Apr 10, 2025

# Explant *in natura* and cryopreserved myometrial tissue for *in vitro* cell culture of Primary Smooth Muscle from Myometrium of *Ovis Aries* ($PSMo_{24}$)

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**Protocol status:** Working
**We use this protocol and it's working**

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**Keywords:** uterus, muscle, reproduction, sheep, culture, cell culture of primary smooth muscle, primary smooth muscle from myometrium cell, primary smooth muscle from myometrium, studying primary smooth muscle, tissue culture, specific muscle cell, immediate acquisition of uterine tissue, myometrium cell, muscle cell, myometrial tissue, psmo24 cell culture, myometrium of ovis ary, primary smooth muscle, uterine tissue samples from ovis ary, cell culture, uterine tissue sample, establishment of psmo24 cell culture, uterine tissue, fragments of myometrial tissue, myometrium, subsequent cell explantation, standard in cell biology study, tissue sample, cell biology, cell biology study, fresh cell, similar to the in vivo state, cell passage, cell proliferation, physiological mechanisms with other cell type, muscle, uterus, protocol for cryopreservation, comprehensive understanding of cellular function, human biotechnology, vivo state, tissue, cell

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## Disclaimer

The information presented in this article aims to provide general knowledge on the topic discussed. The use of this information is the sole responsibility of the reader. The author is not responsible for any direct, indirect, or consequential damages resulting from the use or interpretation of the data presented here.

## Abstract

Since the first *in vitro* cell culture was conducted in 1907 by the American Ross Granville Harrison to the present day, cell culture has established itself as an essential tool for basic and applied research, covering areas such as cell biology, molecular biology, physiology, animal and human biotechnology, among others. Cell culture is widely recognized as the standard in cell biology studies due to its ability to mimic the cellular environment in a way similar to the *in vivo* state. In the context of studying primary smooth muscle from myometrium cell, beyond their reproductive importance, these cells share physiological mechanisms with other cell types, allowing for a more comprehensive understanding of cellular function and specific muscle cells. One of the main challenges in establishing primary culture is the immediate acquisition of uterine tissue. To overcome this limitation, this study describes the development of a methodology for primary smooth muscle from myometrium of *Ovis Aries* (**PSMo$_{24}$**) and establish a protocol for cryopreservation of myometrial tissue followed by subsequent cell explantation. This approach aims to maximize the uterus' biological potential and minimizing tissue waste, which is traditionally used only in fresh form. Moreover, given the restrictions on animal's experimentation, tissue culture stands out as an alternative method, providing valuable knowledge to evaluate substances. This contributes to the reduction or replacement of animal experimentation, aligning with ethical and scientific principles in biological studies. For the establishment of **PSMo$_{24}$** cell culture, uterine tissue samples from *Ovis Aries* were collected and subjected to enzymatic digestion. Simultaneously, fragments of myometrial tissue were cryopreserved, thawed, and explanted for cell culture. Ten cell passages were performed, along with quantification, morphology analysis, cytotoxicity assessment, antioxidant capacity evaluation, and RNA extraction. Here, we validated the protocol for **PSMo$_{24}$** from fresh cells and cryopreserved tissue for explant by demonstrating the morphology, viability, and cell proliferation, indicating new possibilities for their aplication in studies using reproductive and muscle cells, as well as in pharmacological or bioproducts tests in animals and humans.

## Attachments

PNG

Imagem8.png

10.8MB

## Guidelines

The propose a methodology for the primary culture of smooth muscle cells from the myometrium of *Ovis aries* (**PSMo$_{24}$**), emphasizing the use of both fresh and cryopreserved tissue. The protocol includes uterine tissue collection, enzymatic digestion, cell explantation, cell passaging, and morphological, functional, and molecular analyses. The aim is to optimize the use of uterine tissue, reduce the use of animals in research, and expand the applications of these cells in reproductive, muscle, and pharmacological studies.

## Materials

Silica Resin **Carolina Biological Supply** **Catalog #**C33426

| | Reagents | Company | Code |
|---|---|---|---|
| | ABAP (Ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfônico), solução a 1 mM) | Sigma-Aldrich | 440914 |
| | Amphotericin B Solution | Sigma-Aldrich | A2942 |
| | Antibiotic/Antimycotic | Sigma-Aldrich | A5955 |
| | Collagenase from Clostridium histolyticum | Sigma-Aldrich | C9891 |
| | DAPI - 4′,6-Diamidine-2′-phenylindole dihydrochloride | Sigma-Aldrich | D9542 |
| | DCFH-DA 2',7'-Dichlorofluorescin diacetate | Sigma-Aldrich | D6883 |
| | Dimethyl sulfoxide - DMSO | Sigma-Aldrich | D2650 |
| | Dulbecco's Modified Eagle's Medium/High Modified | Sigma-Aldrich | 56436C |
| | Ethylene glycol | Sigma-Aldrich | 324558 |
| | Fetal Bovine Serum | Sigma-Aldrich | F7524 |
| | Formaldehyde solution | Sigma-Aldrich | 818.708 |
| | Glycerol | Sigma-Aldrich | G7757 |
| | Ham' Nutrient Mixture F12 | Sigma-Aldrich | 51651C |
| | Hanks' Balanced Salt solution | Sigma-Aldrich | H6648 |
| | HEPES | Sigma-Aldrich | H6147 |
| | MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) | Sigma-Aldrich | CT01-5 |
| | penicilina/estreptomicina | Sigma-Aldrich | P4333 |

| | Reagents | Company | Code |
|---|---|---|---|
| | Phalloidin 488 | Sigma-Aldrich | 49409 |
| | Phosphate Buffered Saline | Sigma-Aldrich | 806544 |
| | Quercetin Solution | Sigma-Aldrich | Q4951 |
| | Sacarose | Sigma-Aldrich | 50389 |
| | Sodium Bicarbonate | Sigma-Aldrich | S5761 |
| | Sodium chloride | Sigma-Aldrich | S5886 |
| | Solução de azul de tripano a 0,4%. | Sigma-Aldrich | T6146 |
| | Triton X-100 | Sigma-Aldrich | T8787 |
| | Trypsin-EDTA | Gibco | T2610 |
| | Absolute Ethanol | Êxodo Científica | AG07145 RA |
| | Isopropyl alcohol | Êxodo Científica | AI09965 RA |
| | Tri Reagent | Sigma-Aldrich | BCCB016 3 |
| | Chloroform | Êxodo Científica | C09877R A |

Reagents for Primary Smooth Muscle Cell Culture from Ovis aries Myometrium

| | Material For Cell Culture (Sterile) |
|---|---|
| | Vacuum Filter System for Bottles |
| | Syringes (10 mL and 20 mL) |
| | Sterile Pipettes |
| | Sterile Filter (if necessary) |

| | Material For Cell Culture (Sterile) |
|---|---|
| | Sterile Falcon Tubes (15 mL and 50 mL) |
| | Sterile 0.22 µm Filter |
| | Shoe Covers |
| | Scalpel Handle |
| | Scalpel Blade |
| | Petri Dish |
| | Parafilm |
| | Paper Towel |
| | Mask |
| | Glass Tip |
| | Glass Rod |
| | Glass Bottles – Blue Cap |
| | Falcon Tube Racks |
| | Disposable Gown |
| | Disposable Gloves |
| | Cell Culture Bottles |
| | Bottle with Alcohol |
| | Beaker |
| | Aluminum Foil |
| | 1-Liter Volumetric Flask |

Material for cell Culture to Primary Smooth Muscle Cell Culture from Ovis aries Myometrium

| | Equipment | Company |
|---|---|---|
| | Inverted Microscope | Bel photonics |

| | Equipment | Company |
|---|---|---|
| | Heating Plate | Ted |
| | Flow 1 (Biological Safety Cabinet) | Pachane pa400eco |
| | Flow 2 (Biological Safety Cabinet) | Pachane pa70 |
| | Refrigerator | Consul frostfree |
| | Freezer | Coldlab |
| | Incubator 1 | Binder |
| | pH Meter 2 (Fixed) | Hanna edge pH |
| | Water Bath | Benchmark scientitic mybath 8l |
| | Balance | Bel engineering |
| | Magnetic Stirrer | Fisotom |
| | Ultrasonic Bath | Solidsteet |
| | Vortex Mixer | Nova |

Equipment for cell Culture to Primary Smooth Muscle Cell Culture from Ovis aries Myometrium

## Safety warnings

This protocol does not involve hazardous procedures; however, it is essential to follow standard biosafety practices to protect both the researcher and the cellular material. The use of appropriate personal protective equipment (PPE). Additionally, all procedures should be conducted under aseptic conditions in a cell culture laboratory to prevent contamination and ensure the integrity of the biological material.

