

Effect of microcurrent electrical tissue stimulation on equine tenocytes in culture

Yi-lo Lin, DVM, MSc; Hugo Moolenaar, PhD; P. René van Weeren, DVM, PhD; Chris H. A. van de Lest, PhD

Objective—To determine effects of microcurrent electrical tissue stimulation (METS) on equine tenocytes cultured from the superficial digital flexor tendon (SDFT).

Sample Population—SDFTs were collected from 20 horses at slaughter.

Procedure—Tenocytes were isolated following outgrowth from explants and grown in 48-well plates. Four methods of delivering current to the tenocytes with a METS device were tested. Once the optimal method was selected, current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. Cell proliferation, DNA content, protein content, and apoptosis rate were determined.

Results—Application of microcurrent of moderate intensity increased cell proliferation and DNA content, with greater increases with multiple versus single application. Application of microcurrent of moderate intensity once or twice increased protein content, but application 3 times decreased protein content. Application of current a single time did not significantly alter apoptosis rate; however, application twice or 3 times resulted in significant increases in apoptosis rate, and there were significant linear (second order) correlations between current intensity and apoptosis rate when current was applied twice or 3 times.

Conclusions and Clinical Relevance—Results of the present study indicate that microcurrent affects the behavior of equine tenocytes in culture, but that effects may be negative or positive depending on current intensity and number of applications. Therefore, results are far from conclusive with respect to the suitability of using METS to promote tendon healing in horses. (*Am J Vet Res* 2006;67:271–276)

The SDFT is the supporting structure of the horse's limb that is most susceptible to injury,¹ and the mid-metacarpal region of the SDFT is particularly prone to stress-induced injuries.² Tendon injuries heal slowly and imperfectly, resulting in repair tissue that is biomechanically inferior to normal tendon.^{3,4} In undamaged tendons, there is no endotendinous cellular activity in the central part of the tendon,⁵ although tendons have

an intrinsic capacity for repair.⁶ Tendon repair is accomplished primarily by resident tendon fibroblasts or tenocytes, which are the principal cellular components of tendon tissue.⁷ Tenocytes produce both the fibrillar and the nonfibrillar components of the extracellular matrix⁸ and play an important role in initiating regenerative responses following injury or degeneration.

Electrical current is known to influence biological processes at the cellular level, and devices that deliver various types of currents to tissues with the aim of engendering a therapeutic effect have been developed since the end of the 19th century.⁹ Most recently, techniques that use currents in the microampere range, which are similar to the currents generated during physiologic processes such as the depolarization of cell membranes, have been introduced.¹⁰ Electrical current has been reported to alleviate pain, probably by altering β -endorphin concentrations,¹¹ and speed up wound healing by increasing cell proliferation.¹² Reported improvements in fracture healing associated with electrical current¹³ might reflect the effects of electrical stimulation on the sequential events of osteoblast differentiation¹⁴ and mineral formation.¹⁵ Circulation can also be enhanced by increasing the concentration of the vasodilator nitrogen oxide by means of electrical stimulation.¹⁶

Depending on the condition being treated and the administered dose, the effects of electrical current may be positive or negative. Electrical current may stimulate cells to proliferate but inhibit proliferation after a certain threshold.¹⁷ High electrical currents will result in overt tissue damage and provoke cell necrosis, but lower currents may stimulate cells to go into apoptosis.¹⁸ Although apoptosis does not cause the inflammation associated with necrosis,¹⁹ it may still be an unwanted phenomenon during tissue healing.

