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Original Article

In Vitro Evaluation of Equine Fibroblast-Like Synoviocytes Viability Treated with Doxycycline

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Abstract

Objective- The purpose of the present study was to investigate the viability of equine fibroblast-like synoviocytes (FLSs) treated with doxycycline.
Design- Experimental study.
Animals - FLSs from metacarpophalangeal joints of six skeletally mature horses.
Methods- FLSs were established from synovial fluids of healthy joints. The cells were treated with varoius concentrations (1, 5, 10, 50, 100, 150, 300, 400 µg/ml) or without doxycycline for 48-hour. Viability of FLSs was determined using MTT assay and the trypan blue dye exclusion method.
Results- No significant differences were observed between viability of FLSs cultures treated with doxycycline until 150 µg/ml and control group (P>0.05). Doxycycline at 300 and 400 µg/ml significantly decreased FLSs viability (P<0.05). FLSs viability was 74.28% and 59.07% in 300 and 400 µg/ml, respectively, when measured by the MTT assay. Also FLSs viability at 300 and 400 µg/ml of doxycycline were 68.10% and 43.26%, respectively, with trypan blue exclusion method.
Conclusion and Clinical Relevance- These findings demonstrated that doxycycline was not toxic for equine FLSs at concentration ≤150 µg/ml *in vitro*. Further studies are needed to investigate the safety, efficacy and detrimental effects of doxycycline in equine joints.
Key words: Doxycycline, Equine fibroblast-like synoviocytes, MTT assay, Trypn blue, Viability.

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Introduction

Osteoarthritis is a chronic multifactorial joint disease which is known as the most frequent causes of lameness in horses.[1-3] The major pathological characteristic of osteoarthritis is the irreversible destruction of the joint tissues especially articular cartilage.[1,4,5] In osteoarthritis, elevated production of proteinases especially matrix metalloproteinases (MMPs) by synovial tissue is associated with progression of osteoarthritis.[1,6] MMPs are a family of zinc and calcium-dependent proteinases that facilitate tissue remodeling.[6] Levels and activities of different types of MMPs increase in equine arthritic joints.[3,7,8] Treatments of osteoarthritis are primarily limited to symptomatic relief but have no capability to protect articular cartilage from further degeneration processes.[1,9,10] Therefore, it seems that preventing, or even retarding of cartilage matrix degradation through

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effect on MMPs actions is one of the important aims in osteoarthritis.[4,11,12] Doxycycline is a tetracycline family antibiotic which suppresses the MMPs at multiple levels. Several studies have demonstrated the beneficial effects of doxycycline administration in different arthritic diseases.[13] Because of side-effects, systemic administration of doxycycline for treatment of osteoarthritis is not practiced routinely in horses.[14] Although intra-articular administration of therapeutic agents has several advantages over systemic delivery, some of these drugs can induce tissue irritation.[15,16] Therefore, it is critical to estimate cellular damages in joints where the drugs are locally administrated. All intra-articular tissues contribute to joint health.[16] Because the synovial membrane is important in the maintenance of joints, the cellular damage arising from intra-articular injections of any therapeutic agents must be evaluated on synovial cells as well as other component.[2,17]

In the present study, the *in vitro* effect of various concentrations of doxycycline were evaluated in cultured equine fibroblast-like synoviocytes viability, using the 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and trypan blue exclusion. To the best knowledge of the authors, no study to date has investigated the cytotoxicity of doxycycline in equine FLSs.

Materials and Methods

Culture of cells from synovial fluid

Synovial fluid samples were aspirated with syringes containing sodium heparin (Heparodic, Caspiantamin, Gilan, Iran) from metacarpophalangeal joints using aseptic techniques from six skeletally mature (4-7 years old) horses after getting permission of the owners. All horses were free of lameness with the normal metacarpophalangeal joints based on clinical and radiographical evaluations. Each sample was centrifuged at 600 g for 30 minutes to be clear from cells. The cells were then resuspended in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO BRL, Eggenstein, Germany) low glucose supplemented with 10% fetal calf serum (FCS, GIBCO BRL, Eggenstein, Germany) and 1% penicillin streptomycin solution (GIBCO BRL, Eggenstein, Germany), and seeded into 60 mm culture dishes. The dishes were placed in a humidified atmosphere with 5% carbon dioxide at 37°C for 24-hour after which cells were washed with phosphate buffered saline (PBS, GIBCO BRL, Eggenstein, Germany) to remove non-adherent cells. Growth medium was replaced every 2 to 3 days and confluent FLSs were passaged using trypsin-EDTA solution (0.5% trypsin, 0.2% EDTA in PBS). At passage two, the equivalent number of cells from each FLSs were pooled and plated in 75 cm^2 flasks and grown in supplemented media until third passage. Then cells were detached from the flasks by trypsinization. FLSs at fourth passage were seeded at a density of 5×10^4 cells per well containing the 1 mL culture medium of 24-well plates and stored in an incubator at 37 °C with 5% CO2 until observance of 70% cellular confluence per well culture.

