic group significantly increased ($P\leq0.0001$) with a comparison to other groups, while, between control and therapeutic groups, there were no significant differences. So, it can be concluded that enrofloxacin increases apoptosis in chondrocytes and decreases their numbers. Enrofloxacin use in growing lambs even at recommended therapeutic dose is not completely safe on articular cartilage. Moreover, higher doses of enrofloxacin induce severe changes in lamb articular cartilage.
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Introduction

Fluoroquinolone antibiotics are commonly used to treat a variety of infections, including urinary tract, respiratory tract, gastrointestinal tract, skin, bone and joint infections (1). Enrofloxacin is one of fluoroquinolones, characterized by a fairly good safety and tolerability profile, good bioavailability and a strong antibacterial effect with low minimal inhibitory concentrations (2) that is metabolized to ciprofloxacin in body as active metabolite (3). The use of quinolones is restricted because their toxic effects on articular cartilage (4). Initial papers describing quinolone-induced chondrotoxicity in growing animals were published 30 years ago (5). Then arthropathy induced by the quinolones was described in juvenile animals of multiple species such as dogs (6,7), rats (8), rabbit (9), guinea Pigs (10) and chicken (4). Quinolones are contraindicated in Children and adolescents in growth phase because they may damage weight-bearing joints

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(5). Also in veterinary Medicine in immature dogs, particularly those of large breeds (2) and in juvenile horses and in neonates (11), the use of quinolones is restricted. Histological changes usually are detectable such as chondrocytes loss, matrix degeneration, Cavitation of articular Cartilage (6,7) and loss of proteoglycans (5). Several studies suggest that quinolones alter proteoglycans content and thereby may alter collagen fibrillogenesis (4,7,12). Sox9 is a transcription factor that is essential for chondrocyte differentiation and cartilage formation. It also regulates the synthesis of some matrix components after birth (13). Apoptosis possibly plays a role in fluoroquinolone – induced arthropathy (5). It was suggested that apoptosis has to be considered as a final event in the pathogenesis of fluoroquinolone induced tendinopathy (2). A group of cysteine proteases denoted caspases, play a central role in apoptosis. Caspase-3 is one of the most important caspases (14). There are few published reports about the influence of quinolones on sheep articular cartilage (15), But mechanisms of articular cartilage damage due to consumption of enrofloxacin and its pathophysiology especially cellular and molecular aspects are not been made clear. So lambs were chosen for this study due to their similarity to human subjects with respect to skeletal development kinetics, biomechanical loading and activity cycles (15) to investigate the effects of enrofloxacin on histochemistry, immunohistochemistry and molecular changes in growing lamb articular cartilage and evaluate some possible mechanisms involved these changes.

Materials and Methods

Twelve, 2-month-old male clinically healthy lambs were studied. The lambs purchased a farm, were placed in the animal house of Veterinary Medicine Hospital of Shahid Chamran University, one week before administration of the medication. All animals experienced routine animal husbandry. Lambs were randomly divided into 3 treatment groups of 4 animals each. Group 1 (control group) received distilled water in equal quantities of the most enrofloxacin volume. Group 2 (therapeutic group) received 5 mg/kg of enrofloxacin (EnRo-10%; T products LTD, Liverpool, U.K.). Group 3 (toxic group) received 35 mg/kg of enrofloxacin. The drug was subcutaneously administrated daily for 15 days. All lambs treated daily as described for 15 days. All doses were given between 1 pm and 3 pm. Twenty four hours after the last dose, all lambs were slaughtered ritually.

