

Enrofloxacin Effect on Histomorphologic and Histomorphometric Structure of 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 in animals. Fluoroquinolones cause some lesions in articular cartilage of growing mammals so with due attention to clinical importance of enrofloxacin, comprehensive study for cellular and molecular damaging events in articular cartilage due to consumption of this drug has been designed and in this paper, histological part has been presented. Thus the aim of the present study was investigation of enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. Twelve, 2-month-old male lambs were divided 3 groups; control group, therapeutic group: received 5mg/kg enrofloxacin subcutaneously for 15 days and toxic group received 35 mg/kg enrofloxacin subcutaneously for 15 days. Control group received distilled water subcutaneously. Twenty four hours after the last dose the animals were euthanized and stifle joint was dissected. Distal femoral and proximal tibial extremities were grossly examined then routine histological sections were prepared and evaluated by light microscope. Macroscopic changes as erosion and flap were seen on distal femoral and proximal tibial articular cartilages only in toxic group. Histological changes included decrease of matrix proteoglycans, total cartilage thickness and chondrocyte numbers. Also presence of spindle-shaped cells, fissure in articular cartilage matrix and increase of empty lacuna were observed in toxic group while chondrocyte numbers significantly decreased and empty lacuna increased in therapeutic group with comparison to control group. In conclusion, results of this study indicate that the use of enrofloxacin in growing lambs at recommended (therapeutic) dosage is not completely safe on articular cartilage. However higher doses of enrofloxacin induce sever changes in lamb articular cartilage.

Key words: Enrofloxacin • Articular cartilage • Lamb

INTRODUCTION

Fluroquinolones are antimicrobials used extensively in human and veterinary medicine [1, 2]. Enrofloxacin is a useful fluoroquinolone agent in veterinary medicine [3] because of its rapid, broad – spectrum bacterial activity at therapeutic concentrations, infrequency of bacterial resistance and post antibiotic effect *in vitro* [4]. The use

of quinolones is restricted because their toxic effects on articular cartilage [5]. Initial papers describing quinolone induced chondrotoxicity in growing animals were published 30 years ago [6]. Then arthropathy induced by the quinolones was described in juvenile animals of multiple species such as dogs [7-11], rats [8, 12-14], rabbit [8], guinea Pigs [15] and chicken [5]. Quinolones are contraindicated in Children and adolescents in growth

phase because they may damage weight-bearing joints [6]. Also in veterinary Medicine in immature dogs, particularly those of large breeds [16] and in juvenile horses and in neonates [2], the use of quinolones is restricted.

Chondrotoxic effects of quinolones in animals cause gait abnormalities but these are reversible [6]. Histological changes usually are detectable such as chondrocytes loss, matrix degeneration, Cavitation of articular Cartilage [7, 9] and loss of proteoglycans [6]. Effect of enrofloxacin on chicken articular cartilage was studied by Maslanka *et al.*; they found histologic changes included spindle shaped chondrocyte and loss of matrix proteoglycans. They concluded that quinolone-induced arthropathy is considerably less expressed in birds than in mammals [5].

There are few published reports about the influence of quinolones on sheep articular cartilage [17]. 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, we decided to study detail of this process at one part of this study is reported in present paper. Thus the aim of this experimental study was to determine effect of enrofloxacin on histomorphologic and histomorphometric structure of lamb articular cartilage.

MATERIAL AND METHODS

Twelve, 2-month-old male clinically healthy Arabian lambs were studied. The lambs purchased at 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% inj.; special (T products LTD, Liverpool, U.K.). Group3 (toxic group) received 35 mg/kg of enrofloxacin. Drug was subcutaneously administrated daily for 15 days. All lambs treated daily as described for 15 day. All doses were given between 1pm and 3 pm. The behaviors of all animals particularly in view of gaiting and lameness were monitored during treatment. Twenty four hours after the last dose, all lambs were euthanatized by intravenous injection of saturated potassium chloride solution. Immediately after euthanasia, the stifle joint were dissected and articular cartilage of the distal femoral and

