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RESEARCH ARTICLE

Developing a Novel, Efficient and Cost Effective Tissue Injury Model *(in-vitro)* on Equine Tendon Fibroblasts

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ABSTRACT

Tendinopathy or tendon degeneration in equine is an illness caused by inflammation or injury. Being a difficult tissue to repair, tendon damage can cause physical disabilities. Successful tendon regeneration requires an appropriate *in-vitro* model for tissue injury. Current study was planned to compare the effect of two inflammatory mediators: prostaglandinE2 (PGE2) and hydrogen peroxide (H_2O_2) on equine tendon fibroblasts. Tendon fibroblast cultures were subjected to two different doses of PGE2 (100ng/ml and 200ng/ml) and H_2O_2 (500µM and 1mM). Compared with untreated tendon fibroblasts, all doses of PGE2 and H_2O_2 reduced cell viability markedly. Viability was assessed by trypan blue staining method and crystal violet staining. Lactate dehydrogenase (LDH) release was measured to assess injury and cell proliferation by MTT assay. In comparison of dose, 100ng/ml of PGE2 and 500µM of H_2O_2 showed optimal results, whereas, higher dose exhibited highly reduced cell viability and increased cytotoxicity, which may cause difficulty in studying tendon injury *in-vitro* model. These findings suggest that both PGE2 and H_2O_2 have equal potential to cause tendon fibroblasts injury. Therefore, H_2O_2 can be used as cost-effective *in-vitro* model for tendon degeneration studies.

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INTRODUCTION

Tendon is the tough connective tissue, which is affected severely from repetitive loading or mechanical stress resulting in Tendinopathy, especially in achilles tendon which is strongest tendon (Avella *et al*., 2009; Sobhani *et al*., 2013; de Cesar *et al*., 2018; Nikolikj *et al*., 2018). Tendinopathy results in chronic pain, weakening of the tissue and delayed healing, architecture destruction, disorganized collagen fibers, chondroid cellular metaplasia, deposition of calcifications and angiogenesis (Alfredson and Lorentzon 2000; Maffulli *et al*., 2003; Riley 2004; Sharma and Maffulli 2005; Arya and Kulig 2010; Maffulli *et al*., 2010).

There are limited *in-vitro* methods to test equine tendonitis as compared to *in-vivo*. Significance of inflammation is still debated in tendon disorders and it is considered that inflammatory mediators may trigger to increase long-term degenerative mechanisms (Dakin *et al*., 2012). PGE2 is an inflammatory mediator which possesses the ability to degrade tendon matrix which reduces the proliferation rate and collagen production of tendon fibroblasts (Cilli *et al*., 2004; Zhang and Wang 2010; Kawahara *et al*., 2015).

Oxidative stress is another cause of development of many diseases as it involves in DNA and protein damage (Singh *et al*., 2015, Toyokuni, 2016). Hydrogen peroxide (H_2O_2) is a form of reactive oxygen species (ROS), which is normally produced in cells during respiration and metabolism (Barbosa *et al*., 2013). Interaction of H_2O_2 with intracellular ions starts a chain reaction to produce more reactive radicals, which causes DNA and protein damage in cells (Jomova and Valko, 2011; Shen *et al*., 2017).

Purpose of present study was to compare the effects of H_2O_2 and PGE2 on viability of tendon fibroblasts, as well as shedding light upon the suitable dose for an optimum injury in tendon fibroblasts *in-vitro*.

MATERIALS AND METHODS

Ethical statement: All procedural steps of the experimentation were performed by following the guidelines provided by Research Ethical Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan after thorough approval from competent authority.

Collection of equine tendon: Tissue samples of Achilles tendon were obtained from adult horses (n=6), which were subjected to euthanasia for clinical reasons at University of Veterinary & Animal Sciences. Immediately after euthanasia, area above the hock was sterilized with 70% ethanol and an incision of about 3 inches was given to the skin with sterile surgical blade no. 11 to expose Achilles tendon. Tendon tissue (30 mm^2) was cut carefully with blade, washed and stored in Dulbecco Modified Eagle Medium (DMEM) and shifted to cell culture laboratory.

