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PŮVODNÍ PRÁCE

NiTi Alloys Exposure Alters miR-124 Expression in Physiological and Osteoarthritic Osteoblasts

Expozice NiTi slitinám mění expresi miR-124 u fyziologických a osteoartrotických osteoblastů

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ABSTRACT

PURPOSE OF THE STUDY
Nitinol (NiTi) is a biomaterial widely used in medicine based on super-elastic and shape memory properties. miR-124 has a key role in inflammatory process, osteoblasts differentiation, and mineralization. The aim of study was evaluating the differences in gene expression of miR-124 of human physiological osteoblasts (HOB) and human osteoarthritic osteoblasts (OSBA) as a response to NiTi alloy in different heat treatments.

MATERIAL AND METHODS
The cells were cultivated with NiTi discs with/without addition of bacterial lipopolysaccharide (LPS) for 72 hours. MicroRNAs were isolated, underwent reverse transcription and were analyzed by RT-PCR.

RESULTS
As a response to LPS, HOB overexpressed miR-124, while in OSBA expression change did not occur. Overexpression was also observed in both cell lines as a response to hydrogen and helium treated NiTi discs. HOB expressed significantly higher amount of miR-124 than OSBA as a response to hydrogen treatment of NiTi discs. In addition, hydrogen treatment caused significantly higher expression in HOB than LPS. The combination of NiTi disc and LPS treatment in HOB didn't cause any expression changes. Comparing to LPS-only treatment, the expression in HOB with combination of LPS and alloy was significantly lower. In OSBA, the expression was increased by the combination of LPS and hydrogen disc, in case of helium disc, the expression was decreased.

CONCLUSIONS
In conclusion, human physiological and osteoarthritic osteoblasts respond to NiTi alloy with both surface (hydrogen and helium atmosphere) treatment by overexpression of miR-124. The effect of LPS as inflammatory modulator suggests the presence of an "anti-inflammatory preconditioning" in osteoarthritic osteoblasts, as physiological osteoblasts overexpression was significantly higher.

Key words: nitinol, osteoblast, miR-124, lipopolysaccharide.

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INTRODUCTION

Nitinol (NiTi), is a biomaterial composed of nickel and titanium widely used as a joint replacement material because of its super-elastic properties. When the material is mechanically loaded, it deforms and gains a new state. After releasing the pressure, the new state is unstable, and it returns to the original state. Nitinol as a super-elastic material is a part of a larger family of shape memory alloys. Shape memory is the ability of a material to restore its original shape after being deformed and heated (15). Despite these beneficial assets of NiTi alloy, there are still concerns about biocompatibility of the material caused by the leakage of nickel ions, which are well known for their carcinogenic and cytotoxic effects (18).

Several publications have appeared in recent years documenting severely harmful effects of nickel ions. Ni^{2+} has an impact on intercellular adhesion molecules and the studies showed that the amount of adhesive molecules and cytoskeletal proteins, for instance, VE-cadherin and actin molecules were decreased in Ni^{2+} exposed endothelial cells suggesting disruption of the adherent junctional structures (11). Presence of the nickel ions has been linked to an increased level of oxidative stress within exposed cells. Analysis of the redox cycle of Ni^{2+} proposes that Ni^{2+} binds to intracellular ligands, such as imidazole nitrogen in a histidine molecule. That causes its conversion to Ni^{3+} and creates a hydrogen peroxide or other reactive oxygen species (ROS) (3). The increased amount of ROS, which are generated by an in-

teraction of nitinol and cell proteins, can alternate miRNA expression and result in pathological processes (12).

The surface of the material undergoes oxidation processes and creates a layer composed predominantly of titanium oxides. Numerous studies provided information about the toxicity of oxidation products of titanium. Titanium dioxide (TiO_2) nanoparticles released from the surface of joint replacement material are well known for causing double-strand breaks (10). They can also cause increased production of ROS, which are potentially harmful to cells (19).

ROS lead to an inflammatory response or cell death. In both of this occasion, miRNAs are released from the cells. Recent studies indicate that several miRNAs are participating in physiological and pathological processes including inflammatory response (20). miRNAs are short (±22 bp long) non-coding RNA, which are participating on a gene expression regulation. There are several ways to achieve gene regulation by miRNA. miRNA can bind to mRNA with an absolute complementarity and tags mRNA for degradation. If miRNA binds incompletely it causes repression of a translation process (1). The importance of a regulatory role of miRNA is potentiated with the fact that there are approximately 3 700 miRNAs in human cells targeting the expression of numerous gene (8).

miR-124 is an inductor of autophagy-related cell death via targeting signaling cascade of EZH2-STAT3 (9). The role of this miRNA in the inflammatory process was described in retinal inflammation, where the overexpression of miRNA was associated with retinal inflammation and photoreceptors death (2). On the contrary, the overexpression of miR-124 in the pathophysiology of Parkinson's disease leads to inhibition of neuroinflammation by regulation of MEKK3/NF-κB pathway (24). The same effect is observed in traumatic brain injury in microglia (5).

