

Dzhyvak V. G., Dovhalyuk A. I., Paliy I. R., Klishch, I. M. Research of stimulating efficiency of blood allogeneic plasma fractions on cell proliferation in vitro conditions. *Journal of Education, Health and Sport*. 2020;10(8):559-569. eISSN 2391-8306. DOI <http://dx.doi.org/10.12775/JEHS.2020.10.08.068> <https://apcz.umk.pl/czasopisma/index.php/JEHS/article/view/JEHS.2020.10.08.068> <https://zenodo.org/record/4302478>

The journal has had 5 points in Ministry of Science and Higher Education parametric evaluation. § 8.2) and § 12.1.2) 22.02.2019.

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The authors declare that there is no conflict of interests regarding the publication of this paper.

Received: 04.08.2020. Revised: 14.08.2020. Accepted: 31.08.2020.

## RESEARCH OF STIMULATING EFFICIENCY OF BLOOD ALLOGENEIC PLASMA FRACTIONS ON CELL PROLIFERATION IN VITRO CONDITIONS

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### Abstract

We conducted a comparative experiment on the effect of platelet rich plasma and platelet poor plasma of allogeneic blood of rats on the proliferative activity of different rat cell lines in experimental conditions in vitro. Rat cells such as umbilical cord mesenchymal stem cells, dermal fibroblasts, and fetal myogenic cells were used in the experiment. To compare the proliferative activity of cell lines, the density of the cell population - confluent - was estimated as the percentage of filling the bottom area of the culture vessel with attached and

spread cells. As a result of the research it was found that plasma enriched with platelets in the form of 5% supplement to the growth medium has a pronounced stimulating effect on the proliferative activity of fibroblasts, but less effective in the case of cell culture of mesenchymal stem cells. Platelet poor plasma has a physiological effect on the cell cultures of mesenchymal stem cells and rat fibroblasts similar to fetal calf serum. Myogenic rat cells have a high proliferative potential and do not require additional stimulation of platelet rich plasma and platelet poor plasma at the concentrations studied by us.

**Key words: platelet rich plasma; platelet poor plasma; mesenchymal stem cells; in vitro**

### **Introduction**

In recent years, to stimulate the repair of various injuries or trauma, doctors are increasingly turning to promising means of regenerative medicine, based on the use of autologous or allogeneic cells and tissues. Currently, there are scattered data on the effectiveness of platelet-rich plasma (PRP) in wound healing processes in the treatment of pathologies of joints, muscles, tendons [1, 2]. Interest in PRP -therapy due to the fact that this group of patient's blood contains white blood cells so a number of biologically active substances - growth factors, cytokines , enzymes that can effectively stimulate regenerations uw damaged body tissues [3 - 6]. Platelets play an exceptional role in hemostasis, inflammation, cell proliferation and differentiation. Platelet granules contain at least 60 biologically active substances that stimulate cell chemotaxis, cell proliferation and differentiation, angiogenesis, immunomodulation and remodulation [7 - 10]. Among these active substances for reparative tissue regeneration the most important are growth factors - cellular mitogens: fibroblast growth factors, transforming growth factors, bone morphogenetic proteins, insulin-like growth factors, vasculo-endothelial, epidermal growth factors, etc. [1, 13]. The use of PRP involves a multiple increase in the concentration of growth factors at the point of application to ensure a rapid and complete regeneration process by stimulating cell proliferation.

The platelet-depleted plasma fraction (PPP) is often overlooked by researchers. However, platelet-free blood plasma is also known to be a source of hormones, hormone-like growth factors, and transport proteins for transport. In addition, there are molecules to create an extracellular biomatrix that promotes cell adhesion to the bottom of culture vessels in vitro cultures [4]. Therefore, for the growth of cells in vitro, a necessary addition to the growth medium is rich in biologically active substances, the blood serum of cattle embryos. Today,

mesenchymal stem cells (MSCs) are considered by scientists as a promising material for cell therapy and regenerative medicine. Human stem cells (SC) are isolated from bone marrow, umbilical cord blood, adipose tissue, endometrium, tooth pulp, amnion, umbilical cord and the like. [14, 15].