The mechanism by which microcurrent affects tissues remains largely unclear, although activation of signal-transducing pathways is a likely candidate. Effects on nitric oxide synthase, which regulates nitric oxide production, may play a role, as nitric oxide is known to promote the healing process of tendons.²⁰ Microcurrent application increases concentrations of transforming growth factor- β 1 and insulin-like growth factor II, which are associated with bone cell proliferation.^{21,22} Electrical fields may play a role in extracellular communication, and stimulation of progenitor cells by microcurrent has been shown to speed up the process of endochondral ossification.²³ The phase of the cell cycle may be important to the effects of microcurrent, as cell cycle phase seems to influence the receptivity of

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From the Departments of Equine Sciences (Lin, van Weeren) and Biochemistry and Cell Biology (van de Lest), Faculty of Veterinary Medicine, State University of Utrecht, Utrecht, The Netherlands; and the Department of Physiotherapy, University of Professional Education, Utrecht, The Netherlands (Moolenaar).

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Address correspondence to Dr. Lin.

SDFT Superficial digital flexor tendon
METS Microcurrent electrical tissue stimulation

the cell to extracellular signals. Generally, cells in the G_0/G_1 stage undergo division when they receive signals that instruct them to enter the active phases of the cell cycle.²⁴

Changes in electrical potential of the tissue may also play a role in the effects of electrical current. Trauma affects the electrical potential of damaged cells, and an injured area has higher electrical resistance than the surrounding tissue, resulting in decreased electrical conductivity through the injured area and decreased cellular capacitance.²⁵ Exogenous microcurrent might augment the endogenous current flow and allow the traumatized area to regain its capacitance. Total resistance of the injured tissue would thereby be reduced, allowing physiologic amounts of bioelectricity to enter the area and reestablish homeostasis.

A noninvasive method for improving the quality of repair tissue or shortening the recovery time in horses with tendon lesions would be of great value in veterinary medicine. Potentially, microcurrent electrical devices would be good candidates. However, given the ambiguous effects of electrical currents on tissues, there are potential adverse effects as well. For instance, microcurrent treatment might be far from innocuous if it were to enhance apoptosis, which could be expected to adversely affect tendon healing. Studies of the effects of microcurrent on calf osteoblast-like cells²⁶ and rat tendon fibroblasts¹⁸ have been reported, but relatively little is known about the effects at the cellular level in general, and no specific data exist on the effect in equine tenocytes. The purpose of the study reported here, therefore, was to determine the effects of microcurrent electrical stimulation on equine tenocytes in culture. We hypothesized that microcurrent application would not only promote cell proliferation and increase DNA and protein synthesis, but would also increase the apoptosis rate.

Materials and Methods

Tenocyte culture—Tenocytes were obtained by means of an explant technique, as described.^{27,28} Briefly, SDFTs without macroscopic lesions were collected from 20 horses at the time of slaughter (mean \pm SD age, 7 ± 2 years), and samples were taken from the central core of the mid-metacarpal region of each tendon. Samples from various horses were mixed, and tissue fragments of approximately 1 mm^3 were placed in tissue-culture-grade flasks (25 cm^2). Initially, 5 tissue fragments were added to each flask, but during subsequent studies, 10 fragments were added to each flask to increase cell production. Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and streptomycin-penicillin (50 U/mL) was used for culture medium. Cultures were incubated in a humidified incubator with 5% CO_2 at 37°C . Medium was refreshed twice weekly.

Explant-derived cells took approximately 4 days to emerge from the tissue fragments and another 2 weeks to become confluent. At confluence, the explants were removed, and the cells were transferred to larger flasks (75 cm^2). Cells between passages 2 and 5 were used for electrical current stimulation experiments. For these experiments, cells were trypsinized and seeded at a density of $3 \times 10^4/\text{mL}$ in 48-well plates and grown until they were nearly confluent ($9 \times 10^4/\text{mL}$). Cells were supplied with serum-free

medium 24 hours before application of electrical current to synchronize them in the G_0/G_1 phase, as described.²⁹

METS device—A METS device^a that delivered a pulsating direct electric field was used in the study. The waveform consisted of a brief monophasic square pulse (duration, 0.8 milliseconds) followed by exponential decay to base level. The pulse frequency was 150 Hz. The device delivered a constant current; that is, it automatically changed voltage as necessary to deliver the same current when peripheral resistance changed.

