European Review for Medical and Pharmacological Sciences 2020; 24: 12041-12049

Higher frequency electrical stimulation enhanced myloglossus satellite cell differentiation by upregulating expression of Pax7 mRNA, MyoD, myogenin and MyHC protein

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Abstract. – OBJECTIVE: We investigated the effect of electrical stimulation (ES) of varying pulse frequency on differentiation and proliferation of canine myloglossus satellite cells *in vitro.*

MATERIALS AND METHODS: Cellular viability and proliferation were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay and flow cytometry fluorescence-activated cell sorting analysis. Cellular differentiation and expression of mark molecule were assayed by Real Time-PCR and Western blot.

RESULTS: With increasing frequency ES, we found a significant increase in Myod (r=0.988, *p*<0.0001), myogenin (r=0.988, *p*<0.0001), MyHC-slow (r=0.988, *p*<0.0001), MyHC-fast (r=0.875, *p*<0.0001) protein expression, and Pax7 mRNA expression (r=0.712, *p*=0.001).

CONCLUSIONS: Pax7 mRNA expression and MyoD, myogenin, and MyHC protein expression were increased with increment of electrical stimulation frequency in myloglossus muscle satellite. Higher frequency ES enhanced myloglossus satellite cell differentiation, not proliferation and viability.

Key Words:
Satellite cell, Myloglossus, Electrical stimulation, Frequency, Dog, Differentiation, Proliferation.

Introduction

Functional neuromuscular stimulation (FNS) has been proposed as a potential therapy for obstructive sleep apnea syndrome, recurrent laryngeal nerve paralysis, and denervated muscle atrophy[1,2]. Various pacemakers produced by the principle of functional electrical stimulation are also being gradually applied in clinical practice. Muscle pacemakers, such as paralyzed cricoarytenoid muscle pacemaker, paralysed detrusor of bladder pacemaker, and apnea muscle pacemaker have entered clinical trials and been used in clinic. The effects of electrical stimulation by pacemakers on muscles, both paralytic and normal, have not been elucidated, especially as the cytological changes are more ambiguous. In recent years, studies have found that muscle satellite cells are the main source of muscle regeneration undergoing electrical stimulation. *In vitro*, the application of electrical stimulation (ES) partially attenuates muscle atrophy and changes satellite cell activity[3]. Continuous ES of muscle also bring about conversion of muscle fiber isoforms *in vivo*[4-6].Therefore, we carried out the research based on myloglossus satellite cell by electrical stimulation in order to provide useful information on the optimal electrical stimulatiom pattern which can be achieved in head neck muscle pacer application.

In adult healthy muscle fibers, satellite cells normally do not proliferate and differentiate. In response to injury or ES, myogenic satellite cells start to proliferate and differentiate[3,7,8]. Following injury and ES, satellite cells initially express Pax7, Myf-5, MyoD, desmin, and myogenin which are mark molecule of satellite that proliferate and differentiate. Subsequently, the expression of structural muscle genes, such as myosin heavy chain,

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is triggered[9-12]. *In vivo,* continuous ES of rabbit fast muscle at 10 pulse per second (pps) results in complete conversion of muscle fibers to the slow phenotype[13]. Muscles receiving 2.5 Hz short-term stimulation were almost indistinguishable in histologic appearance from control muscles. If muscles received long term 2.5 Hz ES, fast myosin isoforms were found to predominate[4].

ES pattern may impact the process of satellite cell differentiation and proliferation *in vivo* and *in vitro*[4,13,14]. However, the optimal ES frequency remains unknown, although likely important[4,13]. We aimed to investigate the effect of ES of varying pulse frequency on differentiation and proliferation of head and neck muscle satellite cells in large animals *in vitro.* We will present the finding that satellite cell differentiation and proliferation may be modulated by varying ES frequency in different stages[15].

Materials and Methods

This study was approved by the Guangzhou Medical University Research Ethics Committee Review Board before conducting this investigation (No. Protocol: GDREC2013151A).

