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SHAPE OF CALCIUM PHOSPHATE BIONS DEFINES A PROATHEROSCLEROTIC SHIFT IN CYTOKINE SECRETION PROFILE OF ENDOTHELIAL CELLS

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ABSTRACT

Study aim. To investigate whether the shape of calcium phosphate bions (CPB) affects their endothelial toxicity via evaluating the cytokine secretion profile of endothelial cells upon the exposure to either spherical or spindle-shaped CPB.

Material and methods. For the experiments, we used an immortalized human vein endothelial cell line EA.hy 926. Cells were seeded into 6-well plates (3*105 cells) with the further: 1) addition of 100 μL either spherical CPB, spindle-shaped CPB, or 1x phosphate buffered saline (PBS) upon 1 h following culture for 24 h (non-confluent cell culture); 2) culture for 44 h and subsequent addition of 100 μL either spherical CPB, spindle-shaped CPB, or PBS following culture for 4 h (confluent cell culture). Upon the collection of cell culture supernatant (n=11 wells per group), the levels of proatherosclerotic cytokines (interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, IL-23, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and soluble vascular cell adhesion molecule (sVCAM)-1) were measured utilizing an enzyme-linked immunosorbent assay.

Results. In a non-confluent cell culture, exposure to spindle-shaped CPB increased the secretion of several proatherosclerotic cytokines (IL-1β, IL-10, IL-12, IL-23, IFN-γ) compared to either spherical CPB-treated or control cells. In a confluent cell culture, exposure to either of CPB types decreased the release of IL-1β, IL-10, and IFN-γ; however, their concentration was still higher upon the exposure to spindle-shaped CPB in comparison with exposure to spherical CPB. Discriminant analysis and principal component analysis demonstrated that the cytokine secretion profile of spindle-shaped CPB-treated endothelial cells significantly differed from those of either spherical CPB-treated or control cells.

Conclusion. Spindle-shaped CPB induce the secretion of proatherosclerotic cytokines by endothelial cells compared to spherical CPB; this suggests higher endothelial toxicity of spindle-shaped CPB.

Keywords: *atherosclerosis, triggers, bions, endothelial cells, endothelium, inflammation, cytokines, cytokine secretion profile, interleukin-6, interleukin-8.*

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INTRODUCTION

Bions are a family of mineral-organic nanoparticles, synthesized under conditions of supersaturation of biological fluids (blood serum, urine, saliva, bile, amniotic fluid and others) by various ions as a result of chemical interactions: 1) cations, or salts, containing cations; 2) phosphate anion or salts, containing it; 3) certain proteins (albumin, fetuin-A, osteonectin and several others) [1]. Depending on forming cation, bionsare divided into calcium phosphate, magnesium phosphate, manganese phosphate, strontium phosphate, barium phosphate and the others [1], of which only a calcium phosphate bion (CPB) is accurately formed in the human body [2-4]. It was suggested [1] and experimentally proved [5], that the CPB are one of the mechanisms for maintaining mineral homeostasis, preventing direct tissue calcification, when serum environment is saturated with ions of calcium and phosphorus. At the same time it was revealed that CPB have a toxic effect on the culture of immortalized venous and lymphatic endothelial cells, triggering their apoptosis and stimulating the secretion by them of proatherosclerotic cytokines of interleukin (interleukin, IL-6) and IL-8 and on the intima of the abdominal aorta of rats, causing its concentric or eccentric hypertrophy [5]. In this regard, the hypothesis was proposed, that protecting the body from the "greater evil" – massive ectopic calcification, CPB nevertheless damage healthy endothelium, potentially becoming one of the triggers for the development of atherosclerosis [5-8]. Earlier in the experiment it was shown, that with moderate oversaturation of serum medium with ions of calcium and phosphorus, spherical CPB are formed, and with a strong oversaturation – needle-shaped CPB [9]. However, it remains unclear whether this determine proatherosclerotic action of CPB.

One of the triggers for the development of atherosclerosis is auto- and paracrine secretion of the so-called proatherosclerotic cytokines (IL-1β, IL-6, IL-8, IL-12 and others) by endothelial cells, which are directly exhibited by CPB, emerging in the blood, while previously our group demonstrated increased secretion by endothelial cells, IL-6 and IL-8 due to exposure of CPB [5]. A relatively new term «proatherosclerotic cytokines» means cytokines where it's generally pleiotropic effects somehow contribute to the development of atherosclerosis [10-14]. The aim of this study was to compare the profile of proatherosclerotic cytokines secreted by endothelial cells under the influence of equal concentrations of spherical CPB and needle-shaped CPB.