**The aim of this study** was to compare the effect of PRP and PPP of allogeneic blood of rats on the proliferative activity of different rat cell lines in vitro.

### **Materials and methods**

The experimental study was conducted on the basis of the Central Research Laboratory (certificate of certification № 001/18 dated September 26, 2018) and the interdepartmental training and research laboratory (certificate of certification /132 / 17 issued on December 29, 2017) I. Horbachevsky Ternopil National Medical University, Ministry of Health of Ukraine.

Obtaining primary cultures of fibroblasts, myogenic cells and MSC of rat umbilical cord.

Rat cells such as umbilical cord mesenchymal stem cells (MSCs), dermal fibroblasts, and fetal myogenic cells were used in the experiment. MSCs were obtained from the umbilical cord and amnion of rats in late gestation. Primary cultures were obtained from umbilical cord, skeletal muscle and skin, washed from the blood with sterile HBSS buffer solution (Gibco) with the addition of 1% penicillin-streptomycin (Sigma). Next, an enzymatic method was used to dissociate the cell mass and obtain viable MSCs. To do this, tissue samples were crushed with a scalpel into fragments of 0.5-2 mm<sup>3</sup>, transferred to centrifuge tubes with 2 ml of growth medium DMEM / F12 Advanced (Gibco) and 0.2 ml of collagenase I (Gibco) at a concentration of 0.075 mg / ml and mixed, preventing flotation. Tubes with primary material and collagenase were incubated in a 37 ° C thermocouple for 70 min (umbilical cord), 75 min (subcutaneous muscle), and 85 min (pieces of skin), stirring thoroughly every 15 min. After fermentation, 4 ml of growth medium was added to the tubes, pipetted and centrifuged for 5 min at 300 g. The procedure was repeated twice. The precipitate obtained was resuspended in 7 ml of DMEM / F12 Advanced with the addition of 10% fetal calf serum (ECT) (Gibco) and placed in culture vials. Cultivation was carried out in a CO<sub>2</sub> incubator at a temperature of 37°C and a CO<sub>2</sub> concentration of 5%.

Obtaining PRP, PPP was performed under sterile conditions in two steps: in the first stage, the blood was centrifuged for 10 min at 1600 rpm, the plasma fraction was collected,

and centrifuged again for 10 min at 2100 rpm. The transparent fraction of platelet-free plasma PPP was separated from PRP.

Growing the cultures. Experiment bookmark.

The studied cell cultures: MSC from the umbilical cord, skeletal muscle and fibroblasts before the bookmark of the experiment (passages I-III) were grown on DMEM / F12 medium with the addition of 5% ECT. The experiment was performed on the 4th passage in triplicate. For each replication, 12 culture vials (25 cm<sup>2</sup>) were placed (4 vials for each cell line). 7 ml of 250,000 cell suspension was added to each vial. The number of cells was counted using a hemocytometer using a vital dye trypan blue. Evaluation of the intensity of cell proliferation was performed on 1,3,7 days of cultivation, analyzing the density of the cell population - confluent. Analyzed the growth of cell cultures after 1, 3 and 7 days of the experiment using an inverted microscope "Delta Optical" (Poland). To compare the proliferative activity of cell lines, the density of the cell population - confluent - was evaluated as the percentage of filling the bottom area of the culture vessel with attached and spread cells. The design of the experiment included negative control - cell growth on DMEM / F12 growth medium without additives, positive control - DMEM / F12 with the addition of 5% fetal calf serum (ECT) and two experimental groups: I - DMEM / F12 with the addition of 5% PPP and II - DMEM / F12 with the addition of 5% PRP.

In working with animals, the rules of handling experimental animals were followed in accordance with the EU Council Directive 2010/63 / EU on compliance with regulations, laws, administrative regulations of the EU on animal protection, which are used for scientific purposes [16, 17].

Statistical processing of research results was performed using an Excel computer program using Student's t-test.

