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Murine model of neuromuscular electrical stimulation on squamous cell carcinoma: Potential implications for dysphagia therapy

Gary Linkov, BS[1], **Ryan C. Branski, PhD**[2], **Milan Amin, MD**[2], **Natalya Chernichenko, MD**[1], **Chun-Hao Chen, MD**[1], **Gad Alon, PhD**[3], **Susan Langmore, PhD**[4], **Richard J. Wong, MD**[1], and **Dennis H. Kraus, MD**[1,*]

[1]Head and Neck Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York [2]Department of Otolaryngology, New York University Medical Center, New York, New York [3]Physical Therapy and Rehabilitation Sciences, University of Maryland, School of Medicine, Baltimore, Maryland [4]Department of Otolaryngology–Head and Neck Surgery, Boston University Medical Center; Speech Language Hearing Department, Boston University, Boston, Massachusetts

Abstract

Background—Dysphagia is a potential consequence of treatment for head and neck cancer. Neuromuscular electrical stimulation (NMES) has evolved as a treatment option, with the goal of improved swallow function in patients with chronic dysphagia. However, the effects of NMES on tumorigenicity are unknown and often confound the initiation of this therapy, potentially limiting its efficacy in treating patients with head and neck cancer.

Methods—Squamous cell carcinoma was grown in the flank of athymic, nude mice. Mice were randomized into treatment and control groups; the experimental group received daily NMES directly to the flank for 8 days.

Results—Tumor volumes, recorded on days 0, 3, 7, and 10, demonstrated no significant differences between groups on each day of measurement. Immunohistochemical analysis of apoptosis, proliferation, and vascularization also failed to demonstrate statistically significant differences between treated and untreated groups.

Conclusions—NMES does not promote the growth of underlying tumor in our model. These data may provide preliminary evidence that applying electrical stimulation over the muscles of the anterior neck does not increase the risk of tumorigenicity. Early initiation of NMES in this challenging population may be feasible from an oncologic standpoint.

Keywords

NMES; electrical stimulation; VitalStim; squamous cell carcinoma; dysphagia

Electrical stimulation has been widely applied in medicine to combat microorganisms,[1] enhance wound healing,[2] and treat tumors,[3] as well as promote strengthening and motor control of skeletal muscles.[4,5] Common rehabilitation practice is to place electrodes on the

*Corresponding author: D. H. Kraus, Head and Neck Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York. krausd@mskcc.org.

skin to stimulate nerves supplying muscle groups of interest to increase strength.[6,7] Neuromuscular electrical stimulation (NMES) has been used for decades on a wide array of muscle groups, but it was investigated for dysphagia rehabilitation in neurologically impaired patients in the late 1990s,[8,9] and in 2002, the U.S. Food and Drug Administration (FDA) cleared a device for the treatment of dysphagia. Several meta-analyses have since evaluated the effect of NMES on swallowing rehabilitation and revealed a small but significant summary effect size.[10,11]

More recently, investigation of NMES has focused on its effects as an adjunct to traditional swallowing therapy in patients receiving treatment for locally advanced head and neck cancer.[12] However, the FDA applies a general warning to all NMES devices to avoid stimulating over areas of malignancy.[13,14] The concern is that applying electrical stimulation to the anterior neck may stimulate tumor growth if there is residual disease or recurrence following treatment. It is common practice to wait at least several months after radiation and/or surgical treatment before initiating NMES to minimize or resolve dysphagia. A drawback to delaying therapy is that by the time NMES has commenced, the patient typically has chronic dysphagia,[15] making the condition more difficult to manage. If NMES in the setting of active malignancy proves not to enhance tumorigenic activity, then perhaps its benefit for dysphagia therapy can be enhanced by starting therapy earlier in the rehabilitation period.

To our knowledge, there has never been an animal or human study investigating the effects of skin surface electrical stimulation (as in NMES) on an underlying malignancy, located deep to the skin surface but aligned with the location of electrical stimulation. Based on our review of the literature pertaining to electrodes inserted directly into tumors and resulting in tumor destruction,[3, 16–21] we hypothesized that NMES will not increase underlying tumor burden.