MATERIAL AND METHODS

Artificial synthesis of spherical CPB and needle-shaped CPB

Spherical CPB were synthesized by sequential addition of 9.9 μl 0.5 M $CaCl_2$ (Sigma-Aldrich) and 21.5 μl 0.2 M Na_2HPO_4 (Sigma-Aldrich) in 1319 μl of the medium Needle, modified by Dulbecco (DMEM, Dulbecco's Modified Eagle's Medium, Gibco) containing 150 μl (10% of the total volume) of fetal calf serum (Gibco). Needle-shaped CPB were synthesized with successive addition of 16.5 μl 0.5 M $CaCl_2$ and 37.5 μl 0.2 M Na_2HPO_4 936 μl of DMEM medium, containing 10 μl (1% of the total volume) of fetal calf serum. After a short mixing by vortex, the tubes 1.5 ml (Eppendorf) with reagents for the synthesis of CPB were incubated at +37°C (MCO-18AIC, Sanyo) for 24 h with further centrifugation at 200,000 x g and +4°C for 1 h (Optima MAX-XP, Beckman Coulter). In order to obtain a working solution for addition to cells, spherical CPB precipitate was dissolved in 300 μl and needle-shaped CPB precipitate – in 1500 μl of a single phosphate-saline buffer (FSB, 1X phosphate buffered saline, Gibco). That allowed to reach an optical density (OD) of 0.5 Mcfarland standard (MF), which is a minimally measurable and pathophysiologicallyrelevant value of concentration of CPB in the solution. All the above procedures were performed under sterile conditions. Measurement of OP was carried out on a microplate spectrophotometer "Uniplan" (AIFR-01, Picon) at the wavelength of 650 nm.

Exposure of endothelial cells of needle-shaped CPB and spherical CPB

For experimentsit was used to culture of immortalized venous endothelial cell of the human line EA.hy 926, kindly provided by Dr. Cora-Jean S. Edgell (University of North Carolina at Chapel Hill.). This cell line is hybridoma, which was obtained by merging the endothelial cells of the human umbilical vein (human umbilical vein endothelial cells, HUVEC) with cells of adenocarcinoma of human lungs of line A549 and retains the basic morphological and functional features of the venous endothelial human cells [15]. Cells were cultured in the DMEM/F12 (Gibco) medium with 10% of fetal calf serum (Gibco), 2% solution of hypoxanthine- aminopterin-thymidine (Gibco), 1% solution of HEPES-buffer (Gibco), 1% solution of L-glutamine-penicillin-streptomycin (Gibco) and 0.4% solution of amphotericin B (Gibco). All experiments with cells were performed under sterile conditions at 37°C, 5% CO_2 and high humidity (MCO-18AIC, Sanyo).

Due to the fact, that the stability and response of endothelial cells to external influences greatly depends on the integrity of their monolayer [6-8], cytokine profile secreted by the cells was assessed in two cell models: sparse model (<50% of cell confluency in the culture dishes) and confluent model (> 90% of cell confluency in the culture dishes). In sparse models the cells were planted in 6-hole tablets (3*105 cells per hole, 1900 μl of the medium in the hole) with the addition to them after 1 hour of 100 μl of spherical CPB (0.5 UF), needle-shaped CPB (0.5 UF) or PBS (phosphate buffered saline) with further incubation for 24 h. In a confluent model, cells similarly were planted in 6-hole tablets (3*105 cells per hole, 1900 μl of medium in the hole) and cultivated for 44 h, after which 100 μl of spherical CPB (0,5 McF), needle-shaped CPB (0,5 McF) or PBSwere added with further incubation for 4 h. At the end of the incubation, the supernatant was taken from holes (n=11 holes for each group) using an automatic single-channel dispenser (Lenpipet), which was divided into aliquots with a volume of 400-500 μl in four test tubes with a volume of 1.5 ml and stored at -40°C (Sanyo) until it was time to measure the profile of proatherosclerotic cytokines, secreted by cells (IL-1β, IL-6, IL-8, IL-10, IL-12, IL-23, tumor necrosis factor (tumor necrosis factor, TNF)-α, interferon (interferon, IFN)-γ, soluble vascular cell adhesion molecule-1 (soluble vascular cell adhesion molecule, sVCAM-1)) by method of enzyme immunoassay by means of appropriate kits of Abcamcompany (ab46052, ab46027, ab46032, ab46034, ab46143, ab64708, ab46087, ab46025, ab46118) in accordance with the manufacturer's instructions. The selection of the above cytokines was due to their secretion by endothelial cells and central role in the development of atherosclerosis [10-14]. Each aliquot of supernatant was thawed and used for analysis no more than once. The measurement of outcomes was conducted on a microplate spectrophotometer "Uniplan" at a wavelength of 450 nm.