Immediately after ritually slaughtering, the stifle joint were dissected, and articular cartilage of the distal femoral and proximal tibial extremities were investigated. Then articular cartilage samples including subchondral bone were collected and fixed in 10% buffered formalin (Merck, Germany) for 4 weeks. Twenty four hours after primary fixation, the fixative was changed. The tissues were decalcified in 5% formic acid (Merck, Germany) for 2 weeks, and the decalcification fluid was changed every day regularly. After decalcification, washing in water for 24 hours was performed. The tissue blocks were dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin. 5-μm-thick sections were used for conventional histology. Toluidine blue stain was used to demonstrate proteoglycans (16). The content of proteoglycans were evaluated by the scoring system used by Maslanka *et al.*; Uptake of toluidine blue scored from 0 to 5. Normal stain uptake got point 0, lack uptake in the matrix surrounding cartilage canal 1, slight reduction 2, moderate reduction 3, severe reduction 4 and no dye got point 5 (4). For immunohistochemistry, the deparaffinized and hydrated sections were incubated in methanol containing 0.3% H2O2 for 15 min at room temperature to blocking endogenous peroxidase activity. The sections were then treated with citrate buffer (*p*H=6) for 15 min at 98 ºC as antigen retrieval and then incubated overnight at 4 ºC with primary antibody (Anti-collagen type-Π antibody, Millipore, USA) diluted 1:100 in phosphate buffered saline (PBS, *p*H=7.4). After twice washing with PBS, the sections were incubated with secondary antibody biotinylated anti-mouse IgG at 1:100 for 30 min that existed in the ABC staining system (Santa Cruz Biothechnology Inc., USA). Then the specimens were incubated peroxidase-conjugated avidin-biotin for 30 min at room temperature. After washing, the sections incubated with diaminobenzidine (DAB substrate) as chromogen and counterstained with haematoxylin. Three immunohistochemical sections from each animal were blindly assessed by 3 workers, and staining intensity was estimated using a semiquantitative method based on the study was described by Hollaner *et al.*, (17). No staining was recorded as zero. Traces of staining mainly in pericellular sites was graded as 0.5. Definitive staining restricted to the superficial layer and upper mid zone was recorded as 1. Diffuse staining of the superficial and mid zones with pericellular staining of the upper deep zone was graded as 2. Similar staining to 2 that extended throughout the depth of deep zone was designated 3. Staining of most of the extracellular matrix throughout

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the depth of the cartilage, with the loss of staining in the superficial zone associated with fibrillation, was recorded as 4. Photographs were taken using Dinocapture 2.0 (Dino-Lite and Dino-Eye, AnMo Electronics Corporation, Taiwan).

RNA was isolated using combined method (18). In this method, the RNeasy mini Kit (QIAGEN, Germany) was used. To avoid contamination with genomic DNA, RNA samples were treated with RNase-free DNase I (QIAGEN). NanoDrop was used to measure the amount of RNA. RNA purity was verified in agarose gel. Primer sequences and specifications are given in (Table 1).

Table 1. Primer sequences and specifications

Gene	Sequences	TM °C	Length	References
Sheep GAPDH F	5'-TGTCCGTTGTGGATCTGACC-3'	53.87	20	Shiwang *et al*., 2004
Sheep GAPDH R	5'-CGTACCAGGAAATGAGCTTGAC-3'	53.57	22	Shiwang *et al*., 2004
Sheep Sox_9 F	5'-ATGTAGTGTATCACTGAGTC-3'	48.9	20	AbouMerhi *et al*., 2001
Sheep Sox_9 R	5'-AGATTAAGGTCTGTCAGTGG-3'	51.3	20	AbouMerhi *et al*., 2001
Sheep Cas-3 F	5'-AGAAGTCTGACTGGAAAACC-3'	56.5	20	Green wood *et al*., 2009
Sheep Cas_{-3} R	5'-GTCTCAATACCACAGTCCAG-3'	56.8	20	Green wood *et al*., 2009

For cDNA synthesis, 1 microliter of RNA template (2 ng to 2 µg) and 1 µL of random nonomer primer mix (QIAGEN) were added to 0.2 mL Eppendorf tubes in order to conduct cDNA synthesis. The tubes were then kept in the thermal cycler for 5 min at 65 °C. A master-mix (total volume of 10 µL) was prepared for cDNA synthesis. Next, this master-mix was added to each Eppendorf tube. cDNA was obtained by keeping these tubes in the thermal cycler at 42°C for 60 min and at 70°C for 5 min.

The lyophilized form primer was diluted with RNAse/DNAse-free water in according the protocol. Ten microliters of qPCR SYBER Green master-mix, 3 µL forward and 3 µL reverse primers (especially for each gene), 3 µL RNAse/DNAse-free water and 1 µL of cDNA were added into the Eppendorf tube to prepare RT-PCR mix. The RT-PCR mix was then kept in the thermal cycler at 95°C for 2 min in order to achieve enzyme activation. Afterwards, the mix was subjected to reaction at 95°C for 15 sec for denaturation, at 57 °C for 1 min annealing and at 60°C for 30 sec for extension (50 cycles). The obtained data was analyzed using the comparative threshold cycle (Ct), in which the formula 2 - ΔCT was used as ΔCT = CT of target gene - CT of housekeeping gene (normalization) and ΔΔCT = ΔCT of samples - ΔCT of the calibrator (control).

