Effects of platelet-rich plasma on mesenchymal stem cells isolated from rat uterus

Polina Vishnyakova [Corresp., Equal first author, 1, 2], **Daria Artemova** [Equal first author, 1], **Andrey Elchaninov** [1, 3], **Zulfiia Efendieva** [4], **Inna Apolikhina** [1, 4], **Gennady Sukhikh** [1], **Timur Fatkhudinov** [2, 5]

[1] National Medical Research Center for Obstetrics, Gynecology and Perinatology Named after Academician V.I. Kulakov of Ministry of Healthcare of Russian Federation, Moscow, Russia

[2] People' s Friendship University of Russia, Moscow, Russia

[3] Pirogov Russian National Research Medical University (RNRMU), Moscow, Russia

[4] I. M. Sechenov First Moscow State Medical University of Ministry of Health of Russia (Sechenov University), Moscow, Russia

[5] Scientific Research Institute of Human Morphology, Moscow, Russia

Corresponding Author: Polina Vishnyakova
Email address: vpa2002@mail.ru

Background. Platelet-rich plasma (PRP) is a valuable source of growth factors widely used in regenerative medicine. Recently, PRP was used for to treat infertility caused by refractory thin endometrium. Mesenchymal stem/stromal cells (MSCs) of endometrium are an essential cellular component responsible for extracellular matrix remodeling, angiogenesis, cell-to-cell communication, post-menstrual tissue repair etc. We examined the effects of PRP on the MSCs isolated from rat uterus and compared them with the effects of ordinary plasma (OP).

Methods. MSCs were isolated from rat uterus by enzymatic disaggregation. Flow cytometry analysis was used for the immunophenotyping of the primary cells. The ability of MSCs to differentiate in osteo-, chondro- and adipogenic directions was assessed with differentiation-induced media. Immunocytochemistry assay was performed for Ki-67 and vimentin staining. Autophagy and apoptosis markers as well as the level of MMP9 and ERα was assessed by western blotting.

Results. After 24 h incubation of MSCs proliferation index was significantly higher for PRP compared to OP and complete growth medium. The treatment with PRP and OP enhances activation of autophagy while OP also induced up-regulation of stress-induced protein p53 and extracellular enzyme MMP9. Significantly elevated level of MMP9 production was observed in both OP group compared to complete growth medium and PRP. These results indicate the practical relevance and validity of PRP usage in treatment of infertility.

Effects of platelet-rich plasma on mesenchymal stem cells isolated from rat uterus

Vishnyakova Polina [1,2#*], Artemova Daria [1#], Elchaninov Andrey [1,3], Efendieva Zulfiia[4], Apolikhina Inna [1,4], Sukhikh Gennady [1] and Fatkhudinov Timur [2,5]

Author affiliations:

[1] National Medical Research Center for Obstetrics, Gynecology and Perinatology Named after Academician V.I. Kulakov of Ministry of Healthcare of Russian Federation, Moscow, Russia

[2] Peoples' Friendship University of Russia, Moscow, Russia

[3] Pirogov Russian National Research Medical University (RNRMU)

[4] Institute of Professional Education of the I. M. Sechenov First Moscow State Medical University of Ministry of Health of Russia (Sechenov University)

[5] Scientific Research Institute of Human Morphology, Moscow, Russia

# – equal contribution

Corresponding Author:

Polina A. Vishnyakova, PhD

Laboratory of regenerative medicine, Research Center for Obstetrics, Gynecology and Perinatology, Ministry of Healthcare of the Russian Federation, 4, Oparina street, Moscow, 17513, Russia. Tel: +79150658577.

Email: vpa2002@mail.ru or vishnyakovapolina@gmail.com

Abstract

Background. Platelet-rich plasma (PRP) is a valuable source of growth factors widely used in regenerative medicine. Recently, PRP was used for to treat infertility caused by refractory thin endometrium. Mesenchymal stem/stromal cells (MSCs) of endometrium are an essential cellular component responsible for extracellular matrix remodeling, angiogenesis, cell-to-cell communication, post-menstrual tissue repair etc. We examined the effects of PRP on the MSCs isolated from rat uterus and compared them with the effects of ordinary plasma (OP).

Methods. MSCs were isolated from rat uterus by enzymatic disaggregation. Flow cytometry analysis was used for the immunophenotyping of the primary cells. The ability of MSCs to differentiate in osteo-, chondro- and adipogenic directions was assessed with differentiation-

induced media. Immunocytochemistry assay was performed for Ki-67 and vimentin staining. Autophagy and apoptosis markers as well as the level of MMP9 and ERα was assessed by western blotting.

Results. After 24 h incubation of MSCs proliferation index was significantly higher for PRP compared to OP and complete growth medium. The treatment with PRP and OP enhances activation of autophagy while OP also induced up-regulation of stress-induced protein p53 and extracellular enzyme MMP9. Significantly elevated level of MMP9 production was observed in both OP group compared to complete growth medium and PRP. These results indicate the practical relevance and validity of PRP usage in treatment of infertility.

Introduction

Platelet-rich plasma (PRP) is plasma with a platelet concentration above normal (Theoret & Stashak, 2014) The increased platelet counts in PRP deliver increased amounts of the growth factors enclosed within the platelet granules, including platelet-derived growth factors (PDGF: PDGF-AA, PDGF-BB, and PDGF-AB), transforming growth factors (TGF), insulin-like growth factors (IGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), connective tissue growth factor (CTGF), fibroblast growth factors (FGF) (Lubkowska, Dolegowska & Banfi, 2012) etc. PRP is also rich in certain plasma proteins e.g. fibrin, fibronectin and vitronectin (Marx, 2019). For the platelet degranulation and fibrinogen cleavage, plasma samples are usually activated by the addition of calcium chloride (alternatively, thrombin or collagen) or by applying freeze/thaw cycle(s) (Cavallo et al., 2016; Maffulli, 2016). The view of PRP as a potential therapeutic agent for tissue regeneration was initiated by Marx et al. (Marx et al., 1998). In 1998, these authors found that the addition of PRP to a bone graft during the bone-milling phase of its preparation enhanced the rates of bone formation in the treatment of

