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The effect of a fibroblast growth factor, insulin-like growth factor, growth hormone, and Biolaminin 521 LN on the proliferative activity of cat stem cells

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Abstract

The wide use of cell technologies in clinical practice requires a large amount of cell material, which has led to improvement in culture conditions, making it possible to obtain more cell material in a shorter period of time. Thus, the purpose of our paper was to study the effects of different concentrations of an insulin-like growth factor (IGF-1), a fibroblast growth factor (FGF-2),| a growth hormone (rhGH), and Biolaminin 521 LN (LN 521) on the proliferative activity and genetic stability of stem cell cultures derived from the cat bone marrow, adipose tissue, and myocardium. Cell cultures for the experiment were obtained from the adipose tissue, bone marrow, and myocardium of a cat. Differences were found in the effects of the various growth promoters on the proliferative activity of cells in the culture. The IGF-1 demonstrated a positive effect on the proliferative activity of all cultures. The addition of the rhGH to the bone marrow-derived cell culture increased the size of the cells and decreased the proliferation index relative to the control group. The addition of the growth factors to the culture medium did not significantly increase the number of cells with altered karyotype in any of the cultures relative to the control group.

Cell culture, cytogenetic analysis, adipose tissue, bone marrow, myocardium, feline

Cell therapy in veterinary practice is a new experimental method of treating animals suffering from diseases of the musculoskeletal system, pathologies of the immune system, diabetes and other diseases that are accompanied by cell damage (Chomik et al. 2016; Mazurkevych et al. 2017, Zubko and Frishman 2009; Irfan and Ahmed 2015; Moga et al. 2016). The vast majority of research conducted in this direction concerns practical aspects of the use of cells. At the same time, the objects of research are mainly those types of animals for which the use of cell therapy will be most economically justified, in particular horses of sports breeds. However, many studies are also being conducted on animals of other species: rats, mice, rabbits, cats, dogs, pigs, and goats. Most of them are designed to work out approaches to the use of cell therapy in the treatment of humans, which brings animal research to a higher level.

When choosing the direction of experimental research in the application of cell therapy, first of all, it is necessary to take into account the prevalence of a particular pathology among a certain type of animal and the possibility of using the most effective approaches in their treatment. It is well-known that the use of cell therapy in experimental models of damaged myocardium has shown a positive effect on tissue repair and restoration of heart activity (Smits and van Geuns 2003; Hill et al. 2005), so at the experimental level,

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cell technologies are already actively used in cardiology for myocardial infarction and cardiomyopathy in humans. Since heart disease is common in cats, affecting about 15% of animals (Payne et al. 2015, Wagner et al. 2010), the use of stem cells as therapy is quite relevant. However, the problems associated with quickly obtaining the necessary amount of cat cell material for transplantation have not yet been fully resolved. For this purpose, it is necessary to study not only different sources of stem cells, but also the improvement of methods of their cultivation.

Bone marrow, as a well-researched source of stem cells, is the most commonly used. Different types of cells capable of division can be isolated in *in vitro* conditions (Mazurkevych et al. 2018): haematopoietic stem cells (HSC) (Pontikoglou et al. 2011), mesenchymal stem cells (MSC) (Mazurkevych et al. 2016), endothelial progenitor cells (Nolan et al. 2007), and pluripotent (Wiedemann et al. 2012) and multipotent (Beltrami et al. 2003) stem cells.

Researchers are increasingly focusing on adipose tissue as a source of stem cells, as it is possible to isolate HSC (Han et al. 2010), MSC (Mahmoudifar and Doran 2015), endothelial progenitor cells (Xue et al. 2010), and pre-adipocytes (Mazurkevych et al. 2018). Regional SC from various organs, necessary for the repair of damage, may also be considered a source of stem cells. A group of researchers headed by Beltrami et al. (2003) have broken new ground in myocardium recovery by confirming the presence of stem cells in the heart of adult animals (Bearzi et al. 2007).

