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Satellite cell functional alterations following cutaneous burn in rats include an increase in their osteogenic potential

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ABSTRACT

Background: Significant consequences of severe burn include skeletal muscle atrophy and heterotopic ossification (HO). The cellular mechanisms underlying either of these conditions are not known. Whether the functionality of satellite cells stem cells resident in skeletal muscle is affected by changes in circulatory factors following burn was determined to better understand their role in atrophy and HO.

Materials and methods: Serum (20%) from sham-treated animals or burned animals (40% total body surface area full-thickness burn) was used to culture satellite cells isolated from either sham or burn animals. Satellite cells were separated based on fiber type (i.e., fast-twitch or slow-twitch in some cases). To gain greater insight into the potential role for satellite cells in controlling muscle mass following burn, the effect of serum taken from burn animals on satellite cell proliferation, migration, and myogenic differentiation was evaluated. Osteogenic differentiation was assessed to evaluate the potential of satellite cells to contribute to HO.

Results: Burn serum (BS) increased the proliferative capacity of cells from fast-twitch muscle, and the migratory capacity of satellite cells taken from both fast- and slow-twitch muscles. BS increased both the myogenic and osteogenic differentiation of satellite cells taken from both sham and burn animals.

Conclusions: The unexpected increase in myogenic functionality of satellite cells with BS is difficult to rectify, given the degree of atrophy that occurs. However, the increased osteogenic capacity of satellite cells with BS suggests they may play a role in burn-induced HO.

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1. Introduction

A significant consequence of cutaneous burn is a rapid and severe atrophy of skeletal muscles that has deleterious effects on long-term outcomes and quality of life [1]. It has been well-documented that elevations in metabolism and catabolism in addition to protein wasting in skeletal muscle is associated with decreased muscle mass [1,2]. In addition to burn-induced muscle atrophy, a less frequent but extremely problematic complication that accompanies burn is the ectopic formation

of bone, or heterotopic ossification (HO). A commonality between both burn-induced atrophy and HO is that they occur distant from the burn injury, implying that circulating factors are responsible, at least in part, for the deleterious consequences. The cells involved and the role of circulating factors in the manifestation of either of these complications that accompany severe burns are not fully understood.

Satellite cells are stem cells that reside between the basal lamina and plasalemma of adult skeletal muscle that have been especially recognized for the role they play in regulating

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skeletal muscle mass in response to local injury [3]. They have also been recognized as having a role in diseases, including cachexia and sarcopenia, where muscle mass may be negatively regulated by systemic factors that contribute to disease [4–6]. *In vivo*, the ability of satellite cells to facilitate changes in muscle is dependent on several different components, including their activation, proliferation, differentiation, and migratory capabilities. We previously reasoned that since satellite cell activation is increased in conditions where hypertrophy occurs, fewer satellite cells would be activated in response to burn where atrophy occurs [7]. Contrary to the hypothesis, satellite cell activation was increased in response to acute burn *in vivo* and when cultured with serum taken from burned rats *in vitro* [7]. This observation supports the idea that burn alters circulating factors that can impact satellite cell functionality. Given their role in muscle homeostasis relies heavily on their proliferation, migration, and differentiation of their progeny, muscle precursor cells [8], a logical speculation is that one of these processes that occur subsequent to activation are diminished. Based on our previous observation of increased activation in the presence of burn serum (BS) it is likely that these processes are also sensitive to the effect of circulating factors altered with burn.

Similar to muscle atrophy, HO can occur at sites distant from the burn injury, and may also result from the profound systemic changes that accompany burn. The stem/progenitor cell(s) responsible for this pathology has yet to be definitively identified; however, the idea that satellite cells may play a role in HO following burn has been suggested [9]. Given the quantity and distribution of satellite cells throughout all skeletal muscles and their osteogenic capacity, satellite cells are uniquely situated to play a role in the development of HO [10,11]. Despite the physical locale and osteogenic capacity for satellite cells and the supposition that they could potentially play a role in HO following burn, such a phenomenon has yet to be documented.