Statistical analysis

Statistical analysis of the results was performed using the programs GraphPad Prism 6 (GraphPad Software), Excel 2013 (Microsoft) and Statistica 13 (Dell). Intergroup comparison of the measured levels of each of the nine cytokines were performed by one-way variance analysis. In case of detection of statistically significant differences (the probability of rejecting the true null

hypothesis P≤0,05) between the groups, a subsequent pairwise comparison of the groups was carried out, using the Tukey criterion. P-values ≤0,05, obtained by the criterion of Tjuki, were considered to be statistically significant. To assess whether the profile of secreted cytokines as a whole is different in three groups, heat maps of cytokines secretion were constructed, and discriminant analysis and the main component method were used. As in the case of single-factor variance analysis, for discriminant analysis and the method of principal components, statistical differences between groups were recognized as significant at P≤0,05.

THE RESULTS OF THE STUDY

Experiments performed on sparse cell model, showed that, in contrast to spherical CPB, needle-shaped CPB induced secretion of a number of cytokines (IL-1β, IL-10, IL-12, IL-23, IFN-γ) compared to control cells (Fig. 1). In addition, needle-shaped CPB increased the level of secreted sVCAM-1 and reduced concentrations of secreted TNF-α in comparison with spherical CPB (Fig. 1). Spherical CPB as well as needle-shaped CPB in a similar extent increased the secretion of IL-8 compared to control cells (Fig. 1)

At the same time, on the confluent cell model, the exposure of both spherical CPB and needle-shaped CPB caused a significant decrease in the secretion of IL-1β, IL-10 and IFN-γ, but their level was higher when exposed to needle-shaped CPB than when exposed to spherical CPB (Fig. 2). Conversely, the concentration of secreted IL-6 in the exposure of both spherical CPB and needle-shaped CPB in comparison with control cell cultures increased, but did not differ between the types of CPB itself (Fig. 2). The level of TNF-α and IL-8 was similar to that of control cells in exposure to needle-shaped CPB, but lower than that of spherical CPB exposure (Fig. 2). The concentration of IL-12, IL-23 and sVCAM-1 in exposure to both types of CPB was significantly lower than in the absence of exposure, without significant differences between needle-shaped CPB and spherical CPB (Fig. 2).

To collectively visualize the result of the impact effects of spherical CPB and needle-shaped CPB, heat maps of cytokine release by endothelial cells on both cellular models (Fig. 3) have been compiled, which suggested that the profile of secreted cytokines as a whole varies significantly under the influence of spherical CPB and especially needle-shaped CPB.

To test this hypothesis, multidimensional statistical analysis methods were used: discriminant analysis and the method of principal components. Both of these methods confirmed that the profile of the cytokine secreted by the cells was significantly different in all three groups and under the influence of the needle-shaped CPB was significantly further from that of the control cells compared to the spherical CPB (Fig. 4a, b), with the main contribution to this difference being made by IL-8, IL-10 and TNF-α (Fig. 4c).

DISCUSSION

Previously, it was found that with a moderate supersaturation of the serum environment with calcium and phosphorus ions, a spherical CPB forms, and with a strong supersaturation – a needle-shaped CPB form [9], which may indicate the possibility of the formation of a spherical form in the blood of a person with moderate hypercalcemia / hyperphosphataemia and needle-shaped CPB with severe hypercalcemia / hyperphosphataemia. However, despite a number of published studies, demonstrating the cytotoxic and, in particular, endotheliotoxic effect of the CPB [5, 16-18], no studies have been conducted of the effect of the form of CPB on their toxicity for cells. Since the CPB is considered as a potential trigger for the development of atherosclerosis [5-8], and the shift in the profile of cytokines released by endothelial cells released to the proaterosclerotic under the imfluence of CPB is one of the effects that determines their endotheliotoxicity, in this study, the dependence of the secretion of proterosclerotic cytokines of endotheliocytic forms of artificially synthesized CPB.