It is known from literature data that fibroblast growth factor (FGF-2) is able to stimulate mitosis of human bone marrow cells (Ratajczak et al. 1996) and support proteins associated with pluripotency in cat embryonic cells (Zhou et al. 2019). In turn, insulin-like growth factor (IGF-1) is a weak mitogen for most human cells cultured (Niedźwiedzka 2000; Jakubczak et al. 2014). The growth hormone (rhGH) promotes cell proliferation, in particular, there is evidence of its positive effect on human mesenchymal stem cells (Li and Wang 2008; Waters and Brooks 2012). Biolaminin 521 LN (LN521) is a natural laminin for human pluripotent stem cells that has a positive effect on their proliferation (Rodin et al. 2014).

Due to the differences in the cell composition of cultures obtained from different sources and the lack of data in the available literature on the effect of various growth stimulants on the proliferative activity of cat cells, our goal was to study the effect of IGF-1, FGF-2, rhGH at various concentrations, and LN 521 on the proliferative activity and genetic stability of stem cell cultures obtained from the bone marrow, adipose tissue and myocardium of a cat.

Materials and Methods

Animal experiments were subject to the 'General Ethical Principles of Animal Experiments' approved by the National Congress on Bioethics (Ukraine) and they were in compliance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes (European Convention 1986). All manipulations with animals were carried out with the prior consent of their owners. Adipose tissue-derived stem cell cultures (ATSCC) were obtained from the subcutaneous adipose tissue of adult cats during planned hysterectomy using a standard method with our own modification (Clark et al. 2017). Bone marrow-derived stem cell cultures (BMSCC) were obtained mainly from the marrow of the femoral bones of adult cats using a standard method (Maciel et al. 2014). Myocardium-derived stem cell cultures (MSCC) were obtained from the heart of dead kitten foetuses that remained following veterinary-assisted delivery, modified by the explant method (Freshney 2005).

The cell mass was cultured until a confluence of 90–95% was reached, in a standard medium of 80% DMEM (Dulbecco's Modified Eagle Medium); 20% FBS (Foetal Bovine Serum); and 10 μl/cm^3 antibiotic-antimycotic (Sigma, USA) in a CO_2 incubator at 37 °C and 5% CO_2. Cells were removed by a standard method with a solution of 0.25% trypsin/EDTA (ethylenediamine tetraacetic acid) (Sigma, USA) (Freshney 2005).

Cells in the third passage were used in the experiment. The cells were passaged at a ratio of 1:5 (400 thousand cells/cup). The cells were cultured in a standard medium consisting of 80% DMEM, 20% FBS, and 10 μl/cm^3 antibiotic-antimycotic (Sigma, USA), with the addition of 1) IGF-1 (Sigma, USA) at concentrations of 10, 20 and 50 ng/ml (n = 3); 2) FGF-2 (Sigma, USA) at concentrations of 10, 20 and 50 ng/ml (n = 3); 3) rhGH

(Sigma, USA) at concentrations of 10, 20 and 50 ng/ml; 4) LN 521 (BioLamina, Switzerland) according to the manufacturer's instructions. Cells were counted after a confluence of 95–100% was obtained (BMSCC – 2 days, ATSCC – 3 days, MSCC – 2 days). Additionally, the proliferation index was determined: PI = PP/PK, where: PI – proliferation index; PP – number of cells after passage; PK – number of initially seeded cells. Cell cultures were microscopically analysed using an Axiovert 40 inverted microscope (Carl Zeiss, Germany).

Additionally, cytogenetic analysis of cell cultures obtained from the cat bone marrow, adipose tissue, and myocardium was performed. The studies were performed on 50 metaphase plates in each of the 3 studied samples of each group. To obtain the chromosome preparation, a modification of the standard cytogenetic method was used (Freshney 2005). The slides were stained using the Leykodif 200 kit, according to the manufacturer's instructions. The metaphase plates were analysed using a Leica DMR microscope (Germany) at 400× and 1000× magnification.