To gain better insight into the potential role for satellite cells in controlling muscle mass following burn, the effect of serum taken from burn animals on satellite cell proliferation, migration, and myogenic differentiation was evaluated. Given the well-documented role in muscle mass, we hypothesized that proliferation, migration, and or myogenic differentiation would be decreased in the presence of BS. To evaluate the potential of satellite cells to contribute to HO they were also treated with serum from sham or burn animals and their osteogenic potential measured. We conclude that muscle precursor cell functions subsequent to activation are not diminished following burn, however, their osteogenic capacity following burn is enhanced in the presence of BS, the latter having significant implications for the role of satellite cells in HO following burn.

2. Materials and methods

This study has been conducted in compliance with the Animal Welfare Act and the implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and was conducted in the animal facility at the US Army Institute of Surgical

Research. Rats were housed individually in a temperature-controlled environment with a 12-h light/dark cycle.

2.1. Burn and serum collection

Male Lewis rats (approximately 350 g; Harlan, Houston, TX) were anesthetized with continuous 1.5%–3% isoflurane (Forane; Baxter Healthcare Corp., Deerfield, IL) in 100% oxygen using a nose cone. Satellite cells were isolated from rats that underwent sham operation or burn injury ($n = 2$ per group). Rats in both sham and burn groups were shaved on the dorsal and ventral surface of the trunk. Animals in the burn group received a 40% total burn surface area by immersing the dorsum in 100°C heated water for 10 s and ventral surface for 10 s according to the modified Walker–Mason burn model [7,12]. Burned rats were resuscitated with 20 mL intraperitoneal Ringer's lactate solution immediately following the burn. Both sham and burn animals received buprenorphine (0.05 mg/kg, q12 h subcutaneously) for 24 h after injury. At 48 h after burn or sham burn, animals were euthanized and the serum was collected using serum separation tubes (BD Vacutainer SST; Franklin Lakes, NJ) and kept in -80°C . All serum was used within 30 d and passed through 0.2 μm filter before application.

2.2. Satellite cell isolation and culture

Satellite cells were isolated from the predominately fast-twitch muscles (tibialis anterior, extensor digitorum longus, plantaris, and gastrocnemius) or slow-twitch muscles (soleus) from both hind limbs of rats 48 h after burn or sham burn using methods similar to that previously described [13,14]. For the proliferation and migration experiments fast-twitch and slow-twitch muscles were treated separately. For differentiation assays only fast-twitch muscles were used. Briefly, following euthanasia, muscles were minced and digested for 1 h at 37°C with 1.25 mg/mL protease type XIV (Sigma; St. Louis, MO) in phosphate-buffered saline (PBS) (pH 7.2), followed by differential centrifugation and a pre-plate on tissue culture-treated dish for 2 h in pre-plate medium (Dulbecco's modified eagle medium; Invitrogen, Grand Island, NY) containing 10% horse serum 1% antibiotic-antimycotic mixture, 0.5% gentamicin, pH 7.2) (Invitrogen, Grand Island, NY) to increase myogenic purity. Cells were then seeded on chamber slides or culture plates precoated with 0.1% Matrigel (Sigma; St. Louis, MO) and cultured in growth medium (GM) (Ham's F-10 medium containing 20% fetal bovine serum and 1% antibiotic-antimycotic mixture, 0.5% gentamicin, pH 7.2). We have previously reported that this methodology yields a high percentage of myogenic cells (>90%) that is unaffected by the burn injury [7]. Where appropriate, cells were passaged at 80% confluence, and cells obtained from each animal were seeded on three separate wells of Matrigel-coated tissue culture plates for each condition.

2.3. Proliferation

At the second passage, 1×10^4 satellite cells isolated from fast or slow skeletal muscles taken from sham or burn animals were seeded on Matrigel-coated 96-well plates in Ham's F-10 medium containing antibiotics (1% antibiotic-antimycotic

mixture, 0.5% gentamicin) and either 20% fetal bovine serum (FBS), 20% sham serum (SS), or 20% BS ($n = 6$ wells per condition). Forty-eight hours later, proliferation was measured using the WST-1 cell proliferation assay as per the manufacturer's recommendation (Millipore, Billerica, MA). Briefly, 10 μ L WST-1 reagent was added, the plate was incubated in 37°C for 2 h, and the absorbance at 440 nm read with a SpectraMax M2 microplate reader (Molecular Devices, LLC, Sunnyvale, CA) with software SoftMax Pro 4.7.1. Results for satellite cells treated with sham and BS were normalized to the absorbance of satellite cells cultured in FBS on the same plate as an internal control.