MATERIALS AND METHODS

Cell line and laboratory animals

The murine SCC7 cell line is a cutaneous squamous cell carcinoma (SCC) that arose from the C3H/HeJ mouse. SCC7 cells were grown in vitro in minimal essential medium (MEM) containing 10% fetal calf serum and 1% penicillin and streptomycin. Cells were maintained in a 5% CO_2-humidified incubator at 37°C. In vivo experiments were performed under an approved animal protocol by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Four- to 6-week-old female athymic nude mice were anesthetized with inhalational isoflurane. SCC7 cells (1×10^7) in 50 μL of phosphate-buffered saline (PBS) were injected into the subcutaneous flanks. A palpable, single solid tumor reliably developed within 4 days. On day 4 following inoculation, mice were sorted by tumor volume and divided into 2 groups ($n = 7$ per group), whereby each group (treated and control) had an approximately equal starting tumor volume.

NMES murine model

NMES was delivered via a battery-powered device, using a dual-channel electrotherapy system having symmetric biphasic pulsed current at a fixed pulse rate of 80 Hz and fixed pulse duration of 700 μs (Vital Stim Model 5900; Chattanooga Group, Hixon, TN). Three NMES devices were used to treat up to 6 mice simultaneously (monitored individually). On each day of treatment, the mice were subjected to isoflurane anesthesia induction in a standard chamber, temporarily taken out of the chamber for electrode placement, and then placed back into the chamber for the duration of stimulation. The skin sites of electrode application were cleaned with an alcohol wipe daily. The electrode assembly was designed

for application with the specific NMES device used (2.1-cm round active area). The only modification made to this assembly was to cut across the adhesive area between the 2 electrodes to apply each electrode separately. One electrode was positioned directly over the palpable subcutaneous flank tumor, whereas the other was placed on the abdomen, in the same axis line as the first electrode to ensure current movement through the tumor (see Figure 1).

Treated animals received 30 minutes of daily NMES for a total of 8 days, with a 2-day break interval after the first 5 days of treatment. Control animals underwent the same procedures, but the electrodes were not connected to the current source. Stimulation intensity was applied and recorded daily for each animal, as the minimum necessary for visible muscle contraction around the top electrode. The minimum intensity was maintained throughout each treatment session. Tumor volumes and animal weights were recorded on days 0, 3, 7, and 10. Tumor volume based on caliper measurements in 2 dimensions was calculated by the modified ellipsoidal formula: Volume = 1/2(length × width^2). Animal weights were measured using a simple scale.

Tissue preparation and immunohistochemical analysis

Animals were euthanatized 2 weeks following inoculation (1 day after the last NMES treatment) via inhalational CO_2, when the tumor burden reached the maximal volume allowed by our protocol. Following necropsy, 5 tumors from each group were bisected along their greatest diameter and fixed in ice-cold freshly made 4% paraformaldehyde. Tissues were fixed overnight at 4°C, washed in PBS at 4°C 2 times for 30 minutes each, dehydrated in increasing concentrations of ethanol, and paraffin embedded. Tissue sections were taken at a thickness of 4 μm. Immunohistochemical analysis was performed at the Molecular Cytology Core Facility of Memorial Sloan–Kettering Cancer Center (Discovery XT processor; Ventana Medical Systems, Tucson, AZ). The following primary antibodies were used: anti-Ki67 (Novocastra Reagents, Leica Microsystems, Buffalo Grove, IL); anti-cleaved-caspase-3 (Cell Signaling Technology, Danvers, MA), anti-MECA-32 (hybridoma bank). Positive and negative controls were run in parallel for each antibody. Images of each slide were captured at ×10 magnification (Quantum Medical Imaging, LLC, Ronkonkoma, NY). Percent positivity of each antibody was calculated using imaging software (Meta-Morph Software, Molecular Devices, Sunnyvale, CA) by dividing the area of positive cells by the total area of all cells. The final count per group (treated or control) was an average of 3 high-power fields for each of the 5 tumor samples.

Statistical analysis

Statistical analysis was done using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA), and 2-tailed Student's *t* tests were applied. Error bars were calculated in terms of SE.