human mandibular defects. Nowadays, PRP is widely used in cosmetology, dentistry, sports medicine and surgery (Yuksel et al., 2014; Maffulli, 2016; Patel et al., 2016). PRP could be applied to the site of injury, mixed with a bone graft, layered over the graft upon its placement in the body, sprayed onto the surface of tissue, applied over the graft or used as a biological membrane (Civinini et al., 2011). In a recent study by Kim et al., intrauterine administration of the autologous PRP improved the implantation and pregnancy outcomes for the patients with refractory thin endometrium resulting in ≥ 2 failed *in vitro* fertilization cycles (Kim et al., 2019). These results are encouraging: they indicate that the PRP effects on the refractory thin endometrium may have a potential for the assisted reproductive technology. Endometrium is a dynamic structure composed of simple columnar epithelium with uterine glands, and the underlying stroma (Pertschuk, 1990) with blood vessels, nerves, collagen and reticular fibers, and a variety of stromal cells (Aplin, 2018). Isolation of mesenchymal stem/stromal cells (MSCs) from endometrium (Chan, Schwab & Gargett, 2004; Chan & Gargett, 2006) allows elucidation of tissue-specific functions and markers of these cells thus opening new prospects of their usage. As actively proliferating cells, MSCs play an important role in the tissue homeostasis of endometrium — they are involved in the extracellular matrix remodeling, angiogenesis, cell-to-cell communication, post-menstrual tissue repair, etc.(Mutlu, Hufnagel & Taylor, 2015; Arutyunyan et al., 2016) The use of rat endometrial MSCs makes a good model for studying the action of PRP in the perspective of infertility treatment. Despite the expanding use of PRP in clinical practice, exact molecular mechanisms of the PRP action on cells are largely unclear. In current study, we examine the effects of PRP on the MSCs isolated from rat uterus. We compare them with the effects of ordinary plasma (OP).

Materials & Methods

Ethical disclosure

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. The study was approved by the Ethical Review Board at the Scientific Research Institute of Human Morphology (Protocol №.15, 9th of December, 2019).

Animals. The outbred eight-week-old female Sprague-Dawley rats of 250–300 g weight were obtained from the Institute for Bioorganic Chemistry branch animal facilities (Pushchino, Moscow region, Russia). All experimental work involving animals was carried out according to the standards of laboratory practice (National Guidelines No. 267 by Ministry of Healthcare of the Russian Federation, June 1, 2003), and all efforts were made to minimize the suffering. The study was approved by the Ethical Review Board at the Scientific Research Institute of Human Morphology (Protocol №.15, 9th of December, 2019). The animals were adapted to the laboratory conditions (23°C, 12 h/12 h light/dark, 50% humidity, and ad libitum access to food and water) for 2 weeks prior to the experiments. In adult female rats, the stage of the estrous cycle was determined by taking a vaginal smear. The smear was stained with methylene blue and the stage was determined by assessment of cellular composition of the smear. The uterus was dissected at the stage of metestrus after euthanasia in CO_2-chamber; the blood was collected by puncture from the heart. Animals served only as a source of the uterus and autologous plasma; therefore, no experimental conditions and endpoints were used.

PRP and OP preparation. The blood was collected in tubes with 2 ml of heparin (5000 IU/ml) and 800 μl of 10% sodium citrate. PRP was obtained based on a protocol developed by Yazigi et al. (Yazigi Junior et al., 2015) The blood was centrifuged at 400 g for 10 min, the plasma was collected in a new tube and centrifuged again at 4 °C, 400 g for 10 min. After centrifugation,

70% of the supernatant (the platelet-poor plasma, PPP) was discarded. The remaining fraction PRP, which according to the modern classification belongs to L type (L-PRP), was preserved. The platelet counts were determined on a TC20 Automated Cell Counter (Bio-Rad, USA) and constituted 50×10^6 platelets/ml on average. To obtain OP, the blood was centrifuged at 4 °C, 2000 g for 15 min, and the supernatant was collected. The prepared OP and PRP were aliquoted, frozen and stored at -20 °C. Before use, PRP was activated by adding 10% $CaCl_2$ (10 µl of per 200 µl of PRP) according to the protocol by Messora et al.(Messora et al., 2011)

The protocol for obtaining MSCs from rat endometrium. The primary cell cultures were obtained based on the protocol by De Clercq et al. with modifications (De Clercq, Hennes & Vriens, 2017). The dissected uterus was minced with scissors in Hank's Balanced Salt Solution (HBBS) and transferred into 0.25% trypsin. The tube was incubated at 4 °C for 1 h, then at 22 °C for 1 h, and finally at 37 °C for 15 min with periodical shaking. The supernatant was taken and the solid bulk was transferred to a solution of collagenases type I and type IV in 0.05% trypsin-EDTA solution (collagenase I:collagenase IV:trypsin-EDTA, 1:1:10), incubated at 37 °C for 30 min, and passed through a 70 µm cell strainer. The material remaining on the strainer was transferred to a fresh solution of collagenases with 0.05% trypsin-EDTA, incubated at 37 °C for 15 min, and passed through a strainer again. The resulting suspension containing the cells of interest was centrifuged at 300 g for 5 min at 22 °C. The pellet was resuspended in HBBS supplemented with 1% FBS, centrifuged again, and resuspended in complete growth medium (DMEM/F-12 supplemented with 10% FBS, L-glutamine and penicillin/streptomycin) for cultivation. The obtained cells were verified for compliance with the minimal criteria for MSCs issued by the International Society for Cellular Therapy(Dominici et al., 2006) (adhesion to untreated plastic, specific profile of surface antigens, and *in vitro* differentiation towards

