Decreased Expression of Proliferating Cell Nuclear Antigen Is Associated with Dexamethasone Inhibition of the Proliferation of Rat Tendon Fibroblasts

WEN-CHUNG TSAI, FUK-TAN TANG, MAY-KUEN WONG, HSIAO-CHENG YEN, and JONG-HWEI S. PANG

ABSTRACT. ***Objective.*** To investigate the effects of dexamethasone (Dex) on the proliferation of cultured rat Achilles tendon fibroblasts at concentrations typically used for local injection treatment.

Methods. Fibroblasts cultured from rat Achilles tendons were treated with Dex at concentrations of 0, 10^{-4}, 3×10^{-4}, and 10^{-3} M. [^{3}H]thymidine incorporation was used to measure the rate of cell proliferation. mRNA expression of proliferating cell nuclear antigen (PCNA) and cyclin kinase inhibitor $p21^{CIP1}$ was determined by reverse transcription-polymerase chain reaction (RT-PCR). The protein levels of PCNA and $p21^{CIP1}$ were investigated by Western blot analysis.

Results. An initial inhibitory effect on tendon fibroblast proliferation was observed at a concentration of 10^{-4} M. Further, a significant decline in [^{3}H]thymidine incorporation as a function of Dex concentration was noted ($p = 0.019$). RT-PCR results revealed that PCNA mRNA expression was inhibited after Dex treatment. Western blot analysis of PCNA protein also revealed Dex downregulation. Gradual declines in the levels of PCNA mRNA expression and PCNA protein as a function of Dex concentration were noted. The expression of $p21^{CIP1}$ both at mRNA and the protein levels remained constant.

Conclusion. These results suggest that Dex inhibition of the proliferation of rat tendon fibroblasts is associated with a $p21^{CIP1}$ independent decrease of the PCNA gene expression. (J Rheumatol 2002;29:2397–402)

Key Index Terms:
DEXAMETHASONE
TENDON
FIBROBLAST
PROLIFERATING CELL NUCLEAR ANTIGEN

Corticosteroids are commonly used to treat soft tissue rheumatic disorders. Isolated cases of tendon or ligament rupture following local corticosteroid injection, however, suggest that corticosteroid treatment may produce a deleterious effect on tendon strength, or impair the healing process[1-3]. Whether rupturing is a corticosteroid-mediated side effect or related to the underlying disease process remains unanswered. Extensive *in vivo* and *in vitro* studies have attempted to elucidate the mechanisms responsible for side effects of local corticosteroid injection on tendon structure.

Tendon structure consists mainly of dense collagen arranged in a linear fashion with a basic cellular component, i.e., fibroblasts (tenocytes). Fibroblasts are the source of collagen production, protein mediators of repair, and matrix proteoglycans[4,5]. In the regenerative phase of tendon injury, fibroblasts actively proliferate, being responsible for the tissue's abundant deposition of extracellular matrix.

Animal studies investigating the effects of local steroid administration on injured tendons or ligaments often reveal an associated delay in the healing process[6-8]. The mechanism underlying corticosteroid effect on fibroblast proliferation remains uncertain, with conflicting results from different laboratories[9-12]. Most *in vitro* studies have used tumor (or transformed) cell lines or fibroblasts of other tissue origin[9-12]. It is possible that cells of differing origins do not respond identically to corticosteroids. The actual effect and molecular mechanism of corticosteroid action on tendon fibroblast proliferation remains undetermined.

Proliferating cell nuclear antigen (PCNA), the auxiliary protein of DNA polymerases, δ and ε, is essential for DNA replication and repair[13]. With exponential cell growth, PCNA mRNA is present in all phases of the cell cycle with a 2 to 3 fold increase during the S phase[14]. Blocking PCNA production inhibits cell division, indicating that PCNA plays a pivotal role in the process of cell proliferation[15]. The relationship between the proposed effect of dexamethasone (Dex) on tendon fibroblast proliferation and PCNA has not previously been mentioned in the literature.

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Submitted September 21, 2001; revision accepted May 10, 2002.

We sought to determine the effect of Dex on the proliferation of fibroblasts cultured from rat Achilles tendons and its association with PCNA gene expression.