RESULTS

In Vivo assessment

Initial tumor volumes, immediately prior to NMES, were 105 ± 15 and 110 ± 13 mm^3 for the control and treatment groups, respectively ($n = 7$ per group). The final tumor volumes at day 10 post–treatment initiation, were 1676 ± 213 and 1201 ± 264 mm^3 for the control and treated groups, respectively ($n = 7$ per group). This difference did not achieve statistical significance ($p = .21$). In addition, no statistically significant differences were observed in tumor volume throughout the duration of treatment (Figure 2A). Animal weight remained stable throughout the course of treatment, with an average weight of 24 ± 1 g for each group (Figure 2B). The mean stimulation intensity for all mice combined was 2.5 ± 0.3 mA over the course of treatment (Figure 2C). Stimulation intensities ranged from 1.5 to 4 mA. There

was fluctuation within this range for any mouse over the treatment course. All mice tolerated the treatment without any observed adverse events, including skin irritation.

Explanted tumor assessment

The effects of NMES on apoptosis (Figure 3A), proliferation (Figure 3B), and vascularization (Figure 3C) were then quantified via immunohistochemistry on paraffin-embedded tumor sections with the appropriate markers. Although increased apoptosis (33 ± 12% vs 16 ± 10%) and vascularization (4.6 ± 1.2% vs 2.4 ± 0.2%) were observed in the experimental group (Figures 4A, 4C), these differences were not significantly different ($p = .31$ and .13, respectively). In addition, no differences were observed with regard to proliferation (Figure 4B): 36 ± 4.8% for NMES and 37 ± 1.1% for control ($p = .85$).

DISCUSSION

The ability to swallow after treatment for head and neck cancer is a function of tumor stage, anatomic site, and treatment. Surgical treatment can drastically alter the patient's ability to swallow, but if this is the only treatment then swallow function may improve over time. Radiotherapy, with or without additional chemotherapy and/or surgery, is commonly used with patients with advanced-stage head and neck cancer. Radiotherapy causes acute problems in swallowing because of associated pain, edema, xerostomia, and loss of taste. Late complications, appearing after 3 months, are also common and may cause a permanent dysphagia, primarily due to the fibrosis formed throughout the radiated region. As a result, quality of life can be affected significantly by treatment modality combinations.[22] Moreover, the presence of tumor itself, as well as the treatment, can result in neuromuscular damage affecting any stage of the swallowing mechanism.[23]

Dysphagia rehabilitation for patients with head and neck cancer has primarily focused on preventative strategies in addition to traditional therapy including posture, compensatory maneuvers, and exercise to treat the swallow disorder and help the patient to achieve optimal swallowing function.[24] More recently, NMES has been proposed as an adjunctive intervention for the treatment of dysphagia in patients with head and neck cancer.[15] To date, there have been only a few published clinical studies investigating the efficacy of NMES for patients with head and neck cancer undergoing surgical and/or radiation treatment.[12,25] In 1 double-blinded, randomized case control study, the experimental group consisted of 14 patients randomized to 30 minutes of NMES plus 30 minutes of traditional swallowing training for 5 days per week for 2 weeks.[12] The control group was composed of 12 patients receiving sham stimulation plus traditional swallowing training. The authors found that patients showed greater functional dysphagia scale (FDS) scores following NMES combined with conventional rehabilitation treatment than following conventional rehabilitation treatment alone. There were several limitations to this study: the sample size was small, the subject population was heterogeneous in terms of oncologic treatment received, there was no mention as to the elapsed time between the completion of cancer treatment and the start of rehabilitation, and no objective measures of swallowing function were reported (eg, fluoroscopy or endoscopy study).

An ongoing multi-institutional clinical trial seeks to compare electrical stimulation plus aggressive swallowing exercise to sham electrical stimulation with aggressive swallowing exercise to determine the efficacy of NMES in treating dysphagia in patients who have received postoperative radiation therapy for head and neck cancer. In this trial, NMES is initiated at least 3 months after radiation therapy for head and neck cancer, and patients must be disease free prior to beginning the study,[26] in compliance with the aforementioned FDA warning against NMES application to an area of active malignancy. A potential consequence of delayed rehabilitation is that by the time treatment starts, dysphagia is of a more chronic

nature, and thus less easily rehabilitated. In fact, by 3 months after radiation treatment, the acute inflammatory effects have largely resolved and tissue at this stage begins to become fibrotic and rigid, with resultant loss of function.[15]

