

Research letter

## Effect of the needles with different nanostructured carbon coatings on the osteoporotic bone tissue in the experiment

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**Abstract:** The article presents the comparative analysis of osseointegrative properties of different nanostructured carbon (a-C and CN<sub>0.25</sub>) coatings of the needles which is based on a study of regulating bone remodeling growth factors as well as a marker of apoptosis and bone resorption TNF-related apoptosis-inducing ligand (TRAIL).

**Material and Methods** — The experiment was performed on 64 male rats of the Wistar line. Animals were immobilized by resection of the hind limb bones in proximal part of the right calf in order to simulate immobilization osteoporosis (IOP). Needles with carbon nanostructured CN<sub>0.25</sub>-coatings were implanted for the rats of the first group (32 rats), needles with diamond-like nanostructured a-C were implanted for the rats of the second group (32 rats). On 30<sup>th</sup>, 60<sup>th</sup> and 90<sup>th</sup> day after implantation we determined the concentrations of bone morphogenetic protein 2 (BMP-2), transforming growth factor beta (TGF-β), TRAIL and the bone matrix resorption marker RatLaps in rats serum by ELISA.

**Results** — The level of osteogenic growth factors concentrations was higher in group with a-C-coatings in comparison to that in group with CN<sub>0.25</sub>-coated needles (BMP-2 on the 90<sup>th</sup> day, TGF-β1 on the 60<sup>th</sup> and 90<sup>th</sup> days), probably because of more pronounced cells and intercellular matrix elements adhesion to a-C-coatings. CN<sub>0.25</sub>-coatings demonstrated more pronounced activation of the bone resorption factor: TRAIL – a marker of apoptosis and a cytokine that promotes the activation of resorptive cells osteoclasts was higher in all observed periods.

**Conclusion** — Thus, the results of the study indicated that effect of nanostructured a-C and CN<sub>0.25</sub> coatings with various surface topography and chemical composition on the process of bone remodeling was different.

**Keywords:** nanostructured coatings of needles, immobilization osteoporosis, BMP-2, TGF-β, TRAIL.

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### Introduction

Hypokinesia is a stress factor for the body. In the absence of mechanical loading, the gravity vector of bone tissue changes, which has effect on bone remodeling [1, 2].

Already on the 30<sup>th</sup> day of immobilization the experiment showed changes in the metabolic activity of osteogenic cells, on the 90<sup>th</sup> day of immobilization osteoporotic changes of bone tissue were noted [3, 4].

The most effective method for patients' treatment with fractures and non-unions under immobilization osteoporosis (IOP) is transosseous osteosynthesis by G.A. Ilizarov (TOS) [5-7]. However, when standard metal fixators from medical steel were used, already on the 14 days after implantation an increase of dystrophic and sclerotic changes in the near-spine region of bone tissue were noted. These changes led to micro-mobility of the fixators [8, 9]. Therefore, it is important to search for the methods of improvement the osseointegrative properties of the fixators used for the TOS.

Currently, carbon nanostructured coatings of metal fixators, which possess osteoinductive properties, are actively investigated and applied in traumatology [10-12]. The research on the bone defects replacement with titanium implants covered by nanostructured coatings revealed a different effect of a-C and CN<sub>0.25</sub>-coatings, which vary in chemical composition, surface topography and the method of application, on the activity of osteogenic cells [13].

It is of interest to analyze the regulating bone tissue regeneration mechanisms in implantation of needles with a-C and CN<sub>0.25</sub>-coatings. Regeneration of bone tissue is a complex process. It is important role of growth factors and cytokines because they coordinate intercellular interactions, effect the differentiation, proliferation, and activity of bone cells [14].

Aim of study: comparing the dynamics of growth factors and cytokines, regulating bone tissue remodeling during implantation of needles with nanostructured carbon diamond-like a-C-coatings and carbon CN<sub>0.25</sub>-coatings, ligated by nitrogen atoms.

## Material and Methods

### *Characteristics and methods of obtaining needles with nanostructured coatings*

The author's development and manufacture of needles for experimental studies on animals was carried out by the employees of the Laboratory of Carbon Nanomaterials of MN Mikheev Institute of Metal Physics of the Ural Branch of the Russian Academy of Sciences (Yekaterinburg, Russia). Original nanostructured coatings (diamond-like a-C-membrane and composite CN<sub>0.25</sub>-membrane, doped with nitrogen atoms) were applied on the needles made of 12X18H9T steel with a given roughness and surface thickness. Coatings differ in chemical composition, electrical conductivity, number of sp<sup>3</sup>-bonds, surface structure. Membranes were deposited by the vacuum-plasma method in the UVNIPA-04 vacuum machine.

### Materials

The experiment was carried out on 64 male Wistar rats at the age of 3 months weighing 120-150 g. The conditions for handling, handling and withdrawal of animals were in accordance with the Council Directive of 24 November 1986 "On the approximation of laws, regulations and administrative provisions of EU member states on the protection of animals used for experimental and other scientific purposes".

