Culture conditions influence satellite cell activation and survival of single myofibers

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Abstract

Single myofiber isolation protocols allow to obtain an in vitro system in which the physical association between the myofiber and its stem cells, the satellite cells, is adequately preserved. This technique is an indispensable tool by which the muscle regeneration process can be recapitulated and studied in each specific phase, from satellite cell activation to proliferation, from differentiation to fusion. This study aims to clarify the effect of different culture conditions on single myofibers, their associated satellite cells, and the physiological behavior of the satellite cells upon long term culture. By direct observations of the cultures, we compared different experimental conditions and their effect on both satellite cell behavior and myofiber viability.

Key Words: satellite cells, proliferation, quiescence, growth factors, cell adhesion, myofibers
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Myofibers are multinucleated muscle cells representing the functional contractile units of skeletal muscle. During embryogenesis, myofibers develop by fusion of mononucleated myoblasts into multinucleated myotubes. Worth highlighting, myofiber growth and muscle repair are lifelong physiological processes. Each muscle fiber is populated by a number of myogenic precursor cells residing between the sarcolemma and the basal lamina, i.d. satellite cells (SC).[1,2] During growth and in response to various stimuli, such as exercise, local injuries or muscular diseases, SC provide postnatal muscle with a remarkable regenerative potential, thus contributing to muscle repair also through the formation of new muscle fibers.[3-6]. To this end, stimulated SC activate, proliferate, and terminally differentiate, by either fusing with preexisting myofibers, or originating new myofibers fusing with one another.[7,8] Single myofiber isolation and culture represents an *in vitro* approach allowing not only to investigate the biology of the fiber itself, but also the behavior of *in-situ* quiescent SC and of their progeny, after their migration from myofibers. In 1977, Bekoff and Betz introduced the first protocol for single myofiber isolation from rat muscle, further improved by Bischoff, and since then adopted by many others.[9-11]. To date, such protocol has proved to be one of the most reliable methods to study rat and mice SC behavior *in vitro*. Recently, several methodological modifications have been proposed aimed to address specific needs.[12,13] The single myofiber isolation technique involving the use of floating myofibers is widely exploited to study quiescent, *in situ* (i. e. beneath the basal lamina) SC. When myofibers are cultured in floating conditions, SC are subjected to virtually no influences other than the myofiber environment.[13,14] Single myofibers can also be cultured in standard, adhering conditions. Substrate stiffness and coating type, though, were shown to influence SC biology and ability to recapitulate muscle regeneration *in vitro*.[15-18]. Medium composition has also been shown to influence SC behavior. Serum concentration plays a key role in SC transition from quiescence to activation.[19-22]. In serum-rich medium, SC quickly activate, proliferate, migrate off the fiber, and differentiate, mimicking the *in vivo* regenerative process which takes place following various stimuli. Conversely, in low-serum medium, SC quiescence endures [21]. Another medium component effective on cell kinetics and adopted in several *in vitro* protocols is the chick embryo extract (CEE), a poorly characterized culture supplement rich in heterogeneous components influencing various cellular signalling pathways.[23] Differently combined, serum and CEE, both rich in growth factors, finely tune muscle stem cell behavior, cell cycle kinetics, and expression of myogenic factors.[24-26] Therefore, it is important to establish the most

appropriate isolation and culture conditions before starting a study. In order to summarize and to better define the working options based on both classical and more recent methods, the present work reports the results obtained observing the behavior of both single myofibers and SC activation, proliferation, differentiation and viability in different culture conditions.

Materials and Methods

Myofiber isolation and culture

Single myofibers were isolated from EDL muscles of 4-8-week-old mice as previously described.[12] The detailed protocol follows in the Practical Guide Section. Briefly, EDL muscles were dissected and incubated in DMEM (Sigma- Aldrich) containing 0.2% collagenase I (Sigma-Aldrich) for 45 minutes. Myofibers were detached by gentle flushing with a glass pipette and washed several times in DMEM containing 1% penicillin streptomycin. Myofibers were then cultured in high glucose DMEM containing 1% penicillin streptomycin (Sigma-Aldrich) (Basal Medium), differently supplemented as detailed in table 1. CEE was prepared as previously described [24, 27, 28]. Matrigel coating was performed according to Keire P [28]. Briefly, thawed Matrigel (Corning) was diluted with ice cold DMEM to 1 mg/ml final concentration. After 15 min in ice, the solution was used to cover 24 well-plate wells. The coated plates were left on ice for 7 min, then excess Matrigel was removed and the coated dishes placed in the incubator for at least 1 hour.