Interestingly, the FDA does not provide references to accompany their warning related to NMES and tumorigenesis. The concern for potentially stimulating the growth of a tumor with electric current may be traced to epidemiologic studies that implicate environmental effects of electromagnetic forces as promoting human cancer,[27,28] and on the wound-healing literature that generally claims increased metabolic activity as the primary mechanism of electrical stimulation.[29,30] However, there is an extensive body of literature, dating back to the 19th century, devoted to electrochemical effects on tumors.[31] The overwhelming majority of these papers highlight a reduction in tumor size with electrical stimulation *directly* into the tumor. Several destruction mechanisms of electrochemical treatment have been proposed, the best supported of which are toxic species produced in the electrochemical reactions during electrolysis,[32] extreme local pH changes,[33] and perturbation of local blood flow.[34] It is likely that a combination of these complex processes plays a role in tumor death. An attempt to resolve the discrepancy between electrical stimulation enhancing healing of chronic wounds and retarding tumor growth suggests that currents may normalize cell proliferation.[35]

These previous studies involved electrode placement either directly into the tumor or immediately adjacent to it under the skin. Until now, perhaps for a lack of clinical application, the effect of surface-applied electrical stimulation on underlying malignancy has never been tested. We developed a mouse model to test this phenomenon to better understand the role of NMES for patients with head and neck cancer receiving dysphagia rehabilitation. Our results indicate no significant differences between the NMES and control groups, based on gross and cellular analysis. Therefore, based on our findings, NMES therapy for dysphagia could be initiated earlier in the treatment paradigm for patients with head and neck cancer. However, clearly, additional investigation is warranted in this regard. Using NMES as a preventative measure, or in the early stages of dysphagia, may enhance its therapeutic effects, since earlier initiation of swallowing exercises has been shown to have more beneficial effects on patients with dysphagia.[36]

Limitations of our study include a pilot-scale mouse model, the testing of only a single cancer cell type, and a relatively brief treatment course. Further studies are warranted to probe the effects of NMES on underlying malignancy, particularly in relation to radiotherapy. However, these preliminary studies are encouraging regarding the potential for NMES to play a more prominent role in dysphagia therapy in patients with either active malignancy or during the course of oncologic therapy.

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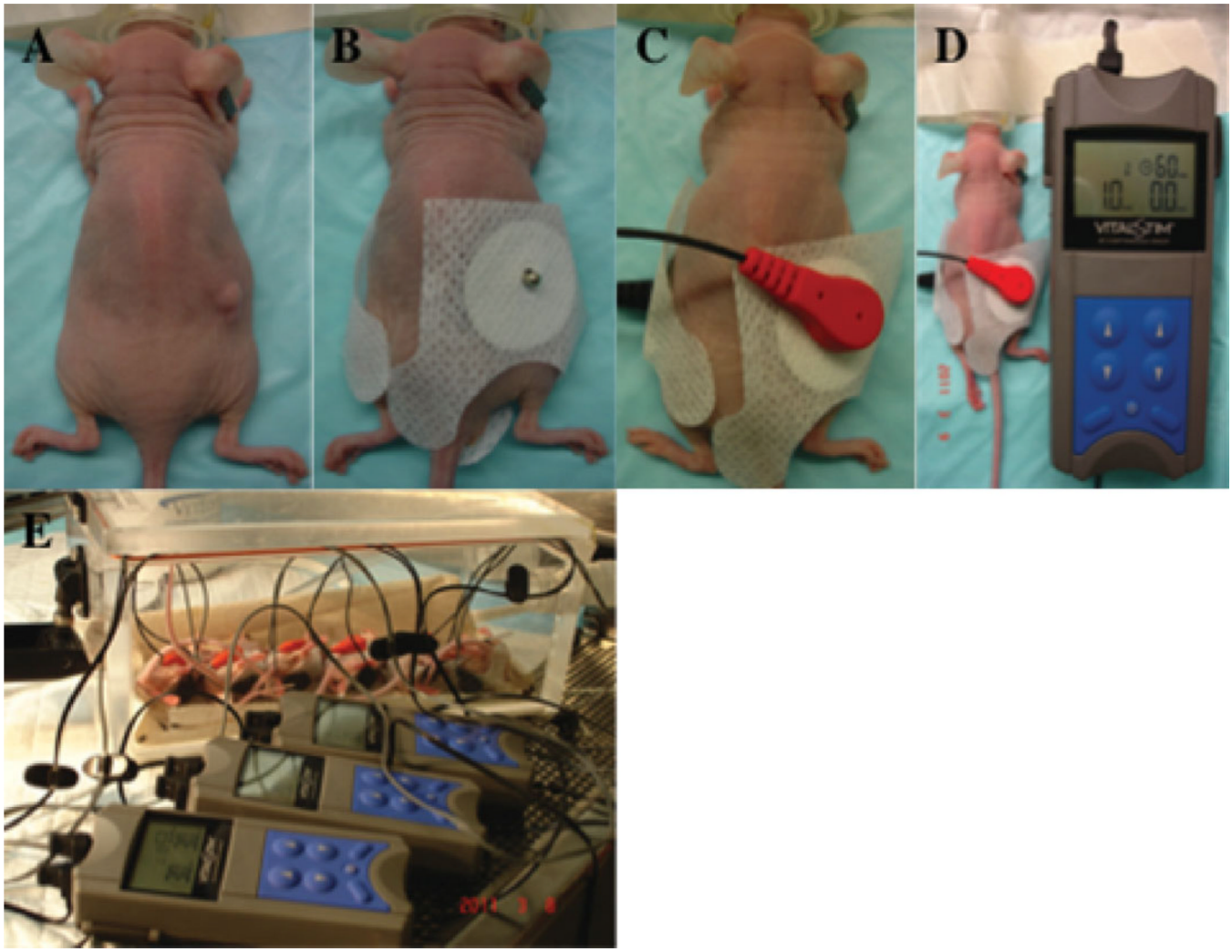