## Ethics statement

The following protocol was submitted to the ethics committee on animal use of the Center for Nuclear Energy in Agriculture from University of São Paulo (CENA/USP/Protocol No. 0005/2022).

# METHOD I - EXPLANT OF FRESH MYOMETRIAL TISSUE FOR THE *IN VITRO* CULTURE OF PSMo$_{24}$ CELLS: PRIMARY SMOOTH MUSCLE FROM THE MYOMETRIUM OF *OVIS ARIES*

3w 3d 1h 25m

1 The myometrial tissue was collected from five non-pregnant Santa Inês ewes, aged between 1 and 2 years, in perfect health condition.

1.1 **Uterus collection**: At the slaughterhouse, use your personal protective equipment and avoid all contaminants in the area. Carefully remove the uterus from the ewe using a sterile blade and place it in a sterile Petri dish.

1.2 **First wash**: Immediately after collection, wash the uterus with 70 % alcohol (± 5 °C ) for 00:00:30 to sterilize the external surface and reduce the presence of contaminants.

1.3 **Second wash**: Wash with **tissue washing medium** go to step #9.2 0.9 % **Sodium Chloride** supplemented with penicillin and streptomycin antibiotics (pen+strep) at ± 5 °C .

1.4 **Cutting of sample I**: In a previously aseptically prepared area at the slaughter site, make an initial large cut of approximately 18 cm ^2 of tissue from each uterine horn, as shown in Figure 1.

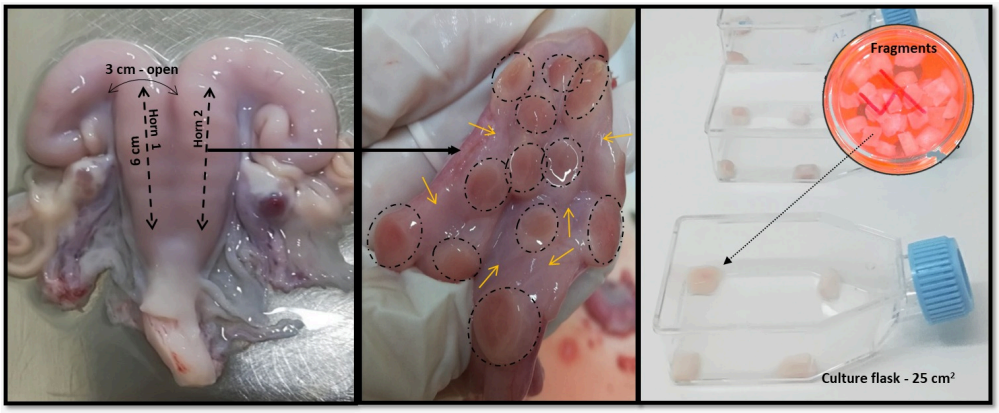


**Figure 1**: Process of obtaining and preparing ovine myometrial tissue for *in vitro* culture. A) Macroscopic visualization of the ovine uterus after dissection; B) Fragmentation of myometrial tissue into explants (indicated by arrows and dotted circles); C) Myometrial explants placed in culture flasks containing specific medium.

1.5 **Transport solution**: Place the sample in a 50 mL Falcon tube with **transport solution,** 0.9 % **Sodium Chloride** supplemented with antifungal at 5 °C go to step #9.3 . Keep the sample for no more than 02:00:00 under these conditions to ensure tissue integrity before proceeding with the next steps of the protocol.

1.6 **Transport:** Transport the samples into a cooler at 5 °C to the laboratory.

Safety information

**Do not expose the sample to extreme temperatures or sudden temperature changes during transport.**

1.7 **Tissue fragmentation**: In the cell culture laboratory, sanitize the Falcon tubes and bring them inside the laminar flow hood. Transfer the sample from the Falcon tube to a sterile Petri dish with **Phosphate Buffered Saline** (PBS) and antibiotics (**cell washing medium** go to step #9.5 ). Cut the tissue into smaller fragments of approximately 0.5 cm ^2 , avoiding the collection of the caruncle area (Figure 1 go to step #1.4 ).

1.8 **Washing of the fragments:** Add several aliquots of 200 µL of antibiotics to a Petri dish. Briefly immerse each fragment for initial disinfection, then transfer the fragments to a new **cell washing medium** go to step #9.5 .

Note

This step is essential to ensure the removal of potential impurities or contaminants.

1.9 **Enzymatic digestion**: Place four fragments in 15 mL Falcon tubes containing a 1 mg /mL collagenase solution. Place the tubes in an incubator at 37 °C with 5 % $CO_2$ and controlled humidity until shaking occurs. 1h 25m

**a. Periodic shaking:** Manually mix the solution every 00:10:00 for a total period of 00:30:00 , up to a maximum of 00:40:00 . Shaking helps the collagenase

digest the tissue so that the cells can be released for subsequent culture.
**b. Visual observation**: Interrupt the digestion as soon as the solution turns white or milky. Avoid allowing the tissue to become viscous or "slimy," which indicates excessive digestion.
**c. Centrifugation:** After digestion, centrifuge the tubes at 1194 x g, 00:05:00 to separate the cells from the collagenase. Discard the supernatant and wash the fragments with PBS to eliminate enzyme residues.

1.10 **Distribution into culture bottle:** Transfer the fragments to a 25 cm² culture bottle and add 4 mL of **culture medium** (Dulbecco´s Modified Eagle´s Medium/High Modified; Ham´s Nutrient Mixture F12; Fetal Bovine Serum; and antibiotics). The medium should cover the fragments shallowly to optimize cell adhesion. Place the bottle in the incubator 37 °C, 5 % $CO_2$. Keep the culture undisturbed for 96:00:00 to allow the cells to begin adhering and proliferating on the fragments. On the fourth day, check the confluence and add 2 mL of **culture medium** go to step #9.7 .

4d

1.11 **Regular medium change:** Starting on the fifth day, perform medium changes and continue every three days to renew nutrients.

**a. Washing**: Using a glass pipette adapted to a vacuum pump, remove the culture medium and add 3 mL of PBS with antibiotics (**cell washing medium** go to step #9.5 ).
**b. Medium replacement:** Add 3-4 mL of **culture medium** go to step #9.7 as the cells detach from the myometrial tissue.

1.12 **Removal of tissue fragments:** Between day 7 and day 10 of culture, remove the tissue fragments to prevent contamination and promote cell expansion. By day 10, cellular cluster should be present.