Monolayer culture of tendon fibroblasts: Collected tendon tissues were washed with phosphate buffer saline (PBS) and cut into small pieces of about 3mm in size, then digested with collagenase type1 (3mg/ml) solution (Sigma) for overnight on rotatory shaker (37°C). Isolated tendon fibroblasts were cultured in DMEM-HG containing 10% FBS, penicillin/streptomycin/gentamycin and amphotericin. These fibroblasts were incubated at 5% CO_2 and 37°C until attain 70-80% confluency and then were subjected to sub-culturing with trypsin-EDTA for passaging.

***In-vitro* induced injury:** At 4th passage of tendon fibroblast culture, *in-vitro* injury was induced by adding two different doses of PGE2 (100ng/ml and 200ng/ml) and H_2O_2 (500µM and 1mM) to the culture medium in 6-well plate. After six hours, medium was recovered from all experimental groups and fibroblasts were washed with PBS.

MTT assay: MTT assay was performed by following protocol used by Amjad *et al*. (2017). Briefly, tendon fibroblasts (1×10^5 in each well) were seeded on 96-well plate for overnight and washed with phosphate buffer saline (PBS) (Sigma). These fibroblasts were incubated with MTT dye solution (25 µl) (Sigma-Aldrich) for 2 hours or until changed to purple colour. 10% sodium dodecyl sulphate (SDS) (Invitrogen, USA) was added to each well and absorbance was recorded at 570nm (Amjad *et al*., 2017).

Cell damage assessment by Lactate dehydrogenase release (LDH): LDH release was assessed by following Wajid *et al.* (2014) protocol and using the kit (AMP Diagnostics, Austria). Briefly, 5µl medium of each group was added in reaction mixtures of kit, to make volume upto 100µl and incubated in 96-well plate for 5 to 10 minutes. Absorbance was taken at 340nm (Wajid *et al*., 2014).

Viability assays: Trypan blue assay and crystal violet assay was performed as described by Maqbool *et al*. (2019) with slight modification. Briefly, 100µl of trypan blue dye solution (Sigma-Aldrich) was added to fibroblasts. After 20 minutes, 5µl of solution was dropped off on hemocytometer and covered by coverslip. Viability was measured by dividing the number of cells excluding trypan blue with total number of cells multiplying by 100 (Maqbool *et al*., 2019).

Statistical analysis: Quantitative data of both groups (control and treatment) was statistically compared to evaluate the effect of different doses of the treatment. Analysis was performed by applying One-Way ANOVA and Tukey's test to compare all pairs of column as it was recommended by Graphpad software. P-value≤0.05 was assumed to be significant.

RESULTS

Obtaining primary and secondary cultures of tendon fibroblasts: Primary tendon fibroblasts isolated from tendon tissue were cultured in DMEM-HG and observed with inverted microscope. After about 48 hours, these isolated fibroblasts started to adhere to plastic surface of culture flasks. After each passage, these cells were appeared to be elongated and somewhat spindle-shaped with slight filopodia. Highest passage was achieved at the subculture level four (Fig. 1).

Estimation of injury induced by PGE2 and H_2O_2: LDH release suggested the level of injury caused in tendon fibroblasts after treatment with doses of H_2O_2 and PGE2. 1mM H_2O_2 (1.854±0.164%) and 200ng PGE2 (2.47±0.125%) showed significant increase in injury or cell death in cultured tendon fibroblasts as compared to control (0.515±0.043%). Whereas lower doses, 500µM H_2O_2 (1.165±0.150%) and 100ng/ml PGE2 (1.39±0.196%), showed an optimal injury level in tendon fibroblasts (Fig. 2).

Tendon fibroblasts prolificacy: MTT assay showed significant decrease in proliferation of tendon fibroblasts on treatment with both doses of PGE2 and H_2O_2. Therefore, 200ng/ml PGE2 (0.385±0.057) and 1mM of H_2O_2 (0.905±0.010) reduced the proliferation at much lower level as compared to the control group (2.321±0.019), which may lead to unbearable toxicity for tendon fibroblasts. Whereas 500µM H_2O_2 (1.318±0.013) and 100ng/ml PGE2 (1.253±0.037), reduced the proliferation rate at an optimal level (Fig. 3). Values of each assay are also given in table below (Table 1).