Development of method of electrical current application—A series of experiments was performed to identify the optimal experimental setup for application of electrical current to cultured tenocytes and to determine the most suitable cell density for assessing the effects of METS. Four methods for electrode placement were tested. With method 1, the electrodes were placed at the bottom of each well, in direct contact with the cells. With method 2, the electrodes were placed just under the surface of the medium in each well. With method 3, the electrodes were placed in separate containers of PBS solution that were connected to the culture wells with agarose bridges to avoid electrolysis. Each bridge consisted of a 5-cm-long, 0.2-mm-inner-diameter capillary filled with sterile 0.1% agarose. With method 4, the electrodes were attached to paper strips^b ($0.5 \times 3 \text{ cm}^2$) soaked in PBS solution that were placed in the culture wells.

Electrical current treatment—After selection of the optimal method for current application, electrical current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells in wells on 48-well plates (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. To assess the effect of cell density, cell concentrations of $4 \times 10^4/\text{mL}$ (50% confluence) and $9 \times 10^4/\text{mL}$ (90% confluence) were used.

Assessment of tenocyte proliferation—To assess the effects of METS on tenocyte proliferation, viable cells were quantified by use of a nonradioactive, colorimetric cell proliferation kit.^c This method is based on metabolism of tetrazolium salts to formazan dye, and the outcome relates directly to the number of viable, metabolically active cells.³⁰ Cell proliferation was measured 24 hours after the end of each specific current treatment. For assessment of proliferation, cells were incubated for 2.5 hours, and spectrophotometric absorbency was measured with a microtitration plate reader^d at a wavelength of 450 nm. The reference wavelength was set at 650 nm.

Determination of DNA and protein contents—To evaluate the effect of METS on DNA and protein contents, cells were rinsed 2 times with PBS solution 24 hours after the end of each specific current treatment and scraped from the plates with a rubber spatula. Cells were lysed by freeze-thawing, placed in liquid nitrogen for a few seconds to destroy the cell membrane, and then stored at -20°C until further analysis. The DNA content was determined as described.³¹ Briefly, a fluorescent dye was added to the lysed cells, and fluorescence was measured with a fluorometer^e immediately after mixing, with excitation at 366 nm and emission at 442 nm. Calf thymus DNA^f was used as a reference. Results were expressed as microgram of DNA per well.

Protein content was measured by use of a bicinchoninic acid protein assay reagent kit.^g Briefly, 25 μL of lysed cells was added to 200 μL of kit reagent, and absorbency was measured with a microtitration plate reader^d at an absorbency of 595 nm after 30 minutes of incubation at 37°C . Bovine serum albumin provided by the manufacturer of the kit was used as a standard. All data were related to results for a negative control sample.

Determination of apoptosis rate—The apoptosis rate was used as a measure of possible cytotoxicity of METS. Apoptotic cells were detected on the basis of annexin V binding to translocated plasma membrane phosphatidylserine, as described.³² Propidium iodide was used to test for loss of cell membrane integrity, which is indicative of necrotic and late apoptotic cells. Briefly, 24 hours after the end of each specific current treatment, culture medium was collected and tenocytes were rinsed briefly with PBS solution, trypsinized, and centrifuged at $2,900 \times g$ for 5 minutes at 4°C . Apoptosis rate was determined by use of an annexin V and propidium iodide double-staining kit,^b following the manufacturer's instructions. The stained cells were then analyzed by means of flow cytometry.¹ The fluorescence intensity of the pulse area signal from 10,000 events was analyzed. Cells that had undergone no electrical treatment were used as negative controls, and cells directly stimulated with 3.5 mA of current were used as positive controls. Viable cells were negative for annexin V and propidium iodide, apoptotic cells were positive for annexin V but negative for propidium iodide, and necrotic cells were positive for annexin V and propidium iodide. Further analysis for cell subpopulations was performed with quadrant gates. In this analysis, the lower left quadrant represented the viable cells; the upper right quadrant represented nonviable, necrotic, or late-stage apoptotic cells; and the lower right quadrant represented apoptotic cells. Relative cell death was calculated by counting apoptotic cells and dead cells together (ie, upper and lower right quadrants) because the terminal phase of cell death in vitro represents a gradual shift from apoptosis to necrosis.

