Enhance of Migration and Proliferation of Cells from Tendon Biopsies by High Voltage Pulsed Current Stimulation

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<Abstract>

The purpose of this study was to examined whether high voltage pulsed current stimulation (HVPCS) enhances the migration and proliferation of fibroblasts from tendon biopsies to provides evidence that the cellular activities of fibroblast are enhanced by HVPCS. Flexor digitorum profundus tendon of chickens were excised, biopsied and cultured in M199 medium for a day. The biopsies through which a cathodal HVPC with 100 pps, 50 V for 30 minutes was passed in medium. A day after treatment, the biopsies embedded in fibrin clot were covered by the addition of 1 ml of M199 medium to the well, and placed in the CO_2 incubator for the duration of the experiment. The migration distance of cells from tendon biopsies were measured at 6 days after treatment, and proliferation of cells from tendon biopsies were measured at 7 days after treatment. The migration distance of cells from tendon biopsies in the HVPCS group demonstrated significantly greater than the shame treated control group ($t=-2.675$, $p<0.05$). Also HVPCS had significantly increased optical density of fibroblasts from tendon biopsies ($t=-2.136$, $p<0.05$). These results indicate that the HVPCS with 100 pps, 50 V for 30 minutes enhanced either the migration and proliferation of fibroblast from tendon biopsies. These results supposed that the HVPCS activates cellular responses in fibroblasts from tendon biopsies. This suggests that enhanced the migration and proliferation of fibroblast by HVPCS may be one of the mechanism involved in tendon healing.

KEY WORDS : electrical stimulation, high voltage pulsed current, fibroblast, migration, proliferation, tendon

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INTRODUCTION

The use of electrical stimulation to promote tissue healing has been reported by many authors. In especially, there has been several reports that high voltage pulsed current stimulation (HVPCS) can accelerate dermal wound healing (Kloth and Feedar, 1988; Feedar, Kloth and Gentzkow, 1991; Griffin et al, 1991) and tendon healing (Owoeye et al, 1987).

Healing of tendon injuries follows the general process of inflammation, proliferation and remodeling that occurs as well as dermal wounds. During the initial inflammatory phase of tendon repair, fibrin and other components of inflammatory cells and cells derived from the tendon. in the subsequent proliferative phase of tendon healing, fibroblasts proliferate, new blood vessels are formed and extracellular matrix is produced, eventually leading to the formation of tendon fibers.

Fibroblasts play a key role in tendon repair process and is the major source of collagen production, protein mediators of repair and matrix proteoglycans (Leadbetter, 1992). Fibroblast derived from the outer tendon surface (epitenon) and from within the tendon (endotenon) migrate into the defect site and make a bridge of the gap between the tendon ends with collagen fibres and fibroblasts (Wang, 1998; Woo et al, 1999). Initially, type III collagen is rapidly laid down in a woven pattern because these disulfide crosslinks can be generated rapidly to provide mechanical strength to the newly synthesized matrix (Liu *et al*, 1995). The remainder of the repair process is characterised by a shift to the deposition of type 1 collagen which continues for an indeterminate period in the final remodelling phase (Leadbetter, 1992). Overall the collagen that is deposited in the proliferative phase, has its fibrils randomly oriented such that the scar appears as a disorganised meshwork of collagen fibers (Tomas et al, 1987).

The migration, proliferation and activity of the fibroblasts may regulated by an exogenous electricity. Brown and Loew (1994) has reported that the fibroblast exhibit persistent, cathode directed motility when exposed to direct current electric fields. Erickson and Nuccitelli (1984), also has reported that the fibroblast migrated towards the cathode of the field by extending lamellipodia in that direction. Fibroblasts are attracted by the negative pole to proliferate and synthesize collagen (Bourguignon et al, 1989). Bourguignon and Bourguignon (1987) have reported that an high voltage pulsed current can stimulate protein and DNA synthesis in fibroblast with polarity, voltage and frequency dependencies.

In this study, we have examined the effects of HVPCS in cell migration and proliferation of fibroblast in vitro model of tendon biopsies derived from digital flexor tendon to provides evidence that the cellular activities of fibroblast from tendon biopsies are enhanced by HVPCS.

MATERIALS and METHODS

Cell Culture

Flexor digitorum profundus tendons were removed from the middle toes of feet collected on ice from chickens that had been slaughtened at Inghams Enterprises Pty Ltd (Burton, South Australia). Chickens were generally 31 to 34 days old. 2 mm biopsies were taken from the tendons using biopsy punch (Stiefel Laboratories Pty Ltd, NSW, Australia) and placed in the wells of 24 well polystyrene tissue culture plates (Cellstar, Greiner Labortechnik Ltd, Gloucestershire, UK) with 5 ml of M199 medium.