129 osteogenic, chondrogenic and adipogenic progeny). To assess the effects of PRP and OP on
130 MSCs, the cells were cultured for 24 h in the medium supplemented with 10% PRP and 10% OP,
131 respectively, instead of FBS. CGM with 10% FBS was used as the control.
132 ***Flow cytometry analysis.*** Immunophenotyping of the cells for the surface and intracellular
133 markers was performed upon reaching 80% confluence. The harvested cells were centrifuged at
134 800 g for 10 min, the supernatant was discarded, the cells were fixed in 2% paraformaldehyde
135 for 15 min at room temperature (RT), diluted with 5 ml of PBS, and centrifuged at 1500 g for 10
136 min. The pellet was resuspended in 1 ml of PBS. For immunostaining, 1×10^5 of fixed cells cells
137 were incubated in 100 µl of Rinsing Solution (Miltenyi Biotec, USA) with primary antibodies to
138 CD90 (ab225, 1/100, Abcam), CD45 (130-107-846, clone REA504, 1/20, Miltenyi Biotec),
139 CD105 (ab107595, 1/100, Abcam), CD34 (PAB18289, 1/100, Abnova) at room temperature for
140 1 h, and subsequently with secondary antibodies — anti-mouse Ig-FITC (ab6785, 1/500, Abcam)
141 or anti-rabbit Ig-PE (sc-3739, 1/100, Santa Cruz) at RT for 1 h in the dark. After the incubation,
142 the cells were washed in PBS, resuspended in 0.5 ml of PBS, and transferred to fresh tubes for
143 the analysis on a FACScan flow cytometer (Becton Dickinson, USA) with the CellQuest
144 software.
145 ***Induced differentiation of MSCs.*** The ability of MSCs to differentiate in osteo-, chondro- and
146 adipogenic directions was assessed at early passages (up to 7). The cells were grown to 70%
147 confluency in CGM, and then the growth medium was replaced with differentiation medium (for
148 differentiation) or CGM (for the controls). Differentiation into adipogenic progeny was
149 accomplished by using StemPro® Adipogenesis Differentiation Kit (Gibco) in the course of 7
150 days. At the end of the differentiation process, the cells were fixed with ethyl alcohol:formalin
151 solution (1:4) for 3 minutes and stained with Sudan III (5.7 mM) for 10 minutes to visualize fat

droplets. For osteogenic differentiation, the medium was supplemented with dexamethasone (10^{-7} M) and ascorbic acid (0.2 mM) in the course of two weeks. At the end, the cells were fixed with 70% alcohol, and for the detection of mineralization sites, the cells were stained with 40 mM alizarin red S solution (pH = 4.7) for 5 min. Chondrogenic differentiation was performed by using StemPro® Chondrogenesis Differentiation Kit (Gibco). After two weeks of differentiation, the cells were fixed with 4% formalin for 1 h and for detection of mucopolysaccharides were stained with 1% alcian blue for 24 h. Samples were analyzed using an Axiovert 40 CFL inverted microscope (Zeiss, Germany) using ZEN software (Carl Zeiss, Germany).

Immunocytochemistry. The MSCs were grown on glass coverslips (Fisher Scientific) coated with gelatin and placed in Petri dishes (35 × 10 mm). For immunocytochemistry, the cells were fixed in 2% paraformaldehyde. The coverslips with fixed cells were treated with 0.1% Triton X-100 for 10 min (cell membrane permeabilization) and washed with PBS. Non-specific binding sites were blocked with 1% BSA in PBS with 0.1% Tween-20 for 30 minutes. The coverslips were incubated with antibodies to Ki-67 (ab15580, 1/100, Abcam) or vimentin (ab8978, 1/250, Abcam) at +4 °C for 24 hours. After incubation, the coverslips were washed with PBS and then incubated with the secondary anti-rabbit-PE antibodies (1/200) or anti-rabbit-FITC antibodies (1/200) in the dark at RT for 1 h and subsequently washed with PBS. To stain the nuclei, the coverslips were incubated with DAPI (0.004 mg / ml) at 37 °C for 10 min. Then the coverslips were washed in PBS and mounted in Aqua-Poly Mount. The photographs were made using a Leica DM 4000B fluorescence microscope (Leica Microsystems, Germany) with the LAS AF v.3.1.0 software (Leica Microsystems, Germany).

Western blot assay. The cells were washed with PBS and lysed in the ice-cold RIPA buffer. The sample was mixed with 2X loading buffer and incubated at 95 °C for 1 minute. The samples

were stored at -80 °C until use and heated for 1 min at 95 °C before loading. The proteins were separated by 10–12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from the gel to PVDF membranes by the semi-wet approach using Trans-Blot® Turbo™ RTA Mini LF PVDF TransferKit (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween (TTBS) at RT for 1 h, then stained overnight with primary antibodies to LC3B (ab48394, Abcam), Bcl-2 (ab32124, Abcam), p53 (ab90363, Abcam), ERα (ab3575, Abcam), MMP-9 (ab38898, Abcam) and GAPDH (sc-25778, Santa Cruz) and subsequently with the HRP-conjugated secondary antibodies (Bio-Rad Laboratories, Inc.). Chemiluminescent signal developed by using Novex ECL Kit (Invitrogen, USA) was visualized in a C-DiGit® Blot Scanner (LI-COR, USA) using the Image Studio™ Acquisition Software (LI-COR, USA). Figure of uncropped membrane is available in Supplementary information (Fig.S1). The relative protein levels were determined via normalization by GAPDH signals.

Statistical analysis. Statistical data processing was performed in the GraphPad Prism 8 (Software GraphPad Software, USA). The normality of distributions was tested by the Shapiro-Wilk criterion. In the case of normal distribution, one-way ANOVA with the Turkey post-hoc test for multiple comparison was used. In the case of non-normal distribution, the Kruskal-Wallis test with the post-hoc Dunn test was used. The differences were considered statistically significant at $p < 0.05$.