Viability of tendon fibroblasts: Viability of tendon fibroblasts was evaluated by two staining methods. Firstly samples were treated with trypan blue stain. After treatment with different doses, viability of fibroblasts was observed to be decreased significantly and more dead cells (dark blue stained) were found on treatment with 200ng/ml PGE2 (54.8±0.85%) and 1mM of H_2O_2 (51.5±1.71%) as compared to untreated group (8.00± 1.29%) and lower doses of PGE2 and H_2O_2 (Fig. 4).

Table 1: Values of all assays performed on administration of H_2O_2 and PGE2

Assays	Control	H_2O_2 500uM	H_2O_2 1mM	PGE2 100ng/ml	PGE2 200ng/ml
MTT	2.321±0.019	1.318±0.013	0.905±0.010	1.253±0.037	0.385±0.057
Trypan Blue (%)	8.00±1.29	34.8±2.95	51.5±1.71	38.3±1.11	54.8±0.85
LDH (%)	0.515±0.043	1.165±0.150	1.854±0.164	1.39±0.196	2.47±0.125
Crystal Violet	84.6±4.33	49.0±1.15	27.3±0.33	52.2±1.20	33.0±1.15

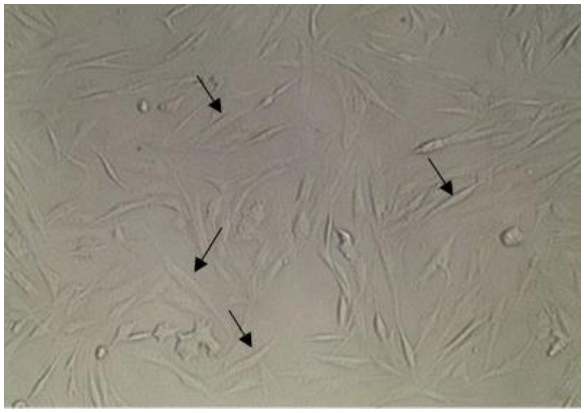

Fig. 1: Morphology of tendon fibroblasts is somewhat spindle-shaped and elongated (indicated as black arrow).

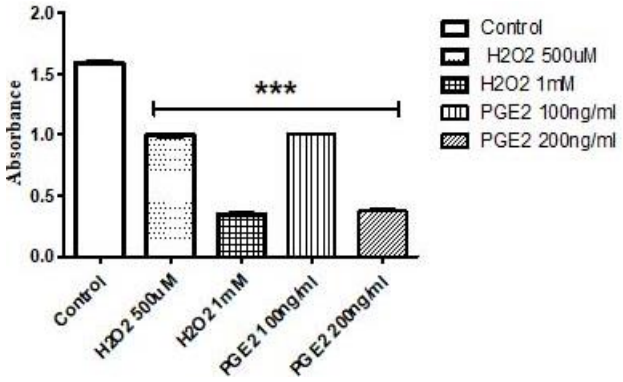

Fig. 3: MTT assay for all groups shows a significant difference between proliferation of control and treated tenocytes. Significantly reduced proliferation was observed in Bar Graphs on increasing the dose for H_2O_2 and PGE2. Star indicates a significant difference from control (*P-value<0.05).

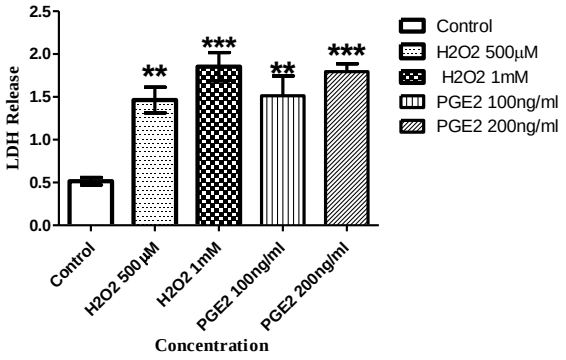

Fig. 2: Graphical representation of release of LDH by injured tendon fibroblasts. Tendon fibroblasts showed increased injury as concentration of H_2O_2 and PGE2 increases. Mean ± SEM was used to express values (*P<0.05 vs. control).