Primary Satellite Cell Isolation and Culture

The Institutional Animal Care and Use Committee at the Guangzhou Medical University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, approved this study. Three female beagles were purchased from Gaoyao Kangda Laboratory Animal Care. The age of the dogs at time of surgery ranged between 3 and 4 months. The dogs were housed in groups of 2-3 animals, separated for feeding twice daily. Animals spend approximately 22 h a day running free in indoor pens. Pen sizes were 8 m^2/2. Each dog was fed dry dog food (SDS Breeder Diet) twice daily approximately. Water was available at all times. All dogs were enrolled in a veterinary preventative medicine program that included a standard immunization series against canine distemper, parvovirus, adenovirus type 2, parainfluenza virus, coronavirus, and rabies.

The animals were anesthetized with ketamine hydrochloride 35 mg/kg, xylazine hydrochloride 5 mg/kg, and acepromazine maleate 0.75 mg/kg administered by intramuscular injection. Subsequently, intramuscular injection of ketamine hydrochloride (17 mg/kg) and acepromazine maleate (0.375 mg/kg) maintained a surgical plane of anesthesia. The heart rate and spot oxygen saturation (SpO2) were monitored throughout the experiment to monitor the animal's state of anesthesia. Animals were euthanized using overdose of intravenous anesthetics agent without recovery from anesthesia after biopsy.

The skeletal muscle biopsy was harvested from the myloglossus muscle of three donor canines. Three dogs biopsy specimens were separately trimmed into smaller pieces along the length of the fibers, and then, digested with 1 g/L collagenase type IV (Gibco, Grand Island, NY, USA), 2 g/L collagenase type I (Gibco, Grand Island, NY, USA), and 2 g/L trypsase (Gibco, Grand Island, NY, USA) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) for 30 min at 37°C. The digested muscles were triturated and filtered through a series of stainless steel mesh filters (diameter 40 μm). The satellite cells released from the muscle were washed twice in phosphate-buffered saline (PBS). The resulting cell pellet was resuspended in DMEM and Ham's F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, Grand Island, NY, USA), 0.5 nM recombinant human FGF2 (Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Gibco, Grand Island, NY, USA), and 0.1 μg/mL streptomycin (Gibco, Grand Island, NY, USA). Canine satellite cells were transferred to a tissue culture flask coated with 0.1% pig-skin gelatin (PSG; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C in a humidified atmosphere of 95% O2 and 5% CO_2.

Immunofluorescence for Satellites Verification and Assessment

The cells were fixed with 4% paraformaldehyde, washed with PBS (KeyGen Biotechnology, Nanjing, China), and permeabilized with 0.1% Triton X-100 in distilled water at room temperature (RT) for 5 min. In order to prevent non-specific binding of the primary antibody, the cells were blocked with 2% horse serum (Gibco, Grand Island, NY, USA) for 30 min at RT. Thereafter, the cells were washed with PBS, incubated with primary antibodies overnight at 4°C, rinsed with PBS, and incubated with secondary antibodies diluted 1:100 in PBS for 1 h at RT. The cells were washed again three times with PBS, before nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; Bio-Rad, Hercules, CA, USA) at a

dilution of 1:20 in PBS and incubated for 3 min at RT. Frozen sections were post-fixed in 4% paraformaldehyde before staining. The primary antibodies used were: anti-desmin 23879 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:500, and anti-Pax7 (Sigma-Aldrich, St. Louis, MO, USA) at 1:500. Staining was visualized and photographed with light microscopy (SP5-FCS, Leica, Wetzlar, Germany).

Electrical Stimulation of Cell Culture

At 70% confluence, each dog muscle satellite cell was harvested and seeded onto 10 six-well dish at a concentration of $1x10^5$ cells/cm^2 (Corning Life Sciences, Tewksbury, MA, USA) which maintained in culture at 5% CO2 and the 37°C medium was changed daily.

The apparatus for ES was composed of two platinum steel electrodes (height 0.8 cm, length 1 cm, separation gap 14-mm). Electrodes were enclosed in an autoclavable Plexiglass cover. The platinum electrodes were immersed and maintained in a perpendicular position onto the bottom of the six-well circular culture dishes. The electrical leads were connected to a generator (Master 9, AMPI, Jerusalem, Israel). The amount of voltage between the electrodes were measured and verified by a signal collection system (BL-420S, Tai-Meng, Chendu, China).