Regarding the role in the osteoblasts, miR-124 is a regulator of mesenchymal stem cells (MSC) differentiation, as osteoblasts are derived from MSC. It is one of the key miRNAs of an adipocyte and an osteoblast differentiation process and also plays a role in mineralization of bone matrix and an osteoblast markers expression (13). This miRNA is also involved in cell response to a lipopolysaccharide (LPS) presence (14) (Fig. 1) and a part of Wnt/β-catenin pathway (4).

Our study is focused on the expression changes of miR-124 in physiological and arthritic osteoblasts as a response to nitinol presence and to determine the involvement of miR-124 in the inflammatory process of physiological and osteoarthritic osteoblasts. The expression changes of these miRNA were observed in nitinol-miRNA and LPS-miRNA interactions.

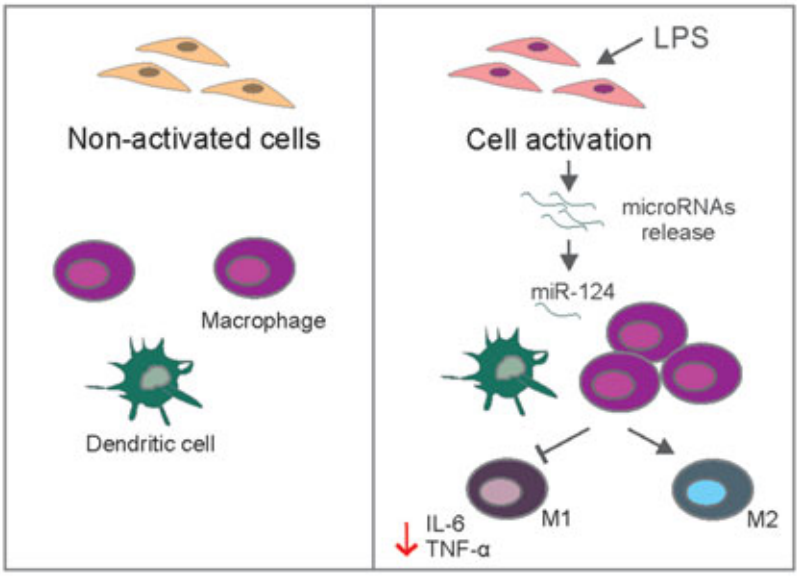

Fig. 1. miR-124 mediates the anti-inflammatory action through targeting STAT3 and TACE. LPS-stimulation upregulates miR-124 expression, which leads to polarization of macrophages to M2 and inhibition of pro-inflammatory M1. STAT3 is critical for IL-6 production in response to LPS. Over-expression of miR-124 results in the reduction of IL-6 at the transcription level and modulation of TNF-α maturation.

MATERIAL AND METHODS

Alloys preparation

Shape memory alloys composed of nickel and titanium with stoichiometric Ni50Ti50 compositions and different surface modifications (hydrogen or helium) were used in this study. The NiTi in a shape of rod contained fully recrystallized bars made from grain of a size ranging from 20 to 40 μm with a homogenous crystalline structure. The rods were cut into 20 mm wide oval-shaped segments, followed by a mechanical burnish of their surface with 600 grit finish. Burnished segments were cleaned in ethanol and distilled water to remove any possible surface contamination and underwent a pressure air drying process. Separate segments were enclosed in quartz capsules containing pure hydrogen or pure helium with a total pressure of 950 mbar and annealed at 860 °C for 30 minutes and then smothered in ice water. Subsequently, the capsules were broken, and the ovals were then shaped into round circle discs with a thickness of 1.5 mm and diameter of 12 mm. As a last step of preparation, the entire surface of the disc was polished with SiC papers with grit sizes ranging from 220 to 800.