Medium	FBS (v/v)	HS (v/v)	HSA/ BSA (w/v)	CEE (v/v)
A	15%	_	_	1%
B	15%	_	_	_
C	_	15%	_	1%
D	_	15%	_	_
E	_	2%	_	1%
F	_	2%	_	_
G	_	_	2%	1%
H	_	_	2%	_

Table 1. *Composition of different culture media obtained by supplementing Basal Medium with the indicated concentrations of: Fetal Bovine Serum (FBS, Sigma-Aldrich), Horse Serum (HS, Sigma-Aldrich), Human Serum Albumin (HSA, Sigma-Aldrich), Bovine Serum Albumin (BSA, Sigma-Aldrich), CEE.*

Practical guide for single myofiber isolation protocol

1. Before starting

1.1. Prepare four petri dishes (60x15mm) per mouse, one for fiber isolation and three for washings. The latter dishes are prepared as follows: cover the dishes with sterile filtered 5% BSA (Sigma-Aldrich) in PBS (Sigma-Aldrich), remove the solution and let the dishes dry in a sterile hood with the lid open for at least 30 min. BSA will prevent fiber attachment to the dish during the isolation and washing processes. Once the dishes are dry, add 4 ml of DMEM (Dulbecco's modified Eagle's medium, high glucose, L-glutamine with 110 mg/ml sodium pyruvate) (Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and store in an incubator at 37°C with 5% CO2.

1.2. Prepare two previously sterilized glass pipettes: one with a large hole for myofiber dissection from the muscle, the other curved, with a tiny hole for single myofiber handling. Use a flame to curve the glass pipet and smooth the edges so as not to damage myofibers. Coat the pipet surface with 5% BSA in PBS solution by aspirating and releasing the solution several times; then, let the pipettes dry for 30 min in a sterile hood.

1.3. Prepare 0.2% Collagenase Type I (Sigma-Aldrich) solution in a pre-filtered sterile DMEM media and store at 4°C.

1.4. If myofibers need to be attached for long-term culture: coat the dishes with matrigel (Corning) according to Keire P 26. Briefly, thaw in ice the required frozen aliquots of matrigel for at least 30 min; add ice cold DMEM to dilute matrigel to 1 mg/ml. Leave the solution on ice for at least 15 min, then use 250-300 µl per well to cover 24 well-plate wells. Leave the matrigel-coated plates on ice for 7 min, then remove excess matrigel and leave the coated dishes in incubator for at least 1 hour. Ten minutes before use, let the dishes dry in a sterile hood with the lid opened. Matrigel coated dishes can be kept in incubator with DMEM for a maximum of 1 week.

Myofibers can be cultured in suspension by using 5% BSA in PBS coated dishes, prepared as described in a. Coating with BSA instead of serum yields less active satellite cells.

2. EDL muscle dissection and digestion

Young (4-8 weeks old) C57/BL6 mice are used to isolate myofibers. EDL muscle is dissected as previously described 9. Briefly:

2.1. Spray hindlimbs with 70% EtOH and remove the skin with scissors.

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2.2. Expose the muscles and cut the TA and EDL distal tendons.
2.3. Holding the TA and EDL distal tendons, pull the muscles up, until EDL muscle is completely visible.
2.4. Holding EDL tendon, gently separate the EDL muscle from the TA, then cut the TA off.
2.5. Expose the proximal tendon of the EDL and cut it, paying attention not to damage the EDL muscle.
2.6. Transfer the EDL muscle into a 15 ml tube containing 2 ml of 0, 2% Type I Collagenase, and incubate it at 37°C either in a water bath without agitation or in an incubator for 45 min. Digestion time may vary depending on the EDL size. Muscle digestion should be checked frequently to avoid over-digestion. Stop the digestion when myofibers start to fray out of the muscle.
2.7. Transfer the digested EDL muscle in a Petri dish containing warm DMEM with 1% pen/strep and proceed with the dissociation of single myofibers.