Figure 1.
NMES murine model. (A) Established SCC7 subcutaneous flank tumor (1×10^7 cells). (B) One electrode placed directly over the tumor, the other placed on the abdomen in the same axis line. (C) Wire leads attached to the electrodes. (D) NMES device connected to electrodes, powered on, and displaying the minimum intensity necessary to observe visible muscle contraction around the top electrode (1.0 mA in this case). (E) Three NMES devices simultaneously stimulating 6 mice in their anesthesia induction chamber. NMES, neuromuscular electrical stimulation; SCC7, a spontaneously arising squamous cell carcinoma (SCC) of C3H mice.

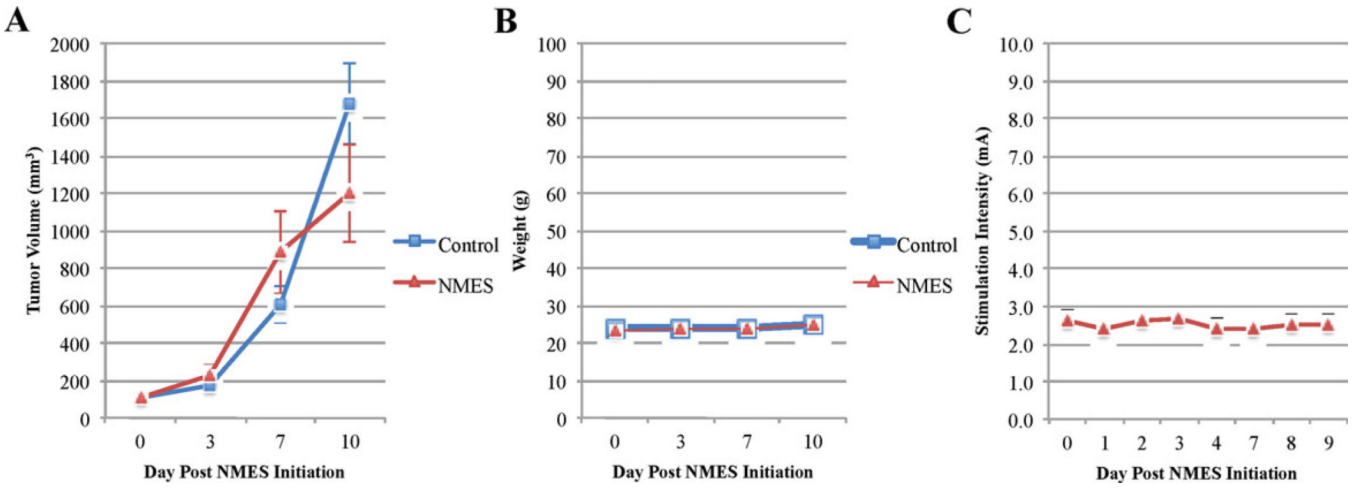

Figure 2.

