Diaphragm Satellite Cells Isolation by Optimized MACS and the Effect of Mechanical Ventilation on their Proliferation and Differentiation Characteristics through MyoD and Myogenin Pathways

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Abstract

Objective: In the present study, we aimed to establish a feasible method to isolate single diaphragm satellite cells from C57 mice, and clarify the effect of mechanical ventilation (MV) on the proliferation and differentiation of diaphragm satellite cells. Moreover, the underlying molecular mechanism was also explored.

Methods: After the dissection of the diaphragm, enzymolysis, and specific antibody selection, single diaphragm satellite cells were harvested from C57 mice receiving 6 h of MV or not with optimized magnetic-activated cell sorting (MACS) approach. The cells were stained with BrdU or labeled with the differentiation antibody MYH3, followed by observation using fluorescence microscopy. The cells were counted from randomly selected visual fields, and the proliferation or differentiation characteristics of the control and MV groups were compared by IMAGE software. Besides, the expressions of MyoD and myogenin were detected by quantitative real-time PCR (*qRT-PCR*).

Results: The single diaphragm satellite cells were successfully purified through MACS using a set of optimized parameters. Generally speaking, 1.5×10^5 cells could be harvested from a single diaphragm. Upon MV, the proliferation rate of diaphragm satellite cells was decreased from 88.74% to 81.92%, while the differentiation rate was increased from 17.94% to 27.58%. Moreover, the expressions of MyoD and myogenin were significantly up-regulated upon MV.

Conclusions: In our current work, an efficient method was successfully established to isolate single diaphragm satellite cells. After MV, the differentiation rate of diaphragm satellite cells tended to increase, and the expressions of MyoD and myogenin were up-regulated. Collectively, our findings provided valuable insights into further research and clinical target treatment.

Background

Mechanical ventilation (MV) can be life-saving but is also associated with adverse effects on the diaphragm muscle [1, 2]. Especially, weaning from MV is a challenging step during recovery from critical illness. Moreover, weaning failure is associated with increased morbidity and mortality, exposing patients to life-threatening complications again [3]. Accumulating evidence has indicated that the prolonged MV (1 week or longer) promotes respiratory muscle weakness due to both contractile dysfunction and atrophy, especially in the diaphragm [4–7], indicating that diaphragm dysfunction plays a key role in weaning failure from MV. Petrof BJ and Vassilakopoulou have termed such a condition as ventilator-induced diaphragmatic dysfunction (VIDD) [8, 9].

The proliferation of diaphragm satellite cells is a promising driving force to reverse VIDD. It is well known that in muscle regeneration, satellite cells are a small population in myogenic stem cells and adult muscle. These cells are mitotically quiescent, while they can be activated following injury, leading to the production of myogenic precursor cells, or myoblasts to mediate the regeneration of muscle tissues [10]. It has been reported that satellite cells expressing paired box protein 7 (Pax7) are the primary myogenic

cells required for skeletal muscle regeneration [11]. Moreover, CXCL12 (C-X-C motif chemokine ligand 12) can facilitate muscle regeneration and Pax7 expression after cardiotoxin-related muscle injury [12]. Previous studies have well documented the importance of proliferation and differentiation of satellite cells in MV-associated respiratory failure. Besides, the myogenic cells in dystrophic muscle display accelerated differentiation [13]. However, it remains largely unexplored whether such a phenomenon also happens in the diaphragm muscle.

Previous studies have shown that pro-myogenic factors or transplantation of muscle satellite cells (MuSCs) can enhance skeletal muscle regeneration [14]. Anti-IL-6R antibody can also promote skeletal muscle regeneration [15]. Heme oxygenase-1 (HO-1; Hmox1) can affect myoblast differentiation by inhibiting myomiRs [16]. Inspiratory muscle training can improve the weaning outcomes in mechanically ventilated patients [17]. Moreover, diaphragm satellite cells are myogenic cells with potentials for proliferation and differentiation [18]. If the targets of MV changing the growth characteristics of diaphragm satellite cells can be clarified, the corresponding well-directed enhancers or inhibitors will be selected, which can help patients recover better. Therefore, the effective isolation of diaphragm satellite cells is the first step of subsequent mechanism research. However, no study has reported an effective approach to obtain diaphragm satellite cells. In the present study, we established an optimized method to purify single diaphragm satellite cells, and the effects of MV on the proliferation and differentiation of satellite cells were also explored. Collectively, our findings provided valuable insights into targeted treatment.