Cell culture

This study contains experiments with human osteoblasts (Sigma-Aldrich Co., St Louis, USA) and human osteoblasts-osteoarthritis (Sigma-Aldrich Co., St. Louis, USA). The physiological osteoblasts isolated from adult bones provide a reliable model for research focused on a human skeletal system, whereas osteoarthritic osteoblasts (osteoblasts that had undergone inflammatory response) provide an excellent model to study the patho-

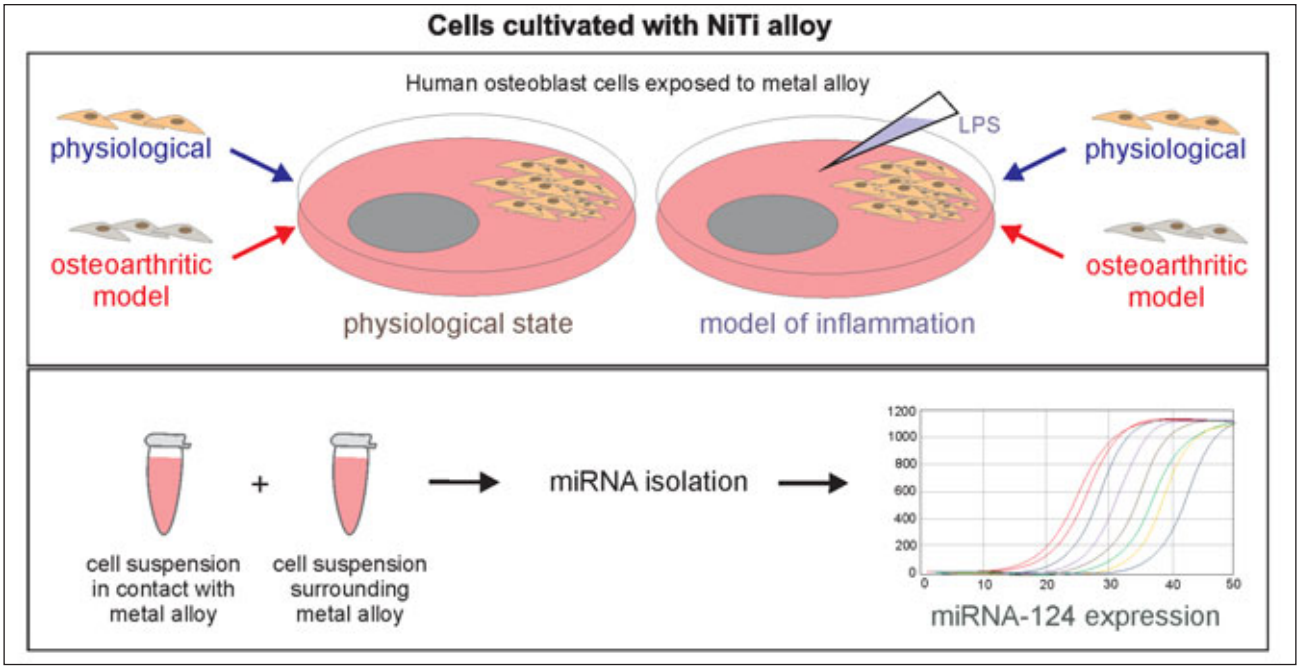

Fig. 2. Human physiological and osteoarthritic osteoblast cells exposed to NiTi alloy in presence or absence of LPS-stimulation (as a model of inflammation).

physiology of osteoarthritis *in vitro*, as they were isolated from the bone of patients with this disease.

Physiological and osteoarthritic osteoblasts were cultured in Dulbecco's Modified Eagle's medium/Ham's Nutrient Mixture F12 (DME/F-12; GE Healthcare) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, USA) and 10 mg/ml penicillin/streptomycin (Sigma-Aldrich Co., St. Louis, USA) and incubated in 5% CO_2 humidified atmosphere. Cells between the fifth and eight passage were used in this experiment. The cell medium was changed every 2–3 days until the required amount of confluence was reached. Cells with a density of 10,000 cells/cm^2 were then seeded in 6-well culture plates containing 2.5 ml of DMEM media. The conditions mentioned above were maintained for the whole duration of the experiment. The metal discs with different surface treatment (hydrogen or helium) were placed inside the wells. Then a fresh medium was added with or without LPS addition with concentration 1 µl of LPS per 1 ml of fresh medium to simulate inflammatory conditions (Fig. 2). Cell lines were incubated for another 72 hours. After this period, the medium was removed, and cells were washed with Hank's Balanced Salt Solution (Sigma-Aldrich Co., St. Louis, USA) to remove dead cells particles and collected into the tubes used for RNA isolation filled with 300 µl of TriPure Isolation Reagent (Roche, Basel, Switzerland).