MATERIALS AND METHODS

Primary culture of fibroblasts from rat Achilles tendon. The Achilles tendon from Sprague-Dawley rats weighing 200 to 250 g was excised. The excised tendon was soaked in povidone-iodine for 3 min and washed twice in phosphate buffered saline (PBS). The tendon was then cut into small pieces of about 1.5–2.0 mm^3 and placed individually in the 6 well culture plates. After 5 minutes of air drying for improved adherence, 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, Utah, USA) with 10% fetal bovine serum (FBS)(Cansera, Rexdale, Ontario, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin was added to each well. The explants were then incubated at 37ºC in a humidified atmosphere with 5% CO_2/95% air until confluence of the cells was reached. Cells were subsequently subcultured with trypsin digestion in a 1:4 dilution ratio. Cells between passage 3 and 6 having proper growth rate and normal fibroblast-shape, were used in this study.

Measurement of DNA synthesis. Cells at 60–70% confluence were synchronized by incubation in serum-free DMEM for 2 days. Cell proliferation was induced by the addition of 10% FBS; Dex (Sigma, St. Louis, MO, USA) at concentrations of 0, 10^{-4}, 3×10^{-4}, and 10^{-3} M was also added. DNA synthesis was measured 18 h after serum stimulation by incubating the cells with [^{3}H]thymidine (1 μCi/ml) for 2 h. The [^{3}H]thymidine incorporation was terminated by rapidly washing the cells with cold PBS, and the cell extracts were prepared by freeze-thaw method. Aliquots of cell extracts were spotted on a GF/C filter and radioactivity incorporation into the DNA was precipitated by incubation in ice cold 10% trichloroacetic acid (TCA), followed by 3 acetone washes. The radioactivity level was determined by scintillation spectrophotometry, and protein content was used to correct the amount of cell extracts used for TCA counting.

RNA isolation. Total cellular RNA was isolated by lysis in a guanidine isothiocyanate buffer followed by a one-step phenol-chloroform-isoamyl alcohol extraction. Briefly, 5×10^6 cells were lysed in 0.5 ml solution D containing 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium sarcosine, and 0.1 M ß-mercaptoethanol with vigorous vortexing. Fifty μl of 2 M sodium acetate (pH 4.0), 0.5 ml of phenol and 100 μl of chloroform-isoamyl alcohol (49:1, v:v) were sequentially added to the homogenate. After 30 seconds of vortexing, the solution was centrifuged at 10,000 μg for 15 min at 4°C. RNA was precipitated by adding 0.5 ml isopropanol to the aqueous phase and storing at –20°C for 1 h. RNA was pelleted by centrifuging the solution at 10,000 μg for 15 minutes at 4°C. After rinsing the RNA pellet in ice cold 75% ethanol, the dry RNA was dissolved in DEPC-treated double distilled H_2O.

Reverse transcription-polymerase chain reaction (RT-PCR). One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) by incubation with 200 u of reverse transcriptase in 20 μl of reaction buffer containing 0.25 μg of random primers, and 0.8 mM dNTPs at 42°C for 1 h. Two μl of the cDNA was used as a template for the PCR reaction. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, monitoring the difference in cDNA loading, and PCR variation. The PCR was performed in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, and 5 u Taq DNA polymerase, for 30 cycles comprising denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Amplification results were assessed using 1.5% agarose gel electrophoresis. Oligonucleotide sequences for the specific primers used in this study are summarized in Table 1.

Western blot analysis. Cell extracts were prepared in a lysis buffer containing Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 2 mM PMSF, and 1% Triton X-100 followed by freeze-thaw method. Protein concentration of the cell extracts was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples with identical protein quantities were then separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Next, the membrane was incubated at room temperature in blocking solution (1% bovine serum albumin, 1% goat serum in PBS) for 1 h, followed by 2 h incubation in blocking solution containing an appropriate dilution of primary antibodies (NeoMarks, Fremont, CA, USA). After washing, the membrane was incubated in PBS containing goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) for 1 h. Membranes were then washed 3 times in PBS and positive signals were developed with either diaminobenzidine (DAKO, Via Real, Carpiateria, CA, USA) or enhanced chemiluminescence (Amershan Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).

Table 1. Oligonucleotides used in PCR.