Stimulation consisted of a biphasic 4-V pulse of 1 ms duration (5 s train time, 3 s on, and 2 s off). The patterns of electrical pulse delivery (0, 2, 10, 40, 80, 160 pps frequency) were used to study the effect of varying frequency. There were no changes in the temperature of the cell culture medium or cell death observed after ES. The analysis was performed after 72 h of ES. We measured cell viability, cell cycle progression and proliferation, and cell differentiation following electrical stimulation.

Cell Viability

We measured cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay (KeyGen Biotechnology, Nanjing, China). A 0.5 mg/mL of MTT solution was added to cell samples and incubated for 3 h at 37°C. The cells were lysed in 90% isopropanol solution. After completely dissolution, the cells were collected and centrifuged at 256 *g* for 5 min. The absorbance of clear solutions was measured with a spectrophotometer (Multiskan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 580 nm. Measurement was repeated twice per specimen. The proportional to the number of viable cells was recorded with the optical density value (OD).

We also evaluated cell survival with a Live/Dead assay (Sigma-Aldrich, St. Louis, MO, USA) after seeding. Briefly, 150 µL calcein (3.5 µM) and ethidium bromide (3.0 µM) in D-PBS (KeyGEN BioTECH, Nanjing, China) were added to the seeded slide, which was incubated for 45 min at RT. After incubation, the labeled cells were observed under fluorescent microscopy[12].

Cell Cycle Progression and Proliferation

The percentage of cells in each cell cycle was determined using Cell Lab Quanta SC (Cell Lab QuantaTM SC MPL, Beckman Coulter, Brea, CA, USA). Cell proliferation was also assayed by evaluating the percentage of S phase cells by fluorescence-activated cell sorting (FACS) analysis. Briefly, subconfluent cultures were harvested and cultured as a single cell suspension (10^5 cells/mL) in semi-solid DMEM containing 1.3% methyl cellulose and 20% FBS (>98% of cells arrest in G0 by 48 h). Satellite cells were collected by trypinization and centrifugation at 256 g for 3 min. The cells were resuspended in 1 mL PBS and collected by centrifugation at 1000 rpm for 3 min. This step was repeated twice. After removing the PBS, the cells were resuspended in staining buffer. Flow cytometric analysis of DNA content was performed twice using propidium iodide (PI) staining and analyzed on FACS Vantage using Cell Lab Quanta SC software.

Total RNA Isolation and quantitative Real Time-PCR for Cell Differentiation

Total RNA was isolated using TRIzol reagent (Gibco, Grand Island, NY, USA) from cultured myloglossus satellite cells. The total mRNA was stored at -80°C.

Reverse transcription and Real Time-PCR was performed using PrimeScript RT Kit (TaKaRa Bio Group, Otsu, Shiga, Japan) and SYBR Premix ExTaq Kit (TaKaRa Bio Group, Otsu, Shiga, Japan). Real Time-PCR reaction mixture composed of 10 µM primer one, 10 µM primer two, 12.5 µL SYBR Premix Ex Taq TM, and 0.5 µL template complementary DNA ribonuclease-free water. Canine-specific primers were used for Pax7, Myf5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Gene Bank, Vipotion Biotechnology Company Design, Guangzhou, China) (Table I). The following conditions were performed for PCR: 1 cycle at 94°C for 2 min, followed by 40 cycles at

94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 1 cycle at 50°C, increasing to 95°C in 0.5°C increments to make a melting curve.

PCR was repeated twice per specimen. We used a Real Time-PCR detection system (Agilent Stratagene Inc., Santa Clara, CA, USA) to detect PCR products. Relative quantitative gene expression was determined by the ratio of Pax7, Myf5 gene concentration to the GAPDH. We used gel electrophoresis to verify PCR products according to fragment size in 2.5% agarose gels containing 0.5 μg/mL ethidium bromide[13].