Results

Characterization of isolated MSCs. The uterus is a non-classical source of MSCs. Given the wide variability of MSC phenotypes and tissue-specific features (Kwon et al., 2016) the first stage of our study was to show the compliance of rat uterine mesenchymal cell cultures to the

established minimal criteria for MSCs, including phenotype profile and the capability of induced *in vitro* differentiation into different mesenchymal lineages. The obtained cultures of fibroblast-like cells were immunophenotyped by flow cytometry for CD90, CD105, CD45 and CD34. The region of interest was selected by relation of forward and side scattering values in a FSC-SSC dot plot diagram (Figure 1 b), reflecting the size and granularity of the cells, respectively. We gated the major pool of single cell events (R1) excluding debris (Figure 1 a). The peaks of fluorescence were distinctly shifted in histograms for the MSC samples stained specifically with antibodies to CD90 and CD105 compared with the controls stained with secondary antibodies only (green curve). We found that 72.7% of the cells were positive for CD90 and 31.8% of the cells were positive for CD105, which indicated correspondence of the obtained cultures to the CD90+CD105+ phenotype. Interestingly, 3.1% and 20.1% of the cells were positive for CD45 and CD34, respectively. To further confirm the compliance, we stained the cultures for vimentin. The immunocytochemical assay revealed the presence of vimentin protein in 100% of the cells (Figure 1 b).

We promoted differentiation of the cell cultures into adipogenic, osteogenic and chondrogenic lineages by using specific combinations of inducers. The adipogenic differentiation was identified by formation of lipid droplets revealed by staining with Sudan III (Figure 1 c). During the osteogenic differentiation, the cells formed characteristic conglomerates, with the foci of calcification revealed by alizarin red staining (Figure 1 c). During the chondrogenic differentiation, conglomerates were also formed by the cells, and the mucopolysaccharide production was revealed by staining with alcian blue (Figure 1 c).

Effects of PRP and OP on cell proliferation and cell death. Effects of PRP and OP on the rat uterine MSC cultures were evaluated after 24 h incubation of the cells in a medium containing

10% PRP or 10% OP instead of FBS. Accordingly, complete growth medium (CGM) with 10% FBS was used as the control. Proliferation was assessed by immunocytochemical staining for Ki-67 following the incubation (Figure 2 a). The percentage of Ki-67 positive nuclei divided by the total number of nuclei (proliferation index) was 18.1% for CGM, 41.9% for PRP, and 21.7% for OP (Fig. 2 b). For PRP, the differences were statistically significant ($p = 0.04$).

To evaluate the activation or inhibition of apoptosis, we analyzed the production of the stress-induced protein p53 and the anti-apoptotic protein Bcl-2. The p53 protein levels were significantly higher in the cells exposed to OP ($p = 0.03$), as compared with the control (CGM) cells (Figure 2 c). Relative levels of Bcl-2 production did not differ among the studied groups.

Autophagy was assessed by the level of production of the autophagy marker LC3B. Western blot analysis revealed elevated production of LC3B protein in the cells exposed to PRP or OP, as compared with the control (CGM, Figure 2 c); the observed effect was statistically significant among studied groups.

Endometrium receptivity: effects of PRP and OP on the matrix metalloproteinase 9 (MMP9) and estrogen receptor α (ERα) production. Zinc-metalloproteinase MMP9 participates in the extracellular matrix remodeling. We estimated potential invasiveness of MSCs as a correlate of MMP9 production. Although elevated levels of MMP9 production were observed in both OP and PRP groups compared to CGM, only in the OP treated cultures the MMP9 upregulation was significant (Figure 3) ($p=0.03$). In addition, we compared the levels of ERα, which can be partially associated with endometrial receptivity. Despite the elevated ERα levels in the PRP and OP treated cultures, the difference was not significant.

Discussion

Administration of PRP for the treatment of thin endometrium has been already introduced to

clinical practice in a pilot study by Kim et al. aimed at increasing the endometrium thickness and accordingly the probability of implantation (Kim et al., 2019). However, the mechanism of the positive effect of PRP on the endometrium thickness remains uncertain. In this study, we attempt to identify signaling pathways activated in the multipotent stromal cells of endometrium under the influence of PRP by using rat endometrium as a model. Endometrial MSCs play a key role in the stroma: they participate in the regeneration of the functional layer of the endometrium due to the presence of receptors for sex hormones and the ability for extracellular matrix remodelling (Mutlu, Hufnagel & Taylor, 2015). These features emphasize the importance of MSCs for receptivity of the endometrium during the implantation period. We isolate primary cultures of stromal cells from the rat uterus and prove that these cells are essentially MSCs by their ability to differentiate in adipogenic, osteogenic and chondrogenic directions *in vitro*. Phenotypic profiles of the isolated primary cultures are also compliant with the CD90+CD105+Vimentin+CD45-CD34- profiles established for MSCs. The presence of cells positive for the CD45 and CD34 markers can be explained by and concomitant presence of hematopoietic cells and high vascularization of the endometrial stroma in the obtained cultures, respectively. Considering a precedent of the 81% CD34- and 97.5% CD34- cultures classified as CD34 negative (Lee et al., 2012), we classify the obtained cultures as CD45 and CD34 negative. The percentage of cells positive for CD105 seems lower than it was expected; however, literature data indicates a substantial variability of this parameter for MSCs from various sources (Ponnaiyan & Jegadeesan, 2014).

In the experiments with plasma treatment, the obtained MSCs were incubated for 24 h in the control medium (the DMEM/F-12 based complete growth medium with 10% FBS) or the medium supplemented with 10% PRP or 10% OP instead of FBS. We observed that under the

influence of PRP the cells proliferated more actively than under the influence of FBS or OP. This observation indicates mitogenic effects of PRP on stromal cells. We also studied the protein production levels for established markers of apoptosis and autophagy. The stress-induced protein p53 is a transcription factor that regulates cell cycle and acts as a tumor suppressor (Labuschagne, Zani & Vousden, 2018). An important function of p53 is to prevent the DNA damage accumulation. In the case of damage to cellular DNA, p53 promotes cell cycle arrest and triggers the emergency DNA repair systems; in the case of extensive DNA damage, p53 triggers apoptosis (Wang, Simpson & Brown, 2015). We show that OP promotes increased production of p53 protein by stromal cells, revealing a certain degree of cellular stress associated with the OP treatment. At the same time, both PRP and OP have no effects on the levels of the anti-apoptotic Bcl-2 protein production by MSCs. Bcl-2 is anti-apoptotic protein localized on the outer mitochondrial membrane, as well as the membranes of nuclear envelope and endoplasmic reticulum (Delbridge et al., 2016). Apparently, Bcl-2 activation is not involved in the effects of PRP and OP.