A day after treatment, 12 biopsies were embedded in fibrin clots. To embed the biopsies in fibrin, the biopsies were first covered with 5 $\mu\ell$ of a 2 mg/ml solution of bovine thrombin (Sigma, Prod. No. T4648, USA), and clots were then formed by covering the biopsies with 50 $\mu\ell$ of a 4 mg/ml solution of bovine fibrinogen (Sigma, Prod. No. F8630, USA). Fibrinogen was dissolved in 0.9% saline that had been warmed to 30℃. Clots were allowed to set for 1 hour in a humidified CO_2 incubator at 37℃. The biopsies embedded in fibrin were covered by the addition of 1 ml of M199 medium to the well, and placed in the CO_2 incubator for the duration of the experiment. The medium was changed everyday.

M199 medium (Sigma, Cat No. M-5017, USA) was used throughout. Each liter of medium contained 10 mls of a solution containing 10,000 units Penicillin, 10,000 μg Streptomycin and 25 μg Fungizone (CSL Ltd., Cat No. 09291501, Vic, Australia). 0.1 mM of ascorbic acid (Fisons Scientific Equipment, UK) was added to the medium to provide conditions that are optimal for collagen production (Conlon et al, 2001). Also, the medium contained 5% Fetal Bovine Serum (CSL Ltd., Cat No. 09702301, Vic, Australia).

High Voltage Pulsed Ccurrent Stimulation (HVPCS)

For expose HVPCS, the biopsies placed in the wells of 6 well polystyrene tissue culture plates (Cellstar, Greiner Labortechnik Ltd, Gloucestershire, UK) filled with 5 ml of M199 medium. Flat and rectangular electrodes (23 x 6 mm) made of stainless steel were fixed at each end of well. The voltage of 50 V, with pulse rates of 100 pps and negative polarity were applied across the well for 30 minutes at room temperature. The high voltage pulsed current stimulator used in this study was the Pulsed High-Volt Stimulator (Intelect® HV2, Chattanooga Group Inc., 4717 Adams Rd., P.O.Box 489, Hixson, TN 37343, USA). The high voltage pulse current stimulator wave form consists of monophasic, twin-peak pulses that have a fixed pulse duration of 65 μs.

Measurement of cells migration from tendon biopsies

Cell migration measurements were carried out by viewing cells in the transparent fibrin clot using a Nikon TMS-F inverted microscope with phase-contrast optics (Nikon Corporation, Tokyo, Japan). Using an eyepiece micrometer, the maximum distance that cells had travelled from e biopsy edge was determined in each of the 4 quadrants surrounding the tendon biopsy. An average migration distance (mm) from each biopsy was calculated from 4 values.

Cell Proliferation Count from tendon biopsies

To determining the viable cells in proliferation assay, the biopsies were removed from the

24 wells, then add 20 $\mu\ell$ of CellTiter 96® (Promega, Prod No. G3580, USA). The cultured media were incubated for 2 hours at 37℃ in a 5% CO_2 atmosphere. After incubation, transfer 100 $\mu\ell$ of medium from 24 well plate into 96 well plate. then the 96 well plates with cultured media were measured on a ELISA reader (EIA 2552, Bio-Rad Laboratories, USA) at wavelength of 490 nm.

Statistical analysis

For comparisons of the distance of cell migration from tendon biopsies between the shame treated control group and the HVPCS group, a Student's *t*-test was used. The Student's *t*-test was also used to compare a difference of proliferation of cell from tendon biopsies between the shame treated control group and the HVPCS group. Significance was deemed to be $p<0.05$. SPSS WIN(ver 7.5) software was used for the analyses.

RESULTS

HVPCS increase of cells migration distance from tendon biopsies

The cells began migrating from biopsies after 3 to 4 days, and migration distances were measured at 7 day after a HVPCS and shame treatmen when cells were in some cases approaching the edge of the clot. In shame HVPCS treated control biopsies, the mean migration distances were 0.81±0.19 mm. In HVPCS treated biopsies had mean migration distance of 1.06±0.28 mm. Student's *t*-test showed a significantly higher the cell migration distance in the HVPCS treated biopsies than control biopsies ($t=-2.675$, $p<0.05$) (Table 1). HVPCS had significantly increased migration of fibroblasts from tendon biopsies into fibrin clots (Fig. 1).

HVPCS increase of cells proliferation from tendon biopsies

The optical densities were measured at 8 day after a HVPCS and shame treatmen. In shame HVPCS treated control biopsies, the mean optical densities were 0.44±0.05. In HVPCS treated biopsies had mean optical densities of 0.49±0.03. Student's *t*-test showed a significantly higher the mean optical densities in the HVPCS treated biopsies than control biopsies ($t=-2.136$, $p<0.05$) (Table 1). HVPCS had significantly increased proliferation of fibroblasts from tendon biopsies (Fig. 2).

Table 1. Group t-test of mean migration distance and optical density of cells biopsies from flexor digitorum profundus

	df	DM	SE	t	p
migration distance	22	-.253875	.096963	-2.618	.016
optical density	22	-.045000	.017555	-2.536	.018

Fig. 1. A comparison of the effect of shame treatment and HVPCS on migration distance of cells from tendon biopsies. Migration distances were measured 6 days after shame and HVPCS treatment. HVPCS had significantly increased migration of fibroblasts from tendon biopsies. Values are the mean and SD (n=12). The symbol * denotes a significant difference ($p<0.05$).

