

DETERMINATION OF THE EFFECT OF IN VITRO CULTURE CONDITIONS ON THE QUALITY OF PORCINE MSCs ISOLATED FROM BONE MARROW

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Abstract

In vitro culture of stem cells makes it possible to conduct studies under strictly defined conditions that can be modified as required. These conditions include selection of an appropriate culture substrate and medium, use of an atmosphere with a high CO₂ content, and establishment of a suitable temperature. This article presents the results of an assessment of the quality of porcine stem cells isolated from bone marrow and cultured in vitro in media of different composition at 37°C and 39°C. Assessment of the effects of the different culture variants on day 7 showed that the highest confluence (90%) and normal morphology were obtained for cells cultured at 39°C in a 1:1 mixture of Dulbecco's Modified Eagle's Medium - low glucose and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham supplemented with 10 ng fibroblast growth factor (Fgf). In addition, cells cultured in this medium showed the highest percentage of viable cells and a low percentage of late-apoptotic and necrotic cells, irrespective of the culture temperature.

Keywords: MSCs, annexin V, phosphatidylserine, apoptosis, pig

Introduction

Maintaining optimal conditions for in vitro culture of stem cells is a key factor affecting their quality. These conditions can be modified by selecting an appropriate culture substrate and medium, using an atmosphere with different CO₂ concentrations, and changing the culture temperature. When selecting a suitable culture medium, the concentrations of glucose and L-glutamine should be considered first. Glucose is the most commonly used carbon source for animal cells in vitro. L-glutamine is used by cells as a nitrogen source, among other functions, for nucleotide biosynthesis. These compounds are the principal substrates from which the cell generates cellular energy (ATP)

through metabolic pathways, which is necessary for growth and development (Salehinejad et al., 2012). Another important factor affecting cell quality is culture temperature. Many studies of porcine cells in vitro use 37°C (Kokubun et al., 2013; Zhang et al., 2024), although the body temperature of pigs is approximately 39°C. It can therefore be inferred that a temperature closer to the body temperature of a given animal species may be closer to the optimum for cell growth and development in vitro. The literature also contains studies in which porcine cells were cultured at this temperature (Romanek et al., 2018). For this reason, analyses were performed to assess the effects of two culture temperatures (37°C and 39°C) on the quality of porcine cells.

Mesenchymal stem cells (MSCs) are capable of differentiating into tissues derived from the mesoderm, such as bone-forming tissue (osteoblasts), cartilage (chondrocytes), and adipose tissue (adipocytes). In addition, because of their easy isolation, high expansion potential, low immunogenicity, susceptibility to genetic modification, and multipotent nature, these cells are used in many cell therapies (Almalki et al., 2017). In mastitis in dairy cows and goats, intravenous or local administration of MSCs is an effective means of reducing inflammation (Ghai et al., 2022; Peralta et al., 2020; Costa et al., 2019). Published studies have demonstrated a positive effect on bone regeneration without complications after MSC therapy in sheep, goats (Elkhenany et al., 2014), pigs (Wilson et al., 2010), and horses (McDuffee et al., 2014; Seo et al., 2014). Cell injection significantly reduced lameness, pain in damaged joints, and swelling in experimental animals (Pacini et al., 2007; Heinola et al., 2013). Overall, injections of both autologous and allogeneic MSCs in the animal species studied are safe and do not cause adverse effects (Renzi et al., 2013; Pacini et al., 2007; Elkhenany et al., 2014; Caminal et al., 2014).

One way to improve the efficiency of in vitro embryo production is to use co-culture with other cells (feeder monolayers) or to culture embryos in media conditioned by cellular secretory activity (conditioned media). Secretory factors produced by MSCs, including haematopoietic and non-haematopoietic growth factors as well as interleukins and chemokines, may beneficially influence the degree of totipotency or pluripotency in developing embryos (Romanek et al., 2018; Opiela et al., 2020).

To determine the effect of in vitro culture conditions on the quality of porcine bone-marrow-derived stem cells, apoptotic changes in the cells were analysed. Apoptosis is a physiological phenomenon important for normal organismal function both during embryonic development and later in life (Gala et al., 2009). The two best-characterised pathways are the extrinsic (receptor) pathway associated with the cell membrane and the intrinsic pathway involving mitochondria. Caspases link these pathways and, depending on the stage of apoptosis in which they participate, are classified as initiator or effector caspases (Stepień et al., 2007). A characteristic feature of apoptosis is alteration of cell-membrane structure, including disruption of the asymmetric distribution of membrane phospholipids. Maintaining membrane lipid composition is important for cell structure and function. The inner leaflet of the cell membrane contains an increased proportion of negatively charged phosphatidylserine. The asymmetric distribution of aminophospholipids is maintained by the activity of an ATP-dependent translocase known as flippase, which transports them into the inner leaflet while other lipids are transported to the outer leaflet. Caspase activation disrupts this process by blocking flippase function, equalising lipid distribution between the inner and outer membrane leaflets and causing PS to appear on the surface of apoptotic cells. The apoptotic signal therefore induces phosphatidylserine translocation (Kiliańska, 2002). Exposure of phosphatidylserine on the cell surface is used to label apoptotic cells with annexin V, which preferentially binds negatively charged phospholipids such as phosphatidylserine (Iaccarino et al., 2011).