Statistical analysis—Measured values were standardized to mean values for control samples (ie, no electrical stimulation) and expressed as mean \pm SD. Effects of METS were tested by means of 2-way ANOVA, followed by the Bonferroni post hoc test. Correlations between DNA content, protein content, cell proliferation, and apoptosis rate were examined by calculation of the Pearson correlation coefficient. All statistical analyses were performed with standard software.¹ Values of $P < 0.05$ were considered significant.

Results

Optimal method of electrical current application—When electrodes were placed at the bottom of each well (method 1), apoptosis was detected immediately after current application, even when a low current (0.2 mA) was used. When electrodes were placed just under the surface of the medium in each well, so that they were not in direct contact with the cells (method 2), apoptosis was seen at low (0.2 mA) and high (3 mA) currents, although effects were dose-dependent. When electrodes were placed in separate containers connected to wells by agarose bridges (method 3), no apoptosis was seen, and it was unclear whether current transmission was effective, as there were no differences between control cells (no current) and treated wells in regard to cell proliferation, DNA content, or protein content. Based on the disadvantages of the previous models, model 4 was generated and appeared to work well. For this reason, attachment of electrodes to paper strips that were placed in the culture wells (method 4) was selected as the optimal method for microcurrent application.

Cell density was associated with severity of detachment and apoptosis caused by microcurrent, with apoptosis rate being lower when cell density was

higher. Therefore, a cell density of $9 \times 10^4/\text{mL}$ (90% confluence) was used.

Effect of METS on cell proliferation, DNA content, and protein content—Application of microcurrent had a stimulatory effect on cell proliferation that was dependent on current intensity and number of current applications (Figure 1). Cell proliferation was significantly increased, compared with the negative control, after a single application of current at 0.5, 1, or 1.5 mA. However, when current was applied twice, cell proliferation was significantly increased by application of current as low as 0.1 mA, and when current was applied 3 times, cell proliferation was significantly increased by application of current as low as 0.05 mA. Application of current multiple times had a greater effect on cell proliferation than did application a single

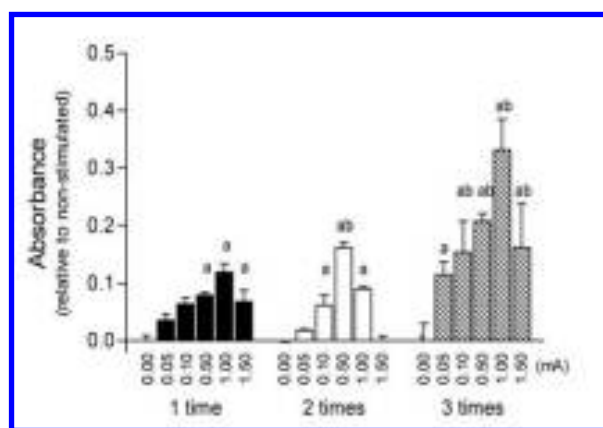


Figure 1—Effect of METS on proliferation of equine tenocytes in culture. Tenocytes were grown in wells on 48-well plates, and electrical current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. Cell proliferation was measured 24 hours after the end of each specific current treatment by use of a commercial kit that measures metabolism of tetrazolium salts to formazan dye. Measurements were standardized by subtracting the mean value of the corresponding control group. Values are expressed as mean; error bars represent SD. a = Significantly ($P < 0.05$) different from value for negative control. b = Significantly ($P < 0.05$) different from value obtained after a single application of current at the same intensity.