Statistical analysis was performed using SPSS version 16. All date was presented as mean±standard error. One-way ANOVA and the LSD test were used to compare the groups. $P \leq 0.05$ was considered to be statistically significant.

Results

Histochemical findings showed that amount of matrix proteoglycans (uptake of toluidine blue stain) was significantly decreased by enrofloxacin particularly with the toxic dose (Figures 1A-1C). This difference was illustrated in (Table 2). Also, multiple small cavities were seen in the surface layer and upper part of mid layer in the toxic group (Figure 1C).

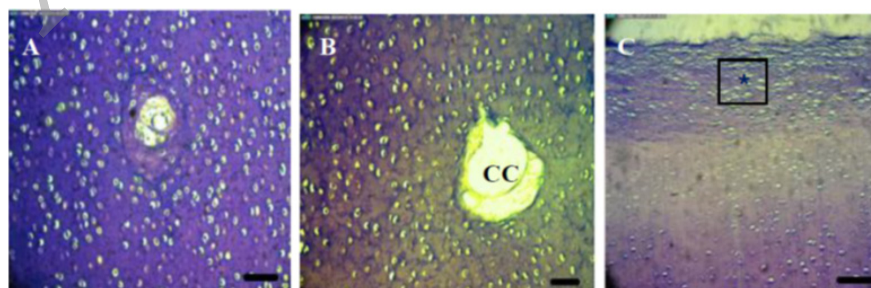

Figure 1. Uptake of toluidine blue by the matrix of the lamb articular cartilage. A: Control group; a regular uptake of toluidine blue by the matrix. B: Therapeutic group; the lack of uptake of toluidine blue by the matrix especially by the matrix surrounding cartilage canal, CC: cartilage canal. C: Toxic group; severe reduction of uptake of toluidine blue by the matrix especially in the middle layer that indicate severe decrease in matrix proteoglycans content. *: Many small cavitations were observed in the superficial and middle layers of the matrix. Toluidine blue staining (bar: 50 µm).

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Table 2. The mean score ± Standard Error of a decrease in toluidine blue stain uptake.

Group	score
Control	$0.3^{*} \pm 0.06$
Therapeutic	$2.1^{*} \pm 0.25$
Toxic	$3.85^{*} \pm 0.21$

*: $P \leq 0.0001$

In immunohistochemistry study, no staining was seen in the negative control samples when only secondary antibody was administered (Figure 2A). Collagen-Π quantity was examined using articular cartilage samples of the control, therapeutic and toxic groups (Figures 2B-2D). Collagen-Π quantity in the articular cartilage was decreased significantly in the therapeutic ($P\leq0.006$) and toxic ($P\leq0.0001$) groups when compared to the control group, while the therapeutic group had no significant difference with the toxic group (Figure 3).

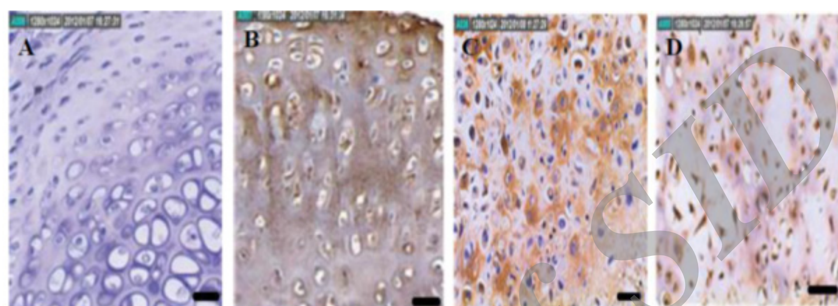

Figure 2. A: Immunoreactivity in the negative control group (only secondary antibody applied to articular cartilage section), B: Immunoreactivity of Collagen-Π in the control group, C: Immunoreactivity of Collagen-Π in the therapeutic group, D: Immunoreactivity of Collagen-Π in the toxic group (bar: 50 μm).