Material and methods

In the experiments, bone marrow donors were Polish Landrace gilts weighing no more than 40 kg. Red bone marrow from the vertebral column served as the reservoir from which bone-marrow-derived mesenchymal stem cells (MSCs) were isolated post mortem. Material was collected at the Chorzów Experimental Station of the National Research Institute of Animal Production. After collection, bone marrow was diluted 1:1 in phosphate-buffered saline (PBS) free of calcium and magnesium ions (Biomed, Lublin, Poland), supplemented with 2% fetal calf serum (FCS). The mixture was layered over Ficoll solution (Ficoll-Paque Plus, Stem Cell Technologies, Canada) to isolate the mesenchymal stem-cell fraction (haematopoietic and non-haematopoietic) and centrifuged for 30 min at 500 g. Cells collected from the interface were washed twice in PBS (Opiela et al., 2016) and then suspended at a density of 50,000 cells/cm² in the appropriate medium. Cells were cultured in an incubator at 37°C or 39°C in an atmosphere containing 5% CO₂ at maximum humidity. The medium was changed every 3 days. Culture was continued for 10 days. To detach cells from the substrate, they were trypsinised with 1 ml of 0.25% trypsin containing 0.01% EDTA and incubated for 5 min at 39°C. Trypsin was inactivated by adding 1 ml FCS. The cells were then centrifuged (5 min, 500 g), resuspended in PBS, and phosphatidylserine was detected by flow cytometry.

The established primary cultures and MSC lines, which retained the ability to self-renew cell subpopulations *ex vivo*, were subjected to immunofluorescence verification to confirm their mesenchymal stem-cell phenotype by identifying an immunophenotype with the appropriate expression profile of surface markers (transmembrane and membrane markers from the cluster-of-differentiation family, CD). The ability of MSCs to differentiate *in vitro* was also verified by identifying the classical cytodifferentiation pathways toward osteoblasts/osteocytes, chondroblasts/chondrocytes, and adipocytes. Only cells positive for CD44, CD90, and CD105 and simultaneously negative for CD14 and CD45 were used in the study.

Basal media used:

1. DMEM with low glucose (Dulbecco's Modified Eagle's Medium - low glucose, D5546), Merck Life Science sp. z o.o., Poznań, Poland
2. A 1:1 mixture of media 1 and 3 (low-glucose DMEM and DMEM/F12)
3. DMEM/F12 (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, D6434), Merck Life Science sp. z o.o., Poznań, Poland
4. Stem MACS™ MSC Expansion Media XF MACS - 130-01-375 - Miltenyi Biotec, Germany.

Medium supplements used:

- Heat-inactivated fetal bovine serum (FBS, Fetal Bovine Serum Heat Inactivated, F4135), Merck Life Science sp. z o.o., Poznań, Poland,
- Antibiotic and antifungal solution (AAS, Antibiotic Antimycotic Solution (100×), Stabilized, A5955), Merck Life Science sp. z o.o., Poznań, Poland,
- GlutaMAX supplement (GlutaMAX™ Supplement, 35050061), Life Technologies Polska sp. z o.o., Thermo Fisher Scientific Gibco, Warsaw, Poland,
- Essential amino-acid solution (BME Amino Acids Solution (50×), B6766), Merck Life Science sp. z o.o., Poznań, Poland,
- Non-essential amino-acid solution (MEM Non-essential Amino Acids (100×) solution, M7145), Merck Life Science sp. z o.o., Poznań, Poland,
- Fibroblast growth factor (Fgf, Fibroblast Growth Factor-Basic, F3685), Merck Life Science sp. z o.o., Poznań, Poland.