### **Results and discussion**

Microscopic analysis of the cell culture at different times of the experiment revealed that the most sensitive to serum or plasma applications were umbilical cord MSCs, because in negative control the growth of these cells was very slow, and within a week of the experiment such cells died completely. Moderate sensitivity to the stimulating effect of biologically active components of blood serum was shown by skin fibroblasts. Myogenic cells were the least dependent on nutrient supplements - they retained their high proliferative activity even in the absence of ECT or blood plasma. The results of microscopic analysis of the confluence of cell populations in the controls and variants of the experiment are summarized in Table 1.

Table 1

Influence of serum and different fractions of rat blood plasma  
on the density of cell populations at different times of the experiment

	Umbilical cord MSC (confluent,%)			MSC of muscle tissue (confluent,%)			Skin fibroblasts (confluent,%)		
	1 day	3 day	7 day	1 day	3 day	7 day	1 day	3 day	7 day
Control – DMEM F12	5±1	2±1	died	50±5	75±7	90±6	40±3	45±5	12±3
Control + 5% ECT	40±3	85±7	100	50±5	85±5	100	40±4	60±5	45±3
5% PPP	35±2	75±7	95±5	45±5	85±7	95±5	50±5	70±5	75±5
5% PRP	30±3	65±5	75±7	45±5	80±5	90±7	45±6	80±7	100

On the seventh day of the experiment, umbilical cord MSCs and myogenic cells reached the maximum cell population density in the positive control and under conditions of PPP addition, whereas in the PRP variant the confluent was statistically significantly weaker in umbilical cord stem cell vials (see Table 1, Fig. 1).