Gene expression analysis

Using TriPure Isolation Reagent (Roche, Basel, Switzerland), RNA from each sample was isolated. The protocol for the isolation process was provided by the manufacturer. The purity of the samples was validated as absorbance of RNA, which was measured by a spectrophotometer at the wavelengths of 260 nm and 280 nm and absorbance ratio 260:280 nm was counted. The spectrophotometer was also used for the measurement of a concentration of RNA in samples.

The isolation of RNA samples was followed by transcription into a complementary DNA (cDNA). The isolated RNA samples were diluted into a concentration of 100 mg/ml of distilled water, then underwent the reverse transcription using First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) with manual provided by the manufacturer.

The final product served as the primary material for a gene expression analysis. RT-PCR was performed in analysis with DEPC water, isolated cDNA, TaqMan Universal MasterMix no UNG (Applied Biosystems), and TaqMan Gene Expression Assays (Applied Biosystems) in a final reaction volume of 20 µl in triplicates. miR-124 primer miR-124 (PN4427975) and housekeep control, RNU6B (PN4427975) were used.

Data analysis

The expression levels were analysed via $2^{-\Delta\Delta Ct}$ method; where ΔΔCT = [(CT target – CT housekeep) sample – (CT target – CT housekeep) control]. Values are expressed as median and 5th and 95th percentile. Mann-Whitney U test was used for the evaluation of miRNA expression levels differences. Statistica v. 10.0 (Statsoft Inc., Tulsa, OK) was used to analyze the data. The values with $P<0.05$ were considered significant.

RESULTS

Physiological state and LPS treatment without contact with metal

The median values of miR-124 expression are listed in Table 1 and shown in Figure 3. The base expression

Table 1. Relative gene expression of miR-124

	HOB*	OSBA*
H	62.25 (58.89; 64.89)	3.25 (1.05; 8.06)
H + LPS	1.22 (1.02; 2.89)	2.91 (2.75; 4.84)
He	12.73 (2.19; 15.24)	6.64 (5.10; 13.87)
He + LPS	0.56 (0.20; 0.70)	0.50 (0.26; 0.94)
LPS	20.23 (3.25; 30.91)	1.32 (0.45; 2.90)

*median (5^{th} percentile; 95^{th} percentile); HOB – human physiological osteoblasts, OSBA – human osteoarthritic osteoblasts, H – hydrogen, He – helium, LPS – bacterial lipopolysaccharide from *E. coli*

in physiological osteoblasts (HOB) was estimated higher compared to arthritic osteoblasts (OSBA).

Compared to control cells, the significant overexpression of miR-124 was detected in HOB cultivated with the addition of LPS ($p<0.01$; R=20.23). On the other hand, there was no significant change in miR-124 expression in osteoarthiritic samples in presence of LPS (R=1.32) (Table 1). The response to the addition of the LPS increased significantly the production of miR-124 in the normal compared to osteoarthiritic osteoblasts ($p<0.05$) (Table 2).

Response to NiTi alloys

In normal human osteoblasts, an increase in expression of miR-124 was detected in cell samples cultivated for 72 hours in a presence of NiTi discs. Comparing the expression changes, the hydrogen surface treatment led to a significant overexpression of miR-124 compared to helium surface treatment ($p<0.05$; R=62.25 vs. R=12.73) (Table 1 and 2).

In osteoarthritic samples, the presence of NiTi discs with the hydrogen and the helium surface treatment caused increase in miRNA expression (R=3.25 vs. R=6.64) (Table 1). However, there was no significant difference observed between these two expressions.

Comparing the normal and the osteoarthritic samples cultivated with NiTi discs, the results suggest that the normal osteoblasts expressed significantly higher amount of miR-124 in the presence of metal with hydrogen surface treatment ($p<0.05$; R=62.25 vs. R=3.25) (Table 2). However, the helium surface treatment did not cause any significant difference in expression.

Response to NiTi alloys and LPS

In the presence of NiTi disc and LPS, the expression changes were not detected in normal osteoblast samples cultivated with either hydrogen or helium surface treatment. In all cases of osteoarthiritic osteoblasts, in the presence of both treatments, only trends in changes of miR-124 expression were observed (Table 1).