The performed experiments demonstrated, that on the sparse

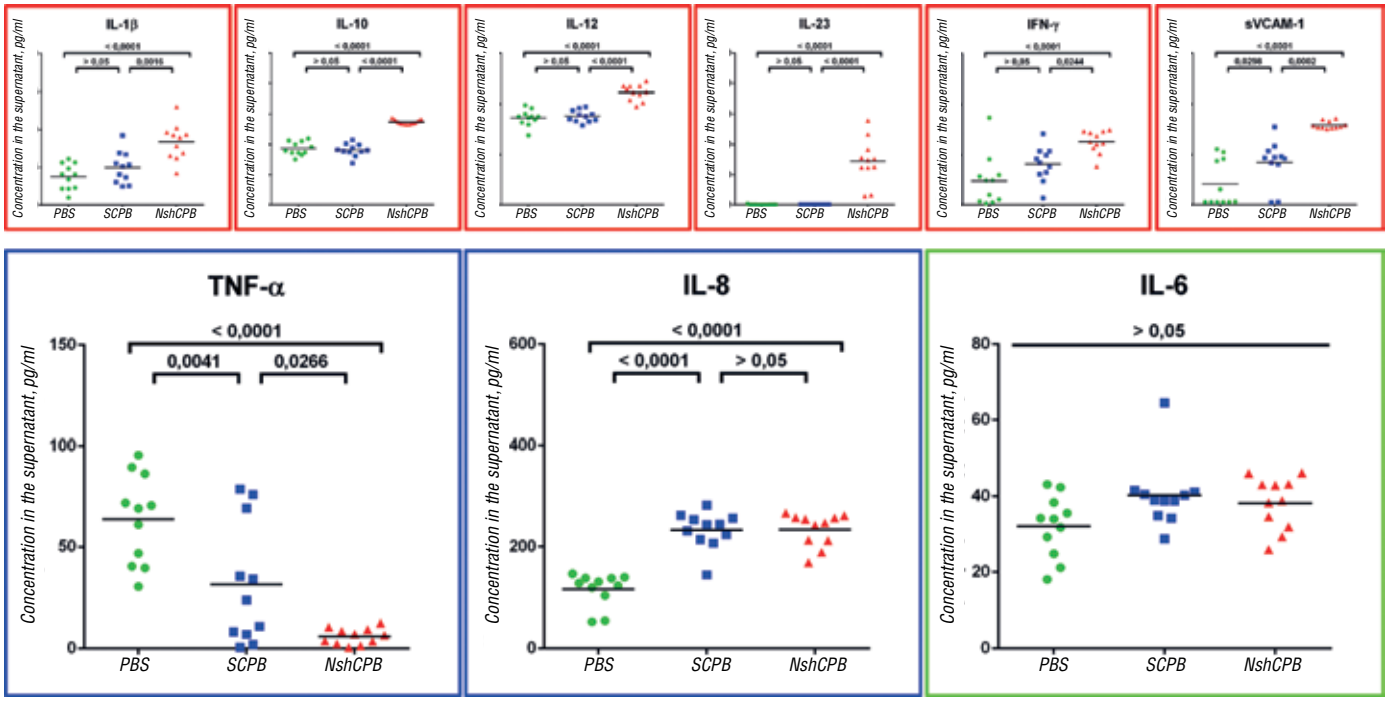

Figure 1. Isolation of cytokines by endothelial cells of the line EA.hy 926 on a sparse cell model under the influence of spherical CPB and needle-shaped CPB

Note: PBS is a phosphate-buffered saline, SCPB – spherical-shaped calcium phosphate bions, NshCPB – needle-shaped calcium phosphate bions, IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, sVCAM-1 – soluble molecule of adhesion of vascular cells-1.

