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Organospecific influence of the extract of cryopreserved piglets' skin fragments

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Abbreviations:
cNPSE – extract of cryopreserved newborn piglets' skin fragments
cPSE – extract of cryopreserved pigs' spleen fragments
FBS – fetal bovine serum
RP – regulatory peptides

Key words: fibroblasts, culture, extract, skin, spleen

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Abstract

Researching mechanisms of peptides' tissue-specific effect is an important task of modern molecular biology, physiology and medicine. In previous studies it was shown that extracts of cryopreserved piglets' skin fragments and pigs' spleen fragments accelerate and normalise the healing of skin wound in experiment. Fibroblasts culture is an appropriate model for studying tissue-specific biological activity of peptide complexes on relevant type of cells. This research was aimed to establish the influence of the extract of cryopreserved newborn piglets' skin fragments (cNPSE) and the extract of cryopreserved pigs' spleen fragments (cPSE) on proliferative and metabolic activity of skin fibroblasts in culture.

Extracts were obtained from cryopreserved skin fragments and cryopreserved spleen fragments. Primary culture of neonatal rat skin fibroblasts was obtained by free cell transfer from skin fragments and subsequent reseeding. Metabolic activity of cells in culture was defined using non-toxic redox indicator AlamarBlue. The number of cells in a well was measured by counting the quantity of cells in wells.

On the 7th day the metabolic activity of fibroblasts, cultured with cNPSE (1 μg/ml final peptide concentration), was higher than control by 1.3 times. Adding of cPSE (in the same concentration of peptides) increased cell metabolic activity by 1.2 times. While incubating fibroblasts in the medium with 2% FBS, a decrease of metabolic activity of cells was observed on the 5th day, and by the 7th day it was 51.2% of the control. At 1 and 1.5 μg/ml final peptide concentration of cNPSE, the metabolic activity of fibroblasts remained at the level observed in the control samples with 10% FBS. Adding cPSE to the incubation medium did not affect the metabolic activity of cells. Increased metabolic activity of cells (initially kept for 30 minutes at 4°C) was observed on the 5th and 7th day in cNPSE presence.

Thus, it was found that adding cNPSE and cPSE to the culture medium of rats' skin fibroblasts increase the metabolic activity of cells. A dose-dependent effect is observed. The addition of cNPSE to the medium with 2% FBS maintains the metabolic activity of fibroblasts at the level observed in the control samples incubated with 10% FBS. Adding extract also increases the metabolic activity of fibroblasts after hypothermic impact on the cells.

INTRODUCTION

Studying the physiological role of regulatory peptides (RP) is one of priority areas of modern science. It was found that these peptides had

a crucial role in maintaining homeostasis and were important components for the functioning of main regulatory systems: nervous, endocrine and immune. RP play substantial role in the implementation of these systems interaction. In case of homeostatic balance disorder, RP determine main parameters for development of compensatory-adaptive responses *(1–2).* RP are derived from almost all tissues and organs. They directly participate in various physiological processes, including the regulation of reparative processes in tissues by either stimulating or inhibiting cell proliferation *(3–5).*

Researching mechanisms of peptides' tissue-specific effect is an important task of modern molecular biology, physiology and medicine. Peptides' tissue-specific effect is mainly identified by the mechanisms of their influence on molecular genetic or biochemical markers of specialized target cells in different organs or tissues *(6–7).* It was established that fractions of low molecular weight components of tissue extracts were characterized by a specific set of peptide-nature substances. Thus, certain groups of tissue-specific peptides can be considered a specific characteristic of the corresponding tissue *(8).* Typically, the active element is represented not by individual components, rather by large number of peptides with variously directed activity.

It has been shown that cNPSE and cPSE accelerate and normalize the healing of skin wound *(9, 10, 11).* Fibroblasts culture is an appropriate model for studying tissue-specific biological activity of peptide complexes on relevant type of cells.

This research was aimed to establish the influence of cNPSE and cPSE on proliferative and metabolic activity of skin fibroblasts in culture.