1.13 **Maximum passage duration:** Maintain the culture of the first passage for no more than 20 days. If confluence is not reached by this period, it is not recommended to proceed. In Figure 2, you can observe the sequence of $PSMo_{24}$ *in natura* until their confluence (80 %).

2w 6d

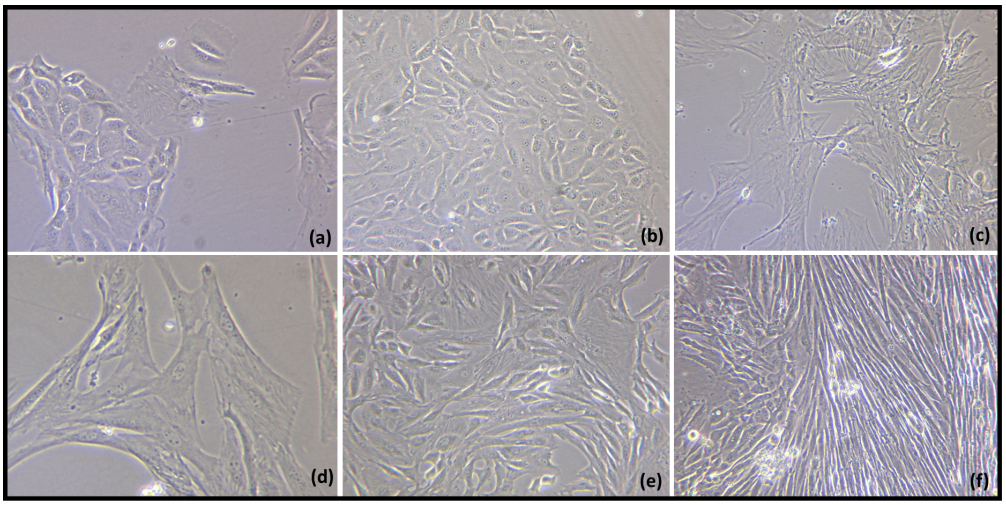


**Figure 2**. Morphology of Primary smooth muscle from the myometrium of *Ovis Aries* (**$PSMo_{24}$**) cells, obtained from fresh tissue, at different stages of in vitro culture. (a) Cells after initial adhesion to the substrate; (b) Initial cell proliferation; (c) Partial monolayer formation; (d) Monolayer at higher confluence; (e) Cells at an advanced growth stage; (f) Cells at 80 % confluence.

1.14

Note

The ruminant uterus caruncles are a dense connective tissue that is highly vascularized, covered by a simple cuboidal or low columnar epithelium. They are located in the uterine mucosa with attachment sites for the cotyledons of the fetal placenta. Therefore, they are not smooth muscle tissue and should not be collected or cultured in this protocol.

Note

Perform at least 4 culture bottles per uterus and/or animal to ensure the growth and storage of future cells.

## METHOD II - DETACHMENT OF $PSMo_{24}$ CELLS-TRYPSINIZATION

1d 0h 14m

2 Once approximately 80 % confluence is reached, remove the culture medium and add 3 mL of **cell washing medium** go to step #9.5 .

2.1 **Addition of Trypsin-EDTA**: Add the **trypsinization medium** go to step #9.8 : PBS + trypsin to the 25 $cm^2$ culture bottle.

2.2 **Incubation:** Place the bottle in the incubator ( 37 °C ; 5 % $CO_2$) for 00:08:00 . Observe under an **inverted microscope** to check if the cells have detached. If there are still adherent cells, incubate for up to a maximum of 00:14:00 . 8m

2.3 **Trypsin inactivation**: Add 3 mL of **culture medium** go to step #9.7 to neutralize the trypsin as soon as the cells are completely detached.

2.4 **Perform the counting:** Count the cells using a Neubauer chamber or an automatic cell counter, ensuring an adequate cell quantity for culture and storage.

2.5 **Counting and viability of Primary Smooth Muscle Cells from Myometrium in the Neubauer Chamber** 1m

After trypsinization of the cells or thawing of the cells, transfer the cell suspension to a sterile Falcon tube. Collect 10 µL of cell suspension + 10 µL of 0.4 % **trypan blue**. Gently mix and let it react for 00:01:00 at Room temperature .

2.6 Clean the Neubauer chamber and coverslip and allow them to dry. Add 10 µL to the chamber, allowing the liquid to fill it by capillary action.

2.7 Count the live (unstained) and dead (stained blue) cells in at least 4 large quadrants and calculate the viability percentage. For quantification, use the following equation:

Note

**Cell Counting = NCC/NQ * Vol * $10^4$ * 2**

Where:
- **NCC:** Total Number of Cells Counted
- **NQ:** Number of Quadrants Counted
- **Vol:** Volume of Cell Suspension Collected (mL)
- **$10^4$:** The correction factor adjusts the calculation to the scale of the volume analyzed in the Neubauer chamber.
- When viability is not performed, remove the dilution factor (**2**).

2.8 **Centrifuge and Separate**: After counting, distribute the appropriate number of cells for reculturing or cryopreservation. Transfer the contents to Falcon tubes and centrifuge at 428 x g, 00:05:00, then discard the supernatant. 5m

> Note
>
> Small flask (P): 25 cm² – 500,000 cells
>
> Medium flask (M): 75 cm² – 1,500,000 cells
>
> Large flask (G): 182 cm² – 3,640,000 cells

2.9 **Part 1 (Criopreservation)**: Add 1 mL of the **cryopreservation solution** go to step #9.10 (Dimehylsulfoxide - DMSO + Fetal Bovine Serum - FBS), place in a cryogenic tube, and place in Mr. Frosty, a cryogenic container that provides a gradual cooling rate of 1 °C /min. After 24:00:00, transfer to the nitrogen tank ( -196 °C ) for indefinite storage. 1d

2.10 **Part 2 (Continuity of Culture)**: Add the acquired cell quantity in 5 mL, 15 mL, or 25 mL of culture medium to a 25, 75, or 182 cm² culture flask, respectively. Return to the incubator for culture until a new confluence is reached.

## METHOD III - SUBCULTURE AND THAWING OF PSMo$_{24}$ CELLS 6m

3 **Subculture of PSMo24 Cell Passages**

After performing the explant and cryopreservation of passage 1, continue with the subsequent passages (maximum of 10). Keep subculturing and changing the culture medium every 3 days, maintaining the culture until the desired cell confluence is reached, which usually occurs between 3 and 9 days. Once confluence is satisfactory, repeat Method II - Trypsinization.

3.1 From this point, the necessary analyses or experimental tests can be performed directly on the cells. To ensure the continuity of the experiment in case of unexpected issues with the cultures, it is recommended to regularly freeze cell passages to enable future subcultures.

3.2 **Thawing of cryopreserved $PSMo_{24}$ cells for subculture**

3.3 Remove the cryotube from the liquid nitrogen tank, keep it at Room temperature for 00:01:00 and then complete the thawing process at 37 °C in a water bath. 1m

3.4 In the laminar flow hood, transfer the entire content of the cryotube ( 1 mL ) into a Falcon tube containing 4 mL of **culture medium** go to step #9.7 .

3.5 Collect 10 µL of the suspension to perform the cell count (item) and determine the appropriate culture flask.

3.6 Centrifuge for 428 x g, 00:05:00 and remove the supernatant without disturbing the cell pellet. 5m

3.7 Add **culture medium** go to step #9.7 to the tube containing the cell pellet and resuspend, homogenizing with back-and-forth movements.

3.8 Place the suspension in the chosen culture flask and complete the medium if needed. Incubate in the incubator with 5 % $CO_2$ at 37 °C .