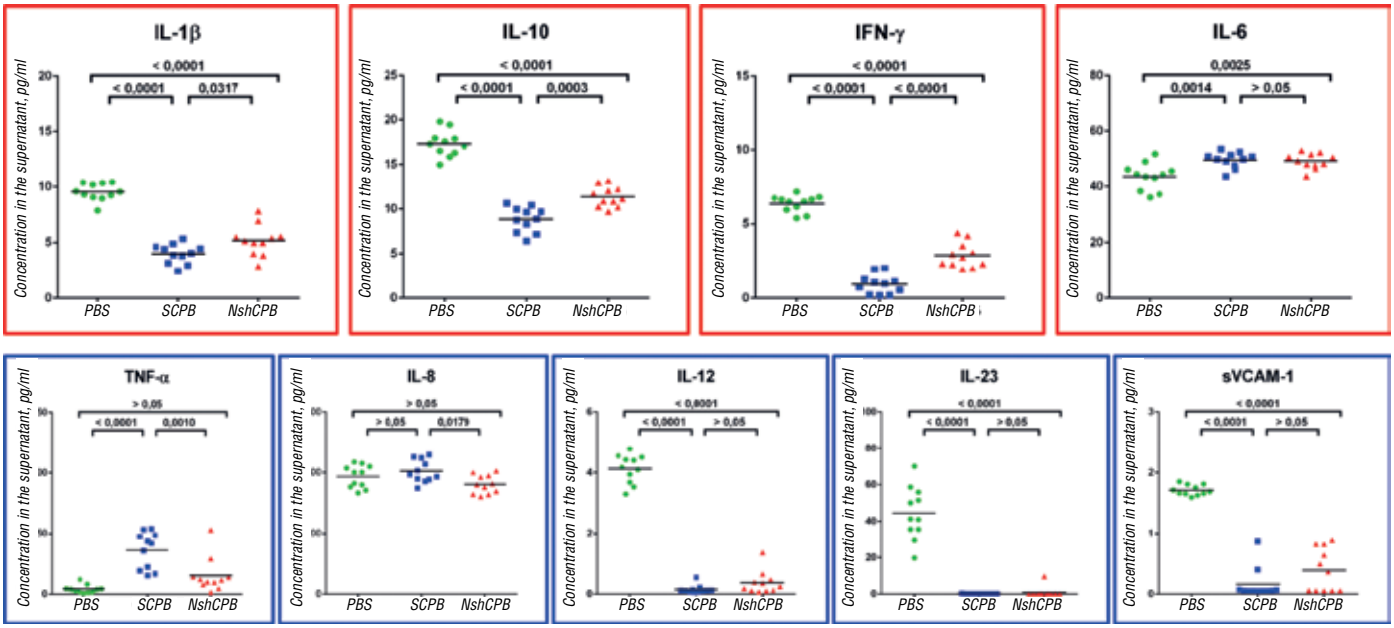

Figure 2. Isolation of cytokines by endothelial cells of the line EA.hy 926 on a confluent cell model under the influence of spherical CPB and needle-shaped CPB

Note: PBS is a phosphate-buffered saline, SCPB – spherical-shaped calcium phosphate bions, NshCPB – needle-shaped calcium phosphate bions, IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, sVCAM-1 – soluble molecule of adhesion of vascular cells-1.

cell model, the exposure of the needle-shaped CPB significantly increased the level of release of a number of pro-atherosclerotic cytokines (IL-1β, IL-10, IL-12, IL-23, IFN-γ) compared to spherical CPB exposure, as well as control cells. On the confluent cell model, the exposure of both types of CPB caused a marked decrease in the release of IL-1β, IL-10 and IFN-γ, but their concentration was still higher when exposed to needle-shaped CPB, than when exposed to spherical CPB. The applied methods of multidimensional statistical analysis (discriminant analysis and main components method) on both cellular models have confirmed that the profile of cytokine secretion by endothelial cells under the influence of needle-shaped CPB is significantly different from that under the influence of spherical CPB and is far from the profile of control cells. Thus, the obtained data indicate a more pronounced shift in the profile of the release of proterosclerotic cytokines by endothelial cells under the influence of needle-shaped CPB in comparison with spherical CPB and, consequently, the greater endotheliotoxicity of the needle-shaped CPB.

Comparison of the results obtained on the sparse and confluent cellular models showed their significant difference, especially noticeable in the application of multidimensional methods of statistical analysis. In a sparse model, the isolation of IL-1β, IL-10, IL-12, IL-23, IFN-γ and sVCAM-1 by cells under the influence of needle-shaped CPB increased in comparison with control cells, and decreased on the confluent model. On a sparse model, the cells exposed to needle-shaped CPB, in comparison with the control cells, were characterized by increased secretion of IL-8, and on the confluent model – IL-6. Finally, on a sparse model, the secretion of TNF-α cells under the influence of needle-shaped CPB decreased in comparison with control cells, and on the confluent model – no. The overall profile of secretion of pro-atherosclerotic cytokines by endothelial cells under the influence of needle-shaped CPB on a sparse model was further from that of control cells in comparison with the profile of cells exposed to spherical CPB, while the opposite was observed in the confluent model. Such differences between the models may be due to different exposure times of the cells of the CPB (24 hours at sparse cell model and 4 hours in the confluent