Gross effects of neuromuscular electrical stimulation (NMES) on subcutaneous SCC7 mouse flank model. Mice were inoculated in the flank with SCC7 (1×10^7 cells) via a subcutaneous route 4 days prior to start of experiment. Seven mice were used for each group, and the data shown are derived from the mean volume μ SE. (A) Tumor volume (mm^3) in mice treated with NMES and controls. No statistical significance between groups ($p = .05$, Student's t test). (B) Animal weight (g) of treated and control mice. (C) Stimulation intensity (mA) of treated mice for each day of stimulation. SCC7, a spontaneously arising squamous cell carcinoma (SCC) of C3H mice. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

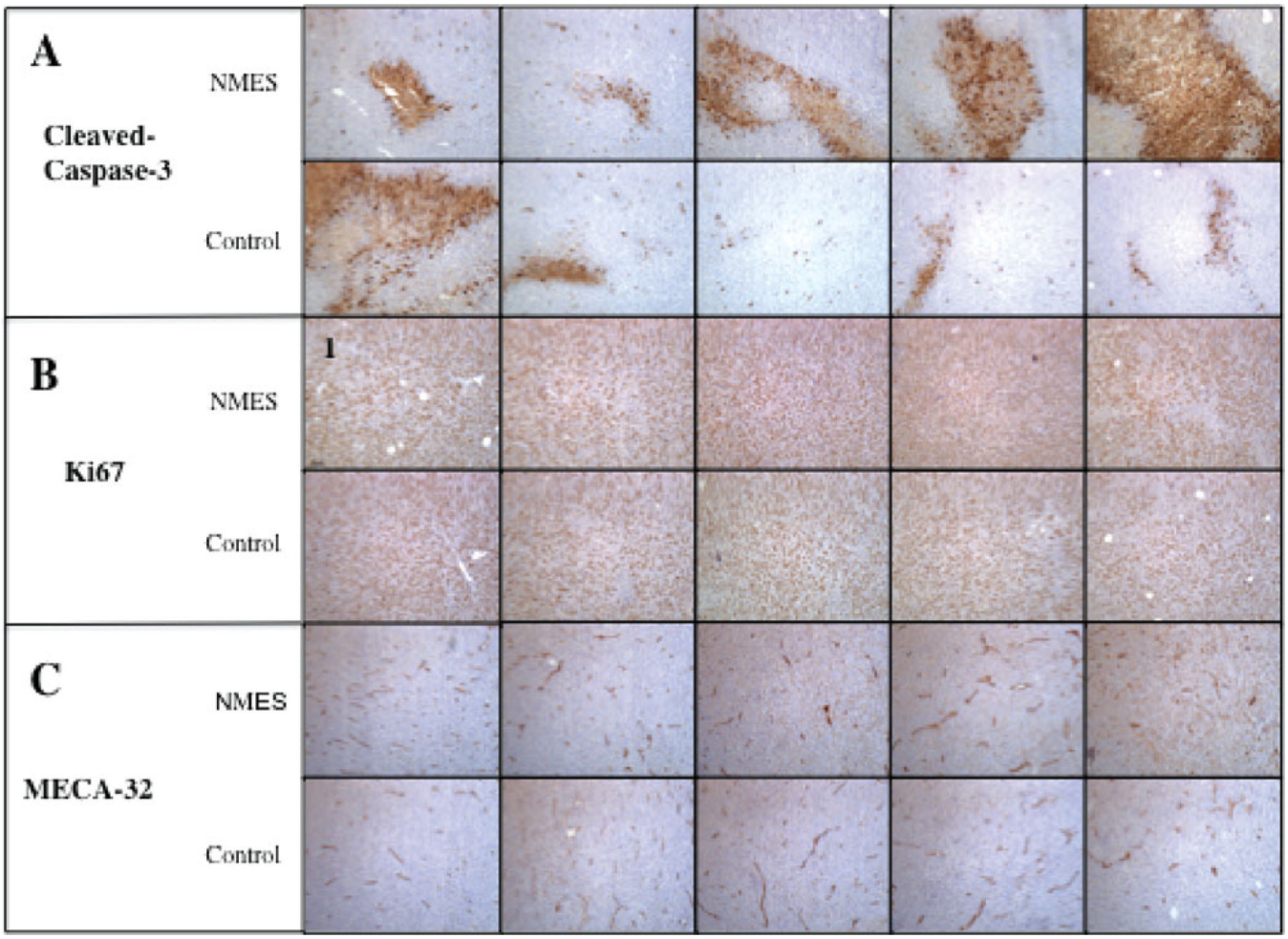

Figure 3.