MATERIALS AND METHODS

The experiments were performed in accordance with the "General ethical principles of animal experiments" approved by the 3rd National Congress in Bioethics (2007, Kyiv, Ukraine) and the Bioethics Commission of the Institute for Problems of Cryobiology and Cryomedicine (IPCC) of the National Academy of Sciences of Ukraine.

Mature pigs' spleen was obtained from the Kharkiv Meat Plant (Kharkiv, Ukraine). Newborn piglets' skin was obtained in vivarium at IPCC (Kharkiv, Ukraine).

Piglets' skin and pigs' spleens were fragmented into 2–5 mg pieces and washed thrice with physiological solution (pH 7.4). Cryoprotective solution of 20% PEO-1,500 was added dropwise to the fragments in 1:1 ratio while carefully stirring the suspension. This suspension was prepacked into 20 ml plastic vials and frozen at 1°C/min cooling rate. Fragments were frozen using UOP-6 programmable freezer (made by "BioCold" Special Designing and Technical Bureau with Experimental Unit of IPCC) down to -70°C with the following transfer into liquid nitrogen. Thawing was performed on water bath at 37-40°C. Cryoprotectant was washed out with physiological solution. Fragments were incubated for 60 min in the same solution. The supernatant was warmed in water bath for 15 min and paper-filtered *(12).* Peptides concentration in the extracts was spectrophotometrically measured at 280 nm wavelength.

Some samples were kept for 30 min at 4°C to investigate the effects of hypothermia on fibroblast culture and afterwards they were returned back to the standard culturing conditions.

Primary culture of neonatal rat skin fibroblasts was obtained by free cell transfer from skin fragments and subsequent re-seeding. Cells were cultured in CO_2 incubator at 37°C and 5% CO_2. In the experiments with extracts the cells of the third passage were used. Fibroblasts were seeded into 24–well plate (PAA, Austria) and cultured in DMEM/F12 (PAA, Austria), supplemented with 10% or 2% fetal bovine serum (FBS) (PAA, Austria), penicillin (100 U/ml) and streptomycin (100 μg/ml). Experimental samples were treated with cNPSE and cPSE with different final concentrations of peptides. Control samples received the equivalent volume of physiological solution.

Metabolic activity of cells in culture was defined using non-toxic redox indicator AlamarBlue (AbD Serotec, UK), which is reduced in the presence of metabolically active cells, forming a fluorescent product *(13, 14).*

AlamarBlue was added in 10% final concentration and the culture was incubated at 37°C for 3 hrs. AlamarBlue fluorescence was assessed using Tecan GENios spectrofluorimeter (Tecan inc., Austria) at 550 nm excitation wavelength and 590 nm emission. Data were processed using XFLUOR4 v.4.50 software. The results were represented as the fluorescence difference between experimental and blank samples, expressed as standard units of fluorescence (stand. units). Blank sample (with no cells) contained 10% solution of AlamarBlue in culture medium.

Visual control of cultures was performed on phase-contrast inverted Meiji Techno microscope (Japan), equipped with computer-connected video camera. The number of cells in a well was measured using BioVision 4.0 software by counting the quantity of cells in 10 vision fields and subsequent re-calculation per well square using relevant calibration.

RESULTS

As Table 1 demonstrates the adding of the extracts to culture medium did not affect metabolic activity of fibroblasts on the 2nd day of culturing. By the 5th day, when adding cNPSE (1 μg/ml final concentration of peptides), the metabolic activity of cells increased by 1.2 times and was statistically significantly higher ($p < 0.05$) than this index in the control cells cultured with no extracts. On

TABLE 1

Metabolic activity of fibroblasts (stand. units) depending on concentration of piglets' skin peptides and pigs' spleen peptides.