Composition of the media used in the experiment:

- ✓ Medium 1: DMEM; 10% FBS; 1% AAS; 1% GlutaMAX; 1% BME; 1% MEM;
- ✓ Medium 1 + Fgf: Medium 1; 10 ng/ml Fgf;
- ✓ Medium 2: DMEM and DMEM/F12 - a 1:1 mixture of the two media; 10% FBS; 1% AAS; 1% GlutaMAX; 1% BME; 1% MEM;
- ✓ Medium 2 + Fgf: Medium 2; 10 ng/ml Fgf;
- ✓ Medium 3: DMEM/F12; 10% FBS; 1% AAS; 1% GlutaMAX; 1% BME; 1% MEM;
- ✓ Medium 3 + Fgf: Medium 3; 10 ng/ml Fgf;
- ✓ Medium 4: Stem MACS™ MSC Expansion Media XF MACS; 10% FBS; 1% AAS; 1% GlutaMAX; 1% BME; 1% MEM.

Detection of phosphatidylserine (PS) and propidium iodide (PI) in MSCs

Fluorescence analyses were performed using the Annexin A5-FITC/7-AAD Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. Positive controls consisted of cells in which apoptosis was artificially induced by 30-min incubation in 3% formaldehyde solution. Negative controls consisted of cells evaluated in a mixture without fluorochrome. To prepared samples containing 5-10 million cells/ml, 10 µl Annexin A5 reagent and 2 µl propidium iodide were added, gently mixed, and incubated for 15 min on ice in the dark. After incubation, 400 µl buffer (1× binding buffer) was added and samples were analysed using CytExpert 2.0 software (Beckman Coulter, USA). In apoptotic cells, membrane lipids are rearranged, resulting in exposure of PS on the outer leaflet of the cell membrane. This phenomenon is used to label apoptotic cells with annexin V, which preferentially binds negatively charged phospholipids, including phosphatidylserine. Propidium iodide (PI) enters cells with damaged membranes and stains red those with compromised cytoplasmic membranes, i.e. necrotic cells or cells in late apoptosis (Trzcińska et al., 2011). The evaluated cells were divided into three groups: necrotic or late-apoptotic cells (green and red fluorescence; annexin V+/PI+), early-apoptotic cells (green fluorescence only; annexin V+/PI-), and viable cells (no fluorescence; annexin V-/PI-).

Statistical analysis

Analyses were performed in at least three biological replicates. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for all calculations.

Results

Confirmation of the mesenchymal phenotype of the established MSC lines

The mesenchymal phenotype of the established MSC lines was confirmed by analysis of selected surface-marker expression and by the ability of the cells to undergo cytodifferentiation into adipocytes, osteoblasts, and chondrocytes (Romanek et al., 2024). Only cells expressing CD44, CD90, and CD105, with no expression of CD14 and CD45, were used in further experiments.

Morphological assessment

On day 7 of in vitro culture of porcine MSCs, colonies with 90% confluence and normal morphology were observed in medium 2 (DMEM and DMEM/F12, a 1:1 mixture) supplemented with Fgf at 39°C (Fig. 2D). At the same temperature and in the same medium but without Fgf, cell confluence was approximately 85% (Fig. 2C). Similar results were observed for cells cultured in medium

2 and medium 2 + Fgf at 37°C (Fig. 1C, Fig. 1D). These cultures also had the smallest number of non-adherent (dead) cells in the medium. Colonies cultured in medium 1 (DMEM) showed approximately 75% and 70% confluence and normal morphology at 39°C and 37°C, respectively (Fig. 2A, Fig. 1A). However, these cultures contained the highest number of non-adherent (necrotic) cells at both temperatures (Fig. 2A, Fig. 1A). Addition of Fgf to medium 1 increased confluence to approximately 80% at both temperatures and reduced the number of dead cells (Fig. 2B; Fig. 1B). Cultures in medium 4 (Stem MACS™ MSC Expansion Media XF MACS) also showed normal morphology, but lower confluence of approximately 70% and 65% at 39°C and 37°C, respectively; the number of non-adherent cells was also low (Fig. 2G, Fig. 1G). The weakest cell growth (50% and 40% confluence) was observed in medium 3 (DMEM/F12) without Fgf at 39°C and 37°C, respectively (Fig. 2E, Fig. 1E). Addition of Fgf to medium 3 increased culture confluence by approximately 10% (Fig. 2F, Fig. 1F), irrespective of culture temperature.