PCNA	
Sense	5′-TTG-TCA-GCA-AGA-CCT-CGC-TC-3′
Antisense	5′-CTG-GGA-TTC-CAA-GTT-GCT-CA-3′
$P21^{CIP1}$	
Sense	5′-CAT-GTC-CGA-TCC-TGG-TGA-TG-3′
Antisense	5′-AGT-GCA-AGA-CAG-CGA-CAA-GG-3′
GAPDH	
Sense	5′-TTC-ATT-GAC-CTC-AAC-TAC-AT-3′
Antisense	5′-GAG-GGG-CCA-TCC-ACA-GTC-TT-3′

Statistical analysis. All data from the [^{3}H]thymidine incorporation study are expressed as mean ± SEM. Comparisons between the Dex treated and control cells were performed using Kruskal-Wallis test. The level of statistical significance was set at a P value of 0.05.

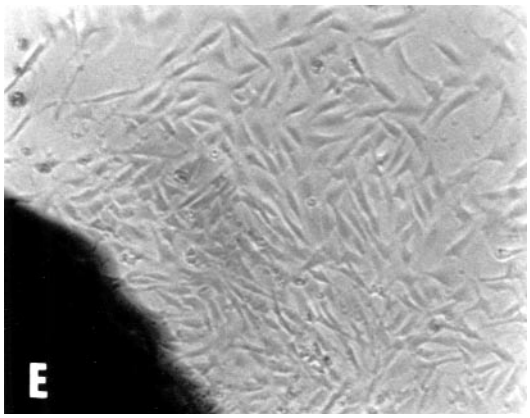
RESULTS

The initial outgrowth of fibroblasts was observed in 2/24 wells, 2 days after plating the tendon explants (Figure 1). Within a 10 day period, the majority of explants (22/24) showed fibroblast outgrowth. Cells started rapid growth after migrating out from the explants, and confluence was reached in 2 weeks. To examine the effect of Dex on fibroblasts derived from tendons, cells at 60–70% confluence were treated with various concentrations of Dex. The photomicrograph of fibroblasts 24 h after treatment is shown in Figure 2. An initial inhibitory effect on fibroblast growth (decreased cellularity) was observed at a concentration of 10^{-4} M. This inhibitory effect became more pronounced in cultures treated with higher concentrations of Dex. Neither significant cell morphological change nor cell death was observed under these conditions.

Results of DNA synthesis assay revealed a gradual decline in [^{3}H]thymidine incorporation as a function of Dex concentration (Figure 3); the inhibition was statistically significant (420.7 ± 18.4 CPM/μg protein for the control group, and 324.0 ± 14.3, 267.7 ± 15.5, and 210.7 ± 17.2 CPM/μg for the cultures treated with 10^{-4} M, 3×10^{-4} M, and 10^{-3} M of Dex, respectively; p = 0.019). The results clearly indicated that Dex decreased the cellularity of tendon fibroblasts by inhibiting DNA synthesis.

To further understand the molecular mechanism for Dex

(A)



(B)

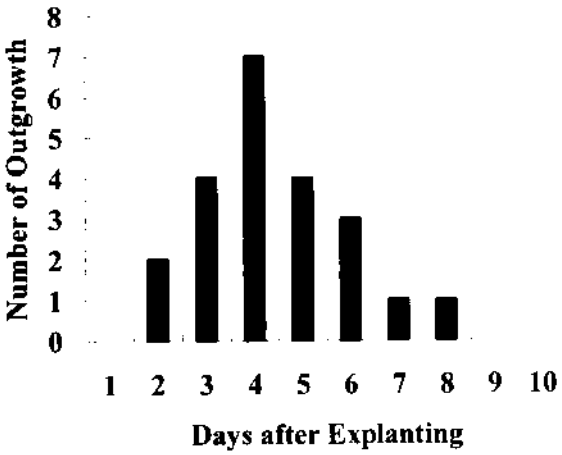


Figure 1. A. Fibroblast outgrowth from rat Achilles tendon explant (E) in the control group (magnification 100×). B. Total number of culture wells with onset of fibroblast outgrowth in the 10 day period.

inhibition of DNA synthesis, the mRNA expression of PCNA in Dex-treated tendon fibroblasts was analyzed. The mRNA expression of PCNA was inhibited dose-dependently after addition of Dex (Figure 4A). The amount of PCNA proteins under the same conditions was measured by Western blot analysis and PCNA protein was identified at size 36 kDa. Results in Figure 4B demonstrated decreased PCNA protein level caused by Dex. The gradual decline in the expression levels of PCNA mRNA and protein was a function of Dex concentration and closely correlated with the inhibition of DNA synthesis in Dex-treated tendon fibroblasts.