Western Blotting

The following antibodies were used for Western blot analysis: desmin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-slow skeletal myosin heavy chain antibody (Abcam, Cambridge, MA, USA); anti-fast skeletal myosin heavy chain antibody (Abcam, Cambridge, MA, USA); anti-MyoD (sc-31942, Santa Cruz Biotechnology), anti-myogenin antibody (sc-576, Santa Cruz Biotechnology, Dallas, TX, USA), and anti-GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). Harvested canine satellite cells were washed twice in cold PBS and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer [(1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentration was measured by the BCA Protein Assay and lysates were stored at -80°C. Total protein supplemented with protease inhibitors (Gibco, Grand Island, NY, USA) were electrophoresed on 5%-12% gradient polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred overnight to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in Starting Block (Bio-Rad, Hercules, CA, USA), and then, incubated overnight at 4°C with primary antibody diluted 1:1000 in starting block. The membranes were washed in Tris-Buffered Saline (TBS) containing 0.1% Tween-20, and then, we incubated the with the secondary antibody (1:7500, KGP, Nanjing, China) for 1 h at RT. After chemiluminescence, quantification of MyHC-slow, MyHC-fast, myogenin, MyoD, and GAPDH was performed using a FluorChem 8900 image system (Alpha Innotech, Miami, FL, USA). Relative quantitative protein expression was determined by the ratio of target protein concentration to the internal control GAPDH.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). Data on cell viability, cycle proliferation, gene mRNA expression, and protein marker expression were analyzed with Spearman's correlation for frequency variables. Student's *t*-test was used to analyze differences in MyHC-slow and MyHC-fast expression between high and low frequency ES. A p-value of <0.05 was considered statistically significant. All statistical analyses were carried out with Statistical Product and Service Solutions (SPSS) version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Myloglossus Satellite Cells Verification and Assessment

Myloglossus satellite cells were verified by multi-staining with anti-Pax7 and anti-desmin (Figure 1). The cells were collected for the next measurement after satellite cell number cultured to more than 90% per dish.

Viability and Proliferation After Increments of ES Frequency

There was no significant correlation between cell viability and ES pulse frequency (Figure 2A). The increment of pulse frequency did not correlate with myloglossus satellite cell percentage in the S-phrase (Figure 2B).

Table I. Primer sequences of real-time PCR.

Primers		Sequence (5′- 3′)	Product length/bp
GAPDH	-F	CTCCCTCAAGATTGTCAGCAATG	93
	-R	CCCTCCACGATGCCGAAGTG	
Pax7	-F	GGCCAGCTGTGGGTCAGCAG	207
	-R	GAGCCGGCCGCATCATCCTC	
Myf5	-F	AGCATCTACTGTCCTGATGGGC	162
	-R	CCGTGCTGGCAACTGGGGAG	

mRNA Expression of Cell Differentiation Gene After Increments of ES Pulse Frequency

The increment of ES pulse frequency significantly correlated with mRNA expression of Pax7 in myloglossus satellite cells (r=0.712, p=0.001), but did not correlate with mRNA expression of Myf5 (Figure 3).

Protein Marks Expression of Cell Differentiation and MyHC Isoforms After Increments of ES Pulse Frequency

The increment of ES pulse frequency was significantly correlated with the protein expression of MyoD (r=0.988, p<0.0001) and myogenin (r=0.988, p<0.0001). The MyHC isoform proteins MyHC-slow and MyHC-fast also significantly correlated with increments of ES pulse frequency (r=0.988, p<0.0001 and r=0.875, p<0.0001, respectively; Figure 4).

MyHC-slow expression was more than MyHC-fast expression after satellite cells were stimulated with high frequency ES, including 40, 80, and 160 pps electrical current (p=0.044). There were no differences between MyHC-slow and MyHC-fast expression after low frequency stimulation including 0, 2, and 10 pps.

Discussion

Continuous pulse ES can attenuate the reduction of fiber cross-sectional area[3], enhance satellite cell activity[3,12], and change myoblast differentiation[16]. On the other hand, functional continuous pulse ES can protect the posterior cricoarytenoid muscle from atrophy by preventing muscle weight loss and type 2 fiber deterioration *in vivo*[2]. Functional biphasic pulse train electrical stimulation in muscles did not change cell culture medium temperature or cause cell death with long term application[2,11], so we used functional train pulse ES to study the effect of pulse frequency on differentiation and proliferation of myloglossus satellite cells in dogs.