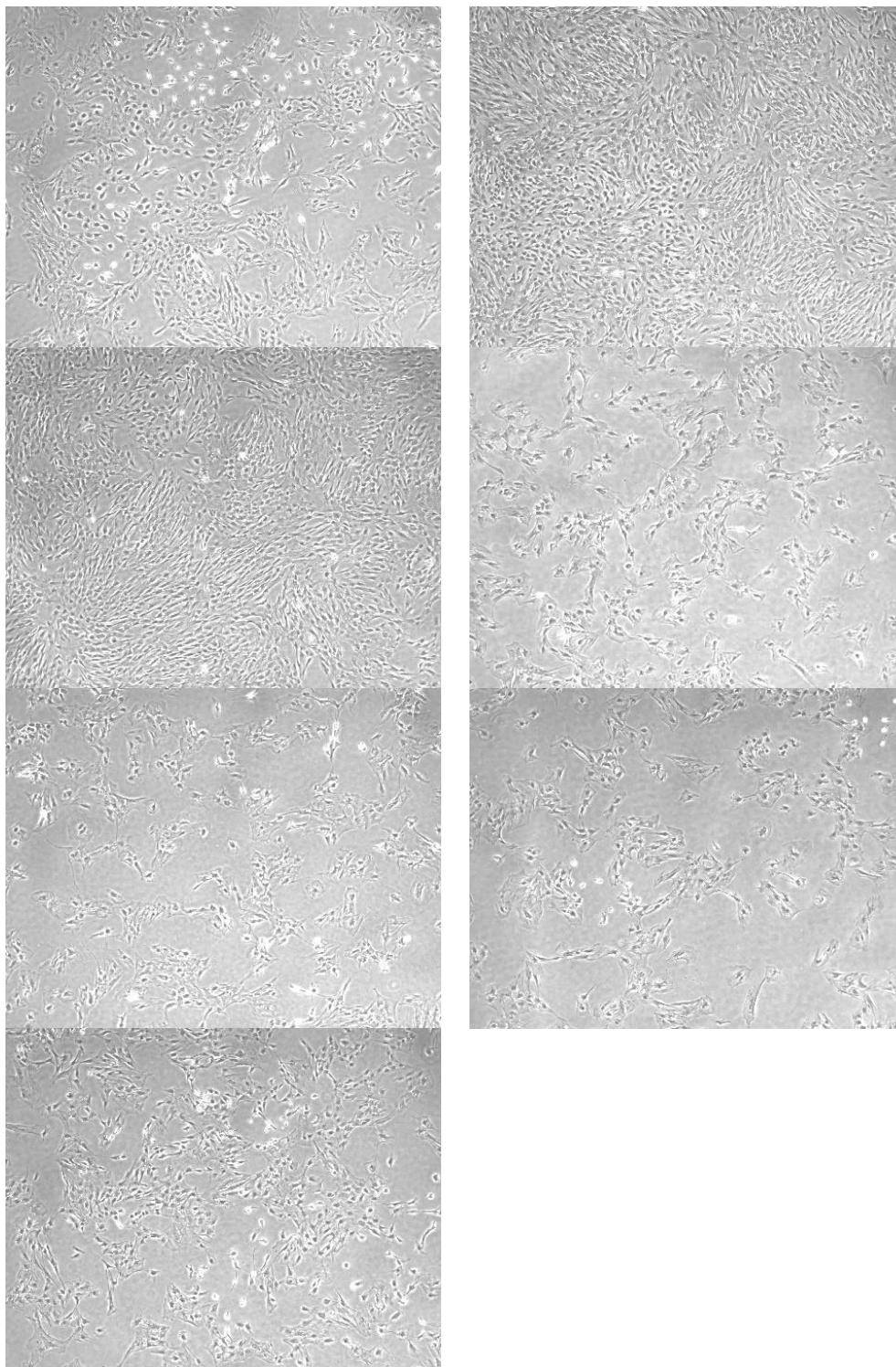


Figure 1. Light micrographs showing the morphology of MSCs on day 7 of in vitro culture at 37°C in the following media: A - 1, B - 1 + Fgf, C - 2, D - 2 + Fgf, E - 3, F - 3 + Fgf, G - 4 (200× magnification)