FLSs treatment with doxycycline

For evaluation of the cytotoxic effects of doxycycline on FLSs, a series of various concentrations of doxycycline (0, 1, 5, 10, 50, 100, 150, 300, 400 µgr/ml) were prepared. For doxycyline preparation, a stock solution of doxycycline hyclate (Sigma-Aldrich, St Louis MO, USA) was made in sterile distilled water at the concentration of 10 mg/ml. The medium was aspirated and cells were washed with PBS and fresh media supplemented with doxycycline solution was added and the plates were incubated for 48-hour at 37 °C in 5% CO. Control samples received only the medium without any doxycycline. The media was refreshed every day.

MTT colorimetric assay

10X MTT stock solution (Sigma-Aldrich, St Louis MO, USA) was prepared at 5mg/mL in PBS and then diluted 10 times with serum free DMEM. The cultures were washed with PBS, then 500 µL of the prepared MTT solution was added to each well and incubated for 4-hour. The MTT reaction was terminated by adding 500 µl acidified HCL isopropyl alcohol. The solution was protected from light and allowed to solubilize thoroughly for 15 minutes. Subsequently, the absorbance of each well was determined using a scanning multiwell spectrophotometer ELISA (Bio-Tek Instruments Inc., VT) at 560 nm.[18] The cell viability was expressed as percentage of control cell growth and obtained from untreated cells harvested in the same 24-well plates. Experiments were performed five times with duplicate to assess for consistency of results.

Trypan blue staining

For evaluating the number of viable cells the trypan blue staining was performed. At the end of the treatment period of 24-well plates (48-hour), with a series of doxycycline concentrations of each extract in 4 replicates the cells were trypsinized and collected cells from each well were centrifuged at 500 g for 5 minutes. An equal volume of freshly prepared solution of 0.4% trypan blue dye (Sigma-Aldrich, St Louis MO, USA) was added to cell suspension and mixed.

12 µL of each sample was spread onto the hematocytometer and the number of viable cells was counted. Cell viability was calculated as a percentage of the respective control values.[19] Experiments were performed five times with duplicate to assess for consistency of results.

Statistical analysis

Data for all groups were presented in mean±SEM. Statistical analyses were performed using one-way ANOVA test to compare among groups and post hoc Tukey's pair-wise comparisons. Significance was set at $p < 0.05$. SPSS version 24.0 (IBM Corporation, Armonk, New York, USA) was used for data analysis.

Results

The MTT assay analysis revealed that there was a significant difference among the experimental groups ($P = 0$, $P< 0.05$). Post hoc comparisons using Tukey's test showed that no significant differences were observed between FLSs cultures treated with (1, 5, 10, 50, 100, 150) µg/ml doxycycline and control group ($P>0.05$). In contrast, doxycycline at 300 and 400 µg/ml significantly decreased FLSs viability compared to control group ($P<0.05$). FLSs viability were 74.28% and 59.07% in 300 and 400 µg/ml, respectively. Significant difference was observed between 300 and 400 µg/ml of doxycycline, too ($P<0.05$) (Fig. 1) (Table 1).

Similar results were observed using trypan blue for FLSs viability among groups ($P = 0.05$, $P< 0.05$). Doxycycline at concentrations 1, 5, 10, 50, 100, 150 µg/ml had no significant effects on FLSs viability compared to control group ($P>0.05$). However, the differences between control group and FLSs treated with 300 and also 400 µg/ml doxycycline were significant ($P = 0$, $P< 0.05$).

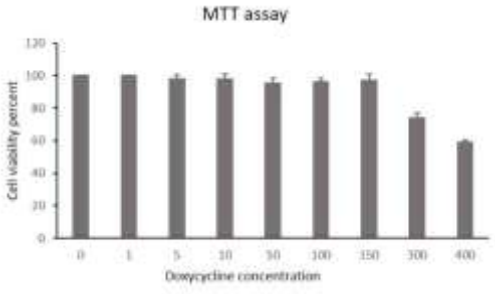

Figure 1. Effect of different concentrations of doxycycline on equine FLSs after 48-hour treatment evaluated by MTT assay.
[a] Significantly different from the control group ($P< 0.05$).
[b] Significantly different from the other doxycycline concentrations ($P< 0.05$).

FLSs viability were 68.10% and 43.26% in 300 and 400 µg/ml, respectively. Also there was a significant difference between 300 and 400 µg/ml concentrations ($P<0.05$), (Fig. 2) (Table 2).