Fig. 2. A comparison of the effect of shame treatment and HVPCS on optical density of cells from tendon biopsies. Optical densities were measured 7 days after shame and HVPCS treatment. HVPCS had significantly increased proliferation of fibroblasts from tendon biopsies. Values are the mean and SD (n=12). The symbol * denotes a significant difference ($p<0.05$).

DISCUSSION

Results from this study show that HVPCS are capable of inducing increases in migration and proliferation of fibloblast from tendon biopsies. We have found that application of 100 pps, 50 V HVPCS for 30 minutes causes increase cell migration distance in negative polarity. Motile cells use chemotaxis, galvanotaxis and haptotaxis in their environment to guide their migration. Galvanotaxis is a guidance of motility by electric fields. Fibroblast exhibit galvanotaxis which migrating towards the negative pole. Galvanotaxis has been documented in many cell types and may play an important role in those cellular responses where local electric fields are present. Erickson and Nuccitelli (1984) have reported that the fibroblasts migrated towards the cathodal end of small electrical field. Soong et al (1990) also have shown that the corneal stromal fibroblasts exhibit cathode directed motility when exposed to direct current. A similar response was observed the murine fibroblasts to migrate towards the cathode (Brown and Loew, 1994). We also have observed that HVPCS with 100 pps, 50 V for 30 minutes enhance the rate of proliferation of fibroblast from tendon biopsies. Electric fields have been reported to promote the proliferation of fibroblast (Gentzkow, 1993). Bourguinon and Bourguinon (1987) have reported that the HVPCS increases in synthesis of protein and DNA in fibroblast. Bourguinon et al (1989) also, reported that the HVPCS triggers the voltage-gated Ca^{++} channel, induces Ca^{++} influx into the fibroblast, and stimulates protein synthesis. Fitzsimmons et al (1989) have reported that the electric field were increased cell proliferation and release of mitogen activity into the cell.

Recently, several mechanisms have been reported for increase of fibroblast activities by electrical stimulation. The electrical properties of cells are determined by their high cell membrane resistance to ionic current. The ionic current act an ionophoretic force on charged proteins and lipids in the cell membrane which exert to redistribution of membrane components. The electrical stimulation can alters the electrical properties of cell membranes. Therefore, the electrical stimulation induces cellular responses including lateral redistribution of membrane proteins such as ion channels and receptors (Brown and Loew, 1994), and changes in intracellular calcium ion concentration. Intracellular Ca^{++} regulates many biological processes including signal transduction cascades, cytoskeletal reorganization, cell orientation and migration, and cell differentiation and proliferation (Bourguinon et al, 1989; Kim et al, 1998; Cho et al, 1999).

CONCLUSION

The results of this study revealed a statistically significantly increased the migration distance of cells, also, increased the proliferation of cells from tendon biopsies. These results indicate that the HVPCS with 100 pps, 50 V for 30 minutes increased either the migration and proliferation of fibroblast from tendon biopsies. These results supposed that the HVPCS activates cellular responses in fibroblasts. This suggests that enhanced the migration and proliferation of fibroblast by HVPCS may be one of the mechanism involved in tendon healing.

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<국문초록>

고전압맥동전류자극에 의한 생검 건의 세포 이동 및 증식 증진

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본 연구는 고전압맥동전류자극이 생검 건의 세포 이동 및 증식의 증진 여부를 규명하기 위하여 시행하였다. 닭의 심지굴근건을 적출하고 직경 2 mm의 생검하여 배양하였다. 생검 건에 100 pps, 50 V로 30분간 음극으로 고전압맥동전류자극한 후 생검 건을 피브린로 피복하고 6일간 배양한 후 세포의 이동거리를 측정하고 7일 후 세포증식능을 측정하였다. 대조군과 고전압맥동전류자극군의 세포 이동 거리 및 흡광도를 t-검정한 결과 고전압맥동전류자극군의 세포 이동 거리가 유의하게 증가하였으며 ($t=-2.675$, $p<0.05$), 흡광도도 유의하게 증가하였다 ($t=-2.136$, $p<0.05$). 이러한 결과는 고전압맥동전류자극이 생검 건 섬유모세포의 성장과 증식을 유발시키고 있음을 보여주고 있으며 이러한 결과는 고전압맥동전류자극이 섬유모세포의 세포반응을 활성화시켰음을 시사하고 있어 고전압맥동전류자극의 건손상 치유 기전의 일부를 제시하였다.

Fig. 1. A comparison of the effect of shame treatment and HVPCS on migration distance of cells from tendon biopsies. Migration distances were measured 6 days after shame and HVPCS treatment. HVPCS had significantly increased migration of fibroblasts from tendon biopsies. Values are the mean and SD (n=12). The symbol * denotes a significant difference ($p<0.05$).

Fig. 2. A comparison of the effect of shame treatment and HVPCS on optical density of cells from tendon biopsies. Optical densities were measured 7 days after shame and HVPCS treatment. HVPCS had significantly increased proliferation of fibroblasts from tendon biopsies. Values are the mean and SD (n=12). The symbol * denotes a significant difference ($p<0.05$).