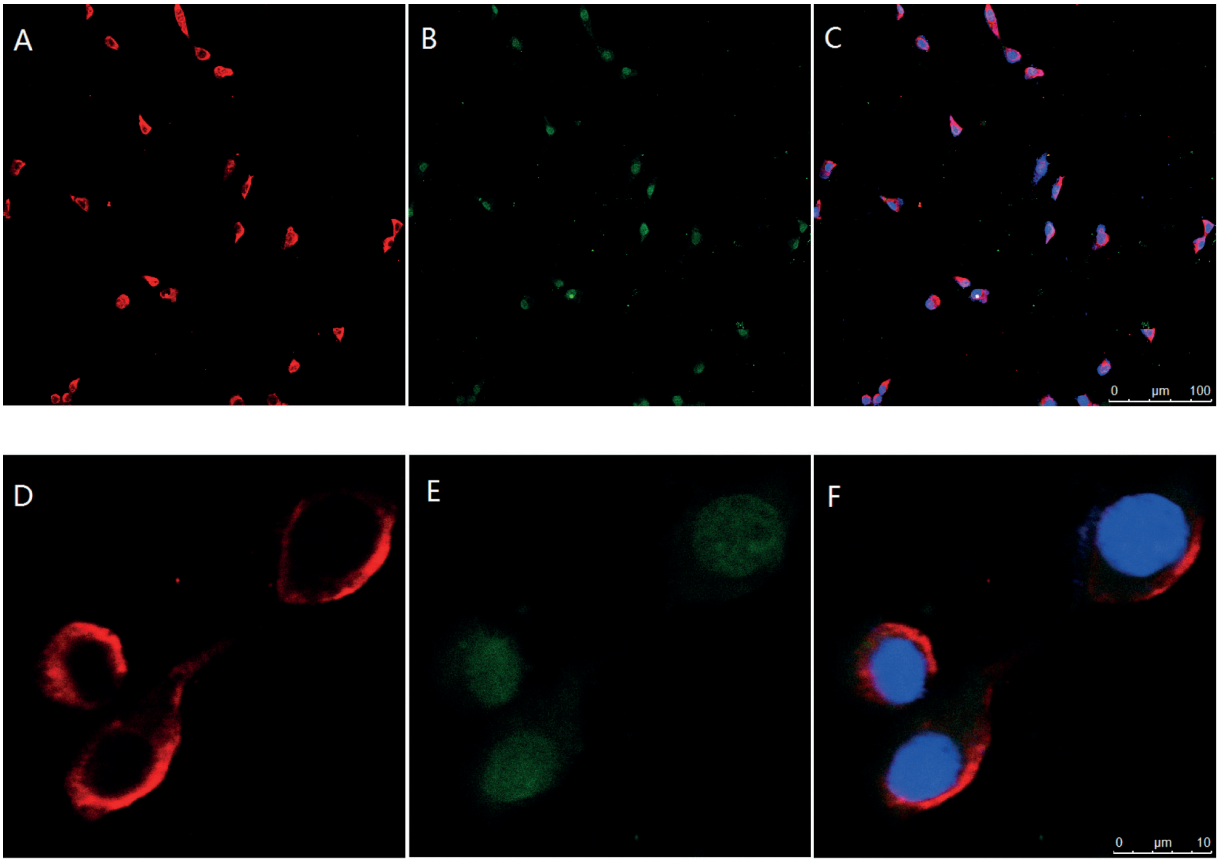

Figure 1. Cultured myloglossus satellite cells from beagles were analyzed and verified by immunofluorescence for desmin (red) (**A, D, C, F**) and Pax7 (green) (**B, E, C, F**) expression using specific antibodies. Nuclei were stained with DAPI (blue) (**C, F**) (magnification: 400×).