According to studies, Dex inhibits the proliferation of mouse L929 cells by increasing the expression of cyclin kinase inhibitor $p21^{CIP1}$ [11]. RT-PCR and Western blot analysis were therefore used to examine the expression of $p21^{CIP1}$ in tendon fibroblasts after Dex treatment. The results demonstrated that the expression levels of $p21^{CIP1}$ mRNA and protein remained constant after Dex treatment (Figure 5A and B).

DISCUSSION

The healing process of an injured tendon can be divided into 3 phases: inflammation, regeneration, and remodeling/maturation[5]. The regenerative phase is characterized by cell proliferation and matrix formation, with tendon fibroblast proliferating and migrating into the repair site. In the remodeling/maturation phase, the regenerated tissue appears in a callus-like formation, mainly due to the proliferation of epitendon cells. Adequate tendon healing obviously requires fibroblast proliferation, as well as production of an appropriate extracellular matrix. Whether the proliferation of tendon fibroblasts is positively or negatively regulated by corticosteroids, however, remains controversial.

Histological and biochemical studies confirm that corticosteroids interfere with the early inflammatory phase of healing, and also the regeneration and remodeling phases[16,17]. However, the underlying mechanism involved in this corticosteroid-induced delay in *in vivo* tendon healing remains to be determined, since *in vitro* studies attempting to describe corticosteroid effects on fibroblast proliferation are contradictory. A related study revealed that Dex enhanced the proliferation of human WI-38 normal lung fibroblasts accompanied by a decrease in the level of $p21^{CIP1}$ (a cyclin kinase inhibitor) gene expression[9]. Dexamehasone was also found to enhance proliferation of murine BALB/c 3T3 embryo fibroblast and increase its connective tissue growth factor expression[10]. By contrast, glucocorticoid induced inhibition of mouse L929 skin fibroblast proliferation due to $p21^{CIP1}$ induction has also been reported[11]. Our current study demonstrates that the expression of PCNA mRNA and protein was decreased as a function of Dex concentration, whereas the expression of $p21^{CIP1}$ mRNA and protein remained unchanged. These contradictory findings may be methodological: the various studies used fibroblasts originating from different tissues or transformed cell lines, which would respond to corticosteroids differently. Further, corticosteroid effects on cell proliferation for normal cells and their virus-transformed counterparts may be totally different[18,19]. Studies on fibroblasts cultured from rat Achilles tendon would therefore be more representative and provide meaningful results to elucidate the actual role of Dex on tendon fibroblasts *in vivo*.

PCNA, initially described as a nuclear antigen for autoimmune disease in patients with systemic lupus erythematosus[20], has been determined to be essential for the coordinated synthesis of leading and lagging DNA strands[21]. Further, it has been demonstrated that PCNA is necessary for simian virus 40 replication[22]. In addition to involvement in DNA synthesis and repair processes, PCNA has been shown to establish interactions with proteins required for

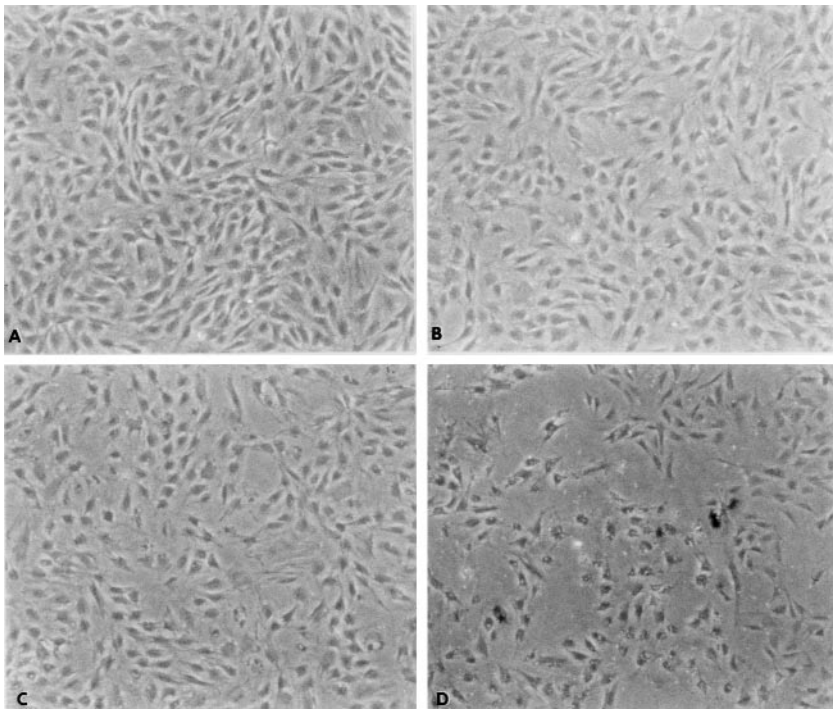


Figure 2. Morphology of rat Achilles tendon fibroblasts 24 h after dexamethasone treatment (40×). A-D. Concentrations of dexamethasone were at 0, 10^{-4}, 3×10^{-4} and 10^{-3} M, respectively.