The results were statistically analysed using Student's *t*-test for significance of differences between means (ANOVA). Differences at $P \leq 0.01$, $P \leq 0.05$, and $P \leq 0.001$ were considered significant or highly significant. The tables show the mean and standard deviation.

Results

Differences were observed in the effects of the various growth promoters on the proliferative activity of cells in the culture.

The addition of IGF-1 to the BMSCC and MSCC culture medium increased the PI in these cultures (Fig. 1A,B). It is important to note that increased concentrations of this growth factor in the medium correlated with an increase in the PI. Thus, at an IGF-1 concentration of 50 ng/ml, the PI of BMSCC and MSCC was 1.4 × and 2.0 × higher, respectively, than in the control group. The reverse was observed for ATSCC: at an IGF-1 concentration of 10 ng/ml, the PI was 1.4 × higher than in the control group (Table 1, Fig. 1C,D), whereas at 50 ng/ml, the PI was 1.8 × lower than in the control group.

FGF-2 was shown to have varied effects on cell proliferative activity, depending on the origin of the cell culture and its concentration in the culture medium. The most significant effect of FGF-2 was demonstrated on MSCC (Fig. 1E,F). When the concentration of this growth factor was 10 ng/ml, the PI increased 2.7 × relative to the control group. A correlation was noted between the increase in FGF-2 concentration and the decrease in the PI in the culture. The promoting effect of FGF-2 in BMSCC and ATSCC was much weaker. The addition of 10 ng/ml increased the PI in the cultures 1.2 × and 1.3 ×, respectively (Table 2). The increase in the concentration of this growth factor in the medium led to a proportional decrease in the proliferation index (relative to the control group).

The addition of rhGH to the BMSCC culture medium decreased the PI relative to the control group. At the same time, a correlation was observed between the increase in the hormone concentration and the decrease in the PI. The addition of 10 ng/ml of rhGH to ATSCC and MSCC increased the PI 1.2 and 1.3 ×, respectively, compared to the control group (Table 3). However, a higher concentration of the hormone in the medium decreased

Table 1. Proliferative activity of stem cells derived from different sources, depending on the concentration of IGF-1 (M ± m, n = 3).

Cell culture	Concentration of IGF-1 in culture medium			Control
	10 ng/ml	20 ng/ml	50 ng/ml	
	Proliferation index			
BMSCC	1.66 ± 0.05*	1.82 ± 0.05***	2.08 ± 0.07***	1.44 ± 0.05
ATSCC	2.67 ± 0.08**	2.08 ± 0.06	1.06 ± 0.09**	1.85 ± 0.11
MSCC	2.41 ± 0.07***	2.66 ± 0.13***	3.65 ± 0.15***	1.81 ± 0.10

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the control group (cells cultured in standard culture medium); IGF-1 - insulin-like growth factor 1; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