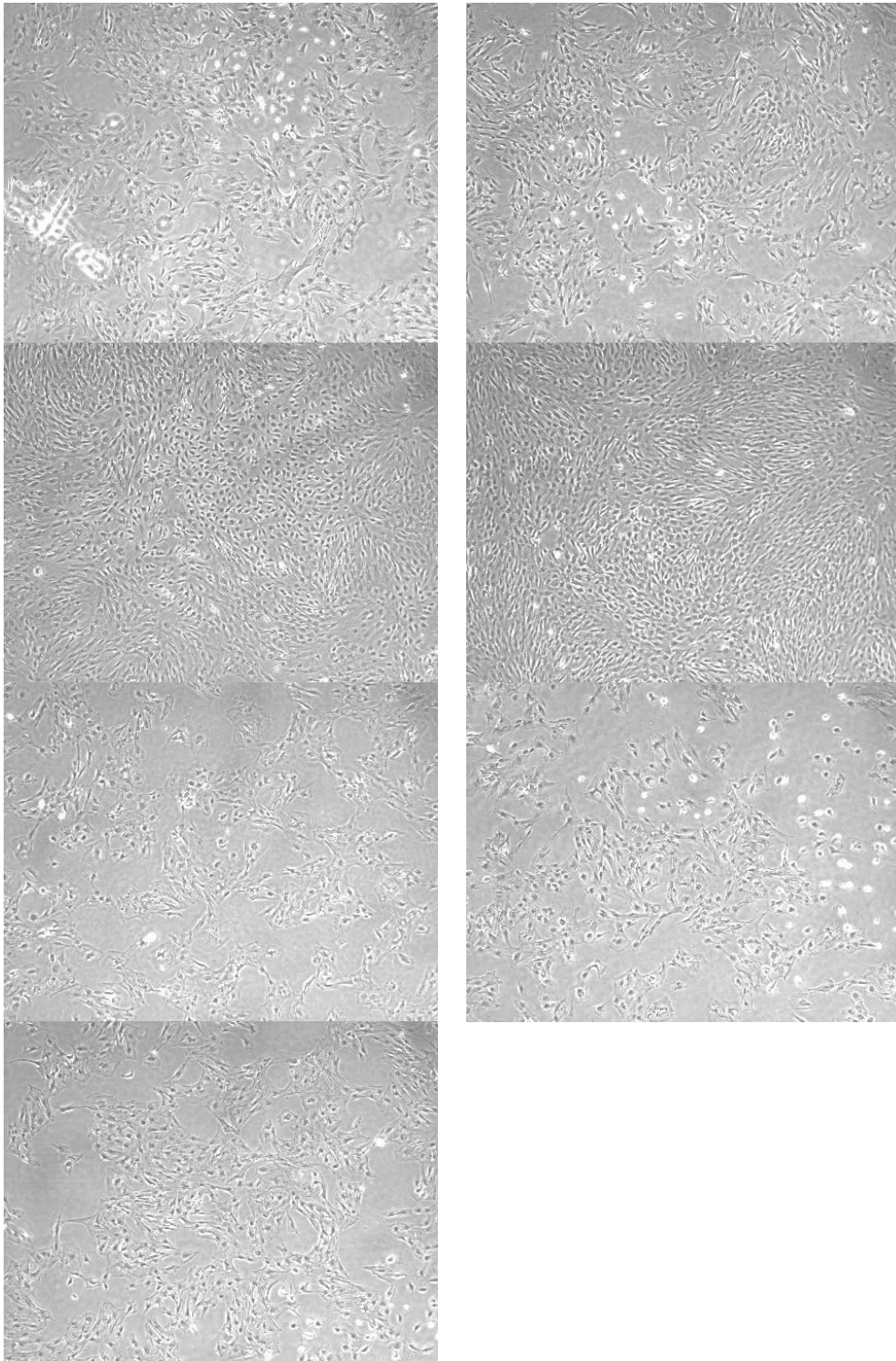


Figure 2. Light micrographs showing the morphology of MSCs on day 7 of in vitro culture at 39°C in the following media: A - 1, B - 1 + Fgf, C - 2, D - 2 + Fgf, E - 3, F - 3 + Fgf, G - 4 (200× magnification)

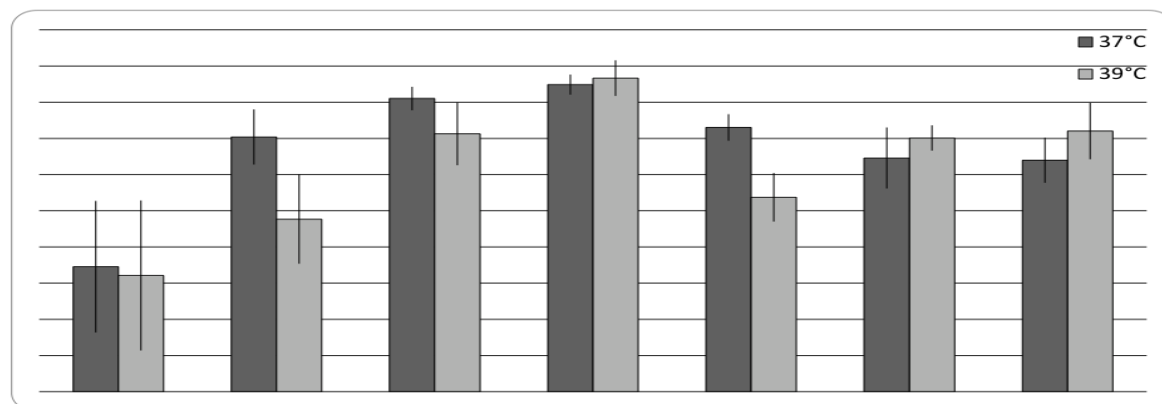
Assessment of PS exposure on the MSC surface