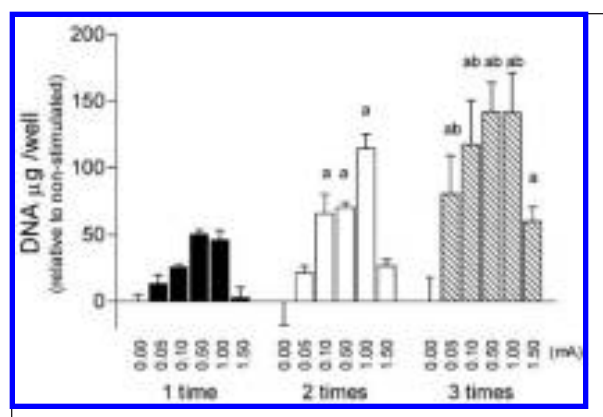


Figure 2—Effect of METS on DNA content of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. See Figure 1 for remainder of key.

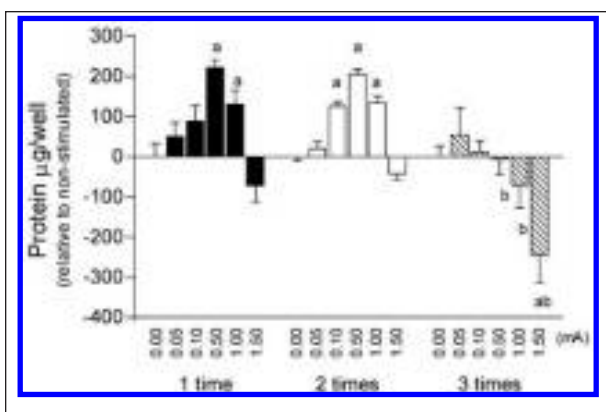


Figure 3—Effect of METS on protein content of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. See Figure 1 for remainder of key.

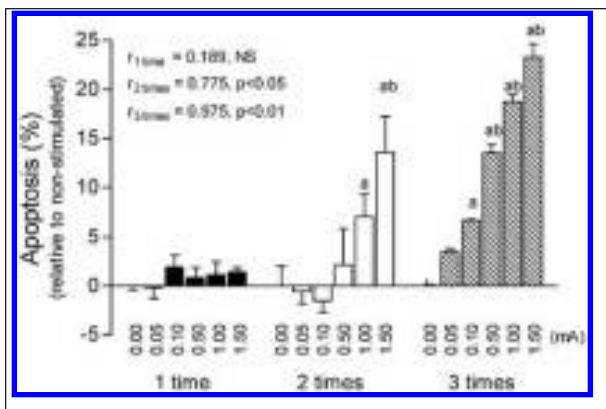


Figure 4—Effect of METS on apoptosis rate of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. See Figure 1 for remainder of key.

time. Cell proliferation was significantly greater after application of 0.5 mA of current twice than after application of the same current only once, and cell proliferation was significantly greater after application of 0.1, 0.5, 1, or 1.5 mA of current 3 times than after application only once.

Similar patterns were found for DNA content (Figure 2), except that application of current a single time did not have any significant effect on DNA content, compared with the negative control. However, application of 0.1, 0.5, or 1 mA of current twice or 0.05, 0.1, 0.5, 1, or 1.5 mA of current 3 times significantly increased DNA content, compared with the negative control. Also, application of 0.05, 0.1, 0.5, or 1 mA of current 3 times resulted in significantly greater DNA content than did application only once.

Protein content was significantly increased, compared with the negative control, after application of 0.5 or 1 mA of current once and after application of 0.1, 0.5, or 1 mA of current twice (Figure 3). However, application of current 3 times resulted in a significant decrease in protein content, compared with application of current only once.

There was a significant ($r = 0.928$; $P = 0.007$) correlation between DNA content after application of cur-

rent a single time and protein content after application of current a single time.