### Design

Immobilization osteoporosis was modeled by amputation of the shin bones of the right hindlimb at in the proximal part (the idea of the IOP model on dogs is Professor SV Gyulnazarova's, 1985, revision of the model in rats by PhD A.Y. Kuchiev, 2008). The surgeries on animals were performed by PhD A.A. Ganzha.

On the 90<sup>th</sup> day of immobilization (by the moment of the formation of osteoporotic changes in bone tissue), the distal metaphysis of the right femoral and the proximal metaphysis of the right tibia were implanted with needles made of 12X18H9T steel with nanocarbon coatings deposited on them. The needles with CN<sub>0.25</sub>-coatings were implanted to animals of the 1<sup>st</sup> group (32 rats); needles with a-C-coating to animals of the 2<sup>nd</sup> group (32 rats). The ends of the needles were processed and immersed in soft tissues. All operations and removal of animals from the experiment were performed under intramuscular anesthesia Xyla Vet 0.05-0.10 ml/kg + Zoletil 1-2 mg/kg. To compare the effect of the coatings on bone remodeling the concentration of key osteogenesis activators – bone morphogenetic protein 2 (BMP-2), Transforming growth factor beta (TGF-β) as well as markers of bone resorption – TNF-related apoptosis-inducing ligand (TRAIL), bone-related degradation products from C-terminal telopeptides of type I collagen in rat (RatLaps) was determined on the 30<sup>th</sup>, 60<sup>th</sup> and 90<sup>th</sup> day after implantation.

### Methods

Determination of the concentrations of the examined parameters was carried out in the blood serum of animals by enzyme immunoassay according to the protocols attached to the kits using controls. We used sets for measure BMP-2, TRAIL of Cloud-Clone Corp. (USA); TGF-β1 of eBioscience (USA); RatLaps of Immunodiagnosystems (Great Britain). Organism species: *Rattus norvegicus* (Rat). The research was carried out on a Stat Fax 3200 analyzer (Awareness Technology, Inc., USA) in combination

with Stat Fax 2600 microplate washer (Medica, USA) and ELMI shaker (ELMI Ltd, Latvia).

Comparison of the groups was performed using the Mann-Whitney test for independent samples. The choice of the criterion is conditioned by the small sample power and the abnormal distribution of the indices (the normality of the distributions was estimated using the Pearson's chi-squared test  $\chi^2$ ). The results are presented as median with lower and upper quartiles – Me (LQ, UQ).

## Results

The dynamics of BMP-2 concentration in the blood serum of animals with various nanostructured coatings appeared to be multidirectional (*Table 1*). In the group with CN<sub>0.25</sub>-coatings (group 1) the concentration of BMP-2 has decreased to the 60<sup>th</sup> and to the 90<sup>th</sup> days, while in the group with the a-C-coatings (group 2) it has increased. On the 90<sup>th</sup> day in the group with a-C-coatings, the level of BMP-2 was significantly higher than in the group with CN<sub>0.25</sub>-coatings in 3 times.

The level of TGF-β1 turned out to be significantly higher in the group with a-C-coatings: on the 60<sup>th</sup> and 90<sup>th</sup> days in 1.6 times (*Table 2*). It's peak concentrations in the group with CN<sub>0.25</sub>-coatings were observed by the 30<sup>th</sup> day. By the 60<sup>th</sup> day the level of TGF-β1 has decreased in 1.9 times.

The positive dynamics of TRAIL was observed in the first group (with CN<sub>0.25</sub>-coatings) by the 90<sup>th</sup> day (*Table 3*). In the group with a-C-coatings, no such dynamics were observed. At all times of observation, the TRAIL level was higher in the group with CN<sub>0.25</sub>-coatings: on the 30<sup>th</sup> day in 1.5 times, on the 60<sup>th</sup> day in 1.9 times, on the 90<sup>th</sup> day – 5-fold.

Also, in analysing the data, a significant negative correlation between the TRAIL level and the bone matrix resorption marker (RatLaps) in the group with a-C-coatings of the needles was found. The Spearman correlation coefficient was -0.552 ( $p=0.009$ ). Correlation between TRAIL and RatLaps in the group with CN<sub>0.25</sub>-coatings was not obtained.

## Discussion

Transforming growth factor beta family plays an important role in reparative regeneration and bone tissue formation. Of these, BMP-2 and TGF-β1 are the key regulators of osteogenesis: they stimulate the differentiation and proliferation of mesenchymal stem cells into osteoblasts [15]. Therefore, higher concentration values of these growth factors in the 1<sup>st</sup> group probably reflect a more pronounced osteoinductive effect of a-C-coatings compared to CN<sub>0.25</sub>-coatings.