We used LC3B as an autophagy marker — this protein is involved in the biogenesis of autophagosomes (Barth, Glick & Macleod, 2010). Upon binding to the membrane, LC3 conjugates with phosphatidylethanolamine lipid (Tanida, Ueno & Kominami, 2008). After the autophagosome formation, LC3-II located in the outer layer is released into the cytosol, while LC3-II located in the inner layer is exposed to hydrolases. LC3B-II is conventionally used as a marker of autophagosomal activity. We observed excessive production of LC3B in MSCs after the exposure to PRP or OP. This observation suggests that PRP and OP contribute to self-renewal of the endometrial stromal cells by enhancing autophagy.

290 Endometrial receptivity results from a combination of different characteristics of the
291 endometrium responsible for the ability to promote implantation. One of the indicators of
292 endometrial receptivity is the level of ERα, as it mediates the action of estrogens preparing the
293 endometrium for implantation by increasing its thickness via the increase in the rates of cell
294 proliferation. In clinical practice, the level of production of ERα can be evaluated by staining of
295 endometrial cells with antibodies to ERα (Glasser et al., 2002), which is expressed by several
296 subpopulations of endometrial stromal cells including MSCs (Zhou et al., 2001). We therefore
297 employed ERα as a marker reflecting the conditional receptivity of endometrium in our model.
298 We observed no significant differences in the level of ERα production upon exposure of the
299 MSCs to PRP or OP, which may indicate that the beneficial effects of these agents are estrogen-
300 independent. However, this finding should be verified on larger samples to exclude individual
301 variations in the estrogen dependence of the plasma treatment effects.
302 In addition to the susceptibility of the endometrium to the action of hormones, a certain degree of
303 its invasiveness is necessary for successful implantation. The trophoblast cells bind to the
304 endometrium, proliferate and penetrate deep into the stroma to come into contact with maternal
305 blood (Bischof, Meisser & Campana, 2000). Progressive remodeling of the extracellular matrix
306 is a hallmark of this process (Bischof, Meisser & Campana, 2002). Expression of matrix
307 metalloproteinases is observed not only in trophoblast, but also in endometrial stromal cells,
308 which provides spatial regulation of trophoblast invasion (Bischof, Meisser & Campana, 2002).
309 In our work, we took MMP9 production as an indicator of the potency of MSCs for extracellular
310 marker remodeling, an important parameter that determines the receptivity of the endometrium.
311 In our experiments, we showed that OP facilitated an increase in the MMP9 production, as
312 compared with PRP and CGM.

In general, the obtained results indicate stimulatory effects of PRP on endometrium in a rat model, as indicated by behavior of MSCs isolated from it. Some other studies also indicate that PRP enhances cell proliferation and differentiation in the uterus (Etulain et al., 2018). Intrauterine administration of PRP to the rats with damaged endometrium has beneficial effects, not only enhancing cell proliferation, thus promoting the tissue regeneration, but also reducing fibrosis (Jang et al., 2017). The effects of OP on endometrium are notably understudied. In our experiments, OP also induced the activation of autophagy in MSCs, with up-regulation of the p53 and MMP9 protein expression. However, the beneficial effects of OP are less pronounced and can be considered intermediate, which indicates the advantages of PRP as a treatment agent for the endometrial dysfunction. This could be explained by obvious increased content of mitogenic substances in platelet granules.

Conclusions

Mesenchymal cell cultures obtained from rat endometrium by the established protocol comply with the essential criteria for multipotent stromal cells (MSCs). The treatment with platelet-rich plasma (PRP) enhances proliferation of these cells with the activation of autophagy. The treatment with ordinary plasma (OP) also activates autophagy with up-regulation of the stress-induced protein p53 and the extracellular enzyme MMP9. Therefore, PRP could be recommended as a preferable means for treating the thin endometrium. PRP usage and possibilities of PRP applications in clinical practice is certainly expanding. At the same time, the number of works on specific mechanisms of the PRP action on specific types of cells is restricted. The establishment of molecular pathways providing cellular division after PRP treatment could expand the range of PRP applications.

Acknowledgements

Special thanks to Natalia Usman for her help in proof-reading of the manuscript.

References

Aplin J. 2018. Uterus-endometrium. In: *Encyclopedia of Reproduction*. Elsevier, 326–332. DOI: 10.1016/B978-0-12-801238-3.64654-8.

Arutyunyan I V., Fatkhudinov TH, El'chaninov A V., Makarov A V., Kananykhina EY, Usman NY, Raimova ES, Goldshtein D V., Bol'shakova GB. 2016. Effect of Endothelial Cells on Angiogenic Properties of Multipotent Stromal Cells from the Umbilical Cord during Angiogenesis Modeling in the Basement Membrane Matrix. *Bulletin of Experimental Biology and Medicine* 160:575–582. DOI: 10.1007/s10517-016-3221-9.

Barth S, Glick D, Macleod KF. 2010. Autophagy: assays and artifacts. *The Journal of pathology* 221:117–24. DOI: 10.1002/path.2694.

Bischof P, Meisser A, Campana A. 2000. Mechanisms of endometrial control of trophoblast invasion. *Journal of reproduction and fertility. Supplement* 55:65–71.

Bischof P, Meisser A, Campana A. 2002. Control of MMP-9 expression at the maternal-fetal interface. In: *Journal of Reproductive Immunology*. Elsevier, 3–10. DOI: 10.1016/S0165-0378(01)00142-5.

Cavallo C, Roffi A, Grigolo B, Mariani E, Pratelli L, Merli G, Kon E, Marcacci M, Filardo G. 2016. Platelet-Rich Plasma: The Choice of Activation Method Affects the Release of Bioactive Molecules. *BioMed Research International* 2016. DOI: 10.1155/2016/6591717.

Chan RWS, Gargett CE. 2006. Identification of Label-Retaining Cells in Mouse Endometrium. *STEM CELLS* 24:1529–1538. DOI: 10.1634/stemcells.2005-0411.