proximal tibial extremities were examined. 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 dehydrate in graded series of ethanol, cleared in xylene and embedded in paraffin. Sections were cut at a thickness of 5µm and stained with hematoxylin and eosin (H&E) for studying morphologic and morphometric features. Toluidine blue stain was used to demonstrate proteoglycans [18]. Photographs were taken using Dinocapture 2.0 (Dino-Lite and Dino-Eye, AnMo Electronics Corporation, Taiwan). Morphometric study was performed with a scaled lens for Olympus light microscope (Tokyo, Japan). Articular cartilage thickness and its layers, chondrocyte and empty lacuna numbers and matrix/cell ratio were measured. For doing these, 3 sections of any animal and in each section, 5 microscopic fields were blindly assessed. The criterions of evaluation such as content of proteoglycans (as demonstrated by uptake of toluidine blue stain) were taken from 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 [5].

Statistical Analysis: The data were analyzed using SPSS Version 17.0 and one way ANOVA. Differences between mean values considered significant at $P < 0.05$.

RESULTS

In the present study, administration of enrofloxacin to lambs with the therapeutic and toxic doses had no clinical symptoms as same as gait abnormalities or lameness.

In macroscopic examination of articular cartilage in the control group, normal structure without any lesion was seen (Figure 1-A). Also in the therapeutic group, it was not found any macroscopic changes with comparison to the control group. But in the toxic group, some lesions such as erosion, flap and excavation were seen on distal femoral (Figure 1-B) and proximal tibial (Figure 1-C) articular cartilages.

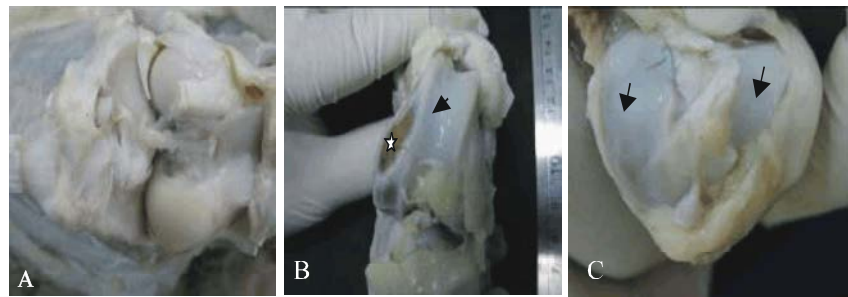


Fig. 1: Macroscopic structure of lamb articular cartilages of the stifle joint. A: control group, B: therapeutic group, C and D: toxic group. Erosion, flap and excavation were seen on proximal tibial and distal femoral articular cartilages (arrows) in the toxic group. () : Articular cartilage with sub chondral bone were collected for histologic sections.

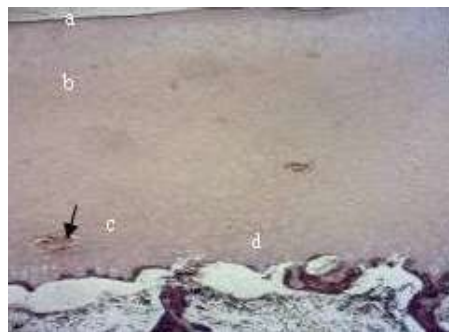


Fig. 2: Histologic structure of normal lamb articular cartilage. a: superficial layer, b: middle layer, c: deep layer, d: calcified layer and e: sub chondral bone. Arrow shows cartilage canal. H&E staining, x4.

In microscopic view of articular cartilage, 4 layers or zones were observed (Figure 2). The uppermost zone was superficial tangential layer. Below that, middle layer with oval shape of chondrocytes was obvious. Under the middle zone, deep layer with hondrocyte columns was observed and finally calcified layer found above the subchondral bone.