Effect of METS on apoptosis rate—Apoptosis rate was dependent on current intensity and number of current applications (Figure 4). Within the range of current used, application of current a single time did not significantly alter apoptosis rate. However, application of current twice or 3 times resulted in significant increases in apoptosis rate, and there were significant linear (second order) correlations between current intensity and apoptosis rate when current was applied twice ($r = 0.775$; $P < 0.05$) or 3 times ($r = 0.975$; $P < 0.01$). Current intensity was not significantly correlated with apoptosis rate when applied only once ($r = 0.189$; $P > 0.05$). There was a significant negative correlation between apoptosis rate and protein content after application of current 3 times ($r = -0.83$; $P = 0.04$).

Discussion

In our attempts in the present study to identify the optimal method for applying microcurrent to tenocytes in culture, we found that allowing for direct contact between the electrodes and the culture medium or the cells themselves resulted in extensive cell death. This phenomenon could have been a result of electrolysis, whereby dissolved positive and negative ions in the medium were discharged at the cathodal and anodal electrodes, respectively, and was probably mediated through formation of H_2O_2 at the anodal electrode. The use of bridges to allow for indirect current transmission avoided this. However, only the paper bridges worked satisfactorily. The agarose bridges most likely had an intrinsic resistance that was high enough to prevent sufficient current to generate a biological effect to pass to the cells.

In the present study, the apoptosis rate was lower when cells that were 90% confluent were used than when cells that were 50% confluent were used. This was not attributed to cells in the denser monolayers receiving less current because the METS device that was used automatically increased the voltage if peripheral resistance changed. However, when cells were 50% confluent, they were fibroblast-like, with more evidence of mitotic and metabolic activity. When cells were 90% confluent, they were spindle shaped and more closely apposed and appeared quiescent and less metabolically active. It is probable that cells in this less active state are less susceptible to stimulation by electric current, but there are other possible explanations for the influence of cell density on effects of microcurrent application. In particular, cell-to-cell contact and communication will be different in close-packed cell populations than in sparser populations. Also, the greater amount of matrix with higher cell density¹⁸ might alter resistance and mitigate the direct effects of current on tenocytes, although it is arguable whether culturing time and conditions used in the present study were sufficient to allow for the formation of substantial differences in amount of extracellular matrix. The presumptive role of matrix in the dissipation of electrical energy may have an important influence on the in vivo effects of METS. Because tenocytes are

embedded in a 3-dimensional network of extracellular matrix components *in vivo*, it can be expected that higher currents or longer application times may be needed to have positive effects *in vivo*. Further study is needed to determine whether effective field strength can be obtained with a treatment regimen that is still practical; what effects intermediate structures, such as skin and fascia, might have on field strength in target tissues; and what effects transducer placement (ie, dorsal-palmar vs lateral-medial vs proximal-distal) might have.

Results of the present study indicate that microcurrent affects the behavior of equine tenocytes in culture. As expected, alterations in cell proliferation, DNA content, and protein content could be detected, depending on current intensity and number of applications. This is in line with previous findings for fibroblasts,³³ chondrocytes,^{34,35} and osteoblast-like cells.³⁶ On the basis of the positive effects on cell proliferation and DNA content, it seems that microcurrent can induce tenocytes to pass from the G₀/G₁ stage to the active phase of the cell cycle.

There was a clear effect of number of current applications on results of the present study. Application of current twice seemed to have an additional stimulating effect on cell proliferation and DNA content, at least at certain current intensities. However, the overall effect was not much different from results of a single application. Three daily applications of current had a somewhat ambiguous effect. Cell proliferation and DNA content were higher, at least at intermediate current intensities, but protein content was negatively affected and apoptosis rate increased substantially.

Overall patterns of changes in cell proliferation and DNA content were similar, which was expected because DNA content is related to cell number. However, results were not identical, possibly because cells in the S and M2 phases have twice as much DNA as do cells in other phases of the cell cycle. Our results also showed that cell proliferation and apoptosis rate may increase at the same time. This may seem contradictory; however, it is conceivable that individual cells respond differently depending on the phase of the cell cycle that they are in at the time of current application.