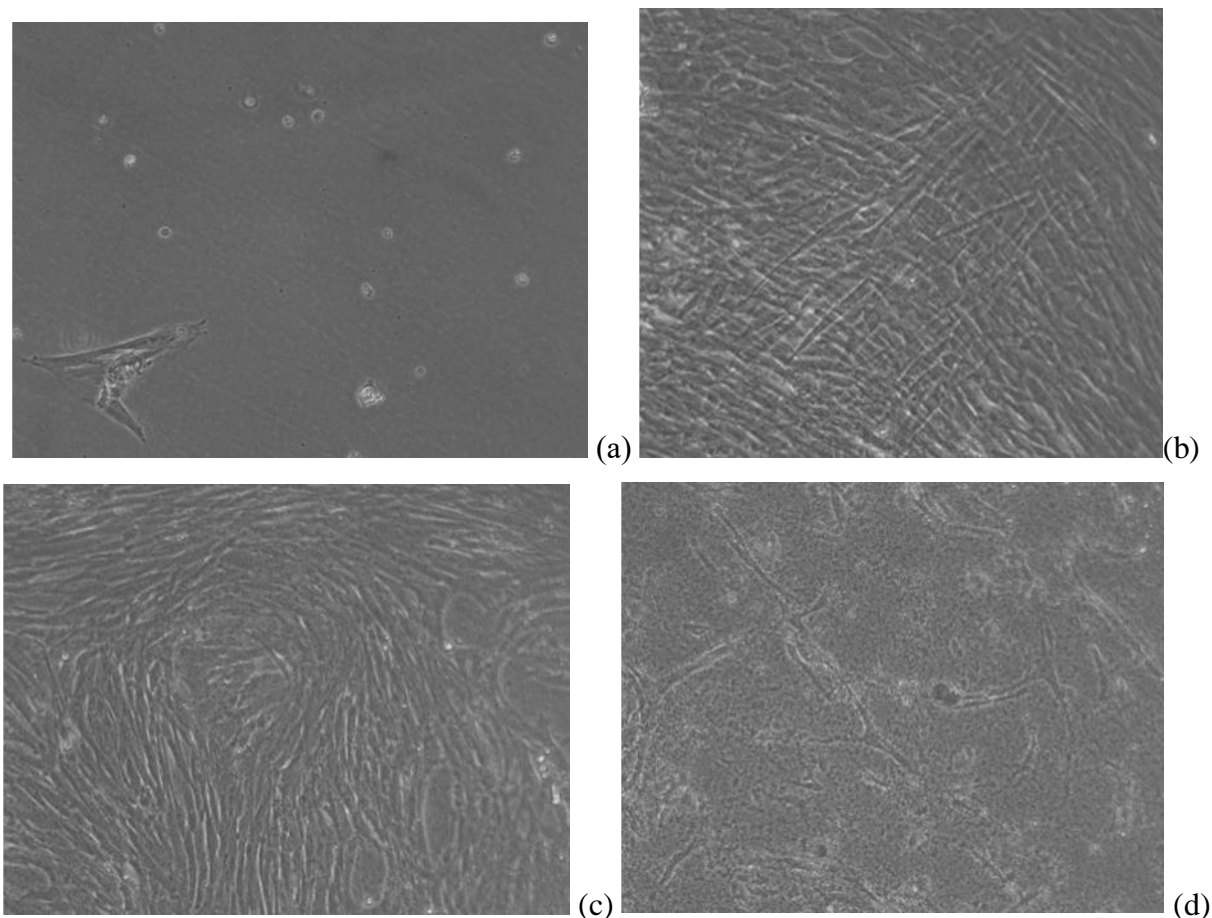


Fig.1. MSC from the umbilical cord (analysis for 7 days): (a) - negative control (without growth factors); (b) - positive control (5% ECT); (c) - PPP; (d) – RRP

The addition of PRP to MSCs of skeletal muscle had a similar stimulating effect to ECT and PPP (Table 1, Fig. 2), but initiated the formation of spheroids in culture (Fig. 2d). Obviously, the biologically active substances of PRP contributed to the detachment of cells from the substrate and the formation of 3-D aggregates from myogenic progenitors. A similar ability to form floating multicellular 3-D spheroids was observed in SiHa and HeLa cell cultures under the influence of human serum, while ECT promoted the formation of spreading monolayers of these cells on culture plastic [12]. It is known that the blood serum of cattle embryos has the necessary substances that promote cell adhesion to the bottom of the culture vessel [12].