No statistically significant differences were observed among the analysed groups in the percentages of late-apoptotic or necrotic cells, early-apoptotic cells, or viable cells. Nevertheless, cells cultured in medium 2 had the highest percentage of viable cells (approximately 90%) and a low percentage of late-apoptotic and necrotic cells (approximately 7-8%), irrespective of culture temperature (Charts 1 and 2). Supplementation of the media with Fgf also improved the quality of cultured cells by increasing the proportion of viable cells and reducing the number of apoptotic cells (early- and late-apoptotic combined). The best quality parameters were observed for cells cultured at 39°C in medium 2 supplemented with Fgf. Three separate experiments were performed to evaluate late-apoptotic or necrotic, early-apoptotic, and viable cells. The results are presented in Table 1. An example of a cytometric analysis for an analysed sample and the positive control is shown in Fig. 3.

Table 1. Percentage of MSCs (%) in late apoptosis or necrosis, early apoptosis, and viable cells $\pm$ SEM

37°C				
No.	Culture type	Viable cells	Early apoptotic cells	Late apoptotic/necrotic cells
1	Medium 1	81.91 $\pm$ 3.64	7.57 $\pm$ 2.54	10.52 $\pm$ 0.85
2	Medium 1+Fgf	89.08 $\pm$ 1.52	4.03 $\pm$ 0.64	6.89 $\pm$ 0.45
3	Medium 2	91.20 $\pm$ 0.65	0.74 $\pm$ 0.03	8.05 $\pm$ 0.43
4	Medium 2+Fgf	91.97 $\pm$ 0.56	1.92 $\pm$ 0.29	6.10 $\pm$ 0.56
5	Medium 3	89.60 $\pm$ 0.73	1.69 $\pm$ 0.36	8.70 $\pm$ 0.47
6	Medium 3+Fgf	87.92 $\pm$ 1.69	1.03 $\pm$ 0.22	11.06 $\pm$ 0.59
7	Medium 4	87.79 $\pm$ 1.25	0.64 $\pm$ 0.09	11.57 $\pm$ 0.75

39°C				
No.	Culture type	Viable cells	Early apoptotic cells	Late apoptotic/necrotic cells
1	Medium 1	81.42 $\pm$ 4.15	6.88 $\pm$ 2.36	11.70 $\pm$ 1.14
2	Medium 1+Fgf	84.53 $\pm$ 2.46	4.41 $\pm$ 0.71	11.06 $\pm$ 0.92
3	Medium 2	89.25 $\pm$ 1.74	1.33 $\pm$ 0.12	9.42 $\pm$ 0.88
4	Medium 2+Fgf	92.33 $\pm$ 0.99	1.12 $\pm$ 0.09	6.55 $\pm$ 0.55
5	Medium 3	85.75 $\pm$ 1.34	0.98 $\pm$ 0.33	13.27 $\pm$ 1.03
6	Medium 3+Fgf	89.02 $\pm$ 0.70	0.77 $\pm$ 0.20	10.21 $\pm$ 0.65
7	Medium 4	89.41 $\pm$ 1.56	0.95 $\pm$ 0.11	9.64 $\pm$ 0.76



Wykres 1. Analiza poziomu ekspozycji fosfatydylseryny na powierzchni komórek MSC – liczba komórek żywych

Diagram 1. Analysis of phosphatidylserine exposure on the surface of MSCs - percentage of viable cells in different culture media

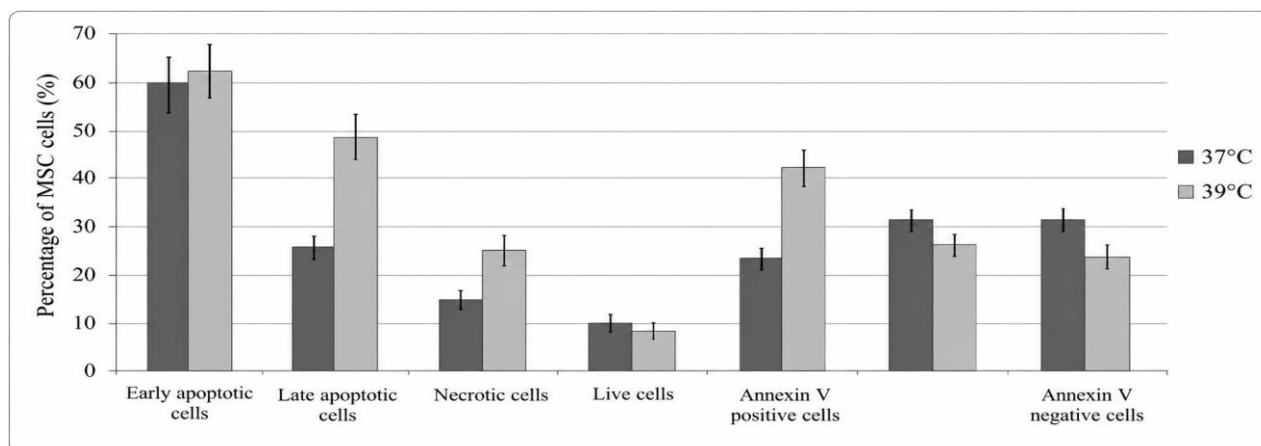


Diagram 2. Analysis of the level of phosphatidylserine exposure on the surface of MSC cells – the number of early and late apoptotic MSC cells (%) in various culture media