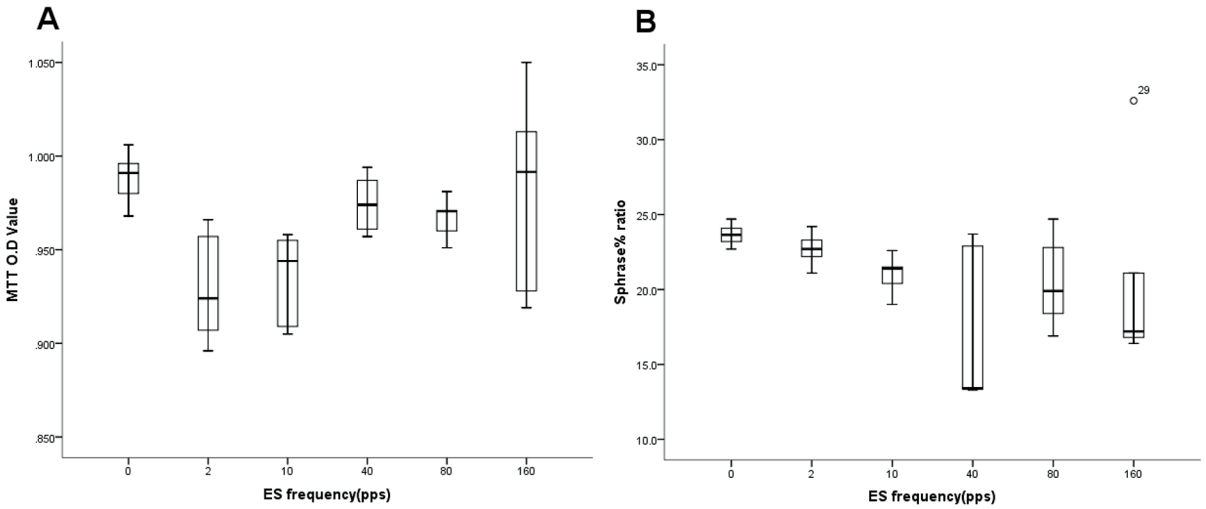

Figure 2. A, The effect of varying ES frequency on dog myloglossus satellite cell viability. The relative MTT OD value of the satellite and myoblast cells did not significantly correlate with ES frequency. **B,** The effect of varying ES frequency on satellite cell proliferation. There were no significant correlations between ES stimulation frequency and percentage of cells in the S-phase.

The myloglossus plays an important role in respiration and mastication[17]. The cultured myloglossus muscle satellite cells were used in this study. Satellite cell myoblasts fuse after 2 days of ES induction[18]. After 3 days of ES, MyoD and desmin expression were increased in muscle progenitor cells, resulting in a shift of MyHC expression[12,16]. Thus, the optimal ES time should be at least 2 days for the study of cell differentiation. We performed the analysis after 72 h of electrical stimulation.

After long-term low frequency ES to innervated muscle, the normal muscle fibers fell and many of the original fibers appeared to have been replaced by adipocytes *in vivo*. Significant fiber loss occurred after prolonged 10 pps ES[4]. Adversely, application of 20 pps ES resulted in denervated muscle and significant increases in