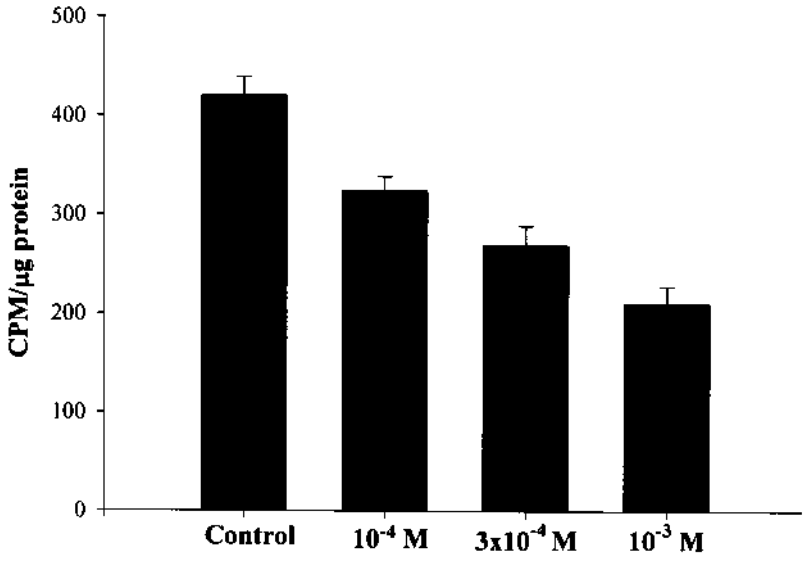


Figure 3. The effect of dexamethasone on the [^{3}H]thymidine incorporation of rat Achilles tendon fibroblasts. A significant decline in the [^{3}H]thymidine incorporation was a function of dexamethasone concentrations ($p = 0.019$, $n = 3$).

control of the cell cycle[23]. To our knowledge, our study documents for the first time that decreased PCNA mRNA and protein expression is associated with corticosteroid inhibition of the tendon fibroblast proliferation. This finding suggests that, at a cellular level, delayed tendon healing after local corticosteroid injection might act through a PCNA related pathway.

Dex concentrations ranging from 10^{-4} to 10^{-3} M were used in the current study, and this range is similar to the typical injection dosage of 4–20 mM[24]. It has been proposed