2.4. Migration

Satellite cell migration was measured using a migration assay kit (Oris Cell Migration Assay; Platypus Technologies, Madison, WI) as per the manufacturer's recommendation. At the second passage, 5×10^3 satellite cells isolated from fast or slow skeletal muscles taken from burn or sham animals were seeded on Matrigel-coated 96-well plates in GM with a stopper (0.3 cm²) to block the cells to the annular region of the well. After 24 h, the stopper was removed and satellite cells were cultured for an additional 24 h in Ham's F-10 medium containing antibiotics (1% antibiotic-antimycotic mixture, 0.5% gentamicin) and either 20% FBS, 20% SS, or 20% BS ($n = 3$ wells per condition). The culture medium was removed, the cells were washed twice with PBS, and then stained with calcein AM (0.1 mg/mL in PBS; Invitrogen, Grand Island, NY), and incubated for 30 min at 37°C. The staining solution was then removed and plate mask was applied to the bottom of the plate so that only annular region was exposed (the area is same as the surface area of the stopper). The fluorescent intensity was determined on a SpectraMax M2 microplate reader with software SoftMax Pro 4.7.1 with excitation at 485 nm and emission at 520 nm, and the results were normalized to the absorbance of satellite cells cultured in FBS on the same plate as an internal control.

2.5. Myogenic differentiation

Equal numbers of freshly isolated satellite cells (passage 0) isolated from predominantly fast skeletal muscles (tibialis anterior, plantaris, and gastrocnemius taken from burn or sham animals) were seeded on separate wells of Matrigel-coated 8-well chamber slides in well plates in Ham's F-10 medium containing antibiotics (1% antibiotic-antimycotic mixture, 0.5% gentamicin), and either 20% FBS, 20% SS, or 20% BS ($n = 3$ wells per condition). Medium was changed 1 and 4 d later. On d 6, the medium was removed, slides were washed twice with PBS, the slides were fixed in 4% paraformaldehyde for 10 min, incubated in 0.3% Triton-X100/PBS for 15 min, and blocked (5% donkey serum in PBS) for 1 h at room temperature, followed by incubation with a myosin heavy chain mouse monoclonal antibody (1:100 dilution; Millipore, Billerica, MA) overnight at 4°C. After three washes in PBS, the slides were incubated with a fluorescence-conjugated secondary antibody (NL-557 anti-mouse immunoglobulin G at 1:200 dilution, NorthernLight; R&D Systems, Minneapolis, MN), washed, and mounted with 4',6-diamidino-2-phenylindole dihydrochloride (Vector Shield

Mounting Medium with 4',6-diamidino-2-phenylindole dihydrochloride; Vector Laboratories, Burlingame, CA) to visualize nuclei.

2.6. Osteogenic differentiation

At the first passage, 1×10^4 satellite cells isolated from predominantly fast skeletal muscles (tibialis anterior, plantaris, and gastrocnemius taken from burn or sham animals) of burn or sham animals were seeded on Matrigel-coated 96-well plates in GM. Forty hours later, GM was replaced with osteogenic medium composed of Dulbecco's modified eagle medium containing, 500 ng/mL human recombinant bone morphologic protein-2 (BMP2; R&D Systems), 10 nmol beta-glycerophosphatase (Sigma; St. Louis, MO), 10 ng/mL rat recombinant fibroblast growth factor (R&D Systems), 1% antibiotic-antimycotic mixture, and either 10% FBS, SS, or BS ($n = 6$ wells per condition). The medium was refreshed every 3 to 4 days. Alizarin red S staining was completed after 3 wk of treatment to determine the amount of osteogenic differentiation and mineralization. Briefly, cultures were washed with PBS and stained with Alizarin red S solution (EMD Millipore, Billerica, MA) for 20 min before photographs were taken. Alkaline phosphatase activity (ALP) was also determined as an index of osteogenic differentiation using an ALP assay kit (QuantifChrom; BioAssay Systems, CA) as per the manufacturer's recommendation. Seven days after seeding cells were washed twice with PBS and treated with 0.2% TritonX-100 (in deionized water) for 20 min, and cell lysates were collected. Thirty μ L of a sample was mixed with 0.17 μ mol of p-nitrophenyl phosphate (p-NPP) substrate solution and the absorbance at 405 nm was read at 0 and 4 min after with a SpectraMax M2 microplate reader with software SoftMax Pro 4.7.1. Results were normalized to protein determined by the BCA assay (Thermo Scientific Pierce BCA kit, Rockford, IL).