Extract addition and concentration of peptides	Time of culturing, day		
	2	5	7
Control with no extract adding	732±33	987±48	1431±73
cNPSE, 0.1 μg/ml	799±38	1031±47	1695±70*
cNPSE, 1 μg/ml	845±45	1204±60*	1875±77*
cPSE, 0.1 μg/ml	766±44	1095±43	1571±86
cPSE, 1 μg/ml	769±42	1137±51	1763±90*

Note: * – differences are statistically significant compared to the control, $p < 0.05$

TABLE 2

Metabolic activity of fibroblasts in 2% FBS presence (% of the control with 10% FBS) depending on concentration of piglets' skin peptides and pigs' spleen peptides.

Extract addition and concentration of peptides, μg/ml		Time of culturing, day		
		2	5	7
Control with no extract addition		86.5	64.1	51.2
cNPSE	0.1	89.1	84.7	79.3
	0.5	98.8	82.5	80.3
	1.0	99.3	97.8	95.9
	1.5	104.8	101.3	94.3
cPSE	0.1	84.2	66.8	50.2
	0.5	84.9	61.6	53.4
	1.0	89.4	62.0	58.3
	1.5	88.7	69.4	59.6

the 7th day the metabolic activity of fibroblasts, cultured with cNPSE (1 μg/ml final peptide concentration), was higher than control by 1.3 times. Adding of cPSE (in the same concentration of peptides) increased cell metabolic activity by 1.2 times. cPSE in peptide concentration of 0.1 μg/ml had no effect on the metabolic activity of cells compared to the control. The increase in metabolic activity of fibroblasts by the 7th day when adding cNPSE is explained by skin peptides' tissue-specific effect on fibroblasts, whereas the biological activity of cPSE may be stipulated by peptides of fibroblasts being present in spleen connective tissue.

While incubating fibroblasts in the medium with 2% FBS, a decrease of metabolic activity of cells was observed on the 5th day, and by the 7th day it was 51.2% of the control (Table 2). At 1 and 1.5 μg/ml final peptide concentration of cNPSE, the metabolic activity of fibroblasts remained at the level observed in the control samples with 10% FBS. Adding cPSE to the incubation medium did not affect the metabolic activity of cells.

On the 2nd day cell number did not depend on the conditions of culturing (Table 3). By the 5th and 7th days, when culturing with 2% FBS and adding cPSE, the number of cells was significantly lower than in control with 10% FBS. Adding cNPSE to culture medium did not change the number of cells in a well in comparison with the control.

FBS contains high amount of different proteins and peptides, which either stimulate proliferative activity of cells or inhibit cell growth. Serum proteins and peptides specifically involved into stimulation of cell division are known as growth factors. The concentration of growth factors in the serum is of a few nanograms or less. Most of these factors are specific to certain cell type and stage of cell differentiation. FBS impact on proliferative and metabolic activity of fibroblasts depends on its concentration in culture medium. Dose-dependent effect of cNPSE on AlamarBlue reduction at a decreased concentration of FBS is identified. Thus, it could indicate that this extract also contains growth factors of fibroblasts.

It was also investigated how addition of the extracts affect metabolic activity of fibroblasts in cell culture under reduced temperature at 4°C. As Table 4 shows, increased metabolic activity was observed on the 5th and 7th day in cNPSE presence. cPSE adding did not change this index.

TABLE 3

Number of cells (x 10^3) per well depending on experimental conditions.

Concentration of FBS and peptides in culture medium	Time of culturing, day		
	2	5	7
10% FBS	10.1 ± 0.6	15.9 ± 0.8	21±1.4
2% FBS	10.4 ± 0.7	11.8 ± 0.6*	13.1±1.1*
2% FBS+1 μg/ml peptides cNPSE	10.2 ± 0.6	14.2 ± 0.7	18.3±1.2
2% FBS+1 μg/ml peptides cPSE	10.1±0.4	11.5±0.8*	12.4±0.9*

Note: * – differences are statistically significant compared to the control (10% FBS), $p<0.05$, the number of cells introduced into a well was 10.0×10^3

TABLE 4

Fluorescence intensity (stand. units) in culture after hypothermia.