3. Single myofiber dissociation

3.1. Under a stereo microscope, use the large bore glass pipette to fill up medium from the dish and flush it on the EDL muscle. Single myofibers will start to fray out of the muscle.
3.2. Repeat the step 1 until myofibers will dissociate from the EDL. To avoid the dish cooling, place it back to the incubator for at least 5 min every 10 min.
3.3. With the small-bore glass pipette, collect individual live myofibers and transfer them to a clean dish containing warm DMEM with 1% pen/strep.
3.4. Repeat this the step 3 twice to separate live myofibers.
3.5. Leave the myofibers in DMEM with 1% pen/strep in the incubator for 1 hour.
3.6. Transfer the myofibers in the dishes properly prepared for the experiment. To avoid myofiber loosing, for downstream analysis only single live myofibers, which appear as long and shining tubular structures, are selected.
3.7. This protocol allows to obtain around 100 vital single myofibers from two EDL muscles.

Microscopy and imaging

Daily observations were performed using an inverted phase-contrast microscope (Nikon Eclipse, TS100). Images were acquired with a Nikon DS-Fi2 camera and NIS Elements version 4.0 Imaging System. Images were assembled using Adobe Photoshop.

Morphometric analysis

SC were counted on images acquired with a 20x objective. Four randomly selected fields were counted for each experimental condition.

Statistical Analyses

Data are presented as mean ± SEM of randomly selected fields for each sample. Statistical significance was determined using two-tailed Student's t-test with significance defined as $p<0.05$ (*).

Results

Effect of FBS on the survival of floating myofibers

We evaluated the effect of FBS on myofiber survival and SC behavior by culturing single myofibers in suspension and comparing 15% FBS-supplemented media (medium A) to the serum-free one (medium G). To prevent fiber attachment, dishes were coated with 5% BSA in PBS. Prevented from adhering, single myofiber survival was compromised (Figure 1). Both in high serum and in HSA-supplemented media, myofibers appeared contracted at day 6 in culture. When myofibers were kept in suspension, SC migration was prevented, with SC

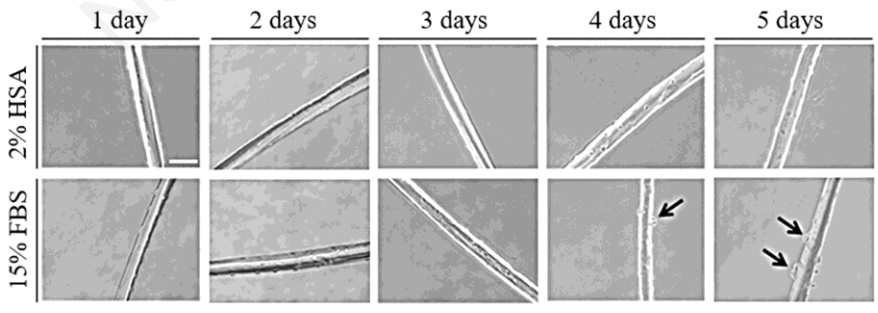

Fig 1. *Evaluation of floating myofibers behaviour. Representative images of floating myofibers cultured with two different media, over time. Arrows indicate SC clusters. Scale bar = 50 micron.*