2.7. Statistics

Results were compared among the treatment groups using three-way (proliferation and migration) or two-way (osteogenic differentiation) analysis of variance procedures using SigmaPlot 12.0 software (Systat Software Inc, San Jose, CA) followed by Tukey *post-hoc* analyses where appropriate. All values are presented as mean \pm SEM. Significance was established at $P < 0.05$.

3. Results

3.1. Satellite cell proliferation

For satellite cells taken from slow-twitch muscle, there was no effect of serum treatment regardless of whether the satellite cells were taken from sham or burn animals (Fig. 1). Satellite cells taken from predominantly fast-twitch muscle had an increase in number when cultured with BS compared with SS ($P < 0.05$; Fig. 1). Satellite cell proliferation in the presence of SS was not different among slow- and fast-twitch muscles taken from sham or burn animals (Fig. 1).

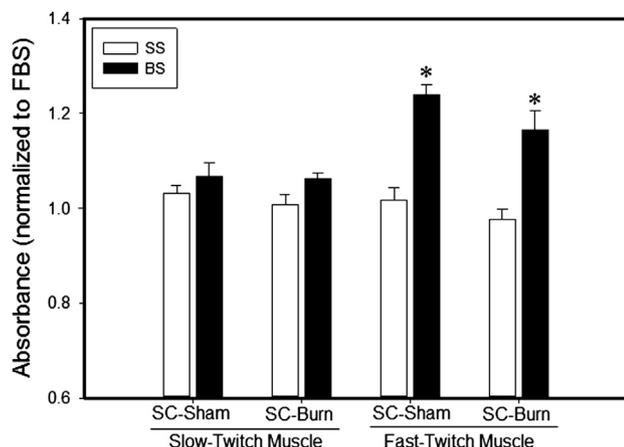


Fig. 1 – BS increases the proliferation of satellite cells isolated from fast, but not slow skeletal muscles. Satellite cells were isolated from fast and slow muscles taken from sham (SC-Sham) or burn (SC-Burn) animals and treated in media containing SS or BS. Proliferation was measured 48 h later. There was no significant difference in proliferation between sham and burn treatment when cells were isolated from slow skeletal muscles. BS treatment increased proliferation of satellite cells isolated from the fast muscles of both burn and sham animals. Bars represent mean \pm SEM for culture wells per group. *Statistically significant difference between BS and SS treatment within fast-twitch muscle, ($P < 0.05$).

3.2. Satellite cell migration

There was a main effect of serum treatment with BS increasing satellite cell migration as compared to SS treatment ($P < 0.05$; Fig. 2). There was also a main effect of cell source on satellite cell migration. Overall, satellite cells derived from slow-twitch muscle had a higher rate of migration compared with satellite cells derived from fast-twitch muscle.

3.3. Myogenic differentiation

Myogenic differentiation was increased when rat serum was used (SS or BS) compared with FBS regardless of whether satellite cells were isolated from sham or burn animals (Fig. 3). Treatment with both SS and BS increased cell fusion and myotube formation compared with treatment with FBS. BS increased myogenic differentiation compared with SS, without obvious differences between muscles isolated from either sham or burn animals (Fig. 3).

3.4. Osteogenic differentiation

Histochemical staining of Alizarin red demonstrated a qualitative increase in osteogenesis in satellite cells from burn animals treated with SS compared with FBS, and compared with satellite cells from sham animals treated with SS or FBS (Fig. 4). BS caused an even greater increase in Alizarin red staining for satellite cells taken from burn animals and was

able to induce osteogenesis in satellite cells taken from sham animals (Fig. 4). ALP followed a trend similar to Alizarin red staining. Satellite cells from burn animals treated with BS had higher ALP activity than all other treatment groups ($P < 0.05$; Fig. 4). For satellite cells from sham animals, BS, but not SS, treatment caused significant increases in ALP activity compared with FBS ($P < 0.05$; Fig. 4).