## METHOD IV - EXPLANTATION OF FROZEN MYOMETRIAL TISSUE FOR *IN VITRO* CULTURE OF $PSMo_{24}$ CELLS

23m

4 **Collection and Preparation of Myometrial Tissue, see item** go to step #1.1 **to** go to step #1.8 .

4.1 **Freezing Process (vitrification or rapid freezing)** 5m

With the tissue fragments prepared, add 2 mL of **cryopreservation medium** (Ethylene glycol - EG, Sucrose - SAC, FBS, and Dulbecco´s Modified Eagle´s Medium - DMEM go to step #9.10 ) to each well of a 24-well plate. Then, immerse two fragments in each well, keeping them submerged for 00:05:00 After the exposure period, transfer four fragments to a cryotube containing 1 mL of **cryopreservation medium** go to step #9.10 and immediately store in liquid nitrogen at -196 °C .

4.2 **Thawing of Myometrial Tissue**

Remove the cryotube from the liquid nitrogen and leave it at Room temperature for about 00:01:00. Then, immediately transfer it to a 37 °C water bath and maintain it until fully thawed, which usually takes 00:02:00. Prepare the sequential washes to be performed with DMEM and Sucrose (0.25 and 0.5 M), referred to as thawing I,II, e III (DMEM-Pure). After preparing the solutions, carry out the sequential washes as indicated: 3m

4.3 **Wash I:**
Place two tissue fragments in each well with 2 mL of thawing solution I go to step #9.11 and keep them for 00:05:00. After this period, transfer the fragments to thawing solution II go to step #9.12. 5m

4.4 **Wash II:**
Keep the fragments immersed in thawing solution II go to step #9.12 for 00:05:00. Then, carefully transfer the fragments to thawing solution III (DMEM-Pure). 5m

4.5 **Wash III**
Keep the fragments in thawing solution III (DMEM-Pure) for 00:05:00 After this time, wash the fragments with **cell washing medium** go to step #9.5 and proceed with the enzymatic digestion, as mentioned in the item go to step #1.9. In Figure 3, we can observe the detachment of cells after the explantation of cryopreserved tissue until it reaches 80 % confluence. 5m

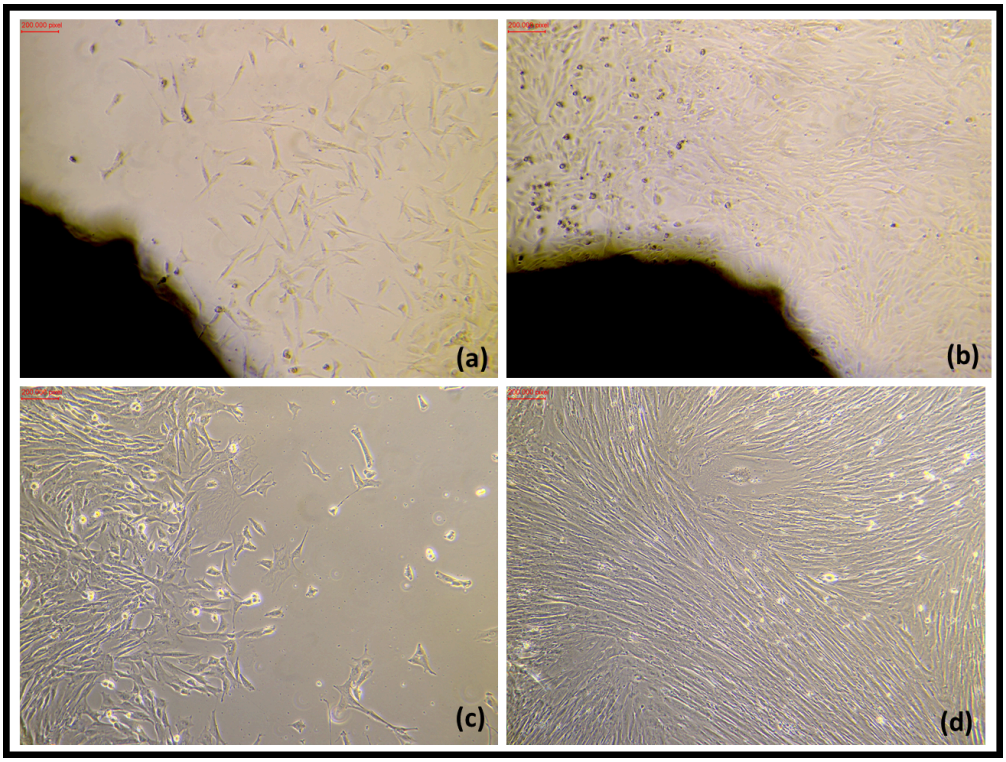


**Figure 3:** Morphology of Primary smooth muscle from the myometrium of *Ovis Aries* (**PSMo$_{24}$**) cell, obtained from cryopreserved tissue, at different stages of *in vitro* culture. (a) Cells emerging from the tissue and proliferating; (b) Initial cell proliferation; (c) Distribution in the culture flask; (d) Cells at 80 % confluence.

Note

After the fragments come out of the water bath, everything that comes into contact with the tissue must be at 37 °C .

## METHOD V - MORPHOLOGICAL VALIDATION BY FLUORESCENCE CONFOCAL MICROSCOPY WITH DAPI AND PHALLOIDIN STAINING OF PSMo$_{24}$ CELLS

3d 5h 50m

5 Culture the PSMo$_{24}$ cells as described in items go to step #3 , go to step #3.2 and go to step #4 . After confluence, perform trypsinization as described in go to step #2

5.1 I a 96-well plate, resuspend the $PSMo_{24}$ cells at a concentration of $2.0 \times 10^4$ cells/well in 200 µL of **culture medium** go to step #9.7 . Incubate the plate at 37 °C in a $CO_2$ incubator for 24:00:00 . 1d

5.2 After 24 hours, observe the cell adhesion under the inverted microscope; Remove the culture medium by carefully inverting the plate, Wash the cells once with pre-warmed PBS at 37 °C , pH 7.4 .

5.3 Apply 100 µL of the treatments of interest, diluted in **serum-free culture medium** ( go to step #9.7 ) in quadruplicate in the 96-well plate.

5.4 Incubate the plate at 37 °C in the $CO_2$ incubator for 04:00:00 or for the necessary duration of the treatment reaction. After the incubation time, remove the treatments by carefully inverting the plate. 4h

5.5 Fix the sample in 3.7 % formaldehyde solution without methanol go to step #9.13 in PBS for 00:20:00 at Room temperature . 20m

5.6 Wash once with PBS.

5.7 Then, permeabilize with 0,3 % Triton X-100 in 1 % Bovine Serum Albumin (BSA) go to step #9.15 for 00:30:00 30m

5.8 Incubate the cells in a dilution of: 1:10,000 of 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) go to step #9.16 and 1:400 of 1:400 of phalloidin 488 go to step #9.17 , prepared in 1 % Triton X-100 in 1 % BSA go to step #9.14 .

5.9 Add 100 µL of a solution containing DAPI and Phalloidin 488 go to step #9.17 to each well of the 96-well plate. Incubate the plate at Room temperature for 01:00:00 , keeping it in the dark to protect the fluorophores. 1h

5.10 Wash once with PBS.

5.11 Add 1 drop per well of the **antifade solution** go to step #9.18 and analyze within 48:00:00 2d

Note

To remove the reagents from the 96-well plate, carefully invert the plate completely.

5.12 Set the inverted confocal microscope to the excitation/emission channels of DAPI (358–461 nm, blue) and Phalloidin 488 (488 nm, green), configuring the acquisition parameters (laser intensity, gain, and resolution) according to the sample. During analysis, identify the green fluorescence to visualize actin filaments in the cytoplasm and the blue fluorescence to examine the integrity of the nuclei. Capture images that shows the cellular organization (Figure 4).