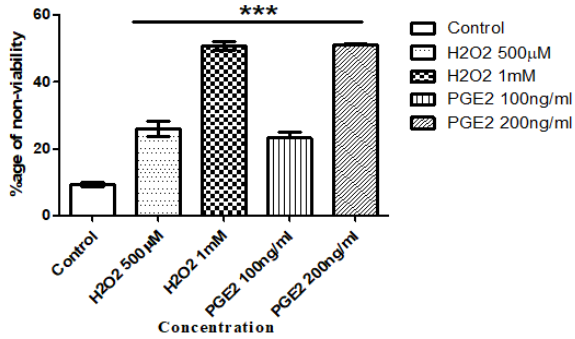

Fig. 4: Percentage analysis of non-viable tendon fibroblasts by trypan blue exclusion assay. 1mM concentration of H_2O_2 showed significant low viability of tenocytes on comparing untreated one and 500µM whereas, PGE2 decreased maximum viability of tenocytes at concentration of 1mM significantly as compared to control. Star shows a significant difference from control. *P-value≤0.05 was considered significant.

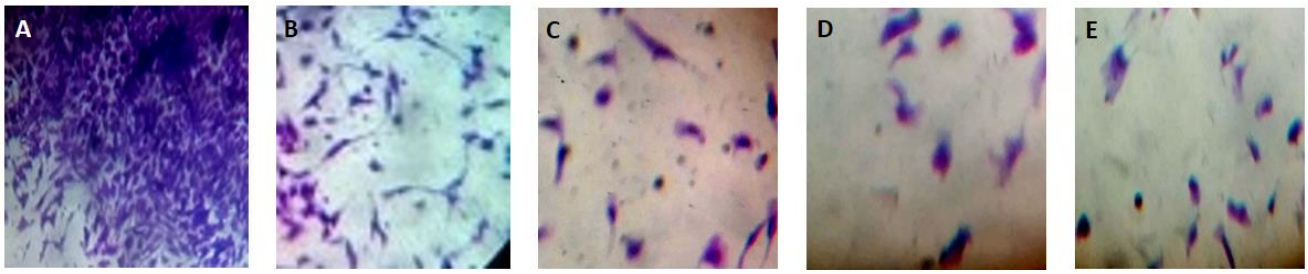

Fig. 5.1: Microscopic view of treated and untreated tendon fibroblasts incubated with crystal violet stain A) untreated tendon fibroblasts, B) and D) showed decreased viability depending on dose of H_2O_2 i.e., 500µM (B) and 1mM (D). Similarly, almost similar decreased viability was noted on treatment with PGE2 100ng/ml (C) and 200ng/ml (E).

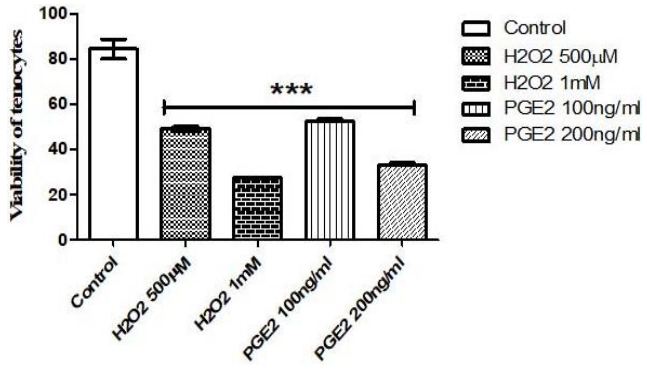

Fig. 5.2: Graphical representation of crystal violet staining results. Significant lower viability on increasing dose of H_2O_2 and PGE2 as compared to control. Values were exhibited as Mean ± SEM (*P<0.05 vs. control).

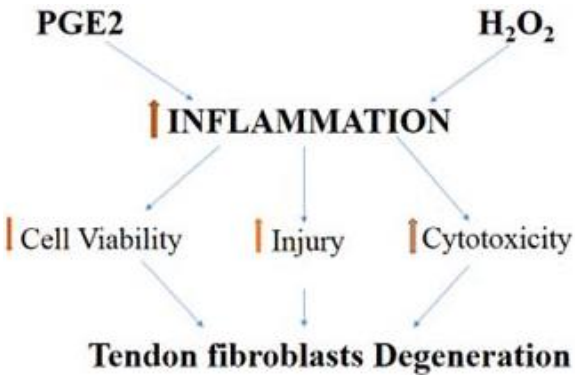

Fig. 6: Summarized results of PGE2 and H_2O_2 on tendon fibroblasts.