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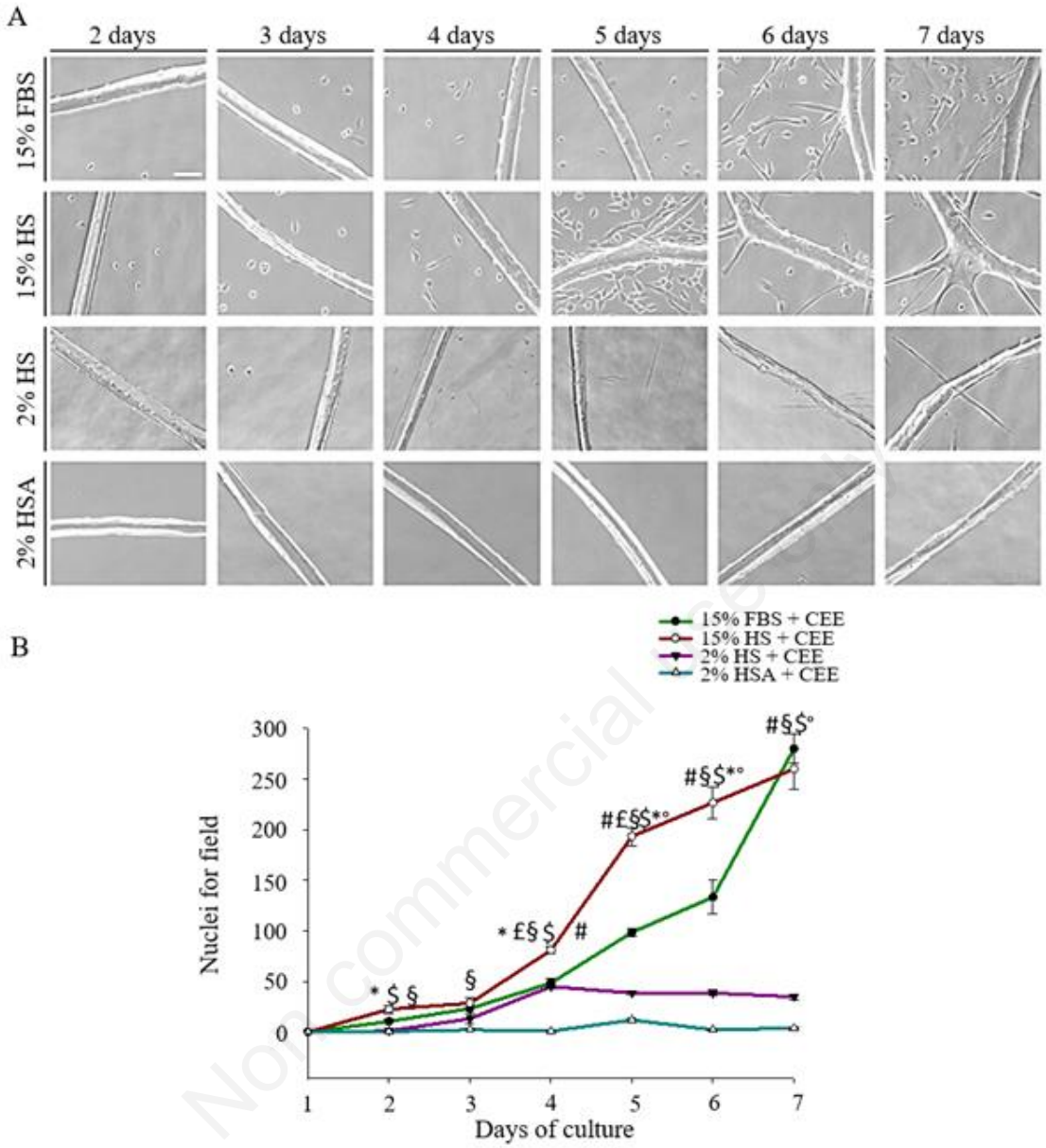

Fig 2. *Evaluation of myofibers behavior in adhesion. (A) Representative images of single myofibers in adhesion on Matrigel coated dishes, cultured with different media, over time. Scale bar = 50 micron. (B) SC quantification in all time point analysed. Data are presented as mean ± SEM. $p < 0.05$ (Student's t-test). * = 15% FBS vs. 15% HS; $ = 15% HS vs. 2% HS; § = 15% HS vs. 2% HSA; # = 15% FBS vs. 2% HSA; £ = 2% HS vs. 2% HSA; ° = 15 FBS vs. 2% HS.*

proliferation being more pronounced in 15% FBS where several SC clusters on myofiber surface were observed, than in serum-free media (Figure 1).

Effect of serum type and concentration in adherent culture

When single myofibers were cultured in adherent conditions (Matrigel-coated dishes) their behavior and survival were strongly modulated by serum type and concentration in the culture media. We evaluated the effect of different culture conditions namely 15% FBS or HS, 2% HS, 2% BSA or HSA on single myofibers in the presence of 1% CEE (media A, C, E, G). Isolated myofibers adhered onto the dishes after about one day of culture. No differences were observed among the different culture conditions at one day after seeding.