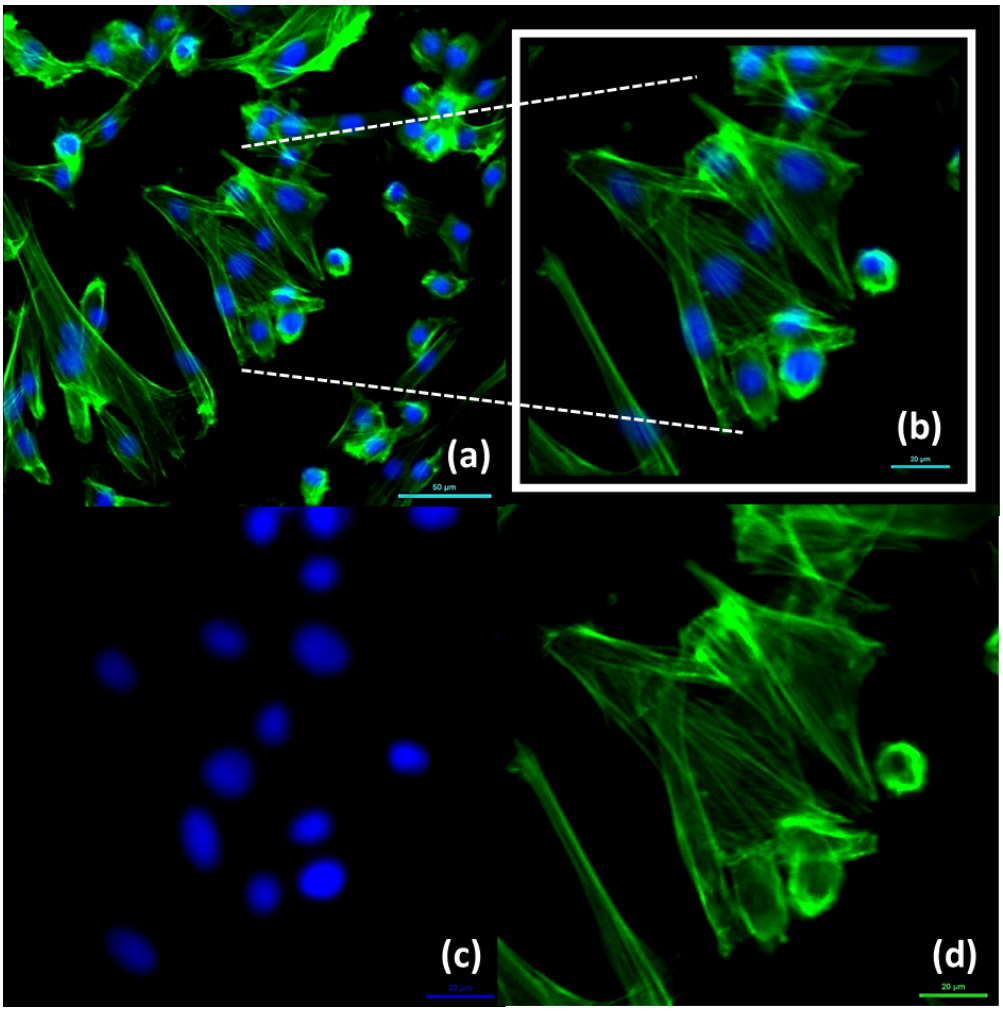


**Figure 4.** Confocal inverted microscopy images of Primary smooth muscle from the myometrium of *Ovis aries* (**PSMo$_{24}$**) cells. (a, b) Confirmation of the characteristic morphology of smooth muscle cells. (c) Nuclei stained with DAPI (blue). (d) Visualization of actin filaments (F-actin, green).

## METHOD VI - EVALUATION OF THE ANTIOXIDANT CAPACITY OF BIOPRODUCTS IN PSMo$_{24}$ CELLS USING DCFH-DA AND ABAP METHODS

1d 5h 25m

6 Culture PSMo$_{24}$ cells as described in items go to step #3, go to step #3.2 and go to step #4. After reaching confluence, perform trypsinization as described in go to step #2.

6.1 Add 5.0 x 10×4 PSMo$_{24}$ cells per well with 200 µL of **culture medium** go to step #9.7 in black clear-bottom plates for fluorescence.

6.2 Incubate the plate at 37 °C in a 5 % $CO_2$ incubator for 24:00:00 . 1d

6.3 After 24 hours, carefully invert the plate to remove the culture medium and wash with 100 µL of PBS to remove non-adherent or dead cells.

6.4 Apply the controls and treatments at the desired concentrations and incubate at 37 °C in a $CO_2$ incubator with 5 % $CO_2$ for 04:00:00 or for the necessary time required for the applied treatment reaction. 4h

6.5 **Negative control**: Untreated cells (DMSO, if used as a vehicle).
**Positive control**: Quercetin, solutions of 25, 50, 100, 175, and 250 µM go to step #9.19 in serum-free medium (final solutions with <2 % DMSO).

6.6 After the treatment exposure time, add **DCFH-DA** (60 µM go to step #9.20 ) for 00:20:00 in an incubator at 37 °C with 5 % $CO_2$. 20m

6.7 Wash the wells with PBS to retain only the antioxidants that entered the cells.

6.8 Add 100 µL of the **ABAP solution** (600 µM go to step #9.21 ) and immediately place it in the microplate reader with a inverted fluorescence detector, programmed to take readings every 00:05:00 for 01:00:00 (a total of 13 readings). Measure fluorescence at 485 nm excitation and 538 nm emission. 1h 5m

6.9 Determine the antioxidant capacity based on the percentage decrease in fluorescence from the area under the curves generated by the 13 points provided by the reader, using the following formula:

Note

**AAC (%) = 1 – AUC of the control / AUC of the sample* 100**

**Where:**
**AAC (%):** percentage of antioxidant capacity (AAC) of the evaluated sample.
**AUC of the control:** Area under the curve of the control (without antioxidant).
**AUC of the sample**: Area under the curve of the sample (with antioxidant).

## METHOD VII - MTT ASSAY (3-(4,5-DIMETHYLTIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE) TECHNIQUE FOR ASSESSING VIABILITY, CYTOTOXICITY, AND PROLIFERATION OF PSMo$_{24}$ CELLS

1d 8h

7 Culture the PSMo$_{24}$ cells as described in go to step #3 and go to step #4. After confluence, perform trypsinization as described in go to step #2.

7.1 In a 96-well plate, resuspend the PSMo$_{24}$ cells at a concentration of 5.0 × $10^4$ cells/well in 200 µL of **culture medium** go to step #9.7.

7.2 Incubate the plate at 37 °C in a $CO_2$ incubator for 24:00:00; 1d

7.3 After 24 hours, observe the cell adhesion under the microscope.

7.4 Remove the culture medium by carefully inverting the plate.

7.5 Apply 100 µL of the treatments of interest, diluted in **serum-free culture medium** go to step #9.7 in quadruplicate in the 96-well plate.

7.6 Incubate the plate at 37 °C in the $CO_2$ incubator for 04:00:00 or for the necessary duration of the treatment reaction. After the incubation time, remove the treatments by carefully inverting the plate. 4h

7.7 Add 100 µL of the 0.3 mg/mL MTT solution per well and incubate the plate at 37 °C in the $CO_2$ incubator for 04:00:00. 4h

7.8 After the incubation period, carefully invert the plate to remove the MTT reagent and add 100 µL of DMSO to each well.