Chan RWS, Schwab KE, Gargett CE. 2004. Clonogenicity of Human Endometrial Epithelial and Stromal Cells1. *Biology of Reproduction* 70:1738–1750. DOI: 10.1095/biolreprod.103.024109.

Civinini R, Macera A, Nistri L, Redl B, Innocenti M. 2011. The use of autologous blood-derived growth factors in bone regeneration. *Clinical Cases in Mineral and Bone Metabolism* 8:25–31.

De Clercq K, Hennes A, Vriens J. 2017. Isolation of mouse endometrial epithelial and stromal cells for in Vitro decidualization. *Journal of Visualized Experiments* 2017. DOI: 10.3791/55168.

Delbridge ARD, Grabow S, Strasser A, Vaux DL. 2016. Thirty years of BCL-2: Translating cell death discoveries into novel cancer therapies. *Nature Reviews Cancer* 16:99–109. DOI: 10.1038/nrc.2015.17.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317. DOI: 10.1080/14653240600855905.

Etulain J, Mena HA, Meiss RP, Frechtel G, Gutt S, Negrotto S, Schattner M. 2018. An optimised protocol for platelet-rich plasma preparation to improve its angiogenic and regenerative properties. *Scientific Reports* 8:1–15. DOI: 10.1038/s41598-018-19419-6.

Glasser SR, Aplin JD, Giudice LC, Tabibzadeh S. 2002. *The endometrium*. Taylor & Francis.

Jang HY, Myoung SM, Choe JM, Kim T, Cheon YP, Kim YM, Park H. 2017. Effects of autologous platelet-rich plasma on regeneration of damaged endometrium in female rats. *Yonsei Medical Journal* 58:1195–1203. DOI: 10.3349/ymj.2017.58.6.1195.

Kim H, Shin JE, Koo HS, Kwon H, Choi DH, Kim JH. 2019. Effect of autologous platelet-rich plasma treatment on refractory thin endometrium during the frozen embryo transfer cycle: A pilot study. *Frontiers in Endocrinology* 10. DOI: 10.3389/fendo.2019.00061.

Kwon A, Kim Y, Kim M, Kim J, Choi H, Jekarl DW, Lee S, Kim JM, Shin JC, Park IY. 2016. Tissue-specific differentiation potency of mesenchymal stromal cells from perinatal tissues. *Scientific Reports*. DOI: 10.1038/srep23544.

Labuschagne CF, Zani F, Vousden KH. 2018. Control of metabolism by p53 – Cancer and beyond. *Biochimica et Biophysica Acta - Reviews on Cancer* 1870:32–42. DOI: 10.1016/j.bbcan.2018.06.001.

Lee D-H, Sonn CH, Han S-B, Oh Y, Lee K-M, Lee S-H. 2012. Synovial fluid CD34− CD44+ CD90+ mesenchymal stem cell levels are associated with the severity of primary knee osteoarthritis. *Osteoarthritis and Cartilage* 20:106–109. DOI: 10.1016/j.joca.2011.11.010.

Lubkowska A, Dolegowska B, Banfi G. 2012. Growth factor content in PRP and their applicability in medicine. *Journal of biological regulators and homeostatic agents*.

Maffulli N. 2016. *Platelet rich plasma in musculoskeletal practice*. DOI: 10.1007/978-1-4471-7271-0.

Marx RE. 2019. In Situ Tissue Engineering. In: Melville J, Shum J, Young S, Wong M eds. *Regenerative Strategies for Maxillary and Mandibular Reconstruction*. Springer International Publishing, 73–86. DOI: 10.1007/978-3-319-93668-0_7.

Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. 1998. Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*. DOI: 10.1016/S1079-2104(98)90029-4.

Messora MR, Nagata MJH, Furlaneto FAC, Dornelles4 RCM, Bomfim SRM, Deliberador TM, Garcia VG, Bosco AF. 2011. A standardized research protocol for platelet- rich plasma (PRP) preparation in rats. *RSBO Revista Sul-Brasileira de Odontologia*.

Mutlu L, Hufnagel D, Taylor HS. 2015. The Endometrium as a Source of Mesenchymal Stem Cells for Regenerative Medicine1. *Biology of Reproduction* 92. DOI: 10.1095/biolreprod.114.126771.

Patel AN, Selzman CH, Kumpati GS, McKellar SH, Bull DA. 2016. Evaluation of autologous platelet rich plasma for cardiac surgery: Outcome analysis of 2000 patients. *Journal of Cardiothoracic Surgery* 11. DOI: 10.1186/s13019-016-0452-9.

Pertschuk LP. 1990. *Immunocytochemistry for steroid receptors*. CRC Press.

Ponnaiyan D, Jegadeesan V. 2014. Comparison of phenotype and differentiation marker gene expression profiles in human dental pulp and bone marrow mesenchymal stem cells. *European Journal of Dentistry* 8:307–313. DOI: 10.4103/1305-7456.137631.

Tanida I, Ueno T, Kominami E. 2008. LC3 and Autophagy. In: Deretic V ed. *Autophagosome and Phagosome*. Totowa, NJ: Humana Press, 77–88. DOI: 10.1007/978-1-59745-157-4_4.

Theoret CL, Stashak TS. 2014. Integumentary System: Wound Healing, Management, and Reconstruction. In: Orsini J, Divers T eds. *Equine Emergencies*. Elsevier Inc., 238–267. DOI: 10.1016/b978-1-4557-0892-5.00019-2.

Wang X, Simpson ER, Brown KA. 2015. p53: Protection against tumor growth beyond effects on cell cycle and apoptosis. *Cancer Research* 75:5001–5007. DOI: 10.1158/0008-5472.CAN-15-0563.

Yazigi Junior JA, dos Santos JBG, Xavier BR, Fernandes M, Valente SG, Leite VM. 2015. Quantification of platelets obtained by different centrifugation protocols in SHR rats. *Revista Brasileira de Ortopedia (English Edition)* 50:729–738. DOI: 10.1016/j.rboe.2015.10.008.