Materials

Xylazine and pentobarbital sodium were provided by McGill University Health Center (Montreal, Canada). Magnetic-activated cell sorting (MACS) kit was obtained from Miltenyi, Germany. Fluorescence microscope was provided by Zeiss Axio ImagerM2, Germany.

Methods

1 MV intervention

The animal-related protocols used in the present study were carried out following the guidelines for animal care and approved by the institutional animal care and use committee (IACUC) of McGill University Health Centre, and the suffering of animals was minimized by all types of efforts according to recommendations proposed by the European Commission (1997). Male C57 mice (10~12 weeks of age) were housed under standard conditions (McGill University Health Center, Montreal, Canada).

Eight mice were randomly divided into two groups, control group and MV group. The mice in the control group were fasted for 6 h, while free access to water was given. The animals in the MV group received 6 h of controlled MV using a previously described protocol. The duration of MV for 6 h was selected based on previous work showing a significant reduction in diaphragmatic force-generating capacity. Briefly, MV

mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg body weight) and pentobarbital sodium (50 mg/kg body weight) and then orally intubated. No neuromuscular-blocking agent was used. The endotracheal tube was connected to a small animal ventilator (Minivent®; Harvard Apparatus, Canada) with the following settings: fraction of inspired oxygen of 0.21 (room air), controlled volume mode with a tidal volume of 10 μ L/mg body weight, respiratory rate of 150 to 170 breaths/min, and positive end-expiratory pressure level of 3 to 4 cm H₂O achieved by placing the expiratory port under a water seal. Animals were placed on a prewarmed homeothermic blanket (Homeothermic Blanket Control Unit; Harvard Apparatus), and the mice received hourly intraperitoneal injections of lactated Ringer's solution (0.10 mL) to compensate insensible losses. Besides, pentobarbital was administered every 1 to 1.5 h to maintain adequate anesthesia as indicated by a lack of animal movement or spontaneous respiratory efforts.

2 Pure diaphragm separation

All the animals were sacrificed using the CO₂ asphyxia method. Diaphragm muscles were dissected, and blood, fat, and tendons were removed. The resultant pure diaphragm muscles were maintained in the F12 medium.

3 Diaphragm muscle digestion

The diaphragm was weighed and chopped into a fine slurry in 800 μ L TC until it could pass through a 10-mL pipette freely. The fine slurry was transferred into a 15-mL Falcon tube and mixed with 9.2 mL TC. The mixture was incubated at 37 °C for 35 min with gentle agitation. Subsequently, the Falcon tube was allowed to stand for 2 min, and 9.5 mL supernatant was collected into a 50-mL Falcon tube containing 8 mL FBS. The above-mentioned steps were repeated three times to completely digest the muscle. Finally, the digested muscle suspension was filtered through an 80-micron nylon cloth and centrifuged at 1,800 rpm (688 g) for 10 min at 4°C. The single cells were resuspended and harvested.

4 MACS isolation

The enzymolysis cell sample was resuspended in 80 μ L buffer per gram tissue, and labeled with the specific antibody in 20 μ L SC isolation at 4 °C for 15 min. MS column was placed on the magnetic rack, followed by pre-equilibration using PBS containing 1% FBS. The cell suspension was applied to the column, and the unlabeled cells were collected.

After centrifugation, the cell pellet was resuspended at a density of 10⁶ cells per 80 μ L buffer, and mixed with 20 μ L anti-integrin α 7 microbeads, followed by incubation at 4°C for 15 min. Another MS column was placed on the magnetic rack, followed by pre-equilibration. Labeled cells were loaded onto the

column. The column was then removed from the magnetic field and washed by 1 mL PBS containing 1% FBS. The target cells (diaphragm satellite cells) were collected.

5 Cell culture and view division

The matrigel medium was prepared and kept at 4°C. After the cell number was determined, the diaphragm satellite cells were seeded into 12-well plates at a density of 1.5×10^4 per well with 400 μ L medium. The cells were cultured at 37 °C for 4 days. Subsequently, the view field of the fluorescence microscope was divided into 8-10 parts to observe for accurate counting (Fig. 2).

6 Immunofluorescence detection

The cells were stained with 0.03 mg/mL BrdU for 24 h, and sequentially incubated with acetone and 1.5 M HCl. Next, the cells were blocked with serum for 1 h. Subsequently, the cells were subjected to incubation with the primary antibody (1:1,000) and secondary antibody (1:500). After fixed with 1:5,000 Hoechst and covered using coverslips, the cells were observed under Zeiss M2 fluorescence microscope. The proliferation characteristics of satellite cells were determined according to the intensity of blue color from BrdU immunofluorescence staining.