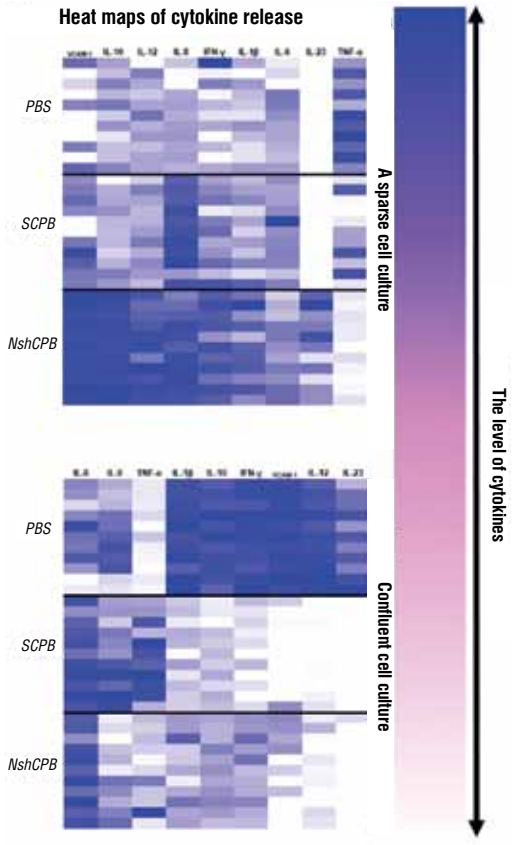

Figure 3. Heat maps of cytokine release by endothelial cells of the line EA.hy 926 under the influence of spherical CPB and needle-shaped CPB

Note: PBS is a phosphate-buffered saline, SCPB – spherical-shaped calcium phosphate bions, NshCPB – needle-shaped calcium phosphate bions, IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, sVCAM-1 – soluble molecule of adhesion of vascular cells-1.

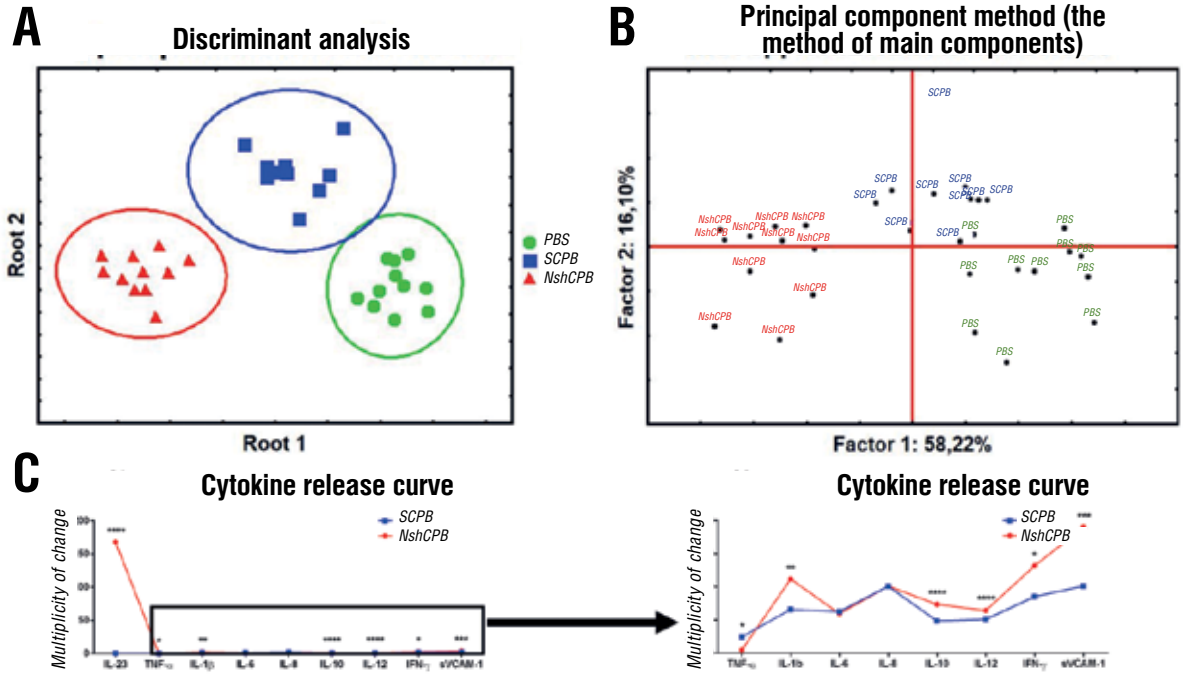

Figure 4. Multi-dimensional statistical analysis of cytokine release by endothelial cells of the line EA.hy 926 on a sparse cell model under the influence of spherical CPB and needle-shaped CPB

Note: PBS is a phosphate-buffered saline, SCPB – spherical-shaped calcium phosphate bions, NshCPB – needle-shaped calcium phosphate bions, IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, sVCAM-1 – soluble molecule of adhesion of vascular cells-1.