4. Discussion

In the current study, we have compared the effects of serum taken from either sham or burn animals on satellite cell proliferation, migration, and myogenic differentiation to test the hypothesis that satellite cells are sensitive to, and inhibited by, the systemic changes that accompany severe burn. In addition to these fundamental processes of satellite cells, their osteogenic differentiation capacity was measured to assess their potential to participate in HO following burn.

The rationale for determining the effects of BS in the current study was based on our previous report, where an increase in satellite cell activation in the presence of BS was observed [7]. Since the satellite cells were taken from the limb muscles distant from where the burn occurred (trunk), a logical assumption was that circulating factors played a part in the unexpected increase in activation. The current study is also based on this putative mechanism to explore subsequent functions of satellite cells that allow them to play an incremental role in regulating muscle mass (i.e., proliferation, migration, and myogenic differentiation). Where possible, satellite cells of slow-twitch and fast-twitch muscles were separated. This was based on the increase in satellite cell activation in fast-twitch muscles after burn *in vivo* and *in vitro*, despite an atrophy of fast-twitch skeletal muscle [7,12]. Contrary to our hypothesis, the treatment of satellite cell cultures with BS caused a significant increase in migration compared with SS for both cells isolated from fast or slow muscles, and an increase in proliferation in cells isolated from fast muscles (Figs. 1 and 2). The increase in proliferation of satellite cells from fast-twitch, but not slow-twitch, muscles in the presence of BS is a phenomenon analogous to preferential increases in satellite cell activation in fast-twitch, but not slow-twitch, muscles [7]. This is in contrast to the previous report where a decreased proliferative capacity was observed *in vivo* in burn animals [15]. A potential explanation to the discrepancy may be that the satellite cell microenvironment present *in vivo* may also contribute to the regulation of satellite cell function in response to burn, negating any changes in the circulation. Nonetheless, the effect of BS treatment on satellite cells from fast-twitch muscle when isolated from their *in vivo* environment suggests there is an inherent difference in their sensitivity. Although there was a decreased migration of satellite cells isolated from fast-twitch compared with slow-twitch muscle, and an increase in migration in the presence of BS, there was no difference between cells taken from sham and burn animals within a fiber type (Fig. 2). At the very least, the observation that BS increased instead of decreased migration supports the idea that migration is not a primary factor in burn-induced muscle atrophy. The robust myogenic differentiation (Fig. 3) was also surprising, and