The total thickness of articular cartilage was significantly decreased in the toxic group when compared with the control and therapeutic groups ($p < 0.05$). These differences was seen for middle layer (Table 1). While therapeutic group had no significant difference with the control group. The number of empty lacuna was significantly increased in the toxic group

Table 1: Mean $\pm$ SD of chondrocyte numbers, thickness of layers, numbers of empty lacuna and matrix/cell ratio in the articular cartilage. The different letters (a: control, b: therapeutic and c: toxic) Show significant difference between groups ($p < 0.05$, $n = 4$).

		Groups		
Parameters	Articular cartilage layers	Control (a)	Therapeutic (b)	Toxic (c)
chondrocyte numbers	superficial	4.46 $\pm$ 0.19 b,c	3.37 $\pm$ 0.17 a,c	2.73 $\pm$ 0.17 a,b
	middle	2.08 $\pm$ 0.11 b,c	1.41 $\pm$ 0.05 a,c	1.04 $\pm$ 0.18 a,b
	deep	1.22 $\pm$ 0.13 c	1.11 $\pm$ 0.21 c	0.74 $\pm$ 0.17 a,b
	calcified	0.74 $\pm$ 0.09 b,c	0.57 $\pm$ 0.02 a	0.5 $\pm$ 0.14 a
Articular cartilage thickness (μ m)	superficial	13.20 $\pm$ 3.18	10.65 $\pm$ 0.26	10.79 $\pm$ 0.41
	middle	77.48 $\pm$ 4.33 c	74.59 $\pm$ 2.72 c	58.57 $\pm$ 1.35 a,b
	deep	32.74 $\pm$ 3.10	32.52 $\pm$ 0.57	34.72 $\pm$ 0.96
	calcified	19.59 $\pm$ 1.39 c	20.64 $\pm$ 1.45	21.65 $\pm$ 0.35 a
	superficial	0 c	0.22 $\pm$ 0.22	0.33 $\pm$ 0.13 a
Empty lacuna	middle	0.04 $\pm$ 0.06 b,c	0.37 $\pm$ 0.06 a,c	0.77 $\pm$ 0.18 a,b
	deep	0.22 $\pm$ 0.12 c	0.32 $\pm$ 0.16	0.51 $\pm$ 0.24 a
	calcified	0.5 $\pm$ 0.08	0.6 $\pm$ 0.12	0.5 $\pm$ 0.18
Matrix/cell ratio	superficial	2.74 $\pm$ 0.33	2.44 $\pm$ 0.42	2.70 $\pm$ 0.57
	middle	3.96 $\pm$ 0.30	3.93 $\pm$ 0.61	3.95 $\pm$ 0.50
	deep	5.06 $\pm$ 0.59 c	4.94 $\pm$ 0.69 c	6.16 $\pm$ 0.61 a,b
	calcified	10.52 $\pm$ 1.12 c	13.07 $\pm$ 0.88 c	25.41 $\pm$ 3.98 a,b
Total thickness (μ m)		142.52 $\pm$ 7.47 c	138.80 $\pm$ 1.64 c	125.95 $\pm$ 1.78 a,b

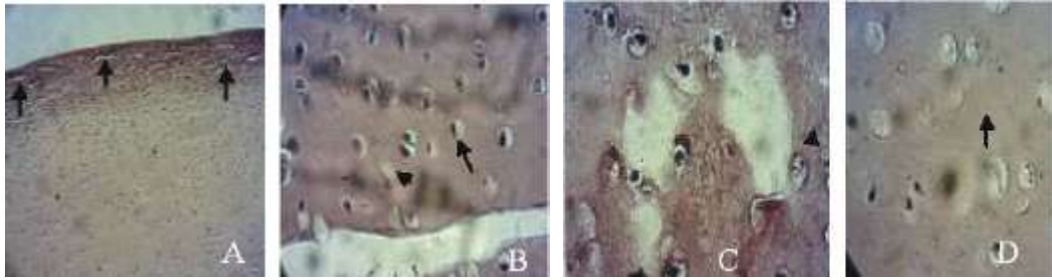


Fig. 3: Microscopical changes in lamb articular cartilage after administration of therapeutic and toxic doses of enrofloxacin. (A) Therapeutic group; multiple small cavitations in the superficial and middle layers (arrows). H&E staining, x10. (B-D); Toxic group. Presence of fissure within the intermediate zone in B, spindle shaped chondrocyte (arrow) and empty lacuna (arrow heads) in B and D and large cavitations in C. H&E staining, x40.