Table 1. Equine FLSs viability after 48-hour treatment with different concentrations of doxycycline measured by MTT assay. There was a significant difference among the experimental groups ($P< 0.05$). Data for all groups are presented in mean±SEM.

Treatment (µg/ml)	FLSs viability (%) Mean±SEM
0 (Control)	100±0
1	100±0
5	97.99±2.74
10	98.10±3.08
50	95.55±3.03
100	96.14±2.26
150	97.23±3.56
300[a,b]	74.28±2.70
400[a,b]	59.07±1.42

[a] Significantly different from the control group ($P < 0.05$).
[b] Significantly different from the other doxycycline concentrations ($P < 0.05$).

Discussion

Doxycycline has different functions such as anti-inflammatory, antioxidant, antitumor, immunomodulatory and MMPs inhibitory in addition to its antimicrobial activities.[20,21] Several *in vitro* and *in vivo* studies suggest that doxycycline has protective activity in arthritic joints via different mechanisms and consider as disease-modifying osteoarthritic drugs (DMOADs).[1,21,22] So it seems that doxycycline may be the ideal drug for treatment of arthritic conditions in horses as well as other animals. According to some side-effects of long-term systemic use of doxycycline, local administration of doxycycline may be more effective and produce fewer side effects compared to systemic delivery.[14] However, before clinical use of doxycycline, *in vitro* evaluation of its effect is required. The purpose of this study was to evaluate the effect of doxycycline on equine FLSs viability.

To the authors' knowledge, this was the first *in vitro* study, to investigate the viability of FLSs treated by doxycycline. Doxycycline did not significantly reduce equine FLSs viability until 300 µg/mL measured by MTT and trypan blue assays. After 48-hour culturing at different concentrations of doxycycline, the FLSs viability was not decreased when the FLSs treated with doxycycline at 1, 5, 10, 50, 100 and 150 µg/mL,

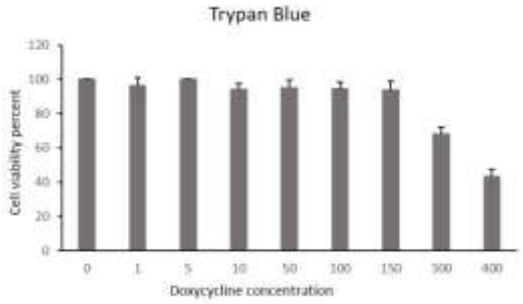

Figure 2. Effect of different concentrations of doxycycline on equine FLSs after 48-hour treatment evaluated by trypan blue exclusion.
a Significantly different from the control group (P< 0.05).
b Significantly different from the other doxycycline concentrations (P< 0.05).

hwoever, significantly decreased at 300 and 400 µg/mL of doxycycline in comparison with control and other doxycycline concentrations.

Karna *et al* used different concentrations of doxycycline (25, 100, 200, 500 µg/ml) in human skin fibroblasts for 24-hour. They observed no significant differences in fibroblasts viability at these concentrations of doxycycline from that of control fibroblasts evaluated by MTT assay. In their study, an increase in the doxycycline concentration to 500 µg/mL resulted in a 25% reduction in the viability of these cells.[23] In another study performed by Myers and Wolowacz, MTT assay evaluation indicated that no significant reduction was observed in viability of human dermal fibroblasts treated with 0, 5, 12.5, 25 and 50 µg/ml doxycycline after 24-hour.[24] In a study performed by Seo *et al*, doxycycline at concentrations between 1-100 µg/ml were examined on periodontal ligament fibroblasts for 24-72-hour and observed that doxycycline at 100 µg/ml significantly decreased cell viability measured by MTT assay.[25]

Different results of these limited studies concerning the effect of doxycycline on cell viability may be due to different types of cells from various origins. Suzuki *et al* evaluated the toxicity of doxycycline on three different cell lines and found a greater sensitivity of the epithelial cells to doxycycline compared to fibroblasts cell lines. Survivals of human gingival epithelial cell line were decreased by treatment of doxycycline at 100 or 300 µM. In contrast, survivals of human gingival fibroblast cell line and periodontal ligament fibroblast cell line were decreased by treatment with doxycycline, at 300 and 1000 µM after 48-hour. They suggested that the differences in the permeability of various types of cells for doxycycline and its retentivity in the cells might be contributed to the various cytotoxic effects among the cell lines.[26]

Table 2. Equine FLSs viability after 48-hour treatment with different concentrations of doxycycline measured Trypan blue exclusion. There was a significant difference among the experimental groups (P< 0.05). Data for all groups are presented in mean±SEM.