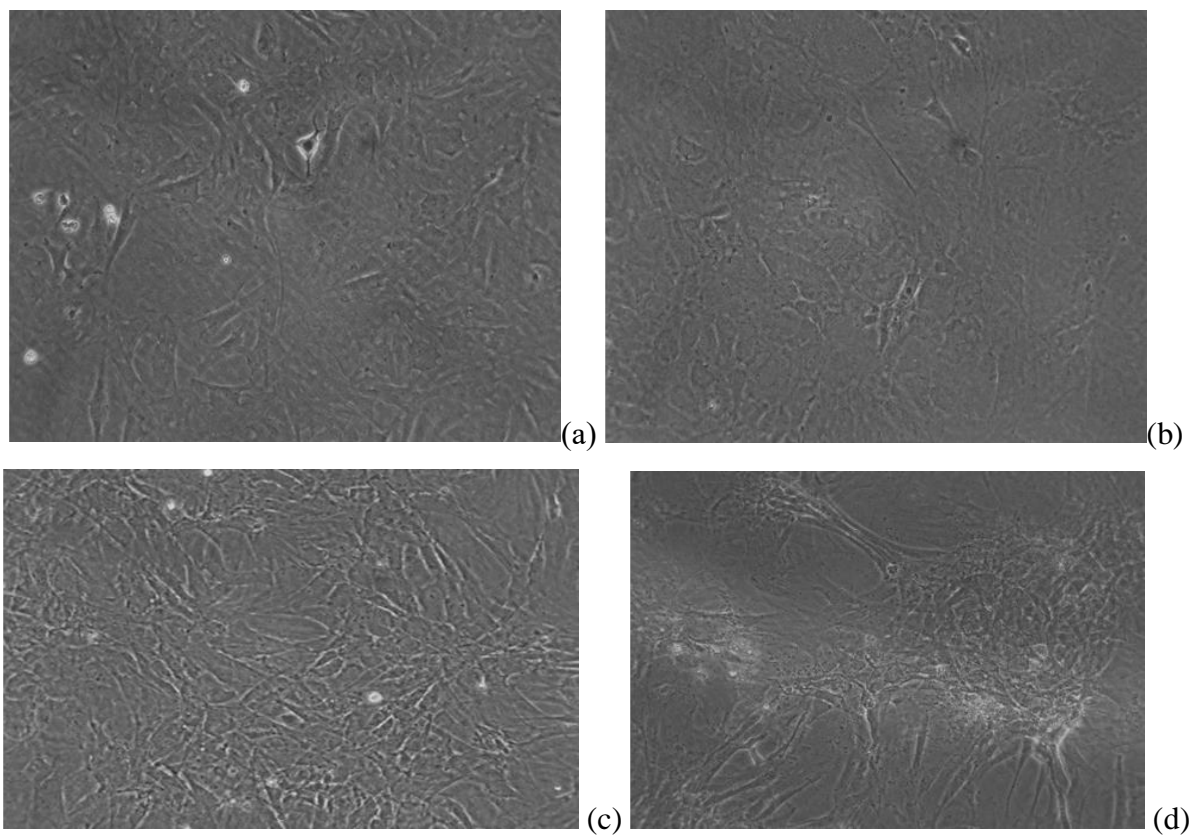


Fig.2. Fetal myogenic cells (analysis on day 7): (a) - negative control (without growth factors); (b) - positive control (5% ECT); (c) - PPP; (d) – PRP

It was found that skin fibroblasts were the most sensitive to the stimulating effect of PRP. This cell line had a weaker proliferative potential than myogenic stem cells and therefore required stimulation with nutrient supplements ECT or blood plasma fractions. On the 7th day of the experiment in the negative control the confluent was about 10%, in the positive control only 45%, while in the PRP variant the density of the cell population reached

100% of the monolayer (Table 1, Fig.3). Therefore, we can assume that in regenerative medicine, the use of PRP is most justified for the healing of wound skin defects.