7.9 Keep the plate at Room temperature, shielded from light, and take it for reading immediately.

7.10 Remove the plate lid and measure the absorbance of each well at 570 nm, including the blanks and negative controls.

7.11 Subtract the absorbance of each well by the average absorbance of the blank.

7.12 Calculate the cytotoxicity using the following equation:

Note

Cell Viability (%)= Abs. Sample - Abs. Control (-) / Abs. Control (+)-Abs. Control (-) x 100

Where:
**Abs** = Absorbance

## METHOD VIII - RNA EXTRACTION PROTOCOL FROM PSMo$_{24}$ CELLS

1h 28m

8 Add 1 mL of Trizol directly to the frozen cells and homogenize using a pipette (up and down motion) until the cells are completely dissolved in the Trizol. Incubate for 00:05:00 at Room temperature ; 5m

8.1 Add 0.3 mL of Chloroform and homogenize using a pipette. Incubate for 00:02:00 to 00:03:00 at Room temperature ; 3m

8.2 Centrifuge for 12.000 x g, 4°C, 00:15:00 ; 15m

8.3 After centrifugation, transfer the aqueous supernatant to a new 1.5 mL Eppendorf tube using a pipette, avoiding the interface. To make this easier, tilt the tube at a 90° angle;

8.4 Add 0.5 mL of Isopropanol to the aqueous phase and homogenize the tube;

8.5 Incubate On ice for 00:10:00 ; 10m

8.6 Centrifuge for 12.000 x g, 4°C, 00:10:00 At this stage, observe the formation of the RNA pellet; 10m

8.7 Discard the supernatant from the pellet by inverting the Eppendorf tube;

8.8 Resuspend the pellet in 1 mL of 75 % Ethanol for the first wash;

8.9 Centrifuge for 7.500 x g, 4°C, 00:05:00 and discard the supernatant using a pipette; 5m

8.10 Resuspend the pellet in 0.5 mL of 75 % Ethanol for the second wash and centrifuge ( 7.500 x g, 4°C, 00:05:00 ); 5m

8.11 Dry the pellet for 00:05:00 to 00:10:00 , ensuring no alcohol droplets remain before proceeding to the next phase; 15m

8.12 Resuspend the pellet in RNase-free water ( 20 µL - 50 µL , depending on the initial size of the pellet);

8.13 Use the Nanodrop spectrophotometer to quantify the RNA , with the 260/280 ratio > 1.80. This step allows precise quantification and purity assessment of the extracted RNA;

8.14 Confirm the integrity of the RNA through electrophoresis and a concentrated agarose gel ( 0.92 g of agarose diluted in 50 mL of **buffer solution**). The tank should be set to 110V and run for 00:20:00 . See Figure 5; 20m

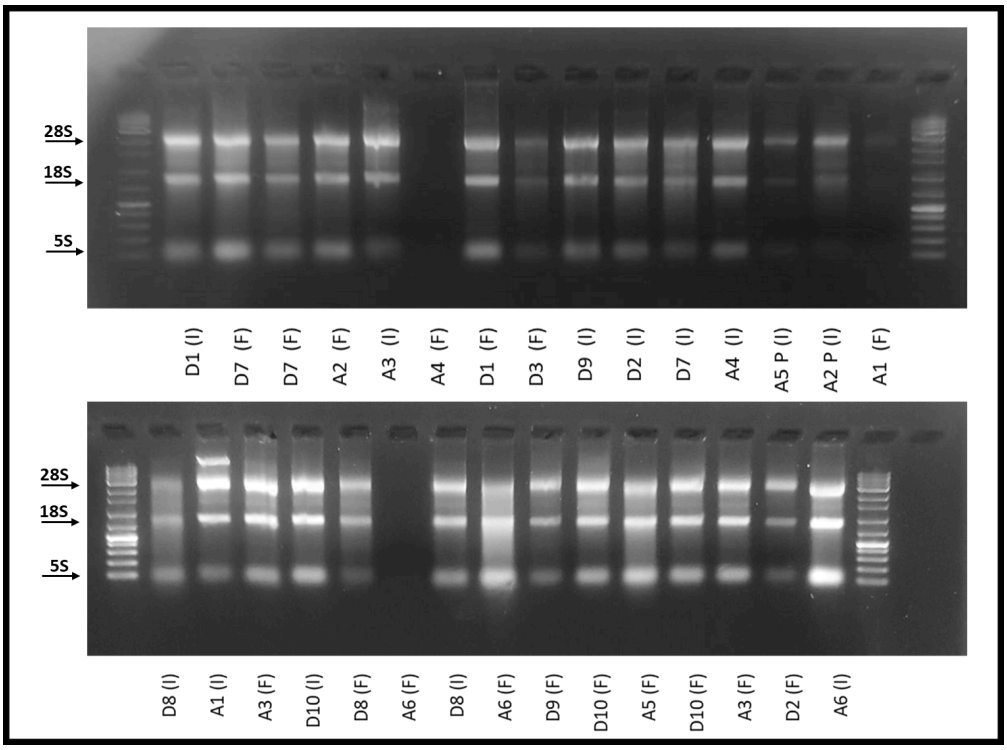


**Figure 5:** Agarose gel electrophoresis showing the quality and integrity of RNA extracted from Primary ovine smooth muscle from the myometrium of *Ovis aries* ($PSMo_{24}$) cells. The molecular weight marker is indicated at the edges of the gel, and the samples represent different experimental conditions.

8.15 Proceed to gene expression approach.

Note

The results obtained in these stages were sent for transcriptome analysis of both the *in vivo* explanted **$PSMo_{24}$ cells** and the cryopreserved tissue. They will be analyzed and submitted for publication.

## METHOD IX - PREPARATION OF MEDIA FOR *IN VIVO* AND CRYOPRESERVED MYOMETRIAL TISSUE EXPLANT

9 The preparation of the media used for tissue collection and explantation should be done the day before collection to ensure that all components are properly prepared and sterile. This step is crucial for the success of the collection and subsequent cell culture. To ensure an appropriate environment for cell growth and viability throughout the process, the following media should be prepared.

9.1 **Sodium chloride (NaCl) 0.9 %**

**Composition:** Sodium chloride (NaCl) 0.9 % - Sterile saline solution.

**Preparation:** Dissolve 9 g of NaCl in 1 L of sterile distilled water to obtain an isotonic saline solution 0.9 %. Filter the solution through a sterile filter and store it in the refrigerator ( 4 °C ).

9.2 **Tissue washing medium - 0.9 % NaCl with 1 % Antibiotics**

**Composition:** 0.9 % Sodium chloride (NaCl) and antibiotics (10,000 units penicillin and 10 mg streptomycin/mL – Pen-Strep).

**Preparation:** In a sterile bottle, add 99 mL of 0.9 % NaCl, then add 1 mL of antibiotics to achieve a final concentration of 1 %. Mix well and store in the refrigerator ( 4 °C ).

9.3 **Transport Medium - 0.9 % NaCl with 1 % antifungal**

**Composition:** 0.9 % Sodium chloride (NaCl) and antifungal (Amphotericin B).

**Preparation:** In a sterile container, add 99 mL of 0.9 % NaCl, then add 1.0 mL of antifungal to achieve a final concentration of 1 %. Mix well and store in the refrigerator ( 4 °C ).

9.4 **Phosphate Buffered Saline (PBS) 1x ( pH 07.4 ).**

**Composition:** PBS (concentrated) powder for 1 liter of distilled or deionized water.

**Preparation:** Add the contents of the PBS envelope (9.07-10.0 g/L) to 1 L of distilled or deionized water. Mix well until the powder is completely dissolved. **Filter the solution** to ensure its purity. Store in the refrigerator ( 4 °C ).