Treatment (µg/ml)	FLSs viability (%) Mean±SEM
0 (Control)	100±0
1	96.23±5.04
5	100±0
10	94.05±3.55
50	94.96±4.95
100	94.83±3.38
150	93.78±5.35
300[a,b]	68.10±4.01
400[a,b]	43.26±4.09

[a] Significantly different from the control group (P < 0.05).
[b] Significantly different from the other doxycycline concentrations (P < 0.05).

Smith and Cock reported that corneal keratocytes are more sensitive than corneal epithelial cells against doxycycline, so that at 120 µM doxycycline killed keratocytes.[27] Di Caprio *et al* investigated that doxycycline at 0.3, 0.6, 1.5 and 3 µg/ml did not exert any significant toxic effect on viability of human keratinocytes after either 24- or 48-hour cellular viability results were similar to controls.[20]

However, *in vitro* tests alone do not demonstrate the potential toxicity of doxycycline on cells. Besides these *in vitro* investigations, in an *in vivo* study, Haerdi-Landerer *et al* reported that intra articular injection of doxycycline at 5 or 10 mg in calves did not evoke clinically relevant inflammatory reactions in joints.[16]

According to this and other investigations, different types of cells have resistance to doxycycline. The present study suggested that doxycycline could be safely used at concentration ≤150 µg/ml in equine FLSs *in vitro*. The cytotoxic activity of doxycycline on equine FLSs was observed at concentrations ≥ 300 µg/ml. However, this short-term study had potential limitations, so further investigations including application of arthritic cells besides healthy cells, evaluation of doxycycline toxicity on both chondrocytes and synoviocytes, or even long-term studies are needed before intra-articular recommendations of doxycycline in horses.

Acknowledgments

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Conflicts of interest

None.

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نشریه جراحی دامپزشکی ایران
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چکیده

ارزیابی توانایی زیستی سینوویوسیت‌های شبه‌فیبروبلاستی تحت درمان با داکسی‌سایکلین در شرایط آزمایشگاهی

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هدف- هدف از مطالعه حاضر، ارزیابی توانایی زیستی سینوویوسیت‌های شبه‌فیبروبلاستی (FLSs) تحت درمان با داکسی‌سایکلین بود.
طرح- مطالعه تجربی.
حیوانات- سینوویوسیت‌های شبه‌فیبروبلاستی اخذ شده از مفاصل قلمی-بندانگشتی ۶ راس اسب بالغ.
روش کار- سینوویوسیت‌های شبه‌فیبروبلاستی حاصل از مایعات مفصلی سالم کشت داده شد. سلول‌ها تحت درمان با داکسی‌سایکلین با غلظت‌های مختلف داکسی‌سایکلین (۴۰۰، ۳۰۰، ۱۵۰، ۱۰۰، ۵۰، ۱۰، ۵، ۱ میکروگرم در میلی‌لیتر و یا بدون حضور داکسی‌سایکلین برای مدت ۴۸ ساعت قرار گرفتند. توانایی زیستی FLSs با استفاده از روش‌های MTT و تریپان بلو ارزیابی شد.نتایج- اختلاف آماری معنی‌داری در توانایی زیستی FLSs درمان شده با داکسی‌سایکلین تا غلظت ۱۵۰ میکروگرم در میلی‌لیتر در مقایسه با گروه کنترل مشاهده نشد ($P>0.05$). با داکسی‌سایکلین در غلظت‌های ۴۰۰ و ۳۰۰ میکروگرم در میلی‌لیتر، موجب کاهش معنی‌دار توانایی زیستی FLSs شد ($P<0.05$). توانایی زیستی FLSs برای غلظت‌های ۳۰۰ و ۴۰۰ میکروگرم در میلی‌لیتر، به‌ترتیب ٪۷۴/۲۸ و ٪۵۹/۰۷ با استفاده از روش MTT بود. همچنین توانایی زیستی FLSs برای غلظت‌های ۳۰۰ و ۴۰۰ میکروگرم در میلی‌لیتر داکسی‌سایکلین، به‌ترتیب ٪۶۸/۱۰ و ٪۴۳/۲۶ با استفاده از روش تریپان‌بلو بود.
نتیجه‌گیری و کاربرد بالینی- تزریق داخل مفصلی داکسی‌سایکلین را می‌توان تا غلظت‌هایی برابر و یا کمتر از ۱۵۰ میکروگرم در میلی‌لیتر در اسب با اطمینان استفاده کرد. مطالعات بیشتری به منظور ارزیابی سلامت، اثربخشی و اثرات مخرب داکسی‌سایکلین در مفاصل اسب نیاز است.

کلمات کلیدی- داکسی‌سایکلین، سینوویوسیت‌های شبه‌فیبروبلاستی اسب، روش MTT ، تریپان‌بلو، توانایی زیستی.