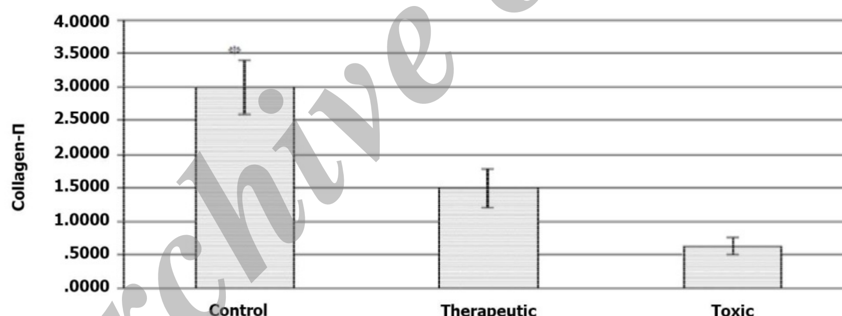

Figure 3. A comparison of Collagen-Π quantity between groups (*: $P \leq 0.006$)

The gene expression levels of sox9 and caspase-3 in the articular cartilage of the examined animals were analyzed using the RT-PCR technique; they were normalized with GAPDH as the reference gene. The achieved data using the formula 2 -$\Delta\Delta C_T$ showed that sox9 expression significantly decreased in the toxic group when compared with the control and the therapeutic groups ($P \leq 0.0001$). Sox9 expression in the therapeutic group had also significant decrease in comparison to the control group ($P\leq0.05$; Figure 4). Caspase-3 expression in the toxic group significantly increased when compared with the control and the therapeutic groups ($P \leq 0.0001$), While the therapeutic group had no significant difference with the control group (Figure 5).

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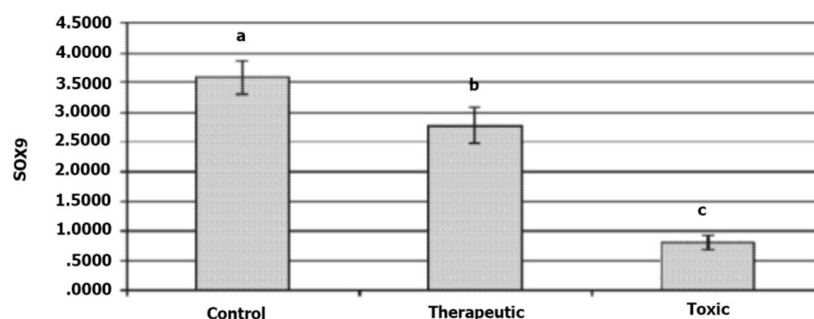

Figure 4. A comparison of sox_9 expression between groups
(a,b,c: significant decrease between groups, $P \leq 0.05$)

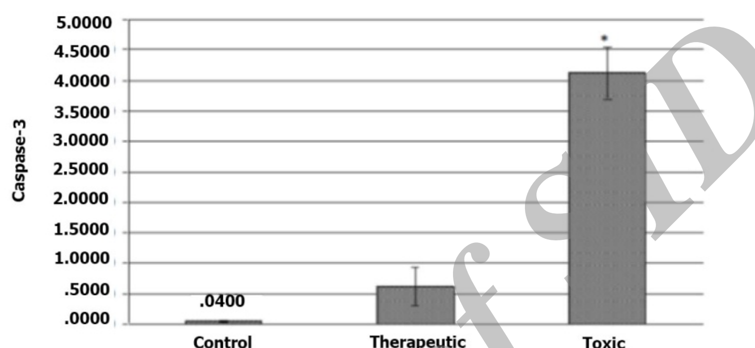

Figure 5. A comparison of Caspase-3 expression between groups
(*:$P \leq 0.0001$)

Discussion

The changes in the histochemical structure of the articular cartilage tissue, the immunohistochemical localization of Collagen -Π, and the gene expression of sox9 and caspase-3 (measured using real-time PCR) of lambs receiving enrofloxacin were analyzed in this study. Enrofloxacin is the most usable agent of fluoroquinolones in veterinary medicine especially in the ruminants that are metabolized to ciprofloxacin in the body as active metabolite (3). However use of this drug especially in young animals had been questionable because of quinolones chondrotoxic effects that have been reported since 30 years ago in some species such as dog (6,7,9,11,19), horse (11) and chicken (4). But in sheep, the only available study involving exposure of lambs to quinolones were done by Sanson *et al*.; They administered ciprofloxacin and gatifloxacin to experimental lambs over a fourteen day interval at a dose designed to reflect those used in pediatric medicine. They concluded that fluoroquinolones did not affect growth velocity in the ovine model. They also refused to suggest chondrotoxicity of quinolones on the lamb articular cartilage (15). But data derived from this study are not very useful to compare with our results because they administered fluoroquinolones with pediatric medicine doses and did not examine articular cartilage by routine histopathological methods. Toluidine blue selectively stain proteoglycans and intensity of the toluidine blue stain uptake is determined by loss of proteoglycans (4,20). Thus, uptake reduction of this dye indicated a decrease of matrix proteoglycans content due to abnormal processes. In the present study were detected moderate to severe reduction of toluidine blue especially in the middle layer of the toxic group. These symptoms indicated a severe decrease in matrix proteoglycans content in the toxic group with a comparison to the mild reduction in the therapeutic group. In the therapeutic group, that loss of matrix proteoglycans was more limited than the toxic group. This change was more detected in the matrix surrounding cartilage channels. This finding was same as results of Melaka *et al*., (4) and may shows the initial site of proteoglycans loss.