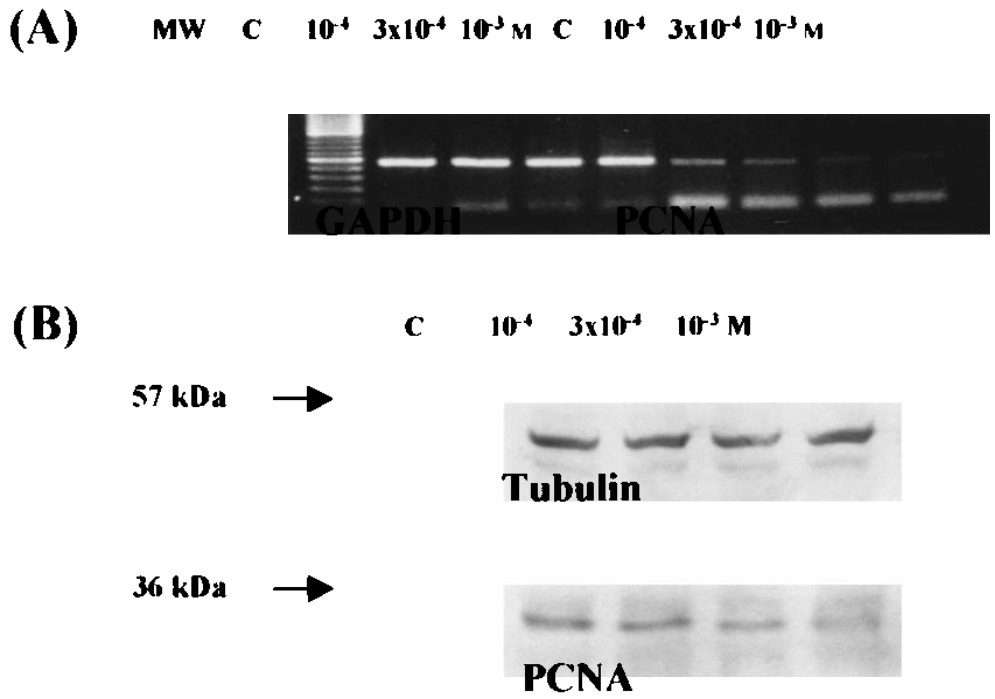


Figure 4. Dexamethasone dose dependently inhibited the expression of PCNA gene 24 h after treatment. A. Gel electrophoresis of RT-PCR products. GAPDH was used as a reference gene. GAPDH and PCNA were identified as 467 bp and 450 bp DNA bands, respectively (MW: molecular weight marker, C: control). B. Western blot analysis of PCNA protein. The tubulin (as an internal control) and PCNA were identified at 57 kDa and 36 kDa, respectively, using specific monoclonal antibodies.

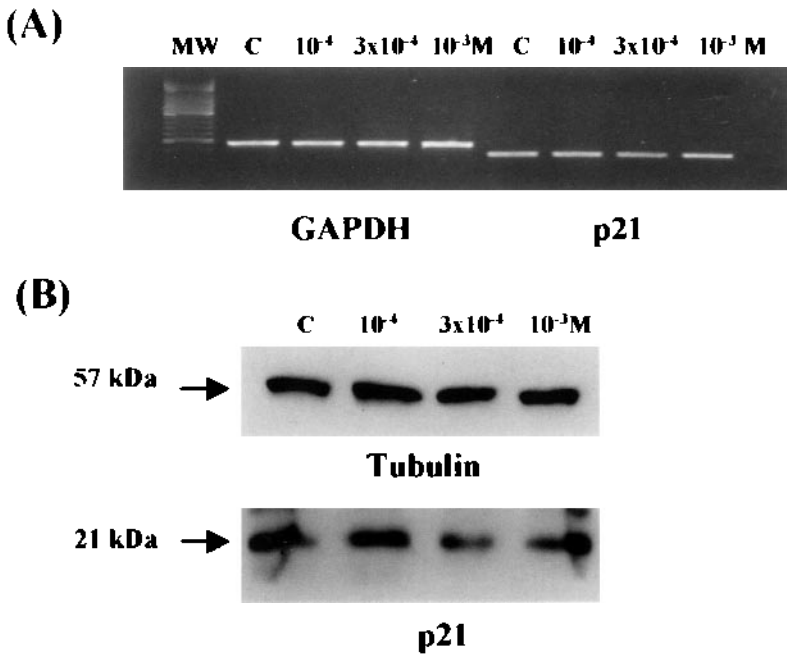


Figure 5. Dexamethasone had no effect on the expression of $p21^{CIP1}$ gene 24 h after treatment. A. Gel electrophoresis of RT-PCR products. GAPDH was used as a reference gene. GAPDH and $p21^{CIP1}$ were identified as a 467 bp and 355 bp DNA bands, respectively (MW: molecular weight marker, C: control). B. Western blot analysis of $p21^{CIP1}$ protein. The tubulin (as an internal control) and $p21^{CIP1}$ were identified at 57 kDa and 21 kDa, respectively, using specific monoclonal antibodies.

that Dex in concentrations above 5.8×10^{-5} M significantly reduced fibroblast proliferation and collagen synthesis, and concomitantly, diminution of the mechanical properties of the rabbit medial collateral ligament[6]. Therefore, to prevent tendon rupture in athletes receiving local steroid injections, caution should be advocated before any early return to the field, and/or resumption of heavy load training.

In conclusion, our study established the primary culture of fibroblasts from rat Achilles tendon and used it as a cell model to investigate the effect of Dex on fibroblast proliferation. The results provide novel information on the molecular events associated with the inhibition of rat tendon fibroblast proliferation in the presence of Dex. The $p21^{CIP1}$-independent decreased expression of PCNA mRNA and protein may account for the inhibition of cell proliferation of Dex-treated tendon fibroblasts.

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