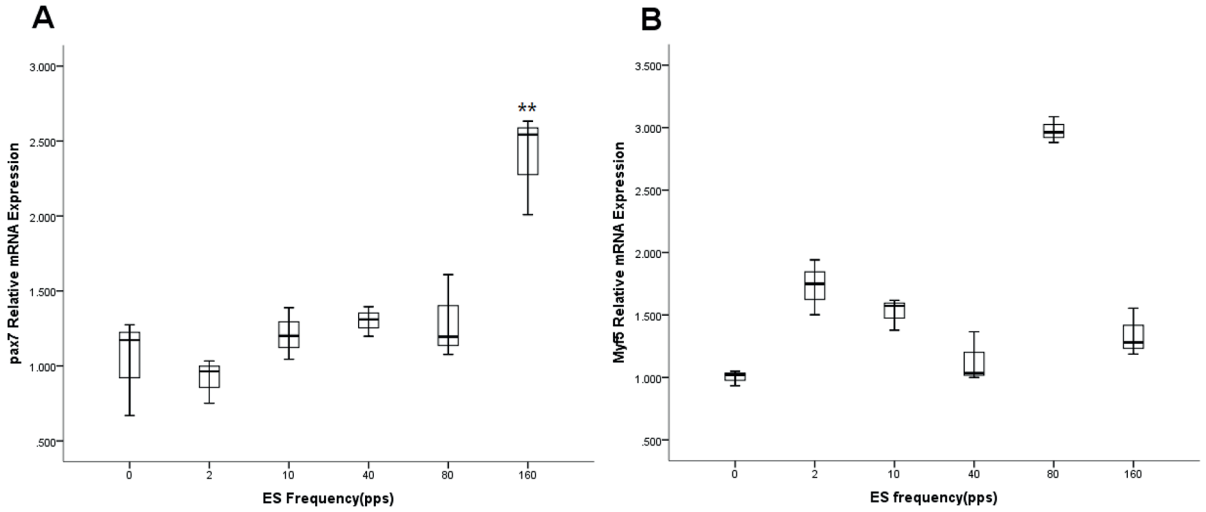

Figure 3. A, The effect of varying ES frequency on differentiation and proliferation of myloglossus satellite cells. The mRNA expression level of Pax7 significantly correlated with ES frequency ($p<0.01$). **$p<0.01$. **B,** There was no significant correlation between mRNA Myf5 expression and ES frequency (pps: pulse per second).

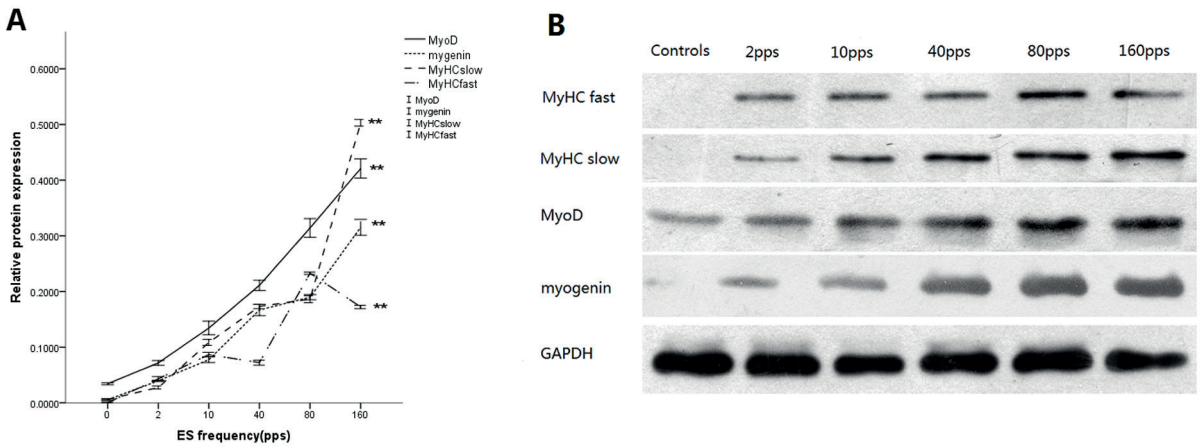

Figure 4. A, The effect of varying ES frequency on differentiation of myloglossus precursor cells. The relative protein expression levels of MyoD, myogenin, MyHC-slow, and MyHC-fast significantly correlated with ES pulse frequency ($p<0.01$). **$p<0.01$. **B,** Western blot confirmed myogenic differentiation enhancement of myloglossus precursor cells with control GAPDH after increasing ES frequency (pps: pulst per second).

muscle wet weight, cross-sectional area, titanic tension, shortening velocity, and power after long term stimulation with bipolar pulses[19]. Satellite cell proliferation increased when underwent 20 pps or 30 pps frequency ES *in vivo*[2,3,20]. Serena et al[12] found that ES did not affect the viability of muscle precursor cells, besides satellite cells *in vitro*. In this study, we investigated the effect of 2, 10, 40, 80, and 160 pps frequency on myloglossus satellite cell viability and proliferation *in vitro*. The MTT and FACS assays were used to measure viability and proliferation of satellite cells. The increment of ES pulse frequency from 2 pps to 160 pps had no correlation with myloglossus satellite cell viability and proliferation. The different niche of satellite cells between *in vivo* and *in vitro* may result in different cell proliferation situation undergoing narrow ranges ES frequency[21]. Frequency variation did not directly change myloglossus satellite cell proliferation, but may indirectly change the cell niche to influence satellite cell proliferation *in vivo*.