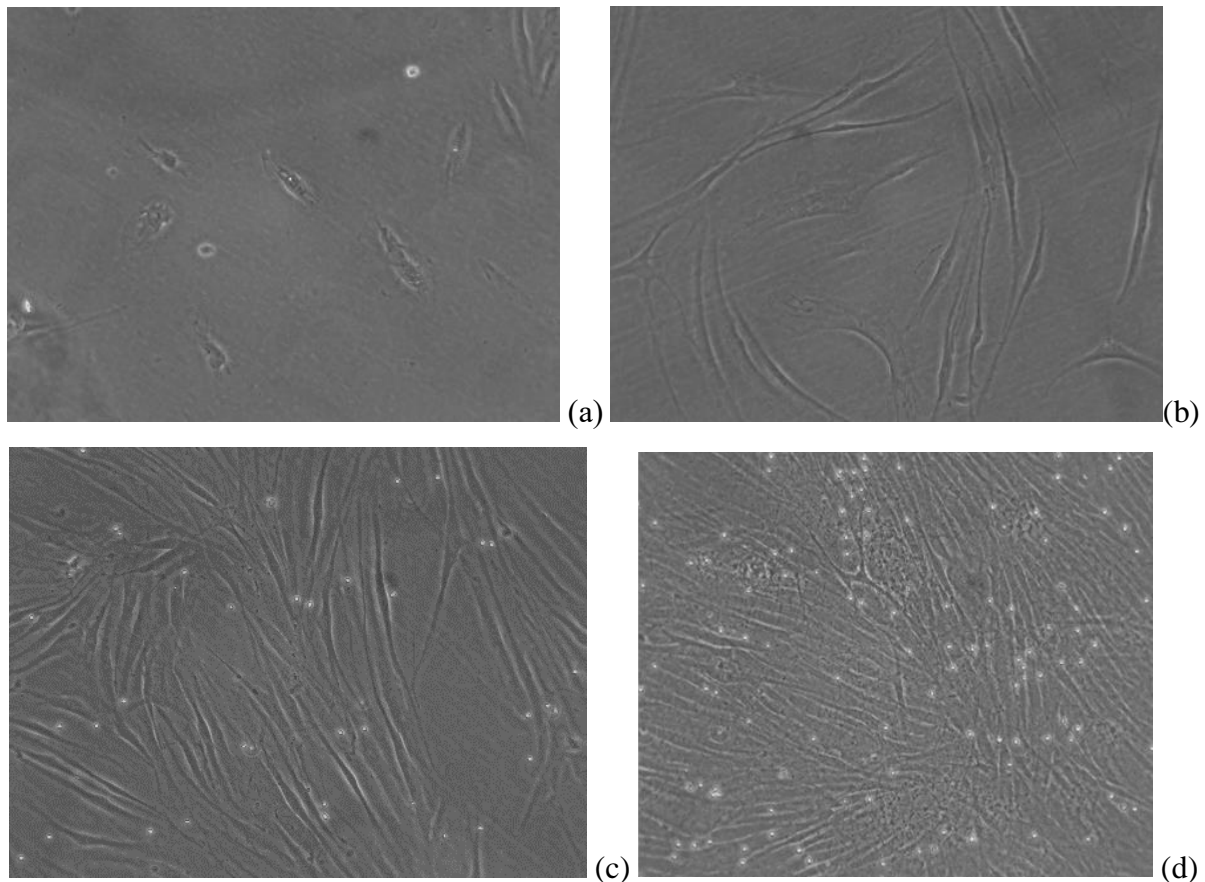


Fig.3. Fibroblasts of fetal skin (analysis for 7 days): (a) - negative control (without growth factors); (b) - positive control (5% ECT); (c) - PPP; (d) – PRP

According to the literature, other studies of the effect of PRP on the growth of multipotent mesenchymal stem cells in vitro are known. Mishra A. and co-authors [18, 21] applied an experimental model in which they studied the effect of 10% PRP on mesenchymal cell proliferation. There was a significant increase in cell proliferation in the study group after 7 days of exposure, an increase in the level of tRNA of the osteogenic marker RUNX2, as well as chondrogenic markers SOX-9 and proteoglycans.

Murphy M.B. and co-authors [19] used PRP, umbilical cord PRP and PPP in cell culture and compared with bovine serum. The experiment showed that while all forms of PRP and PPP have a better proliferating effect on mesenchymal cells than bovine serum, umbilical PRP causes significantly higher and faster proliferation of mesenchymal cells within 7 days of exposure than PRP and PPP.

Kruger J.P. and co-authors [20] investigated the effect of 5% PRP on the culture of progenitor cells derived from subchondral cortico-spongy bone. A significant increase in cell migration was shown, as well as the synthesis of cell matrix, proteoglycans and type II collagen. Subsequently, the authors [6] proved the pronounced stimulating effect of 5% PRP on the proliferation of progenitor cells, the synthesis of proteoglycans and collagen type II polyglycol - hyaluronic matrix.

Mifune Y. and co-authors [21] in their in vitro study showed that the addition of PRP stimulates the proliferation, adhesion and migration of mesenchymal cells derived from muscles. PRP also increases the amount of type II collagen and inhibits cell apoptosis.

Xie X. and co-authors [22] demonstrated that rabbit bone marrow mesenchymal cells have significantly better proliferative activity and higher expression of specific cartilage genes and proteins in the presence of a PRP matrix than adipose mesenchymal cells.

Feng X. and co-authors [23] investigated the effect of platelet lysate on mesenchymal differentiation of umbilical cord blood cells. The authors indicate that a 10% platelet lysate may improve the differentiation of umbilical mesenchymal cells into chondrocytes.

Zaky S.H. and co-authors [24] cultured human mesenchymal cells in three different media: fetal bovine serum + FGF2, fetal bovine serum + 5% PRP, and PRP alone. The authors found the best cartilage population formation in a medium containing only PRP.