Second method used was Crystal violet staining, which showed significantly reduced level of live cells (stained in violet colour) on treatment with 200ng/ml PGE2 (33.0±1.15) and 1mM of H_2O_2 (27.3±0.33) as compared to the untreated fibroblasts (84.6±4.33). 500µM H_2O_2 (49.0±1.15) and 100ng/ml PGE2 (52.2±1.20) reduced the live cells but at lower level as compared to higher doses (Fig. 5.1 & 5.2).

DISCUSSION

Major challenge to study *in-vitro* tendon injuries is to find cost-effective *in-vitro* injury model. There is lack of both *in-vivo* and *in-vitro* model to mimic conditions of degeneration that occur in tendonitis. The current study demonstrated that on treating tendon fibroblasts with 100ng/ml of PGE2, reduced viability and increased injury of fibroblasts was observed. These results were consistent with recent studies in which 100ng/ml PGE2 resulted in decreased proliferation of tendon stem cells (TSCs) and that study also assumed that TSCs on treatment with 100ng/ml dose of PGE2 resulting in decreased number of viable tendon fibroblasts (Zhang and Wang 2010). Another study also showed reduced cell proliferation by 11% on treatment with 100ng/ml of PGE2. It was suggested, that further increase in dose of PGE2 may inhibit the production of collagen by inducing matrix metalloproteinases (MMP-1 and MMP-3) (Cilli *et al*., 2004). PGE2 may also affect mRNA stability which in turn decreases collagen production (Thampatty *et al*., 2006). Another study reported the effect of 500ng of PGE2 in tendon, which caused more degeneration in tendon as compared to saline-injection or needle-stick method. 500ng dose of PGE2 influenced physiologic and toxic effects by loosening the organizations of tendon collagen fibrils. It confirmed our study that higher doses may cause toxicity in fibroblasts (Khan *et al*., 2005). Our study also supported a recent study, which described that induction of higher level of inflammatory mediators like PGE2 decrease the expression of type I collagen and increase type III collagen mRNA in tendon fibroblasts which alternatively enhances the injury (Bauge *et al*., 2015).

H_2O_2 is considered to be a key model for redox modulation, as higher level can increase cellular oxidative stress which can ultimately reduce cell viability and increase apoptosis. Our results showed similar results with a previous report, which used different concentrations of H_2O_2 on tendon-derived stem cells (TDSCs). In this study, 500µM H_2O_2 was used as higher concentration which caused redox modulation and decreased cell viability, abolished colony formation units as well as increased apoptosis in TDSCs (Lee *et al*., 2017). Similarly, oxidative stress was induced in human rotator cuff tendon by H_2O_2 and 500µM H_2O_2 induced oxidative stress (as this is higher than the intracellular physiological level), whereas, much higher dose about 1mM may induce tenocyte necrosis (Doroshow and Davies 1986; Yuan *et al*., 2003). H_2O_2 may result in damage of cell structure and change in function by generating reactive oxygen species (ROSs) (Chen *et al*., 2016). Likewise PGE2, H_2O_2 challenge also promoted higher expression level of both MMP1 mRNA and protein on treatment with human tendon cells in-vitro (Wang *et al*., 2007). Our study was consistent with previously reported studies but 1mM concentration showed much reduced viability of tendon fibroblasts than 500µM. Thus, 500µM demonstrated moderate reduced viability as compared to control group. Our current results outline a possible pathway in tendon degeneration caused by PGE2 and H_2O_2 (Fig. 6).

Conclusions: Present study illustrated that both inflammatory mediators PGE2 and H_2O_2 have almost equal potential to induce injury in tendon fibroblasts *in-vitro*. Depending upon the dose, 500µM of H_2O_2 and 100ng/ml of PGE2 could provide an optimal *in-vitro* injury model for tendon fibroblasts, whereas higher dose may cause deleterious effect to cells. H_2O_2 is cheaper than PGE2 and should be preferred for *in-vitro* tendon degeneration study.

Authors contribution: SS, MJ and SJA conceived the idea, developed the project objectives and executed the project. SS also conducted sampling. AN assisted in data analysis and data interpretation. TY assisted in manuscript write up and editing.

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