Fig. 4: Uptake of toluidine blue by the matrix of the lamb articular cartilage. Toluidine blue staining, x10. (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. (C) Toxic group: sever reduction of uptake of toluidine blue by the matrix especially in the middle layer that indicate sever decrease in matrix proteoglecans content (*). Many small cavitations were observed in the superficial and middle layers matrix.

especially in the middle layer with comparison to the control and therapeutic groups (Table 1 and Figure 3D). Matrix/cell ratio in the deep and calcified layers was significantly increased in the toxic group when compared to the control and therapeutic groups ($p < 0.05$). Amount of matrix glycoproteins was significantly decreased by enrofloxacin particularly with the toxic dose. This difference was illustrated in Figure 4. Also spindle shaped chondrocytes, fissure and cavitations in the articular cartilage matrix were observed in the toxic group (Figure 3B-D) while in the therapeutic group, superficial layer showed small cavitations (Figure 3A).

DISCUSSION

Fluroquinolones represent a class of antibiotics with broad spectrum antibacterial activity. Enrofloxacin is the most usable agent of this class drugs in veterinary medicine especially in the ruminants [1]. 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 [7-11], horse [2] and chicken [5].

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 fluroquinolones did not affect growth velocity in the ovine model. They also refused to suggest chondrotoxicity of quinolones on the lamb articular cartilage [17]. But data derived from this study are not very useful to compare with our results, because they administered fluroquinolones with pediatric medicine doses and did not examine articular cartilage by routine histopathologically methods.

In the present study, we have demonstrated that administration of enrofloxacin to lambs with the therapeutic and toxic doses had no clinical symptoms as same as gait abnormalities or lameness. This result is relatively similar to the others because only few papers described stiffness of gait in animals administrated quinolones [7, 19] and all of them examined dogs. However it seems the most sensitive species among mammals to administration of fluroquinolones are

immature dogs especially those of large breeds as described [16]. That is severely dose and time dependent [11]. It is important to bear in mind that a lack of clinical symptoms does not necessarily indicate a lack of quinolone-induced cartilage damages.

In macroscopic examination of lamb articular cartilage, only in the toxic group some lesions such as erosion, flap and excavation were seen on distal femoral and proximal tibial articular cartilages. To researches of Maslanka *et al.*, the ascertainment of the occurrence of quinolone-induced arthropathy was closely connected with the presence of grossly visible changes such as fluid filled vesicles, erosion and cartilaginous flaps [5, 20].

In microscopic examination, small cavitations were observed in superficial layer of articular cartilage in the therapeutic and toxic groups. While large cavitations, fissure and spindle shaped chondrocytes only in middle layer of the toxic group were detected sporadically. Forster *et al.* and Vormann *et al.* reported cleft formation and cavitations in superficial and middle layer of immature rats articular cartilage especially in proximal tibial extremity [21, 22]. These were due to administration high doses of quinolones and magnesium deficiency regimen. The presence of fissure in middle zone is the most characteristic change of quinolone-induced arthropathy and it has been described in numerous studies [5, 7, 10, 13, 14, 23-25]. In attention to last studies, presence of spindle-shaped chondrocytes seemed to be dose dependent. Changed shaped cells were observed *in vivo* [5, 7] and *in vitro* after treatment in Mg-free medium [26-28]. Egerbacher *et al.* concluded that magnesium deficiency is exerted via integrins in cytoskeletal changes leading to cell shape changes [28].