The use of PRP or PPP involves a multiple increase in the concentration of growth factors at the point of application to ensure rapid proliferation and a full regeneration process, especially when applied to body tissues with an initial low potential for regeneration or insufficient blood supply. In addition to the pronounced mitogenic effect, platelet plasma has a significant anti-inflammatory effect. High efficiency of biological preparations on the basis of platelet-enriched plasma in treatment of sports injuries, at operations, in particular and on joints, and also in stomatology and cosmetology is already proved. Positive experience in the use of PRP in rheumatology in chronic tendinitis of the knee, elbow and ankle joints has been reported. In recent years, PRP is increasingly used in the performance of vertebrodesis, in the treatment of pseudoarthrosis, arthritis, synovitis, lesions of the menisci and articular cartilage. [6.25].

## **Conclusions**

Our studies showed that platelet-enriched plasma in the form of a 5% supplement to the growth medium has a pronounced stimulating effect on the proliferative activity of fibroblasts, but is less effective in the case of cell culture of mesenchymal stem cells. Platelet



poor plasma has a physiological effect on the cell cultures of MSCs and rat fibroblasts similar to fetal calf serum.

The research was performed as part of the initiative research work "Study of the regenerative potential of mesenchymal stem cells in tissue damage in the experiment and prospects for their therapeutic use", the number of state registration of research work: 0120U104146.

### Reference

1. Dzhyvak, V., Klishch, I. Efficacy of platelet-rich blood plasma in induction of muscle tissue healing in an experimental study. Hospital surgery. 2020. №3 (91). P. 36-43. DOI: <https://doi.org/10.11603/2414-4533.2020.3.11461>
2. Bissell L., Tibrewal S., Sahni V., Khan W. S. Growth factors and platelet rich plasma in anterior cruciate ligament reconstruction // Curr. Stem Cell Res. Ther. — 2014. — 10, №1. — P. 19-25.
3. The efficacy of platelet-rich plasma in grafted maxil lae. A randomised clinical trial / M. Badr, P. Coulthard, R. Alissa, R. Oliver // European Journal Oral Implantology. — 2010. — Vol. 3. — №3. — P. 233–244.
4. Achkasov EE, Bezuglov E. N., Ulyanov AA, Kurshev VV Application of platelet-enriched autoplasm in clinical practice // Biomedicine. - 2013. - № 4. - P. 46-59.
5. Influence of different concentrations of platelets in blood plasma on chondrogenesis of cells of pulpal nucleus of rats in vitro / EG Pedachenko, MV Khyzhnyak, IG Vasilieva [etc.] // Journal of the National Academy of Medical Sciences Of Ukraine: scientific journal of the Presidium of the National Academy of Medical Sciences of Ukraine. - 2018. - Volume 24, N 3/4. - P. 232-238. -ISSN 2413-7944
6. Ivchenko DV The use of autologous plasma enriched in platelets to optimize the reparative regeneration of bone tissue in osteopenia / DV Ivchenko, VV Miroshnikov // Pathology. - 2018. - Vol. 15, № 2 (43). - P. 248-258.
7. Platelet-rich plasma injection for chronic Achilles tendinopathy: a randomized controlled trial / R.J. de Vos, A. Weir, H.T. van Schie, et al. // JAMA. — 2010. — Vol. 303. — №2. — P. 144–149.
8. Senzel L., Gnatenko D. V., Bahou W. F. The platelet proteome // Curr Opin Hematol. — 2009. — 5. — P. 329- 333.
9. The influence of different concentrations of platelets in blood plasma on the chondrogenesis of cells of the pulpal nucleus of rats in vitro [Text] / EG Pedachenko, MV

Khyzhnyak, IG Vasilieva [etc.] // Journal of the National Academy of Medical Sciences Of Ukraine: scientific journal of the Presidium of the National Academy of Medical Sciences of Ukraine. - 2018. - Volume 24, N 3/4. - P. 232-238. -ISSN 2413-7944

10. Dzhyvak V., Klishch I., Datsko T., Khlibovska O. Influence of PRP on morphological changes in muscle in the early period after traumatic muscle injury in the experiment. Journal of Education, Health and Sport. 2020;10(6):171-178. eISSN 23918306.