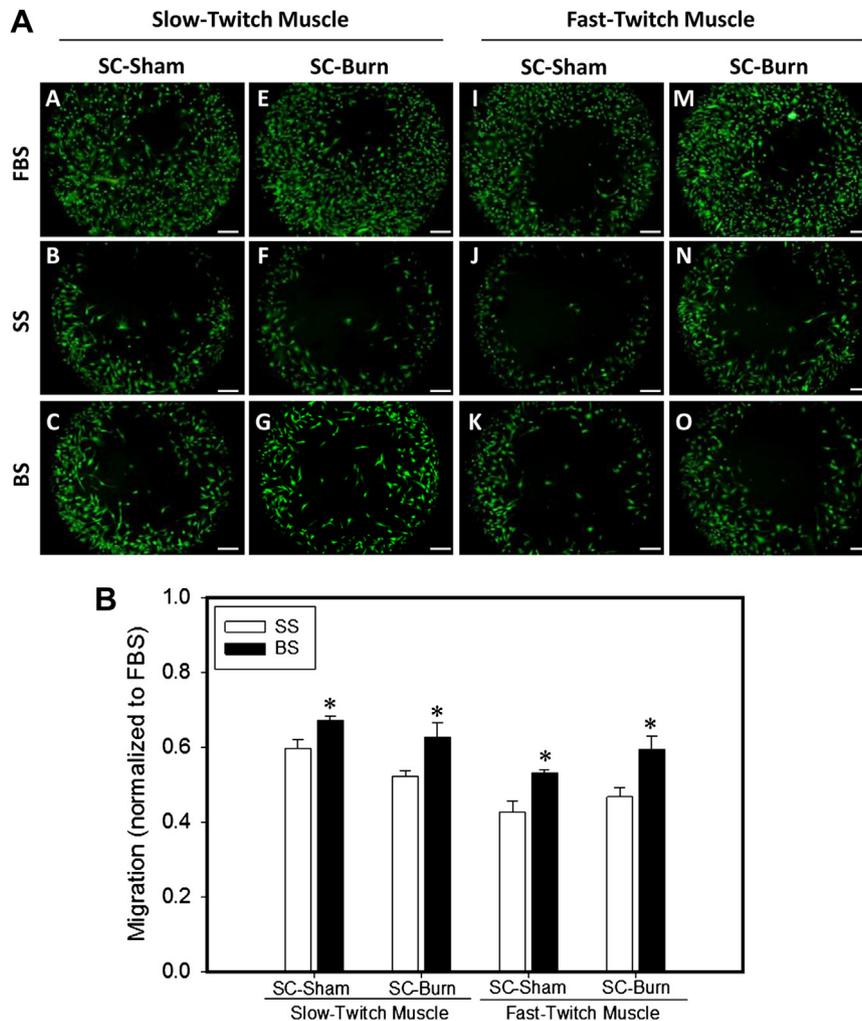


Fig. 2 – BS increases the migration of satellite cells isolated from fast and slow muscles taken from burn and sham animals. (A) Satellite cells were isolated from fast and slow muscles taken from sham (SC-Sham) or burn (SC-Burn) animals and treated in media containing SS or BS. Migration was visualized 40 h later. (B) Quantification of satellite cell migration in “A.” There was a main effect of serum treatment with BS increasing satellite cell migration compared with SS treatment ($P < 0.05$). *Statistically significant difference between BS treatment and SS treatment. Magnification = 4 \times . Bars represent mean \pm standard error of the mean for culture wells per group.

when taken together with the proliferative and migratory observations the overall myogenic capacity *in vitro* appears to be enhanced by burn injury and exposure to BS.

The individual constituents of the BS were not evaluated and cannot be extrapolated from methodology used here. Instead, the net effect of both positive and negative factors in the serum regulated by burn was determined. Several growth factors affected by burn are known to alter satellite cell activity, both positively and negatively, so the attribution of our observations to a single growth factor is not feasible. For example, tumor necrosis alpha levels are elevated in the serum of rats following burn; tumor necrosis alpha increases satellite cell activation and proliferation [16]. Conversely, Insulin-like Growth Factor-1 levels are decreased in the acute phase of burn, but Insulin-like Growth Factor-1 increases muscle mass and decreases atrophy exerting its effects, at least in part, through improving satellite cell proliferation and

differentiation [13,17,18]. The majority of our findings are in response to serum derived from sham or burn animals. These findings should be interpreted with caution since the exposure of cells to serum in culture likely results in a level that is higher than would be seen *in vivo*. Future studies are required to further substantiate the role of circulating factors on satellite cells following burn.

Although the counterintuitive findings herein may simply be an acute response to injury, the discrepancy between increased function in the face of significant losses in muscle mass that are occurring during this phase is not resolved [7]. Indeed, the long-term consequences of this overall increased activity remains to be determined. More specifically, whether the overall capacity of the satellite cells is exhausted at later time points after initial increases in activity, similar to that which is observed with spinal cord injury or denervation will be of significant value [19,20]. With regards to burn, markers of