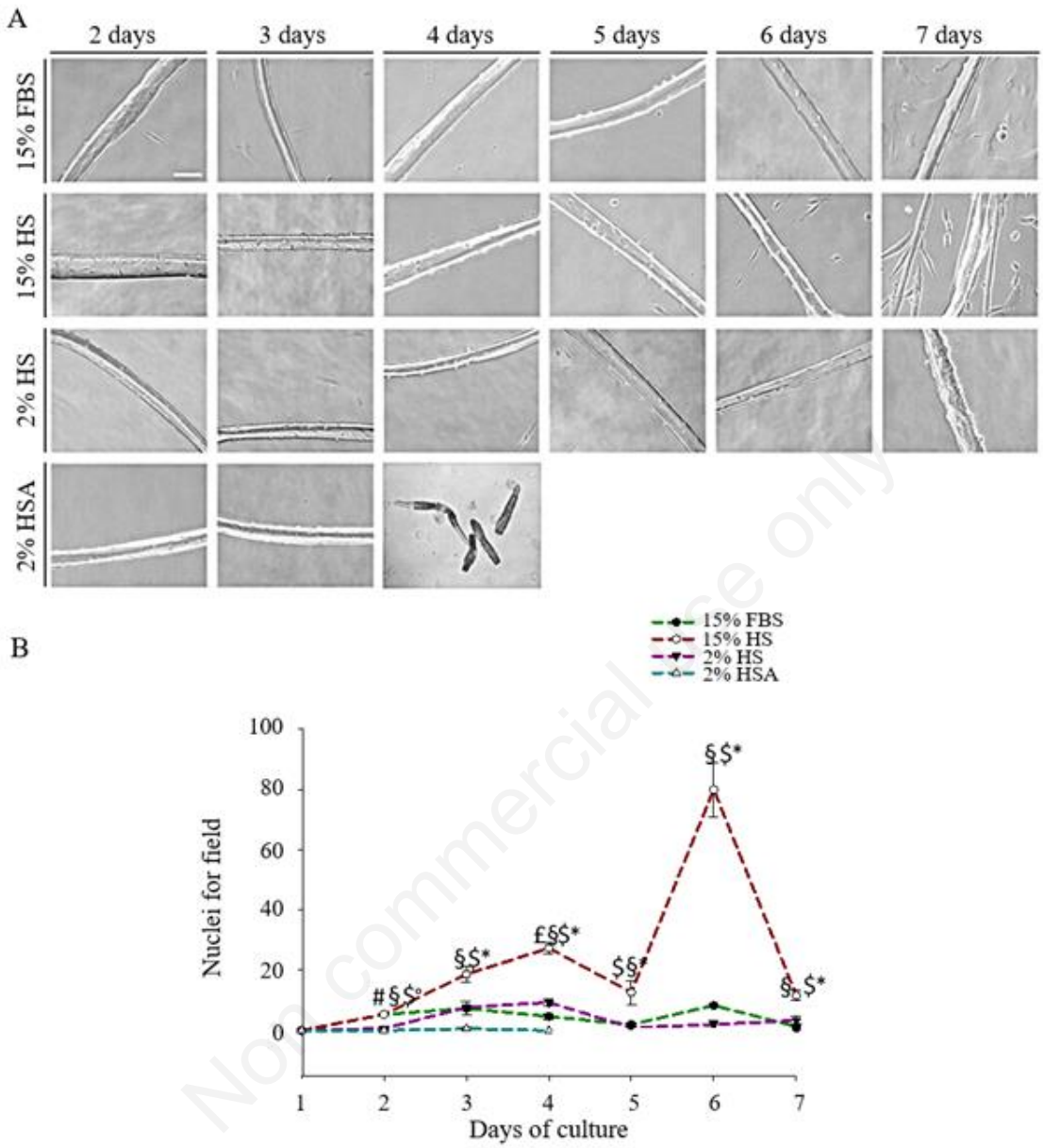

Fig 3. *CEE is necessary for myofiber survivor. (A) Representative images of myofiber cultured with different media depleted of CEE, over time. Scale bar = 50 micron. (B) SC quantification in all time point analysed. Data are presented as mean ± SEM. p < 0.05 (Student's t-test). * = 15% FBS vs. 15% HS; $ = 15% HS vs. 2% HS; § = 15% HS vs. 2% HSA; # = 15% FBS vs. 2% HSA; £ = 2% HS vs. 2% HSA; ° = 15 FBS vs. 2% HS.*

After 2 days in high serum media, SC migration from the parent myofiber occurred, followed by proliferation (Figure 2A). A higher number of SC was present in 15% HS than in 15% FBS (Figure 2B). At later time points, i.e. 5 and 6 days, the difference between 15% HS and 15% FBS became more evident (Figure 2B). At day 7, in 15% HS, virtually all the SC outside the myofibers were found to be fused in myotubes or within the myofibers. Conversely, in 15% FBS the differentiation process was slower and a higher number of the SC outside the myofibers were proliferating, as witnessed by their round shape (Figure 2A). Contrary to high percent serum media, myofibers in 2% HS showed very few SC migrating away after 4 days in culture (Figure 2B). Moreover, SC differentiation was higher in low serum medium, as shown by the presence of small myotubes close to their parent myofibers at day 5 (Figure 2A). Single myofibers in serum-free medium cultured in the conditions, i.e. by using either BSA (data not shown) or HSA (medium G), gave comparable results. SC