9.5 **Cell washing medium – PBS with 1 % Antibiotics**

**Composition:** PBS 1x and Antibiotics (Pen-Strep);

**Preparation:** In a sterile container, add 99 mL of 1x PBS, then add 1 mL of antibiotics to achieve a final concentration of 1 %. Mix well and store in the

refrigerator ( 4 °C ).

9.6 **Enzymatic digestion medium – Collagenase ( [M] 1 mg/mL ):**

**Composition:** Collagenase type I: 100 mg , PBS (1x)

**Preparation:** Dissolve 100 mg or 10 mg of type I collagenase in 100 mL or 10 mL of PBS, respectively, resulting in a [M] 1 mg/mL solution.

9.7 **Culture Medium (DMEM + F-12 + FBS + Antibiotics):**

**Composition:** DMEM (Dulbecco's Modified Eagle's Medium), F-12 (Ham's F-12 Nutrient Mix), FBS (Fetal Bovine Serum), Antibiotics (Pen-Strep).

**Preparation:** Mix 1:1 of DMEM and F-12 to obtain a balanced culture medium rich in essential nutrients for cell growth. Add 10 % FBS and 1 % Pen-Strep. Filter the medium through a sterile 0.22 µm filter and store at 4 °C for no more than 24 hours.

9.8 **Trypsinization Medium**

**Composition:** Trypsin (0.5 % - 10x) and PBS.

**Preparation:** Mix 10 % trypsin with 90 % PBS. Prepare the medium on the day of use.

**Examples of Preparation by Bottle Size:**

**25 cm² flask (5 mL):** 0.5 mL of trypsin + 4.5 mL of PBS
**75 cm² flask (10 mL):** 1.0 mL of trypsin + 9.0 mL of PBS
**182 cm² flask (25 mL):** 2.5 mL of trypsin + 22.5 mL of PBS

9.9 **Cryopreservation medium for $PSMO_{24}$ cells**

**Composition:** FBS, dimethyl sulfoxide (DMSO)

**Preparation:** In the FBS to be prepared, add 10 % DMSO, store in 15 mL Falcon tubes, and freeze at -20 °C until use.

9.10 **Cryopreservation Medium for Myometrial Tissue (Vitrification)**

**Composition:** Ethylene glycol (EG), sucrose (SAC), Fetal Bovine Serum (FBS), and DMEM.

**Preparation:** Prepare a solution containing [M] 6 Mass Percent of ethylene glycol (EG), [M] 0.25 Mass Percent of sucrose, and 10 % FBS, completing the final volume with DMEM.

**Example:**
**Ethylene glycol (EG)**: 41.44 mL (6 M)
**Sucrose**: 8.56 g (0.25 M)
**Fetal Bovine Serum (FBS)**: 10 mL (10 %)
**DMEM**: Complete to a final volume of 100 mL

Mix the solution well to ensure all components are fully dissolved and homogeneous. Filter the medium through a 0.22 µm sterile filter and store at 4 °C for no more than 24 hours.

9.11 **Thawing medium I**

**Composition**: DMEM and sucrose 0.5 M – molecular weight: 342.3 g/mol/L

**Preparation**: In a sterile Falcon tube, dissolve 1.711 g of sucrose and complete to 10 mL with DMEM. Mix well to ensure homogeneity. Filter and store at 4 °C.

9.12 **Thawing medium II**

**Composition**: DMEM and 0.25 M sucrose solution
**Preparation**: In a sterile bottle, dissolve 0.855 g of sucrose and complete up to 10 mL with DMEM. Mix well to ensure homogeneity. Filter and store at 4 °C.

9.13 **Formaldehyde 37 % without methanol**

**Composition**: Formaldehyde 37 %, PBS (1x)
**Preparation**: Add 9.0 mL of 1X PBS. Pipette 1.0 mL of 37 % formaldehyde without methanol. Mix until homogeneous.

9.14 **0.1 % Triton X-100 solution in 1 % BSA**

**Composition**: Triton X-100, BSA, Milli-Q water.

**Preparation**: Weigh 0.1 g of BSA and dissolve in approximately 8 mL of Milli-Q water. Add 10 µL of Triton X-100 (0.1 % v/v). Complete the volume to 10 mL with Milli-Q water and mix well until the solution is homogeneous. Store in the refrigerator.

9.15 **Solution of 0.3 % Triton X-100 in 1 % BSA**

**Composition**: Triton X-100, BSA, Milli-Q water.

**Preparação**: Weigh 0.1 g of BSA and dissolve it in approximately 8 mL of Milli-Q water. Add 30 µL of Triton X-100 (1 %). Complete the volume to 10 mL with Milli-Q water and mix well until the solution is homogeneous. Store in the refrigerator.

9.16 **DAPI solution**

**Composition**: 4′,6-diamidino-2-phenylindole (DAPI), 0.1 % Triton X-100-BSA

**Preparation**: Prepare the stock solution of DAPI according to the manufacturer's specifications. Dilute 1 µL of the DAPI stock solution in 10 mL of the 0.1 % Triton in BSA solution (1:10,000). Mix well and protect from light. Store in the freezer.

9.17 **Phalloidin 488 Solution**

**Composition**: Phalloidin 488, 0.1 % Triton X-100-BSA

**Preparation**: Prepare the stock solution of Phalloidin 488 according to the manufacturer's specifications. Add 25 µL of the Phalloidin 488 stock solution to 10 mL of the 0.1 % Triton X-100 in BSA solution (1:400). Mix well and protect from light. Store in the freezer.

9.18 **Antifade solution**

**Composition**: Glycerol (molecular biology), PBS (1x).

**Preparation**: Add 8 mL of glycerol, then add 2 mL of 1X PBS. Mix well until the solution is homogeneous. Store in the refrigerator.

9.19 **Standard Quercetin Solution (1 mM)**

**Composition**: Quercetin (3.022 mg for 10 mL of solution), solvent (DMSO or ethanol).

**Preparation**: Dissolve 3.022 mg of quercetin in DMSO or ethanol to make 10 mL of a 1 mM stock solution. Store in amber bottles in the refrigerator ( 4 °C ).

**Quercetin Solution Dilutions (25, 50, 100, 175 e 250 µM)**

**Concentrations and volumes**:
- **25 µM**: 0.25 mL of stock solution + 9.75 mL of PBS.
- **50 µM**: 0.5 mL of stock solution + 9.5 mL of PBS.
- **100 µM**: 1 mL of stock solution + 9 mL of PBS.
- **175 µM**: 1.75 of stock solution + 8.25 mL of PBS.
- **250 µM**: 2.5 mL of stock solution + 7.5 mL of PBS.

Store the prepared solutions in the refrigerator ( 4 °C ), protected from light.

9.20 **DCFH-DA Solution 60 µM**

**Composition:** Stock solution of DCFH-DA 10 mM (dissolved in DMSO); PBS

**Prepare the 10 mM DCFH-DA stock solution**: Dissolve 4.86 mg of DCFH-DA in 1 mL of DMSO. Mix until completely dissolved. Store in an amber tube or protected from light at -20 °C for up to 1 month. To prepare the working solution at 60 µM: Add 60 µL of the stock solution (10 mM) to 9.940 µL of PBS. Mix gently to homogenize.

**Storage:** The working solution should be used immediately or stored on ice for a maximum of 2 hours. Always protect the solution from light to avoid degradation.

9.21 **ABAP 600 µM solution**

**Composition:** ABAP (2,2′-azobis(2-methylpropionamidine) dihydrochloride), PBS.

**Preparation:** Weigh 1.44 mg **of ABAP** (corresponding mass for a 600 µM solution in 10 mL). Transfer the powder to a Falcon tube, prepared in a light-protected environment. Gradually add 10 mL **of PBS** ( pH 07.4 ) and mix well until completely dissolved. Ensure that the solution is homogeneous.