Toluidine blue selectively stain proteoglycans and intensity of the toluidine blue stain uptake is determined by loss of proteoglycans [5]. Thus, uptake reduction of this dye indicated decrease of matrix proteoglycans content due to abnormal processes. In the present study, were detected moderate to severe reduction of toluidine blue especially in middle layer of the toxic group. These symptoms indicated sever decrease in matrix proteoglycans content in the toxic group with comparison to mild reduction in the therapeutic group. The sever reduction of matrix proteoglycans could led to presence of other pathological findings such as cleft formation, fissure and cavitations. This alteration is typical for quinolone-induced arthropathy and was described in many papers [5, 23-25]. 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 Maslanka *et al.* [5] and may be show the initial site of proteoglycans loss.

Our findings showed significant decrease in chondrocyte numbers of all layers of the articular cartilage in the toxic group with comparison to the control and therapeutic groups except calcified layer. This significant decrease in the therapeutic group was seen except deep layer Channa *et al.* administered ciprofloxacin (active metabolite of enrofloxacin) to rat pups and detected the reduced count of chondrocytes in the proliferative zone of growth plate cartilage. They concluded that ciprofloxacin effected growth plate cartilage by inhibiting the mitosis in the proliferative zone [29].

Significant increase of empty lacuna was seen especially in middle layer of articular cartilage in lambs received enrofloxacin. Increase of empty lacuna numbers in the articular cartilage could be sign of increased apoptosis or necrosis. Maslanka *et al.* distinguished chondrocytes with shrunken cytoplasm and pyknotic nuclei and necrotic cells in chicken articular cartilage [5]. 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 [30]. Sendzik *et al.*, indicated an up to 15 folds increase of caspase-3 protein in tenocyte following exposure to ciprofloxacin or levofloxacin. They concluded that these changes resulted in pronounced increase of apoptosis [31].

The total thickness of articular cartilage especially in middle layer was significantly decreased in the toxic group. In attention to reduced chondrocyte counting and sever reduction of matrix proteoglycans in the toxic group, decrease of cartilage thickness to be seemed usual. It was important that empty lacuna and decrease in cell counting and matrix proteoglycans were more detectable in middle layer where the most decrease of articular cartilage thickness and presence of fissure and cavitations were observed. Channa *et al.* reported width decrease in the growth plate cartilage of the newly born rats were administered ciprofloxacin. They believed that the decrease in the growth plate width, was due to reduced count of the proliferative cells and diminution in the average size of the hypertrophic chondrocytes in the hypertrophic zone [29].

Significant increase in matrix/cell ratio in deep and calcified layers of the toxic group was due to decrease of cell numbers in these layers and in attention to natural hypocellularity of these layers with comparison to superficial and middle layers. Cell decrease in deep and calcified layers was more effective to increase matrix/cell ratio. On the other hands, it seemed that loss of matrix

components such as proteoglycans was more severe in middle and superficial layers than their below layers.

There are a lot of hypotheses to explain mechanisms of the quinolone-induced chondropathy. More acceptable of these, is lack of magnesium ion due to chelating with the quinolones [5, 11, 20, 22, 26, 32]. 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 [28]. While Maslanka *et al.* suggested enrofloxacin increased proteoglycans degradation [5]. In some studies, increase of matrix metalloproteinases (MMPs) was showed after fluroquinolones administration in tendon [31, 33]. In attention to similarities of tendon and cartilage [5], MMPs especially MMP-3 and MMP-13 were known probably responsible in cartilaginous extracellular matrix degradation [34, 35]. However nowadays, pronounced degradation of extracellular matrix is known as a consequence of the disturbed interaction between matrix and cells and is increased by pronounced increase of apoptosis [6].

In the present study, chondrotoxic effects of enrofloxacin were seen more in the toxic group than the therapeutic group. In available literature, there are several reports describing the influence of various multiple doses of quinolones, more or less exceeding the therapeutically applied doses, on articular cartilage [8, 24, 25, 36]. These studies showed very distinctly a dose dependent incidence of quinolone-induced arthropathy. Thus, the data indicate that degree of severity of fluroquinolones chondrotoxicity depends on volume and number of doses. So higher doses with longer period of use, induce more severe effects.

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

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