Experimental onditions	Time of culturing, day		
	2	5	7
After hypothermia with no extract addition	407±39	621±44	871±52
After hypothermia + 0.1 μg/ml cNPSE	543±54	817±49*	1123±73*
After hypothermia + 1 μg/ml cNPSE	531±60	933±56*	1301±79*
After hypothermia + 0.1 μg/ml cPSE	549±35	693±39	957±62
After hypothermia + 1 μg/ml cPSE	579±29	669±48	975±69

Note: * – differences are statistically significant compared to hypothermia with no extracts, $p<0.05$

DISCUSSION

The effect of peptides on organs and tissues is under continuous investigation using appropriate experimental models. In our study it was found that cNPSE and cPSE manifest definite biological activity in the culture medium of fibroblasts. A dose-dependent effect is observed, and under some experimental conditions a relevant tissue-specific biological activity of peptide extracts is also noted.

The results obtained in our study are consistent with the literature data testifying that natural polypeptide preparations with unidentified structure and synthetic short peptides show both polyfunctionality and tissue specificity *(15-18).* It has been previously shown that tissue-specific peptide, having affinity to cartilage tissue, activates proteins synthesis in mouse fibroblasts culture. Other peptides, whose specificity is directed at bronchial tissue and blood vessels, does not render such effect *(19).* Another research has similar results showing that effects of peptides lead to tissue-specific stimulation of protein synthesis in cells of the organ from which these peptides were isolated *(20).*

It should be noted that the concept of tissue-specific peptides in the scientific literature has no unambiguous interpretation. This is probably related to different approaches in solving this problem and interpretation of research results *(21, 22).* Tissue specificity of peptides is also considered in the notion of structural organisation of proteins of different origin *(23).* Probably, in this case the main function of tissue-specific peptides is the control of proliferation, differentiation and elimination of cells in the corresponding tissue. That is endogenous peptides are involved in the maintenance of structural and functional homeostasis of cell populations that contain and produce these factors In conclusion, it was found that adding cNPSE and cPSE to the culture medium of rats' skin fibroblasts increase the metabolic activity of cells. A dose-dependent effect is observed. The addition of cNPSE to the medium with 2% FBS maintains the metabolic activity of fibroblasts at the level observed in the control samples incubated with 10% FBS. Adding extract also increases the metabolic activity of fibroblasts after hypothermic impact on the cells.