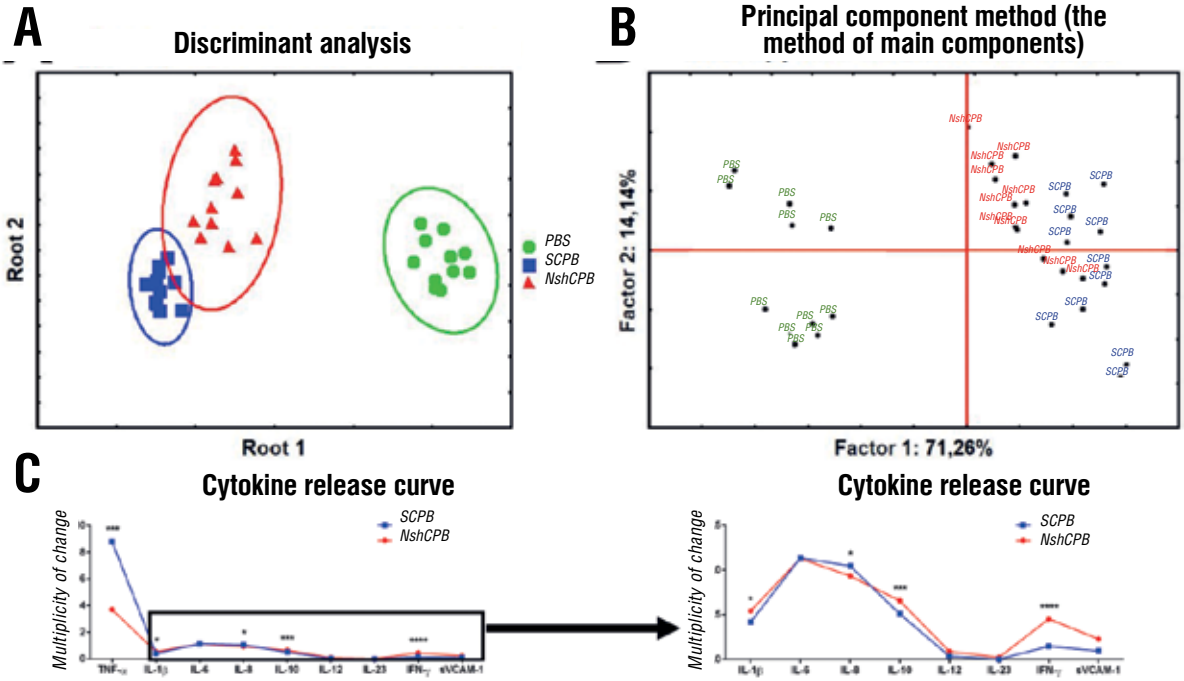

Figure 5. Multivariate statistical analysis of cytokine release by endothelial cells of the line EA.hy 926 on the confluent cell model under the influence of spherical CPB and needle-shaped CPB

Note: PBS is a phosphate-buffered saline, SCPB – spherical-shaped calcium phosphate bions, NshCPB – needle-shaped calcium phosphate bions, IL – interleukin, TNF – tumor necrosis factor, IFN – interferon, sVCAM-1 – soluble molecule of adhesion of vascular cells-1.

cell model), however, with the endothelial cell line immortalized by hybridoma technology, it is pointless to expose its CPB at >90% confluence within 24 hours, because in this case 7-8% of the cells undergo apoptosis even in the control culture (own unpublished observations of the authors), which does not allow to conduct a qualitative experiment. To overcome this lack of modeling of a confluent monolayer of endothelial cells in vitro, it is proposed to use primary arterial endothelial cells, which, due to slow growth, can theoretically be exposed to the CFB for a sufficiently long time, even with high confluence. However, such cell lines are characterized by the complexity of cultivation after isolation and high cost when ordering commercially available standardized cultures, which makes it difficult to conduct such experiments and requires preliminary experiments on classic and widespread immortalized endothelial cell lines such as the EA.hy 926 line used in this study.

Based on the results obtained, it can be concluded that needle-shaped CPB induces an increased secretion of a number of pro-atherosclerotic cytokines (in particular, IL-1β, IL-10 and IFN-γ) by endothelial cells in comparison with spherical CPB, which indicates greater endotheliotoxicity of needle-shaped CPB. To confirm this hypothesis, similar experiments are required to conduct on primary cultures of arterial endothelial cells.