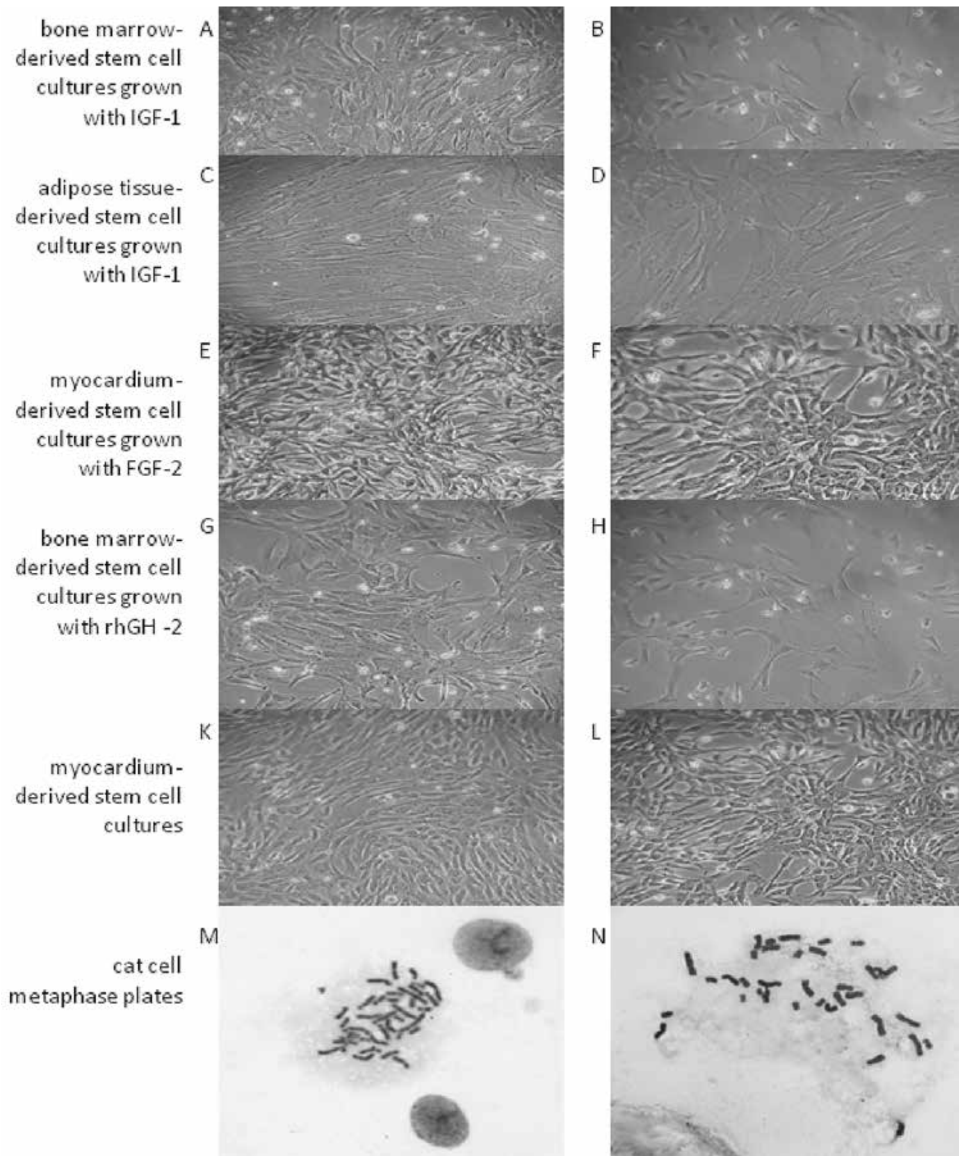

Fig 1. Bone marrow-derived stem cell cultures grown A) with 50 ng/ml IGF-1, B) control (native preparations, × 100). Adipose tissue-derived stem cell cultures grown C) with 10 ng/ml IGF-1, D) control (native preparations, × 100). Myocardium-derived stem cell cultures grown E) with 10 ng/ml FGF-2, F) control (native preparations, × 100). Bone marrow-derived stem cell cultures grown G) with 10 ng/ml rhGH -1, H) control (native preparations, × 100). Myocardium-derived stem cell cultures: K) LN521, L) control (native preparations, × 100). Cat cell metaphase plates: M) normal karyotype, n = 38, N) aneuploidy, n = 35 (Leykodif 200 staining, × 1,000).

Table 2. Proliferative activity of stem cells derived from different sources, depending on the concentration of FGF-2 (M ± , n = 3).

Cell culture	Concentration of FGF-2 in culture medium			Control
	10 ng/ml	20 ng/ml	50 ng/ml	
	Proliferation index			
BMSCC	1.69 ± 0.04 **	1.28 ±0.03*	0.74 ± 0.02	1.44 ± 0.05
ATSCC	2.43 ± 0.05**	1.81 ± 0.06	1.56 ± 0.08	1.85 ± 0.11
MSCC	4.84 ± 0.11***	3.87 ±0.22***	2.51 ± 0.06***	1.81 ± 0.10

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the control group (cells cultured in standard culture medium); FGF-2 - fibroblast growth factor; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

Table 3. Proliferative activity of stem cells derived from different sources, depending on the concentration of rhGH (M ± m, n = 3).