To determine the differentiation characteristics of satellite cells, cells were fixed with 4% PFA and blocked in blocker buffer. Subsequently, the cells were labeled with primary antibody (MYH3, 1:25) and secondary antibody (F-488-IgG, 1:500). Next, cells were fixed with 1:5,000 Hoechst and covered using coverslips. The cells were observed under Zeiss M2 fluorescence microscope, and the differentiation characteristics of satellite cells were determined according to the intensity of blue color.

7 Quantitative real-time PCR (*qRT-PCR*)

Total RNA was isolated from satellite cells using TRIzol reagent (ThermoFisher, USA) and then reversely transcribed into cDNA. The expressions of MyoD and myogenin were assessed using *qRT-PCR* on a StepOne plus system (ABI Instrument, USA). HPRT1 was selected as the housekeeping gene. The primers were designed using Primer3.0, and the primer sequences were as follows: MyoD 5'-AGAATGGCTACGACACCGCC-3', 5'-GCTGTCTGTGGAGATGCGCT-3'; myogenin 5'-GAGGAGCGCGATCTCCGCTA-3'; 5'-GTCAGCCGCGAGCAAATGAT-3'; HPRT1 5'-CATGCCATGGCATAAGTGAAGGTGATC-3'; 5'-TGACAAGCTTGTGTGCCGCCTGGGCACT-3'. Briefly, after an initial denaturation step at 94 °C for 10 min, the amplifications were carried out with 40 cycles at a melting temperature of 94 °C for 30 s, and an annealing temperature of 60 °C for 1 min. The relative expressions of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method.

8 Image software and statistical analysis

Green or blue cells were counted separately, and the ratio was determined. The positive area was calculated with Image software.

All data were expressed as mean $\pm$ SD, and an independent sample t-test was selected to analyze the variables. $P < 0.05$ was considered statistically significant.

Results

1 Optimized isolation for diaphragm satellite cells

Generally speaking, the muscle weight of the diaphragm from C57 mouse was approximately 160 mg, and 1.5×10^5 single diaphragm satellite cells could be harvested from a single diaphragm through MACS using the optimized parameters. These findings laid a preliminary foundation to further investigate the biological characteristics of diaphragm satellite cells and their related mechanisms.

2 MV reduces the proliferation of satellite cells

We found that the cell morphology of satellite cells in the control group was normal, showing obvious proliferation. However, the proliferation of satellite cells in the MV group was significantly reduced, exhibiting apparent disintegration (Figure 3A and 3B).

All the satellite cells from the control or MV group were stained with BrdU after 4 days of incubation. We found that the cell number of the control group was greater compared with the MV group (Figure 3C and 3D), suggesting that the cell proliferation ability in the control group was greater compared with the MV group. However, such finding should be validated using other methods.

3 MV changes the cell morphology of diaphragm satellite cells

All the satellite cells were labeled with specific differentiation antibody after 4 days of incubation. We found that the diaphragm satellite cells in the control group exhibited normal cell morphology, and the nucleus was distributed orderly in fiber bundles. However, the satellite cells of the MV group showed irregular cell morphology, and the nucleus was also irregular and scattered, indicating that the cells of the MV group tended to differentiate (Figure 4C and 4D).

4 MV affects proliferation/differentiation of diaphragm satellite cells

The cell number was counted from randomly selected areas (Fig. 5A, B, C, D). We found that the proliferation ratio of diaphragm satellite cells was decreased by MV from 88.74% to 81.92%, while the differentiation ratio was increased from 17.94% to 27.58% (Fig. 5E).

5 The expressions of MyoD and myogenin are up-regulated upon MV

MyoD and myogenin are promising impact factors on the diaphragm in terms of proliferation or differentiation [Yablonka-Reuveni Z, Anderson JE, 2006]. Fig. 6 shows that the expressions of both above-mentioned genes were significantly up-regulated upon MV for 6 h compared with the control group, supporting the differentiation results of diaphragm satellite cells.