Yuksel EP, Sahin G, Aydin F, Senturk N, Turanli AY. 2014. Evaluation of effects of platelet-rich plasma on human facial skin. *Journal of Cosmetic and Laser Therapy* 16:206–208. DOI: 10.3109/14764172.2014.949274.

Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D. 2001. Estrogen modulates estrogen receptor α and β expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. *Journal of Cellular Biochemistry* 81:144–155. DOI: 10.1002/jcb.1096.

Figure 1

Characterization of the mesenchymal cell cultures isolated from rat uterus.

A: Flow cytometry: a representative forward and side scattering dot plot with the region of interest (R1). The percentages of CD90, CD105, CD45 and CD34 positive cells in R1 are indicated. Green curve corresponds to the control (stained with secondary antibodies only). B: Vimentin protein expression: upper image – negative control (secondary antibodies only); lower image – immunocytochemistry with anti-vimentin antibodies (red) and the nuclei counterstained with DAPI (blue). Bars, 20 µm. C: Induced cell differentiation assay: adipogenic differentiation revealed by Sudan III staining, osteogenic differentiation revealed by alizarin red staining, and chondrogenic differentiation revealed by alcian blue staining. Upper panel – the non-induced control cells, bottom panel – cells after the induced differentiation. Bars, 50 µm.

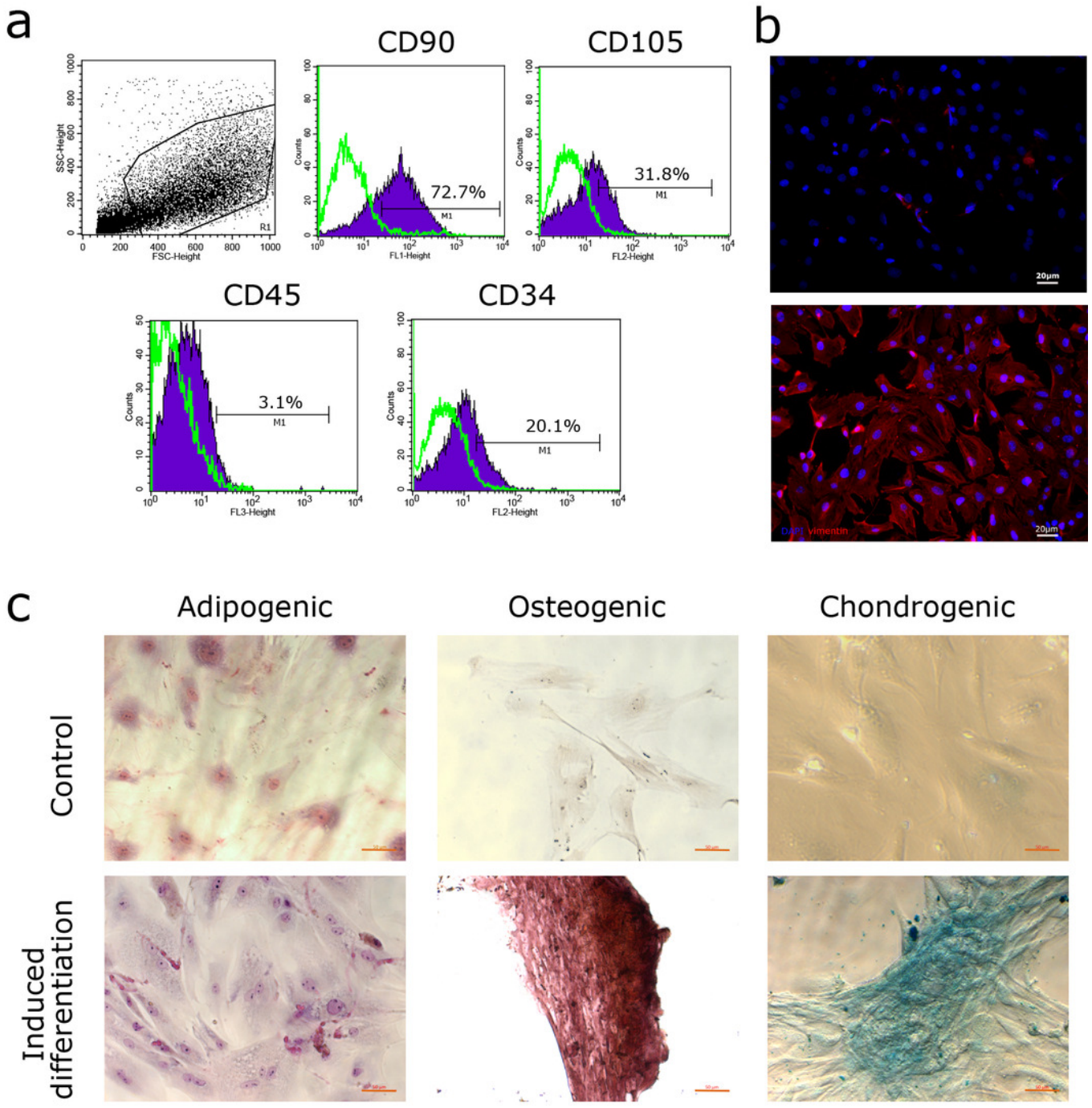

a
CD90
CD105
CD45
CD34
SSC-Height
FSC-Height
R1
Counts
FL1-Height
FL2-Height
FL3-Height
72.7%
31.8%
3.1%
20.1%
M1
b
20μm
DAPI Vimentin
c
Adipogenic
Osteogenic
Chondrogenic
Control
Induced differentiation

Figure 2

The assessment of cell viability and cell death after the 24 h incubation of rat uterine MSCs with complete growth medium (CGM), platelet-rich plasma (PRP), or ordinary plasma (OP).

A - immunocytochemical staining for Ki-67 (green) after 24 h exposure of the MSC cultures to the studied agents; the nuclei were counterstained with DAPI (blue). Bars, 20 μm. B – proliferation index calculated as the number of Ki-67 positive nuclei divided by the total number of nuclei. * - $p < 0.05$ *vs* CGM and OP. C – a representative western blot membrane with the proteins isolated from MSCs after 24 h incubation with the studied agents, stained with p53, Bcl-2, LC3B and GAPDH specific antibodies. Relative protein levels of p53, Bcl-2 and LC3B, normalized by GAPDH level. * - $p < 0.05$ *vs* CGM group.