Immunohistochemistry staining on sections of paraffin-embedded SCC7 tumors. Five tumors were used from each group (NMES and control), per stain. Each image is a representative portion of each tumor. Scale bar in image labeled “1” is 50 μm, and is applicable to all other images. All images were taken under ×10 magnification. Positively staining cells appear brown. (A) Cleaved-caspase-3 is a marker for apoptosis. (B) Ki67 is a marker for proliferation. (C) MECA-32 is a marker for vascularization. SCC7, a spontaneously arising SCC of C3H mice; NMES, neuromuscular electrical stimulation; MECA, mouse endothelial cell antigen; SCC, squamous cell carcinoma.

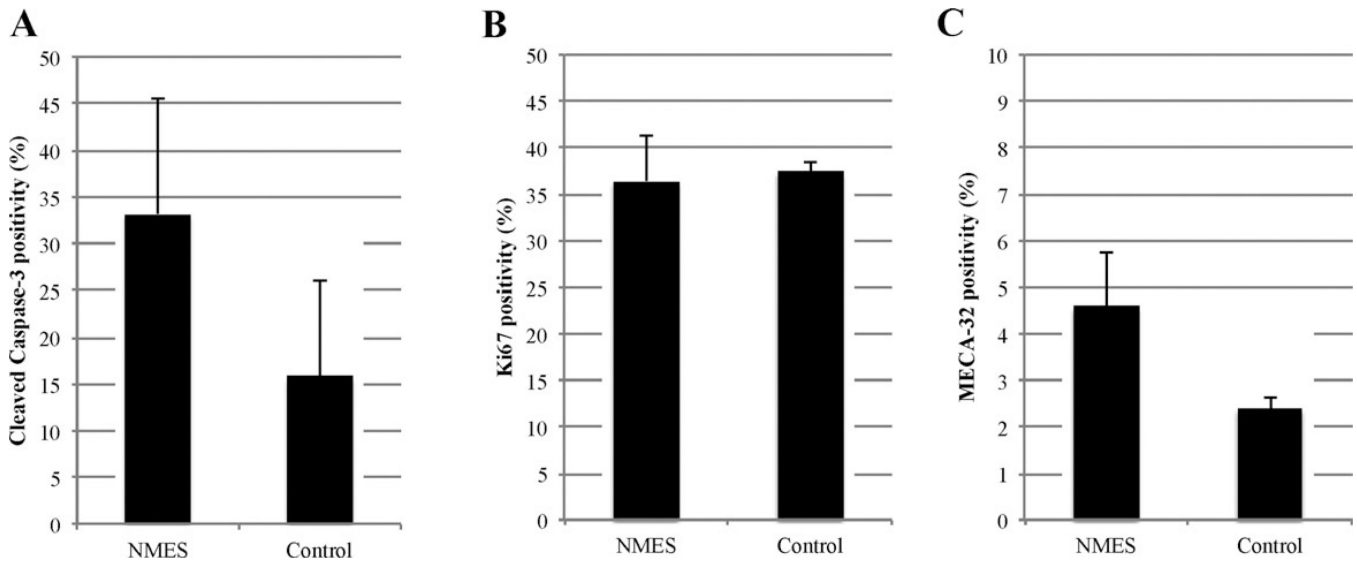

Figure 4.

Cellular effects of NMES on subcutaneous SCC7 mouse flank model. Five tumors were used from each group, per stain. Three high-power fields were counted per tumor. Percent positivity equal to positively stained cells divided by the total number of cells. Data shown are derived from the mean % positivity μ SE. No statistically significant differences were found ($p > .05$, Student's t test). (A) Apoptosis was assessed by IHC with cleaved-caspase-3 and % positivity calculated. (B) Proliferation was assessed by IHC staining with Ki67 and % positivity calculated. (C) Vascularization was assessed by IHC staining with MECA-32 and % positivity calculated. SCC7, a spontaneously arising squamous cell carcinoma of C3H mice; NMES, neuromuscular electrical stimulation; IHC, immunohistochemistry; MECA, mouse endothelial cell antigen.