Discussion

Currently, no reliable approach is available to obtain diaphragm satellite cells, which has greatly restricted further research on relevant mechanisms. In the present study, we aimed to solve this problem with MACS. It is known that MACS can positively select target cells using magnetic beads coated with specific high-affinity monoclonal antibodies [19]. Previous studies have shown that MACS can isolate healthy hematopoietic stem/progenitor cells (HSPCs), astrocytes, and iNKT cells [20-22]. Moreover, target cell isolation through MACS is 4–6 times more efficient than FACS [23]. Through a series of MACS parameter optimization, we successfully established a stable method to obtain single diaphragm satellite cells. The muscle weight of a single diaphragm from C57 mouse was about 150 mg, and 1.5×10^5 single satellite cells could be harvested from it. These findings laid a preliminary foundation to further investigate the biological characteristics of diaphragm satellite cells and their related mechanisms.

MV is a life-saving supportive therapy for patients with acute *respiratory failure*, while it is often referred to as a two-edged sword for patients in cardiogenic shock [24]. MV has been associated with some major complications, such as infection, barotrauma, tracheal injury, and ventilator-induced lung injury [25], especially the ventilator-induced difficulty in weaning [26]. MV can shorten the diaphragm, and induce VIDD, such as damage, disuse, or even weaning failure [27, 28]. A previous study has indicated that diaphragmatic strength and endurance play critical roles in successfully weaning patients from MV [29]. More precisely, MV can cause a sudden interruption of diaphragmatic contractile activity, in which the energetic requirements of the muscle are abruptly reduced [30]. The decreased compound muscle action potential after MV suggests that impaired muscle fiber membrane excitability and/or excitation-contraction coupling participate in such process, leading to disuse atrophy [31, 32]. The relationship between diaphragm muscle strength and ventilation time is descending logarithmically [33]. Taken together, the mechanisms underlying the MV-induced diaphragm damage potentially involve a multistep process, including muscle atrophy, oxidative loads, structural damage, and muscle fiber remodeling. However, such mechanisms have not been well defined.

In the present study, we speculated that the proliferation and differentiation characteristics of diaphragm satellite cells played a critical role in weaning difficulties from MV. It is known that in adult muscle, satellite cells are the myogenic progenitors [13]. In the dystrophic (mdx) mouse model of human Duchenne muscular dystrophy, satellite cells participate in myofiber regeneration [14]. It has been also reported that the atrophy of the diaphragm is faster compared with other skeletal muscles under adverse stimulation [34]. Myofibers isolated from dystrophic muscles show a more rapid differentiation, although there is no statistical significance. Furthermore, myofibers purified from mdx mice exhibit an earlier differentiation compared with the control group (C57 mice). More interestingly, myogenic cells from the dystrophic (mdx) mouse display an accelerated differentiation [13]. In our present study, we also found that diaphragm satellite cells tended to differentiate after 6 h of MV. This result was consistent with the reduced diaphragmatic flexibility, disuse atrophy, and dysfunction after MV.

The interplay between proliferation and differentiation is tightly regulated by the helix-loop-helix family of muscle-specific transcription factors. There are four members in this family, including Myf5, MyoD, myogenin, and MRF4, which are often regarded as myogenic regulatory factors (MRFs) [13]. The latest research has shown that MLL1 can facilitate the proliferation of myoblasts and Pax7-positive satellite cells by epigenetically regulating Myf5 [35]. The myogenic factor MyoD can regulate skeletal muscle differentiation by interacting with a variety of chromatin-modifying complexes [36]. However, bexarotene enhances the differentiation and fusion of myoblasts through a direct regulation of MyoD expression, coupled with an augmentation of myogenin protein [37]. PARP1 depletion can boost the expressions of MyoD targets, such as p57, myogenin, Mef2C, and p21 [36]. In summary, Myf5, MyoD, myogenin, and MRF4 play a crucial role in directing satellite cell function to regenerate skeletal muscle, linking the genetic control of developmental and regenerative myogenesis [38]. In the present study, we partly confirmed that the expressions of MyoD and myogenin in diaphragm satellite cells were significantly increased after 6 h of MV. These findings might help explain the stem cell differentiation tendency and diaphragm dysfunction after MV.

Conclusions

We developed a feasible and efficient approach to isolate diaphragm stem cells through optimized MACS, **which solved the bottleneck in this research field**. Meanwhile, it was clarified that diaphragm stem cells tended to differentiate after MV, and the expressions of MyoD and myogenin were significantly increased. Taken together, our findings provided valuable insights into deeper mechanisms of VIDD.