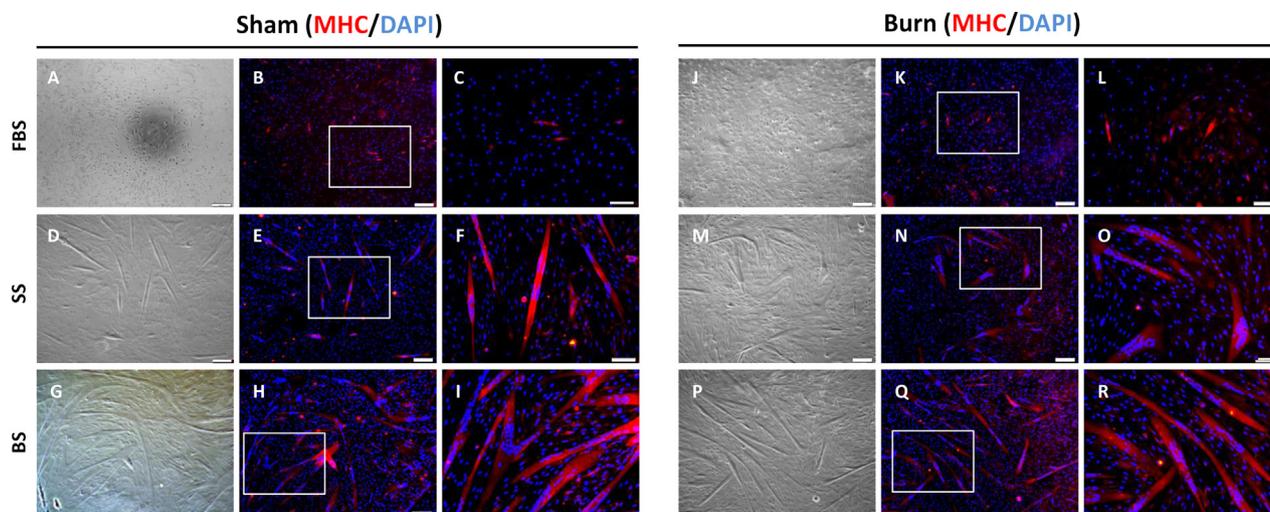


Fig. 3 – Effect of serum treatment on myogenic differentiation. Satellite cells isolated from predominantly fast skeletal muscles taken from either sham (A–I) or burn animals (J–R) were treated with either FBS (A–C, J–L), SS (D–F, M–O), or BS (G–I, P–R) and stained for myosin heavy chain and 4',6-diamidino-2-phenylindole 6 d after seeding. Images A, D, G, J, M, and P are brightfield images corresponding to B, E, H, K, N, and Q, respectively (Bar = 100 μ M). Images in C, F, I, L, O, and R are magnified images of areas indicated by the boxed area in B, E, H, K, N, and Q, respectively (Bar = 200 μ M). Treatment with both SS and BS appeared to increase differentiation compared with FBS with a slight increase in differentiation in BS compared with SS. No apparent differences were observed if cells were isolated from either sham or burn animals.

apoptosis with the capability to induce satellite cell apoptosis have been shown to be up-regulated after injury, which could potentially eliminate the ability of satellite cells to play an integral role in muscle [21].

Another parallel that can be drawn between nerve and burn injuries, in addition to the muscle atrophy, is the presence of HO, with immobilization having an impact on its development in both cases [22,23]. Although generally accepted as a rare occurrence, variations presumably attributable to the population studied and the method of detection, the incidence of HO in burn patients has been reported as high as 13.6% [23]. To date, stem/progenitor cells identified as having a pivotal role in HO following burn, denervation, and spinal cord injury, have yet to be identified. Interestingly, a therapeutic strategy for the treatment of HO for both nerve injury and burn is radiation, a methodology used experimentally to inhibit satellite cells function reducing their contribution to regrowing and/or regenerating muscle [24,25]. Given the apparent similarities between the two models, it may be of interest to rely on the wealth of data collected pertaining to satellite cells in the context of nerve injury to guide future research to delineate mechanisms concerning burn-induced atrophy and HO.

Although the osteogenic capacity of satellite cells has been described [10,11], and is evidenced by the current findings, the possibility that satellite cells may play a role in HO following burn or other traumatic injuries has been largely ignored. This is somewhat surprising, given their abundance in skeletal muscle tissue compared with other stem/progenitor cells and their robust mobilization following injury. The C₂C₁₂ cell line, commonly used as an *in vitro* model for both myogenic and

osteogenic studies for decades, was derived from the serial passage of myoblasts which supports their osteogenic capacity [26]. The potential for a satellite cell contribution to HO may be overlooked because of inadequate culture conditions. In the current study, when satellite cells were treated with the commonly used FBS, osteogenesis was minimal, especially compared with the presence of species-specific serum (serum from either sham or burn rats). There was an appreciable increase in osteogenesis even when satellite cells were cultured in the presence of SS as opposed to FBS, which was even more robust when BS was used (Fig. 4). The main findings that lead us to the conclusion that satellite cells have the potential to play a role in HO following burn were their increases in ALP activity and Alizarin red staining. These metrics are standard indices of osteogenic differentiation and are similar to those used by others when identifying presumptive cells responsible for HO [27]. Therefore, such an observation may present valuable information to aid in delineating mechanisms for this complication.