Collagen -Π is the most fibrillar protein in the articular cartilage that prepares firmness for it (21). In the present study, collagen-Π quantity in the articular cartilage of lambs was analyzed by

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immunohistochemistry. Our findings showed significantly decrease in the therapeutic and toxic groups when compared to the control group While the therapeutic group had no significant difference with the toxic group. Other Immunohistochemical studies indicated that using quinolones increased fibronectin and decreased collagen-Π and integrins (8,20). Sox9 regulates the synthesis of some cartilage matrix components after birth (22), and it is essential for the formation of hypertrophic chondrocytes (23). In this study, Sox9 expression significantly decreased in the toxic group when compared with the control and therapeutic groups. Sox9 expression in the therapeutic group had also significant decrease in comparison to the control group. In spite of our researches, we did not find any study about quinolones effect on Sox9 expression, and it seemed we did it for the first time. So, a decrease of sox9 expression could decrease synthesis of important molecules such as collagen-Π and proteoglycans in the articular cartilage matrix.

Empty lacuna was seen especially in the middle layer of articular cartilage in lambs received enrofloxacin. The increase of empty lacuna numbers in the cartilage could be signed of increased apoptosis (24). Maslanka *et al*., distinguished apoptotic chondrocytes in chicken articular cartilage after enrofloxacin therapy (4). Lim *et al.*, demonstrated that canine tendon cells and chondrocytes treated with 200 µg/ml enrofloxacin for 4 days, exhibited apoptotic features and fragmentation of DNA (2). Apoptosis possibly plays a role in fluoroquinolone – induced arthropathy (4). Therefore, in this study caspase-3 expression compared in treated groups. The significantly increase was observed in the toxic group when compared with the control and the therapeutic groups. So, enrofloxacin increased apoptosis in lamb articular cartilage. Sendzik *et al*., indicated an up to 15 folds increase of caspase-3 protein in tenocytes following exposure to ciprofloxacin or levofloxacin. They concluded that these changes resulted in a pronounced increase of apoptosis (14). On the other hands, an increase of apoptosis in the articular cartilage could lead to a decrease of chondrocytes and subsequently decrease of matrix macromolecules synthesis (25).

There are a lot of hypotheses to explain mechanisms of the quinolone-induced chondropathy. More acceptable of these, is a lack of magnesium ion due to chelating with the quinolones (4,26,27). Lack of extracellular magnesium impairs the function of integrins that regulate matrix protein synthesis such as proteoglycans. Loss of matrix proteoglycans would seem to be caused by decreased synthesis of these compounds (20). While Maslanka *et al*., suggested enrofloxacin increased proteoglycans degradation (4). In some studies, an increase of matrix metalloproteinases (MMPs) was showed after fluoroquinolone administration in a tendon (14,28). In attention to similarities of tendon and cartilage (4), MMPs especially MMP-3 and MMP-13 were known probably responsible for cartilaginous extracellular matrix degradation (29,30). However nowadays, pronounced degradation of extracellular matrix is known as a consequence of the disturbed interaction between matrix and cells and is increased by the pronounced increase of apoptosis (5).

In the present study, chondrotoxic effects of enrofloxacin were seen more in the toxic group than the therapeutic group. In the available literature, there are several reports describing the influence of various multiple doses of quinolones, more or less exceeding therapeutically applied doses, on articular cartilage (9,21,31). These studies showed very distinctly a dose dependent incidence of quinolone-induced arthropathy. Thus, the data indicate that degree of severity of fluoroquinolones chondrotoxity depends on volume and number of doses. So, higher doses with a longer period of use, induce more severe effects.

In conclusion, results of this study indicate although the therapeutic dose of enrofloxacin contrary to toxic dose has no severe effects on lamb articular cartilage, but it is not completely safe. However, future studies with different dose and duration are necessary for lamb and other species.

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