**Storage:** The solution should be freshly prepared before use to ensure its stability. During use, keep the solution on ice and protected from light to prevent degradation.

## RESULTS

10 It was possible to establish the protocol for the cultivation of **PSMo$_{24}$** cells, as well as the protocol for
the cryopreservation of myometrial tissue for later cell explantation.

10.1 The data obtained demonstrated that cells derived from fresh myometrial tissue reached approximately 80 % confluence in the first passage, with an average of 16.7 ± 2.33 days for fresh tissue and 18 ± 0.86 days for cryopreserved tissue (P>0.05). For subsequent passages, from the second to the tenth, the average time to reach confluence was 4.10 ± 0.18 days and 3.43 ± 0.16 days for fresh and cryopreserved tissue, respectively (Figure 6). In the comparative analysis between the two types of explant and the different passages, a significant difference was observed at passage 3 ($p < 0,05$), However, based on the experience obtained in the laboratory, cultures can reach confluence within a range of 2 to 7 days, which is within the reference intervals observed (Figure 6).

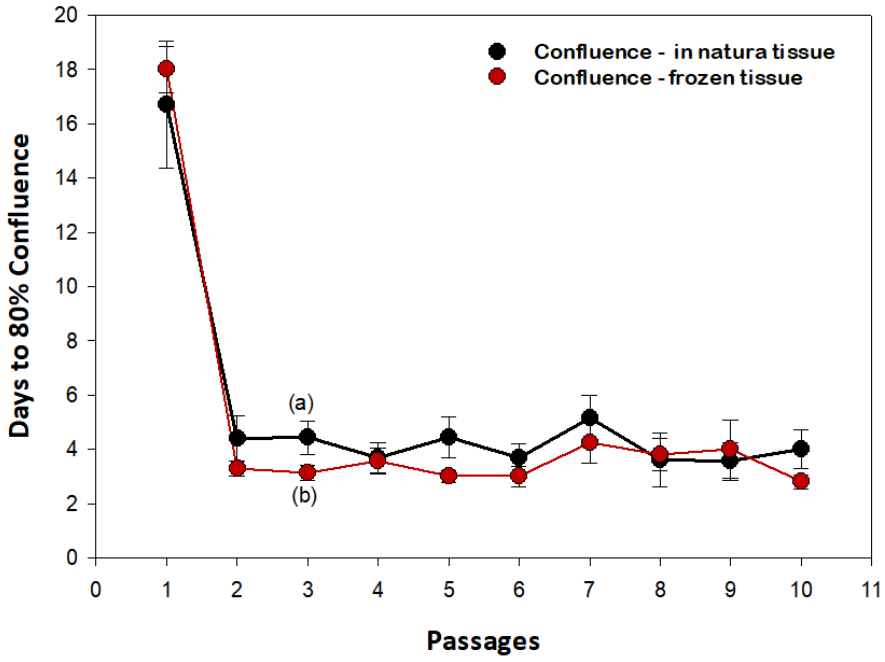


**Figure 6.** Growth scheme of Primary smooth muscle from the myometrium of *Ovis Aries* (**PSMo$_{24}$**) explanted from fresh (in natura) and frozen tissue until reaching 80% confluence.

10.2 The morphological analysis of **PSMo$_{24}$** cells was confirmed by confocal inverted microscopy, as shown in Figure 4 go to step #5.12 . The use of phalloidin, a specific marker for actin fibers, highlighted the organization of the cytoskeleton, allowing the distribution and density of actin fibers within the cells to be observed. Additionally, DAPI staining, which specifically binds to DNA, was used to assess cellular organization and the structural integrity of the nuclei. Through this staining, it was possible to verify the preservation of nuclear morphology, indicating that the cells maintained their protection and structure after culture. These results suggest that **PSMo$_{24}$** cells exhibit a well-organized and intact cellular architecture, which is essential to ensure the quality of cultures and the reliability of experiments.

10.3 Figure 7 shows the quantification of cells per **cm²** during *in vitro* culture. In **PSMo$_{24}$** cells from fresh explants, an increase in cell number was observed during the first three passages, followed by stability in passages 3, 4, and 5. Starting from the sixth passage, there was a decrease in cell density, accompanied by a more random response in subsequent passages, with occasional increases in passages 8 and 10, interspersed by drops in cell numbers, as observed in passages 6 and 9.

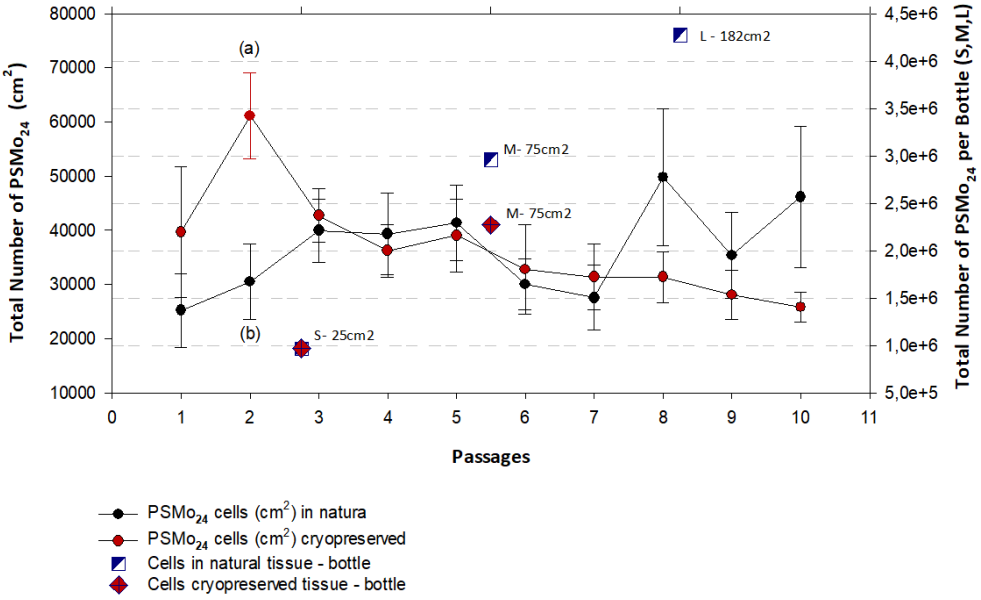


**Figure 7.** Mean and standard error of the mean (SEM) of the total number of Primary smooth muscle from the myometrium of *Ovis Aries* (**PSMo$_{24}$**) cell per cm² (left side of the graph). On the right, mean and SEM of the total number of **PSMo$_{24}$** cells in different flask sizes: small (25 cm²), medium (75cm²), and large (182 cm²), for cells from fresh tissue (blue) and cryopreserved tissue (red).

10.4 In **PSMo$_{24}$** cells derived from cryopreserved tissue explants, an increase in confluence was observed from the first to the second passage, showing a significant difference compared to the cells from fresh tissue explants ($P < 0.05$). However, in the subsequent passages, cell proliferation gradually decreased, with no significant differences compared to the cells from fresh tissue explants ($P > 0.05$).

10.5 Additionally, experiments conducted with the **PSMo$_{24}$** cells throughout the early passages showed satisfactory results, validating the developed experimental model (METHODS V to VIII).

10.6 The results shows that the proposed methodology for culturing primary smooth muscle cells and the protocol for cryopreserving myometrial tissue are viable and have potential for future studies involving reproductive biotechnology, pharmacology, and evaluation of bioproducts in animals and humans.

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