Cell culture	Concentration of rhGH in culture medium			Control
	10 ng/ml	20 ng/ml	50 ng/ml	
	Proliferation index			
BMSCC	1.22 ± 0.04**	1.09 ± 0.04***	0.88 ± 0.04***	1.44 ± 0.05
ATSCC	2.39 ± 0.06	2.08 ± 0.05	1.05 ± 0.06**	1.85 ± 0.11
MSCC	2.36 ± 0.07**	2.20 ± 0.08**	2.07 ± 0.04*	1.81 ± 0.10

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the control group (cells cultured in standard culture medium); rhGH - growth hormone; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

Table 4. Proliferative activity of stem cells derived from different sources, with or without the addition of LN521 (M ± m, n = 3) to the culture medium.

Cell culture	LN521	Control
	Proliferation index	
BMSCC	1.88 ± 0.05***	1.44 ± 0.05
ATSCC	2.35 ± 0.12*	1.85 ± 0.11
MSCC	3.07 ± 0.08***	1.81 ± 0.10

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the control group (cells cultured in standard culture medium); LN521 – Biolaminin 521 LN, BioLamina, Switzerland; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

Table 5. Results of cytogenetic analysis of cat cell cultures grown with IGF-1 and FGF-2.

Cell culture	IGF-1		FGF-2	Control
	10 ng/ml	50 ng/ml	10 ng/ml	
	Normal karyotype, %			
BMSCC	80.0 ± 1.3	-	81.3 ± 0.9	79.3 ± 0.9
ATSCC	-	86.0 ± 1.3	86.7 ± 0.9	88.7 ± 0.8
MSCC	89.3 ± 2.2	-	88.7 ± 0.9	90 ± 1.3

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control group (cells cultured in standard culture medium); IGF-1 - insulin-like growth factor 1; FGF-2 - fibroblast growth factor; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

Table 6. The results of cytogenetic analysis of cat cell cultures after the effect of rhGH and LN521 (M ± m, n = 3).

Cell culture	rhGH, 10 ng/ml	LN521	Control
	Normal karyotype, %		
BMSCC	78.7 ± 0.9	83.3 ± 0.9*	79.3 ± 0.9
ATSCC	86.7 ± 0.9	92.0 ± 1.3	88.7 ± 0.8
MSCC	88.7 ± 1.8	94.0 ± 0.0*	90 ± 1.3

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to the control group (cells cultured in standard culture medium); rhGH growth hormone; LN521 – Biolaminin 521 LN, BioLamina, Switzerland; BMSCC - bone marrow-derived stem cell cultures; ATSCC - adipose tissue-derived stem cell cultures; MSCC - myocardium-derived stem cell cultures

the PI. It should be noted that in the case of addition of rhGH, the cells appeared to be visually larger than in the control group (Fig. 1G,H).

LN 521 positively influenced all cell cultures studied. The PI for BMSCC and ATSCC increased 1.3 ×, and for MSCC 1.7 × relative to the control group (Table 4). It should be noted that the use of LN521 resulted in faster attachment of cells to the culture plastic and strong separation into layers, boosting formation of the monolayer (Fig. 1K,L).

Confirmation of the genetic stability of stem cells is required for further clinical use. As accelerated proliferation may increase the number of cells with altered karyotype in a culture, we carried out a cytogenetic analysis of the cell cultures with the highest PI (Tables 5 and 6, Fig. 1M,N).