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quiescence was preserved in both conditions, as evidenced by the absence of SC clusters on myofibers or SC migrating out of the fibers (Figure 2, A and B). In all the above conditions, and at all the time points analysed, myofibers maintained adhesion to the substrate without showing signs of suffering, such as changes in morphology or in diameter.

Effect of CEE on myofiber survival and SC activation

Another batch of myofibers was cultured in the same media as above, but without CEE (media B, D, F, H). In high serum, CEE-free media, SC proliferation and migration (i.e. activation) was both delayed and reduced (Figure 3, A and B), although SC differentiation and fusion still occurred at day 7 in culture. In the low serum condition, (medium F) SC appeared quiescent over all the time points analysed. However, at day 7, myofibers showed signs of deterioration and lost adhesion to the substrate (Figure 3A). Importantly, in serum-free, CEE-free medium (medium H), myofibers died at day 4 (Figure 3A).

Discussion

In the present study different conditions for culturing single myofibers were implemented to upgrade fragmentary literature data. In agreement with Anderson 2012,[21] we found that low serum concentration preserves the quiescence state of SC in myofibers. Serum free conditions allowed maintaining single myofibers in culture without promoting SC activation; however, after a few days, the fibers were compromised. Albumin addition to the media is necessary and sufficient to avoid myofiber death. Therefore, to better evaluate SC activation induced by growth factors or other ectopic treatments, the use of BSA or HSA-supplemented, serum-free media is suggested. Albumin is the most abundant protein in serum, typically present at 35 mg/ml in mice, accounting for about 60% of the total proteins. A primary function of albumin, besides contributing to oncotic pressure, is to bind, sequester and stabilize a number of important small molecules and ions that (if not complexed) would be unstable or destructive, respectively. In addition, albumin acts as a multifaceted antioxidant thus supporting cell growth *in vitro*.[29] To prevent fiber attachment, plastic dishes are normally coated with HS,[13,30] which stimulates SC activation. Since the mechano-enzymatic fiber isolation protocol is known to partially activate SC, in order to interfere as little as possible with SC activation, we used albumin-coated dishes for both fiber isolation and culture. Media composition influenced SC behavior also when isolated fibers were cultured in suspension. Indeed, after 5 days in culture, myofibers still presented fewer cells migrating in serum-free media, while SC clusters were observed on myofibers in high-serum concentration media. While myofibers in suspension looked healthy until 5 days after isolation, fiber attachment on Matrigel is more suitable for long-term studies, the latter condition allowing cultured myofibers to be maintained in a good living state up to 3 weeks in all the CEE-supplemented media adopted (data not shown). Furthermore, the key role of CEE emerged in all the culture conditions adopted. Derived from chick embryos, CEE contains variable amounts of many growth factors, able to stimulate cell growth and differentiation.[24,29] Usually, a higher CEE concentration is used in growth media compared to differentiation media. Our results emphasize the CEE pivotal role in fiber culture *in vitro*. Indeed, CEE absence caused early fiber death in all the conditions tested. Overall, our results, summing up our direct observations showing that SC and isolated myofiber biology is influenced by different culture conditions, suggest such conditions to be carefully considered upon planning a research project. The direct comparison of very different culture conditions allows, for the first time, to choose the most appropriate medium and surface coating to favour SC quiescence vs. their activation, the maintenance of intact fibers vs the migration of SC, the proliferation vs. the differentiation of SC and last, the use of serum-free medium to investigate the effect of specific factors.[25,26,31]

List of acronyms

DAHFMO - Department of Anatomy, Histology, Forensic Medicine and Orthopedics
SC - satellite cells
CEE - chick embryo extract
EDL - exstensor digitorum longus
DMEM - Dulbecco's modified Eagle's medium
SEM - standard error of the mean
FBS - fetal bovine serum
BSA - bovin serum albumin
HS - horse serum
HSA - human serum albumin

A**uthor's contributions**

AR and AB performed the experiments; all authors contributed to critical analysis of the data and to writing the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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