11. Cole B.J. Platelet-rich plasma: where are we now and where are we going? / Cole B.J., Seroyer S.T., Filardo G. // Sports Health. –2010. – Vol. 2. – No 3. – P. 203–210.

12. Heger JI, Froehlich K, Pastuschek J, Schmidt A, Baer C, Mrowka R, Backsch C, Schleußner E, Markert UR, Schmidt A. Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum. Exp Cell Res. 2018 Apr 1;365(1):57-65. doi: 10.1016/j.yexcr.2018.02.017. Epub 2018 Feb 21. PMID: 29476836.

13. Fishchenko VO The effectiveness of platelet-enriched plasma in limited cartilage damage: a review of basic and clinical studies / VO Fishchenko, OV Fishchenko, MV Rybinsky, S. Yu. Yaremin, VV Hutsol, MM Andrushenko // Bulletin of orthopedics, traumatology and prosthetics. - 2016. - № 3. - P. 63-69.

14. Masurkewitsch A. J., Kowpak W. W., Danilow W. B. (2014) Klitinni tehnologii u weterinarnij medizini. Nawtschal'nij pocibnik [Cellular technology in veterinary medicine. Tutorial]. K.: KOMPRINT.

15. Kovpak VV Proliferative activity of rat mesenchymal stem cells under the influence of culture medium / VV Kovpak, OS Kovpak. // Scientific reports of the National University of Life and Environmental Sciences of Ukraine. - 2016. - № 3.

16. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union, 2010, L276, 33-79.

17. Guide for the care and use of laboratory animals. 8th ed. Washington: The National Academies Press. (2011).

18. Mishra A. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation / Mishra A., Tummala P., King A. // Tissue Eng. Part C Methods.– 2009. – Vol. 15. – No 3. – P. 431–435.

19. Murphy M.B. Adult and umbilical cord blood-derived platelet-rich plasma for mesenchymal stem cell proliferation, chemotaxis, and cryo-preservation / Murphy M.B., Blashki D., Buchanan R.M. // Biomaterials. – 2012. – Vol. 33. – No 21. – P. 5308–5316.

20. Kruger J.P. Human platelet-rich plasma induces chondrogenic differentiation of subchondral progenitor cells in polyglycolic acid-hyaluronan scaffolds / Kruger J.P., Ketzmar A.K., Endres M. // *J. Biomed. Mater. Res. B Appl. Biomater.* – 2014. – Vol. 102. – No 4. – P. 681–692.
21. Mifune Y. The effect of platelet-rich plasma on the regenerative therapy of muscle derived stem cells for articular cartilage repair / Mifune Y., Matsumoto T., Takayama K. // *Osteoarthritis Cartilage.* – 2013. – Vol. 21. – No 1. – P. 175–185.
22. Xie X. Comparative evaluation of MSCs from bone marrow and adipose tissue seeded in PRP-derived scaffold for cartilage regeneration / Xie X., Wang Y., Zhao C. // *Biomaterials.* – 2012. – Vol. 33. – No 29. – P. 7008–7018.
23. Feng X. Effect of platelet lysate on chondrogenic differentiation of human umbilical cord derived mesenchymal stem cells in vitro / Feng X., Tian S., Sun K. // *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi.* – 2011. – Vol. 25. – No 10. – P. 1250–1255.
24. Zaky S.H. Platelet lysate favours in vitro expansion of human bone marrow stromal cells for bone and cartilage engineering / Zaky S.H., Ottonello A., Strada P. // *J. Tissue Eng. Regen. Med.* – 2008. – Vol. 2. – No 8. – P. 472–481.
25. Platelet-rich plasma promotes healing of osteoporotic fractures / L. Chen, X. Yang, G. Huang, et al. // *Orthopedics.* – 2013. – Vol. 36. – №6. – P. 687–694