It is known that BMP-2 stimulates the synthesis of collagen type I, forming the bone matrix, by osteoblasts [16]. In connection with that, the BMP-2 dynamics obtained in the group with a-C-coatings consists the data provided previously (in the group with a-C-coating, the values of the concentration of type I collagen propeptides that are Collagen type I synthesis markers were higher at all terms after implantation) [17].

A crucial role in the bone remodeling regulation plays the TRAIL (apoptosis-inducing ligand). It is known that TRAIL can cause a programmed death of osteoblasts, interacting with death receptors DR4 and DR5 [18]. Therefore, the higher values of TRAIL in the group with CN<sub>0.25</sub>-coatings may indicate more pronounced activity of osteoblasts apoptosis in this group.

**Table 1. Dynamics of BMP-2 concentration in serum of animals, ng/ml**

Groups	30 <sup>th</sup> day after implantation	60 <sup>th</sup> day after implantation	90 <sup>th</sup> day after implantation
1 <sup>st</sup> group	553.90 (482.90, 625.03)	406.80 (323.80, 557.00)	285.90 (251.61, 366.79)**
2 <sup>nd</sup> group	455.30 (381.15, 589.43)	522.30 (225.80, 543.48)	856.60 (771.87, 1082.48)

\* – statistically significant difference (p<0.05) in relation to the 2<sup>nd</sup> group; + – statistically significant (p<0.01) difference in relation to the 30<sup>th</sup> day after the operation. The results are presented as Me (LQ, UQ).

**Table 2. Dynamics of TGF-β1 concentration in blood serum of animals, ng/ml**

Groups	30 <sup>th</sup> day after implantation	60 <sup>th</sup> day after implantation	90 <sup>th</sup> day after implantation
1 <sup>st</sup> group	36.04 (32.47, 39.41)	18.50 (17.26, 19.74)	20 (17.17, 23.91)
2 <sup>nd</sup> group	21.82 (18.57, 35.26)	29.44 (23.45, 35.69)*	31.73 (27.13, 36.32)*

\* – statistically significant difference (p<0.01) in relation to the 1<sup>st</sup> group. The results are presented as Me (LQ, UQ).

**Table 3. Dynamics of TRAIL concentration in serum of animals, ng/ml**

Group	30 <sup>th</sup> day after implantation	60 <sup>th</sup> day after implantation	90 <sup>th</sup> day after implantation
1 <sup>st</sup> group	1.82 (1.78, 1.83)*	1.77 (1.42, 1.98)*	2.15 (1.89, 2.25)*
2 <sup>nd</sup> group	1.25 (1.02, 1.41)	0.91 (0.86, 0.93)	0.37 (0.32, 1.10)

\* – statistically significant difference (p<0.01) in relation to the 2<sup>nd</sup> group. The results are presented as Me (LQ, UQ).

Moreover, TRAIL promotes bone resorption, also under a low bone mineral density. It is known that along with receptors of apoptosis, TRAIL can bind one of the main inhibitors of osteoclastogenesis – osteoprotegerin (OPG). It was also shown that in conditions of weightlessness, the enhancement of osteoclastogenesis is TRAIL-mediated [19, 20]. Thus, the significant negative correlation of the marker of bone resorption RatLaps and TRAIL obtained in the 2<sup>nd</sup> group (with a-C-coatings) (increase in the RatLaps concentration was accompanied by a decrease in the TRAIL level) suggests that when implanting needles with a-C-coatings TRAIL suppresses RANK-RANKL-mediated (*RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand*) osteoclastogenesis in the condition of osteoporosis, possibly through inhibition of phosphorylation of mitogen-activated protein kinase (MAPK) [21].

The results on the more significant osteoinductive properties of a-C-coatings in comparison with CN<sub>0.25</sub>-coatings are consistent with previous studies. In the experiment on the replacement of bone defects with titanium implants with nanostructured a-C and CN<sub>0.25</sub>-coatings applied on them, their various effects on the activity of osteogenic cells were revealed. Morphological data and values of concentrations of regulatory molecules indicated the best properties of a-C-coatings [22].

Previously performed morphological researches of bone tissue when implanting spokes with nanostructured carbon coatings in osteoporotic bone of rats revealed a significant decrease in the diameter of the spinal canal and a decrease in the thickness of the fibrous capsule when using a-C coatings compared to CN<sub>0.25</sub>-coatings [23], which also is consistent with the results of this study.

### Conclusion

The obtained results show differences in the influence of the needles with nanostructured (a-C and CN<sub>0.25</sub>) coatings with surface-differentiated topography and various chemical composition on the process of bone tissue remodeling. Thus, a-C-coatings, probably due to better adhesion to them of cells and elements of the intercellular matrix than to CN<sub>0.25</sub>-coatings, contributed to a more pronounced activation of osteogenic factors

(BMP-2 and TGF-β) and less pronounced degree activation of the bone tissue resorption factor (TRAIL).

**Conflict of interests:** none declared.

### Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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