In summary, the number of non-adherent cells was slightly lower in medium 2 supplemented with fibroblast growth factor at 39°C. In addition, cell growth at 39°C in all tested media reached, on average, approximately 10% higher confluence. Fewer non-adherent cells were also observed at this temperature.

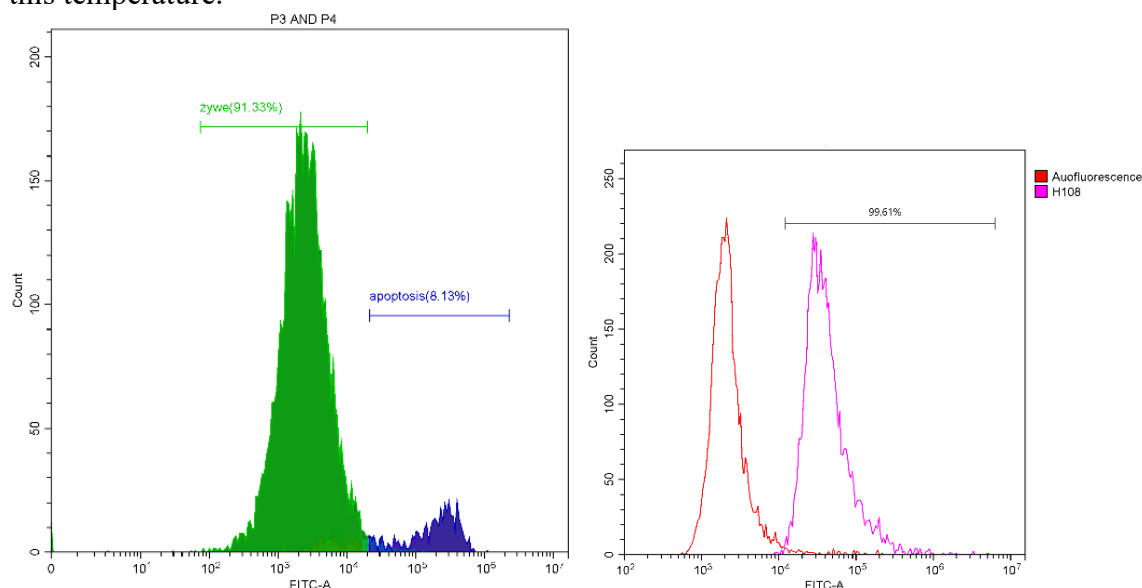


Figure 3A. Flow-cytometric analysis of annexin V-FITC and propidium iodide (PI) expression in immunofluorescently labelled BM-MSC populations. 3B. Flow-cytometric analysis of annexin and propidium iodide expression in cells incubated for two hours with 1 μ M staurosporine (positive control).

Discussion

Maintaining optimal in vitro culture conditions is a key factor affecting the growth and development of cultured cells. Basal culture media of known composition are available and can be supplemented with additional reagents such as L-glutamine, antibiotic/antifungal solution, or growth factors. When selecting a medium for a specific cell type, glucose and L-glutamine are the most

important nutrients for production of cellular energy (ATP), which is essential for cell growth and maintenance. GlutaMAX has been shown to support MSC growth more effectively than L-glutamine (Salehinejad et al., 2012). Commercial media with undisclosed, proprietary compositions are also available for specific stages of cell culture. Given the considerable interest in MSCs in medicine and veterinary medicine, their safety and efficacy in different clinical applications remain key issues. Optimising culture conditions for large-scale MSC production is therefore a priority. The aim of the present study was to establish culture conditions for expansion of porcine MSCs that provide optimal conditions for cell growth while limiting initiation of apoptosis. Our results showed that a 1:1 mixture of DMEM and DMEM/F12 supplemented with 10% FBS and 10 ng Fgf is optimal and suitable for MSC culture and propagation. Under these conditions, culture temperature had no major effect on cell quality. In addition, cultures in DMEM contained more non-adherent cells. This may be because, unlike the other media used, DMEM does not contain HEPES buffer (N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). HEPES can be added to culture medium to provide additional buffering capacity and better control of pH fluctuations (Brodsky et al., 2013). This factor may have resulted in the lower percentage of non-adherent cells in the other media and consequently in higher confluence on day 7. Thus, it cannot be determined whether the lower confluence of cells cultured in DMEM resulted from poorer proliferative capacity under these conditions or from weaker buffering of the medium.