REFERENCES

1. VESNINA L E, GARKOVICH A L, GRITSAY N N *et al.* 2003 Tissue regulatory peptides (theoretical principles and perspectives of practical application) (in Russian) *In:* Kaydasheva I P, Mischenko V P, Ribalchenko V K *(ed).* Zdorovya, Kiev, p 392 p
2. LANCHA A, FRUHBECK G, GOMEZ-AMBROSI J 2012 Peripheral signalling involved in energy homeostasis control. *Nutr Res Rev 25:* 223–248
3. MORO C, LAFONTAN M 2013 Natriuretic peptides and cGMP signaling control of energy homeostasis. *Am J Physiol Heart Circ Physiol 304:* H358–368
4. SURH C D, SPRENT J 2000 Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *JEM 192:* F9–F14
5. MUNIZ L R, KNOSP C, YERETSSIAN G 2012 Intestinal antimicrobial peptides during homeostasis, infection and disease. *Front Immunol 3:* 310
6. DONG C X, BRUBAKER P L 2012 Ghrelin, the proglucagon-derived peptides and peptide YY in nutrient homeostasis. *Nat Rev Gastroenterol Hepatol 9:* 705–715
7. GASSANOV N, BIESENBACH E, CAGLAYAN E *et al.* 2012 Natriuretic peptides in therapy for decompensated heart failure. *Eur J Clin Pharm 68:* 223–230
8. YATSKIN O N, KARELIN A A, IVANOV V T 2009 Peptidomes of the brain, heart, lung, and spleen: Similarity and differences. *Russian Journal of Bioorganic Chemistry 35:* 426–436
9. YERMAKOVA N YU, SHYNDER A V, ROSHAL O D, GALCHENKO S YE, SANDOMIRSKY B P 2010 Effect of animal origin extracts on the healing of cold wounds (in Ukrainian). *Svit medycyny ta biologiyi 1:* 32–36
10. BOGATYROVA O O, BESPALOVA I G, BELOCHKINA I V, GALCHENKO S YE, SANDOMIRSKY B P 2013 Biological effect of extract of cryopreserved newborn piglets' skin fragments in cold injury and when added to fibroblast culture (Abst. from the 25th Conf. Intern. Soc. for Medical Innovation and technology, iSMIT 2013, Baden-Baden,Germany) *MITAT 22(1):* 54
11. BESPALOVA I, BOGATYRYOVA O, SHINDER A, BELOCHKINA I, GALCHENKO S, SANDOMIRSKY B 2013 Organospecific influence of the extract of cryopreserved piglets' skin fragments. Final programme and abstract book (3rd congress of Croatian Physiological Soc. and 1st reg. congress of Physiol. Soc., Zagreb sept. 2013) *Per biol 115(2):* 17
12. GALCHENKO S Y E, SHKODOVSKA N Y U, SANDOMIRSKY B P, GRISCHENKO V I Method for obtaining xenogenic organ extracts. Patent 64381 A Ukraine, IPCA61K35/12. Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. 2003054649. Patent 22.05.2003, Published 16.02.2004. Bulletin 2.

13. AL-NASIRY S, GEUSENS N, HANSSENS M, LUYTEN C, PIJNENBORG R 2007 The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod 22(5):* 1304–1309

14. PATEL H D, ZAVERI A D, ZAVERI D N, SHAH S, SOLANKI A 2013 Comparison of the MTT and Alamar Blue assay for in vitro anti-cancer activity by testing of various chalcone and thiosemicarbazone derivatives. *Int J Pharm Bio Sci 4(2):* 707–716

15. KARELIN A A, BLISHCHENKO E YU, IVANOV V T 1998 A novel system of peptidergic regulation. *FEBS Lett 428:* 7–12

16. KHAVINSON V K H, RYZHAK G A 2010 Peptide regulation of basic functions of the organism (in Russian). *Vestnik RosZdravNadzora 6:* 58–62

17. KVETNOY I M, POLYAKOVA V O, TROFIMOV A V *et al.* 2003 Hormonal function and proliferative activity of thymic cells in humans: immunocytochemical correlations. *Neuro Endocrinol Lett 24:* 263-268

18. UPADHYAY R, GUPTA S, KANUNGO M S 1999 Trans-acting factors that interact with the proximal promoter sequences of ovalbumin gene are tissue-specific and age-related. *Mol Cell Biochem 201:* 65-72

19. PALTSEV M A, KVETNOY I M, POLYAKOVA V O, KVETNAIA T V, TROFIMOV A V 2009 Neuro-immune-endocrine mechanisms of aging (in Russian). *Adv Gerontol 22(1):* 24-36

20. MOROZOV V G, KHAVINSON V K H 1997 Natural and synthetic thymic peptides as therapeutics for immune dysfunction. *Int J Immunopharmacol 19:* 501-505

21. DIAZ D, KREJSA C M, WHITE C C *et al.* 2001 Tissue specific changes in the expression of glutamate-cysteine ligase mRNAs in mice exposed to methylmercury. *Toxicol Lett 122:* 119-129

22. HE B, DECKELBAUM R A, MIAO D *et al.* 2001 Tissue-specific targeting of the pthrp gene: the generation of mice with floxed alleles. *Endocrinology 142:* 2070-2077

23. IVANOV V T, KARELIN A A, PHILIPPOVA M M *et al.* 1997 Hemoglobin as a source of endogenous bioactive peptides: the concept of tissue-specific peptide pool. *Biopolymers 43:* 171-188