It is evident from our results that the effects of electrical current on the function and metabolism of cells is not only positive and stimulating, but may have negative aspects as well. The effects appeared to depend on the intensity of the current that was applied, as shown earlier for fibroblasts from rat skin.³⁷ In particular, our findings suggest that the stimulatory effects on cell proliferation, DNA content, and protein content decrease with application of current at intensities > 1 mA. With a single application of current, the apoptosis rate remained the same throughout the range of current intensities used; however, when current was applied twice or 3 times, there was a significant linear increase in apoptosis rate with current intensity. This implies that cells are less susceptible to a single exposure to relatively high current than to repeated exposure to lower current.

The increase in apoptosis rate found in the present study should not be taken too lightly. Tendon is a tissue with a high ratio of extracellular matrix to cells, and the

tenocytes that are present are solely responsible for the repair process. Loss of even a relatively minor quantity of these cells may severely impair the repair capacity of the tissue. Therefore, any substantial loss of cells as a result of an increase in the apoptosis rate associated with METS may render the whole procedure counterproductive. The negative correlation between protein content and apoptosis rate may be indicative of this. Results of the present study do not yet permit a determination of a detailed scheme for application of METS *in vivo*. However, it seems that 3 consecutive applications is too intensive for cells to cope with. In addition, long-term effects of METS were not evaluated in the present study. Although there are no indications in the literature of delayed cytotoxic effects if the correct dose of electric stimulation is given, this is a possibility that must be investigated in more detail before any clinical application of METS can be recommended.

The mechanism by which microcurrent exerts its effects on cells is not known. There may be a direct effect through influences on transmembrane potential,³⁷ which may result in an increase in permeability of the plasma membrane.³⁸ This could also affect intracellular pathways.³⁹ In this regard, it has been suggested that cells are capable of detecting external electric fields when frequency and phase of the external current match with those of intracellular metabolic oscillators.⁴⁰ Negative effects may be generated through the disruption of cellular membranes, which might be primarily due to large ion shifts. In particular, DNA is a heavily charged intracellular structure whose folded structures and proximity to the nuclear membrane make it susceptible to the effects of current. Damage to DNA will have consequences for cell survival and may result in cell cycle anomalies.³⁸ Protein content may be negatively affected by higher current intensity because of inhibitory effects on amino acid transport.³⁶

The hypothesis of this study was proven to be partially true, but results were not unambiguous. Microcurrent stimulation indeed enhanced cell proliferation and increased DNA and protein synthesis, but only at certain current intensities. At lower and higher intensities, no effects or negative effects were detected. The apoptosis rate also was affected by microcurrent application, but results depended on current intensity and number of applications. The current range used in the present study (0.05 to 1.5 mA) did not have an irreversible effect, as apoptosis rate did not change after a single application of current in this range. However, additional applications of current within a short time apparently influenced the mechanisms of cellular response, as apoptosis rate increased. We conclude that microcurrent may have positive and negative effects on equine tenocytes in culture, depending on application. Although microcurrent is capable of enhancing cell metabolism and therefore may promote tissue regeneration,³⁹ results of the present study are far from conclusive with respect to the suitability of using METS to promote tendon healing in horses.

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- a. APS device MK 1.1 Medeusa, BV Bilthoven, The Netherlands.
 - b. Whatman CHB chromatography paper, Fisher Emurgo BV, Landsmeer, The Netherlands.

- c. Cell proliferation kit II XTT, Roche Molecular Biochemicals, Basel, Switzerland.
- d. Microplate reader, Bio-Rad Laboratories, Hercules, Calif.
- e. LS 2B Fluorimeter, Perkin Elmer Corp, Norwalk, Conn.
- f. Sigma Chemical Co, St Louis, Mo.
- g. BCA protein assay reagent kit, Pierce Chemical Co, Rockford, Ill.
- h. Annexin-V-Fluores staining kit, Roche Molecular Biochemicals, Indianapolis, Ind.
- i. Becton-Dickinson, San Jose, Calif.
- j. Prism, version 4.0, GraphPad Software Inc, San Diego, Calif.

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