Discussion

The structure of IGF-1 (somatomedin C) is similar to that of insulin, which is why this growth factor is able to bind the insulin receptor (Jakubczak et al. 2014; Jakubczak et al. 2017). It is also a mitogen for a majority of cells and can act as insulin (Niedźwiedzka 2000). There is currently no consensus on the effects of IGF-1 on cell proliferation *in vitro*. According to Ren et al. (1999) and Kaplan et al. (2005), IGF-1 is able to promote the growth, proliferation and differentiation of many cell types, including cardiomyocytes, smooth muscle cells and vessel cells, both *in vivo* and *in vitro*, and inhibit apoptosis and cell necrosis. This information correlates with the results obtained in our study in the group of MSCC plates. Li et al. (2007), however, noted that MSCs cultured *in vitro* with IGF-1 at final concentrations of 2.5, 5.0, and 10.0 ng/ml showed no change in the proliferative rate within 48 h. However, in our studies, when IGF-1 was added at final concentrations of 10.0, 20.0, and 50.0 ng/ml in the culture medium, we noted an increase in the proliferation index of BMSCC and MSCC compared to the control, which may indicate a stimulating effect of IGF-1 on these cultures only due to increased concentrations. The opposite effect was observed in the ATSCC, namely, a decrease in the proliferation index with an increase in the concentration of IGF-1 in the medium. The obtained data can be explained by stimulation of differentiation of adipose tissue cells (Boucher et al. 2016) with increased concentration in the medium.

FGF-2 is a multifunctional growth factor influencing various characteristics, such as proliferation induction and cell differentiation. It also influences a wide variety of mesodermal and neuroectodermal cells (Tassi et al. 2001), which explains its promoting effect on the cell cultures in our study. According to Gospodarowicz et al. (1985), this growth factor stimulates the proliferation of endothelial cells, which were present in all studied cultures. According to Hasegawa et al. (2010), FGF-2 is able to both stimulate endothelial cells and make the phenotype of cultured cells more like that of endothelial cells. The inhibiting effect of FGF-2 in the case of increased concentrations in the culture medium is still poorly understood.

LN 521 is a recombinant human laminin-based substrate. According to the manufacturer, it improves the stabilization and homogenization of the humane cell culture. Owing to interactions with appropriate cell surface receptors, laminins activate signalling cascades (such as the PI3K/Akt pathway), thus ensuring the expected cell response and better functionality. LN 521 ensures that cells grow in a homogeneous monolayer that is easy to control, preserving pluripotency and genetic integrity (Rodin et al. 2014). The properties of LN 521 claimed by the manufacturer for humane cell culture have been first experimentally confirmed for cell culture of cat.

The IGF-1 demonstrated a positive effect on the proliferative activity of all studied cultures. The optimum concentration of insulin-like growth factor was shown to be 50 ng/ml of medium for BMSCC and MSCC and 10 ng/ml for ATSCC, causing the proliferation index to increase 1.4 ×, 2.0 ×, and 1.4 ×, respectively, relative to the control group. The optimum concentration of FGF-2 for all studied cultures was 10 ng/ml of medium, and caused the proliferation index of BMSCC, ATSCC, and MSCC to increase 1.2 ×, 1.3 ×, and 2.7 ×, respectively, compared to the control group. The optimum concentration of the rhGH for the adipose tissue-derived and myocardium-derived stem cell cultures was 10 ng/ml of medium, increasing the proliferation index 1.2 × and 1.3×, respectively, relative to the control group. The addition of rhGH to the bone marrow-derived cell culture increased the size of the cells and decreased the proliferation index relative to the control group. LN 521 demonstrated a positive effect on all cell cultures: the proliferation index increased 1.3 × for BMSCC and ATSCC and 1.7 × for MSCC relative to the control group. An increase in the proliferative activity of cells of all the studied cat cultures when adding growth stimulators to the culture medium is not accompanied by a significant increase in the number cells with changes in the number of chromosomes in the culture (in comparison with the control). Culturing cat stem cell cultures by adding of LN 521 causes a decrease in the number of cells with an altered karyotype.

The results obtained during the research will be used in the future to introduce improved methods of culturing cat cell cultures obtained from various sources in veterinary practice for the treatment of heart diseases of this animal species.

Conflict of Interest

We have no conflicts of interest to disclose.

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