The primary cell responsible for HO following burn remains elusive, and the difference in osteogenic potential between satellite cells and other stem/progenitor cells was not determined in the current study. Given the well-documented osteogenicity of mesenchymal stem cells (MSCs) an unexpected finding would be a greater osteogenic capacity of satellite cells as compared to MSCs. However, even when allowing for a greater osteogenic potential of MSCs, the overall abundance, distribution, and responsiveness of satellite cells to an insult in skeletal muscle make them a plausible candidate for causing HO *in vivo*. The concept that some MSCs are pericytes associated with vessels may offer an additional

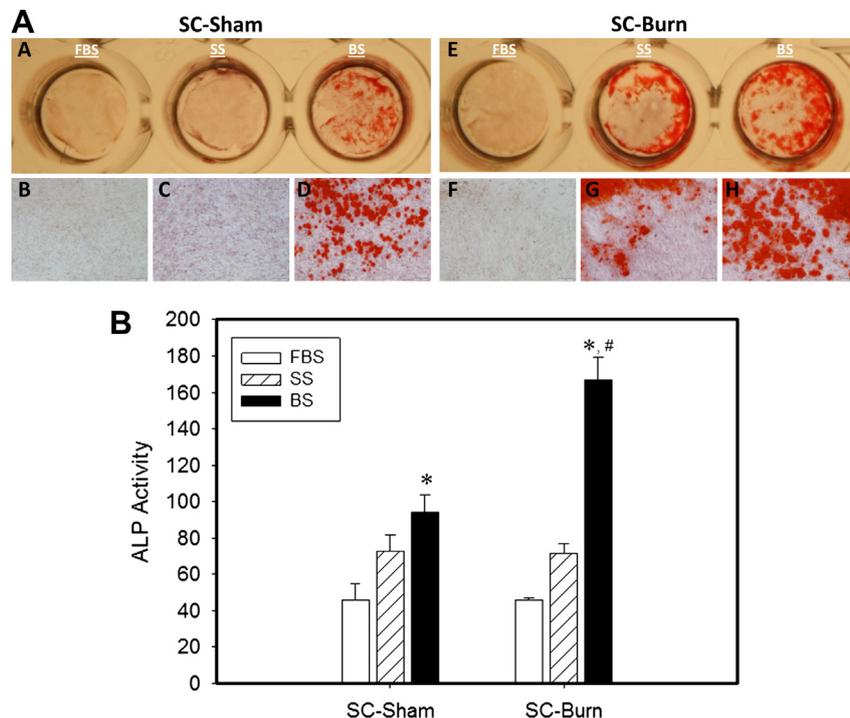


Fig. 4 – Effect of serum treatment on satellite cell osteogenic differentiation. (A) Satellite cells isolated from predominantly fast skeletal muscles taken from either sham (SC-Sham) or burn (SC-Burn) animals were treated with FBS, SS, or BS for 21 d and stained for Alizarin Red S. Figures A and E are representative photographs of an entire well, B–D and F–H are magnified images taken from the culture wells above them (bar = 50 μ M). (B) After 7 d of culture, ALP activity was increased in the presence of BS compared with FBS or FBS and SS for satellite cells from sham and burn animals, respectively. Bars represent mean \pm SEM for culture wells per group. *Statistically significant difference between BS treatment and FBS; #Significantly different from all other treatment groups ($P < 0.05$).

mechanism to support the possibility that MSCs are in a position to play a role in HO [28]. Currently, this possibility is limited simply by the lack of information regarding their activity following burn and other skeletal muscle injuries, especially compared with the wealth of understanding related to that of satellite cells. To effectively address this conundrum, *in vitro* experiments will likely not be the answer as comparisons are subject to logistical constraints. Satellite cells are isolated at a high purity, whereas the percentage of MSCs in an initial isolate, especially in skeletal muscle, is relatively small, requiring extensive culture expansion before osteogenic potential can be quantified. This relatively more extensive *in vitro* manipulation of MSCs makes an extrapolation of their *in vivo* importance for HO more difficult than for satellite cells that can be isolated with a high level of purity [14]. Future work, including improvements in our understanding of muscle biology following burn as well as technological advances, will undoubtedly be critical for these questions to be fully answered.

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The authors declare no conflict of interest.

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