Pax7 and Myf5 play more important roles in satellite cell differentiation[10]. Pax7 is specifically expressed in all quiescent and proliferating satellite cells as canonical biomarker[22]. Myf5 is detected in the majority of quiescent satellite cells and it is one of the key modulators of satellite cell self-renewal[23,24]. In this study, Pax7 mRNA expression was upregulated with increments of ES frequency while Myf5 mRNA expression was not upregulated. Both findings indicate that proliferated satellite cells undergo myogenic differentiation, not self-renewal, with ES frequency increments.

Upon muscle injury, satellite cells initiate the myogenic program by the expression of MyoD, withdraw from their quiescent state, and enter into the cell cycle as activated satellites. Pax (+) MyoD (-) cells are in a quiescent state, Pax7 (+) MyoD (+) cells are in a proliferating state, and Pax7 (-) MyoD (+) cells are undergoing myogenic differentiation followed by cell fusion generated multinucleated myofibers. The modulation of MyoD activity affects the balance between proliferation and differentiation of activated satellite cells[25]. Serena et al[12] demonstrated that satellite cells cultured under electrically stimulation had a higher expression of MyoD ($p<0.01$). Myogenin also reflects terminal myoblast differentiation[26]. In this study, the increment in ES frequency enhanced MyoD and myogenin expression. Using Pax7 and Myf5 genes as mRNA expression markers and MyoD and myogenin as protein expression markers revealed that higher frequencies electrical stimulation enhanced differentiation in satellite cell and precursor cell stages in myloglossus muscle *in vitro*.

Pette and Staron[27] previously showed that low frequency ES to animal limb muscles modulated fiber type expression. Chronic low frequency ES induces a fast-to-slow phenotypic change in the rabbit tibialis anterior (TA) muscle, tongue, and rat leg extensor digitorum longus (EDL) muscle[4,5,28,29]. Phasic 150 pps stimulation led to marked increases of MyHC2B and MyHC2X, as well as decreases in slow MyHC1 *in vivo*[5]. *In vi-*

tro, MyHC2 gene expression was induced by direct ES specifically in the medial adductor muscle cell[6]. ES resulted in advance differentiation and maturation in muscle precursor cells in the mouse hind limb muscle *in vitro*[8,11,12,16,18]. In this study, ES caused increases in MyHC protein, including MyHC-fast and MyHC-slow, but there are no significant difference between MyHC-slow expression and MyHC-fast expression under different frequency electrical stimulation in myloglossus muscle satellite cell *in vitro*. These findings were different than Langelaan et al[16], who reported the initiation of a fast-to-slow transition in MHC expression by ES in mouse hand limb muscle precursor cells *in vitro*. Previously, most researches have concentrated on muscle cells *in vivo*, where it is impossible to comprehend the relationship between individual satellite cell differentiation and ES pattern. Our study, which provided canine myloglossus myoblasts undergoing varying ES frequencies *in vitro*, allows for a comprehensive assessment of satellite cell proliferation and differentiation, as well as myoblast response to varying ES frequency.

Overall, higher frequency ES can enhance myloglossus satellite cell differentiation, but not proliferation and viability. During functional pulse train electrical stimulation, myloglossus satellite cell and precursor cell differentiation was elicited. Higher frequency ES causes more mRNA expression of Pax7 and protein expression of MyoD, myogenin, and MyHC. On the basis of this work, we expect to find out the appropriate electrical stimulation parameters to induce directional differentiation of myloglossus satellite cell in the next step, providing a certain laboratory basis for the clinical development of muscle pacemakers.

Conclusions

The results of this study indicated that Pax7 mRNA expression and MyoD, myogenin, and MyHC protein expression were increased with increment of electrical stimulation frequency in myloglossus muscle satellite. Higher frequency ES enhanced myloglossus satellite cell differentiation, not proliferation and viability.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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