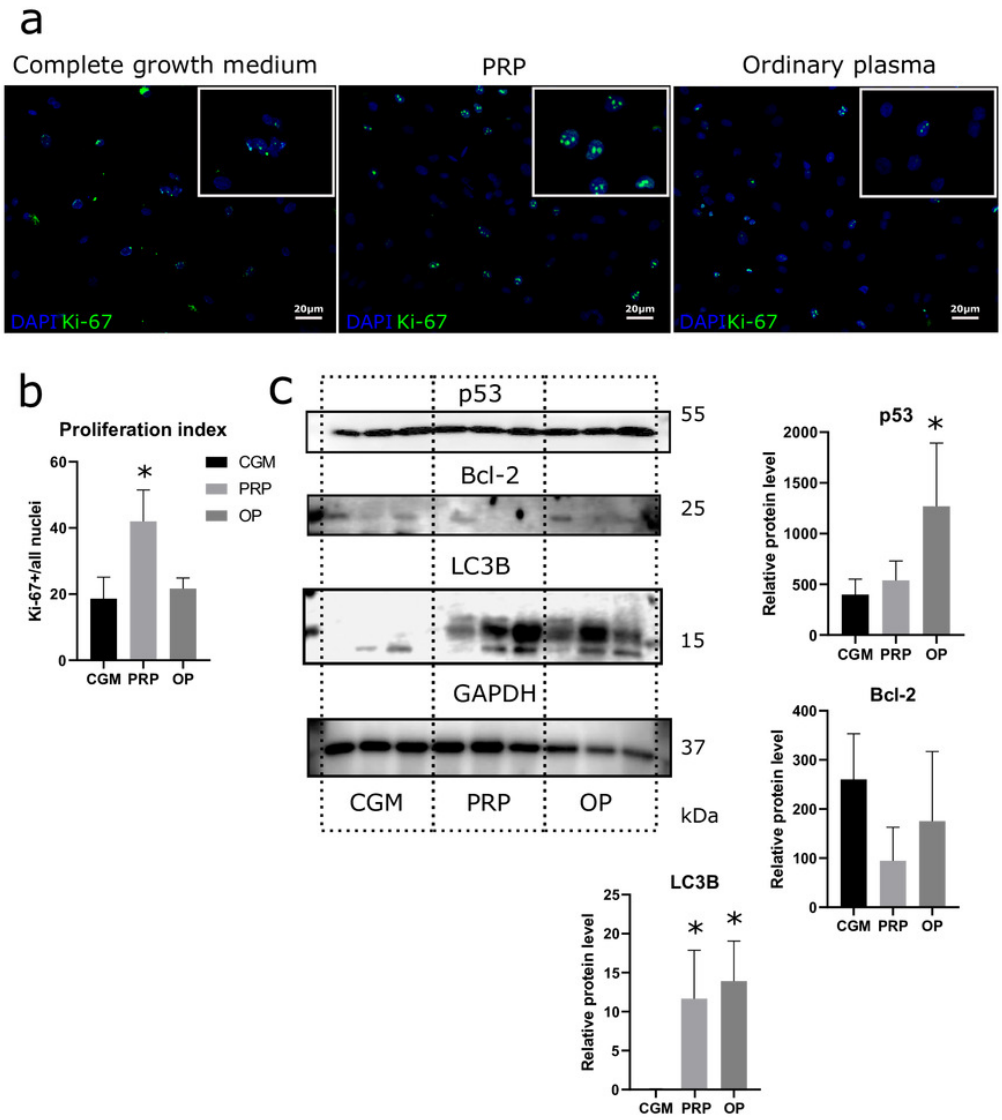
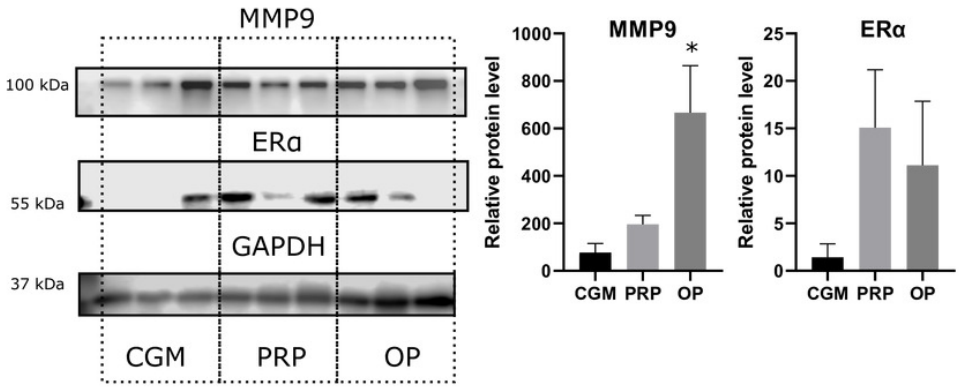

a
Complete growth medium
PRP
Ordinary plasma
DAPI Ki-67
20μm
b
Proliferation index
Ki-67+/all nuclei
CGM
PRP
OP
c
p53
Bcl-2
LC3B
GAPDH
55
25
15
37
kDa
Relative protein level
LC3B

Figure 3

A representative western blot membrane with the proteins isolated from MSCs.

A representative western blot membrane with the proteins isolated from MSCs after 24 h incubation with complete growth medium (CGM), platelet-rich plasma (PRP) and ordinary plasma (OP), stained with MMP9, ERα and GAPDH specific antibodies. Relative protein levels of MMP9 and ERα, normalized by GAPDH level. * - $p < 0.05$ *vs* CGM group.


MMP9
100 kDa
ERα
55 kDa
GAPDH
37 kDa
CGM
PRP
OP
MMP9
Relative protein level
1000
800
600
400
200
0
*
CGM PRP OP
ERα
Relative protein level
25
20
15
10
5
0
CGM PRP OP