Abbreviations

MV	Mechanical ventilation
BrdU	Synonyms: 5-Bromo-2'-deoxyuridine
MACS	Magnetic-activated cell sorting
MYH3	Myosin heavy chain 3
MyoD	Myogenic Differentiation Antigen
qPCR	Quantitative Real-time Polymerase Chain Reaction
VT	Volume of Tidal
VIDD	Ventilator-induced Diaphragmatic Dysfunction
Pax7	Paired box protein 7
CXCL12	Chemokine (C-X-C motif) ligand 12
MuSCs	Muscle satellite cells
HO-1	Heme Oxygenase-1
IACUC	Institutional Animal Care and Use Committee
SPF	Specific Pathogen Free
RR	Respiratory Rate
PEEP	Positive End Expiratory Pressure
TC	solution(50ml TC=42ml F12+4ml 1%Trypsin+4ml 1%Collagenase D)
PBS	Phosphate Buffer Saline
FBS	Fetal Bovine Serum
PFA	Paraformaldehyde
MS	Magnetic Separation
SD	Standard Deviation
CDK	Cyclin-dependent kinases
HSPCs	Haematopoietic Stem/Progenitor Cells
iNKT	Invariant Natural Killer T
Myf5	Myogenic factor 5
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory factors

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

All authors declared no potential conflicts.

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Authors' contributions

Junying Ding performed experiments, conducted data analysis, and drew the manuscript. Qian Li, Feng Liang, and Salyan Bhattacharai contributed to the tests. Qingquan Liu provided related financial support.

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Figures

A

B

C

D

Figure 1

Collection of diaphragm and satellite cells A MV: Inhalation oxygen partial pressure (0.21), VT 10 $\mu\text{L}\cdot\text{kg}^{-1}$, RR 150-170/min, PEEP 3-4 cm H₂O; B Dissection of diaphragm muscle: 150 $\pm$ 30 g/diaphragm muscle; C Diaphragm enzymolysis: 37°C, 30 min, three times; D Isolation of satellite cells through MACS: about 1.5 $\times$ 10⁵ cells/diaphragm;

A

B

Figure 2

View division of fluorescence microscope Green fluorescence stands for cell proliferation (Fig. 2A), and blue fluorescence indicates cell differentiation (Fig. 2B). Slides were observed using fluorescence microscopy. Each selected view was divided into 8-10 parts. To ensure accuracy, the experiment was repeated four times by two researchers.

A

B

C

D

Figure 3

Proliferation assay of diaphragm satellite cells Satellite cells were isolated from the diaphragm muscle of C57 mice with or without 6 h of MV, followed by in vitro cultivation for 4 days (Fig. 3B and A) and 5 days (Fig. 3D and C). Cells were maintained in the culture medium supplemented with 0.03 mg/mL BrdU and allowed to undergo spontaneous proliferation. Cells were immunostained for proliferation assay and counterstained with DAPI to show the nucleus. Initially, the cells showed equivalent density in the control

or experimental group. At the end of the experiment, the cell number was counted in both groups, and the proliferation characteristics of satellite cells were determined. Blue fluorescence represents proliferating cells. Magnification 40×10. A Diaphragm satellite cells in the control group on day 4; B Diaphragm satellite cells in the MV group on day 4; C Diaphragm satellite cells in the control group stained by BrdU after 5 days; D Diaphragm satellite cells in the MV group stained by BrdU after 5 days;

A

B

C

D

Figure 4

Differentiation assay of diaphragm satellite cells Satellite cells were isolated from the diaphragm muscle of C57 mice with or without 6 h of MV, followed by in vitro cultivation for 4 days (Fig. 4B and A). Cells were fixed with 4% PFA and immunostained for differentiation assay. Initially, the cells showed equivalent

density in the control or experimental group. At the end of the experiment, the differentiation of cells was observed under a fluorescence microscope, and the differentiation characteristics of satellite cells were determined. Green fluorescence represents differentiating cells. Magnification 40×10. A Diaphragm satellite cells in the control group; B Diaphragm satellite cells in the MV group; C Diaphragm satellite cells stained by differentiation maker in the control group; D Diaphragm satellite cells stained by differentiation maker in the MV group;

Figure 5

The distributions of proliferating satellite cells or differentiating satellite cells A Proliferation results in the control group; B Proliferation results in the MV group; C Differentiation results in the control group; D Differentiation results in the MV group; E Proliferation and differentiation ratio; The distributions of proliferating satellite cells (Fig. 5A, B) or differentiating satellite cells (Fig. 5C, D) after MV or not in stem cells isolated from the diaphragm. Cells were maintained in the culture medium for 4 days before specific staining. With image software, the proliferation and differentiation ratio was calculated, as shown in Fig. 5E.

Figure 6

The relative expression levels of MyoD and myogenin in different groups. Data are presented as mean $\pm$ SD (n = 3).