Nekanti et al. (2010) found that DMEM/F12 was more suitable than the other media tested for supporting in vitro expansion of MSCs isolated from Wharton's jelly. One of the main differences between the basal media DMEM/F12 and DMEM is glucose concentration. DMEM/F12 contains 3.15 g/l glucose, DMEM contains 1.0 g/l, and the DMEM:DMEM/F12 mixture contains 2.075 g/l. Our analyses showed that 2.075 g/l was the most suitable glucose concentration for cell growth. Cells cultured at the lower concentration (1.0 g/l) also showed satisfactory growth, although they had a higher proportion of non-adherent cells than the other groups. The poorest growth was recorded in medium containing 3.15 g/l glucose. These results differ from those obtained for human bone-marrow-derived MSCs. Pal et al. (2009) showed that MSCs proliferated better while maintaining high expression of surface markers and multilineage differentiation capacity in DMEM/F12. However, cells cultured in medium containing 4.5 g/l glucose showed growth inhibition after passage 5. In low-glucose medium, cells retained population-doubling capacity up to passage 10, but morphological changes appeared with subsequent passages (Pal et al., 2009). Rat bone-marrow-derived MSCs cultured in low-glucose medium (1.0 g/l) showed a shorter colony-formation time, a shorter time to first passage, and normal morphology. These findings indicate that low-glucose DMEM is the most suitable medium for in vitro culture of rat MSCs (Wang et al., 2019). These analyses confirm that glucose concentration is one of the factors affecting MSC proliferation.

It is also known that adding other factors that support cell growth, such as amino acids, vitamins, inorganic salts, or growth factors, has a beneficial effect on maintaining cell proliferative capacity. One such factor is fibroblast growth factor. Supplementation of culture medium with Fgf is known to increase expansion of human MSCs (Gharibi and Hughes, 2012). In the control group without Fgf, MSC numbers increased 10-fold, whereas in the Fgf-supplemented group they increased 25-fold. Clinically, this may allow smaller biopsy samples to be used as a source of MSCs (Gharibi and Hughes, 2012). Solchaga et al. (2010) likewise demonstrated a higher proliferation rate of human MSCs in Fgf-supplemented medium than in control conditions. At every passage, population-doubling time was shorter in Fgf-supplemented cells than in cells cultured without supplementation (Solchaga et al., 2010). Analysis of phosphatidylserine (PS) exposure on the cell surface showed that the analysed culture conditions (37°C and 39°C), different glucose

concentrations, and medium supplements did not cause substantial initiation of apoptosis. Huang et al. (2017) analysed PS exposure in rat MSCs cultured at 37°C in DMEM/F12. Their results showed approximately 91% viable cells and about 9% early/late-apoptotic and necrotic cells (Huang et al., 2017). Similar results were obtained for human bone-marrow-derived stem cells cultured in DMEM at 37°C (de Andrade et al., 2014). Porcine MSCs analysed under the same conditions showed very similar values. Analyses of porcine MSCs cultured at 39°C in low-glucose medium showed $67.2 \pm 14.2\%$ viable cells, $24.3 \pm 12.7\%$ early-apoptotic cells, and $8.5 \pm 4.9\%$ late-apoptotic and necrotic cells (Romanek et al., 2017). In the present study, the percentage of viable cells was higher (over 81%), while the percentage of early-apoptotic cells was lower (approximately 7%). These differences may result from differences in cryopreservation procedures. Cells analysed in 2017 underwent a two-step cryopreservation procedure (2 h at -20°C followed by storage in liquid-nitrogen vapour) without precise control of cooling rate (Romanek et al., 2017). In the current study, cells were cryopreserved using a Nalgene Mr. Frosty Freezing Container (Merck Life Science sp. z o.o., Poznań, Poland), which provides a cooling rate of 1°C/min.

Based on morphological assessment and analysis of PS exposure on the cell membrane, the best conditions for culturing porcine bone-marrow-derived stem cells were a 1:1 mixture of DMEM and DMEM/F12 supplemented with fibroblast growth factor. The results also indicated an advantage of culturing cells at 39°C. The findings suggest that this line of research should be continued. Optimisation of culture conditions should be performed for all cell types and defined separately for each cell type and source species. It should also be remembered that MSCs obtained from different sources differ in viability, plasticity, and expression levels of CD surface markers. Therefore, another important issue is optimisation of culture conditions for cells obtained from sources other than bone marrow.

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