BIBLIOGRAPHY

1. *Wu C.Y., Young L., Young D. et al. Bions: a family of biomimetic mineralo-organic complexes derived from biological fluids. PLoS One. 2013. Vol. 8. No. 9. P. e75501. doi: 10.1371/journal.pone.0075501.*
2. *Price P.A., Thomas G.R., Pardini A.W. et al. Discovery of a high molecular weight complex of calcium, phosphate, fetuin, and matrix gamma-carboxyglutamic acid protein in the serum of etidronate-treated rats. J. Biol. Chem. 2002. Vol. 277. No. 6. P. 3926-3934.doi: 10.1074/jbc.M106366200.*
3. *Jahnen-Dechent W., Heiss A., Schäfer C., Ketteler M. Fetuin-A regulation of calcified matrix metabolism. Circ. Res. 2011. Vol. 108. No. 12. P. 1494-1509. doi: 10.1161/CIRCRESAHA.110.234260.*
4. *Wong T.Y., Wu C.Y., Martel J. et al. Detection and characterization of mineralo-organic nanoparticles in human kidneys. Sci. Rep. 2015. Vol. 5. P. 15272. doi: 10.1038/srep15272.*
5. *Kutikhin A.G., Velikanova E.A., Mukhamadiyarov R.A. et al. Apoptosis-mediated endothelial toxicity but not direct calcification or functional changes in anti-calcification proteins defines pathogenic effects of calcium phosphate bions. Sci. Rep. 2016. Vol. 6. P. 27255. doi: 10.1038/srep27255.*
6. *Gimbrone M.A. Jr., García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ. Res. 2016. Vol. 118. No. 4. P. 620-636. doi: 10.1161/CIRCRESAHA.115.306301.*
7. *Cahill P.A., Redmond E.M. Vascular endothelium - Gatekeeper of vessel health. Atherosclerosis. 2016. Vol. 248. P. 97-109. doi: 10.1016/j.atherosclerosis.2016.03.007.*
8. *Jensen H.A., Mehta J.L. Endothelial cell dysfunction as a novel therapeutic target in atherosclerosis. Expert Rev. Cardiovasc.Ther. 2016. Vol. 14. No. 9. P. 1021-1033. doi: 10.1080/14779072.2016.1207527.*
9. *Young J.D., Martel J., Young D. et al. Characterization of granulations of calcium and apatite in serum as pleomorphic mineralo-protein complexes and as precursors of putative nanobacteria. PLoS One. 2009. Vol. 4. No. 5. P. e5421. doi: 10.1371/journal.pone.0005421.*
10. *Tedgui A., Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol. Rev. 2006. Vol. 86. No. 2. P. 515-581. doi: 10.1152/physrev.00024.2005.*
11. *Kleemann R., Zadelaar S., Kooistra T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovasc. Res. 2008. Vol. 79. No. 3. P. 360-376. doi: 10.1093/cvr/cvn120.*
12. *Ait-Oufella H., Taleb S., Mallat Z., Tedgui A. Recent advances on the role of cytokines in atherosclerosis. Arterioscler. Thromb.Vasc. Biol. 2011. Vol. 31. No. 5. P. 969-979. doi: 10.1161/ATVBAHA.110.207415.*
13. *Ramji D.P., Davies T.S. Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. Cytokine Growth Factor Rev. 2015. Vol. 26. No. 6. P. 673-685. doi: 10.1016/j.cytogfr.2015.04.003.*
14. *Moss J.W., Ramji D.P. Cytokines: roles in atherosclerosis disease progression and potential therapeutic targets. Future Med. Chem. 2016. Vol. 8. No. 11. P. 1317-1330. doi: 10.4155/fmc-2016-0072.*
15. *Edgell C.J., McDonald C.C., Graham J.B. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. U S A. 1983. Vol. 80. No. 12. P. 3734-3737.*
16. *Aghagolzadeh P., Bachtler M., Bijarnia R. et al. Calcification of vascular smooth muscle cells is induced by secondary calciprotein particles and enhanced by tumor necrosis factor-α. Atherosclerosis. 2016. Vol. 251. P. 404-414. doi: 10.1016/j.atherosclerosis.2016.05.044.*
17. *Smith E.R., Hanssen E., McMahon L.P., Holt S.G. Fetuin-A-containing calciprotein particles reduce mineral stress in the macrophage. PLoS One. 2013. Vol. 8. No. 4. P. e60904. doi: 10.1371/journal.pone.0060904.*
18. *Peng H.H., Wu C.Y., Young D. et al. Physicochemical and biological properties of biomimetic mineralo-protein nanoparticles formed spontaneously in biological fluids. Small. 2013. Vol. 9. No. 13. P. 2297-2307. doi: 10.1002/smll.201202270.*