The additional effect of the presence of NiTi alloy to LPS in normal osteoblasts caused downregulation of miR-124 expression in both treatments – hydrogen NiTi alloy samples ($p<0.05$; LPS + H, R=1.22 vs. LPS, R=20.23), as well as in case of helium NiTi alloy samples ($p<0.05$; LPS + He, R=0.56 vs. LPS, R=20.23) (Table 2). However, this effect was not observed in OSBA. The decrease in expression in combined model was proved also comparing NiTi alloy to NiTi alloy with LPS in normal osteoblasts. The downregulation of expression was detected in hydrogen heat treated NiTi ($p<0.05$; H, R=62.25 vs. LPS + H, R=1.22) (Table 2).

DISCUSSION

Our study was focused on miR-124 interaction with the LPS and the NiTi alloy discs with different surface modification, as this miRNA has a regulatory role in the osteoblasts' behavior. Regulatory role of miRNA in osteoblasts life cycle has already been presented in several studies (7, 13). The expression levels in mature osteoblasts in contact with metal alloy, as to our best knowledge, have not been studied yet.

Our results showed that physiological and osteoarthritic osteoblasts both overexpress miR-124 as a response to LPS presence in the medium. These findings correspond with previous studies (21). However,

Table 2. Comparison of miR-124 expressions among the cells cultivated in surrounding of NiTi alloy; A) HOB – human physiological osteoblasts; B) OSBA – human osteoarthritic osteoblasts; C) HOB vs. OSBA – comparing physiological and osteoarthritic osteoblasts

A

HOB treatment1 vs. treatment2		P*
LPS	H	**<0.05**
LPS	He	0.37
H	He	**<0.05**
LPS H	LPS	**<0.05**
LPS H	H	**<0.05**
LPS He	LPS	**<0.05**
LPS He	He	0.07
LPS H	LPS He	0.09

B

OSBA treatment1 vs. treatment2		P*
LPS	H	0.06
LPS	He	0.17
H	He	0.38
LPS H	LPS	0.19
LPS H	H	0.81
LPS He	LPS	0.63
LPS He	He	0.07
LPS H	LPS He	0.07

C

HOB vs. OSBA treatment1 vs. treatment2		P*
LPS	LPS	**<0.05**
H	H	**<0.05**
He	He	0.46
LPS H	LPS H	0.65
LPS He	LPS He	0.42

* Mann-Whitney U test; values with $P < 0.05$ were considered significant; HOB – human physiological osteoblasts, OSBA – human osteoarthritic osteoblasts, H – hydrogen, He – helium, LPS – bacterial lipopolysaccharide from *E. coli*

this miR-124 reaction to LPS contradicts to the pathophysiology of the inflammatory response. During this process, miR-124 downregulates expression of the LPS binding partner TLR-4 (14) responsible for cell reaction. This reaction occurs via TLR-4/STAT3 interaction (25), where the downregulation of TLR-4 leads to the downregulation of STAT3 resulting in a lower expression of IL-6 (20). Based on our previous results IL-6 expression was not significantly changed in the presence of LPS in both cell lines (22). The other role of miR-124 in inflammatory response is the negative regulation of TNF-α expression (21) (Fig. 1).

Connection of miR-124 with inflammation mentioned above, could explain also the different behaviour of osteoblasts to LPS treatment. LPS in the medium caused physiological osteoblasts to express more miR-124 than osteoarthritic osteoblasts, which may be explained by the fact, that osteoarthritic osteoblasts had already undergone the inflammatory process and may have adapted to it – anti-inflammatory preconditioning of osteoblasts.

Hydrogen discs caused significantly higher miR-124 expression in physiological osteoblasts than in osteoarthritic osteoblasts. Even though, the samples pre-treated in the controlled atmosphere of hydrogen exhibit lower release of Ni ions compared to helium pre-treatment. Our previous data suggest that lower Ni-ion released rates are likely based on passivation of NiTi surface layers by an influx of hydrogen and can contribute to the stabilization of the surface and sub-surface layers (17). But the hydrogen time-related release and reaction processes are studied. Iuchi et al. described out that molecular hydrogen regulates gene expression by oxidation processes (6). In addition, titanium oxides, that forms a passivation layer on the surface of material used in this study (17), are also causing increased production of reactive oxygen species with a harmful effect on the cells (19). Numerous studies regarding the mechanism of this process suggest an involvement of the nuclear factor-E2-related factor signaling pathway. This pathway is responsible for the increased amount of ROS (23). Other suggest decreased enzyme activity of the glutathione peroxidase and the superoxide dismutase (26). Further studies confirmed that titanium oxide induces the activity of alkaline phosphatase (ALP) (16). On the contrary, miR-124 is the negative regulator of ALP gene expression (13).

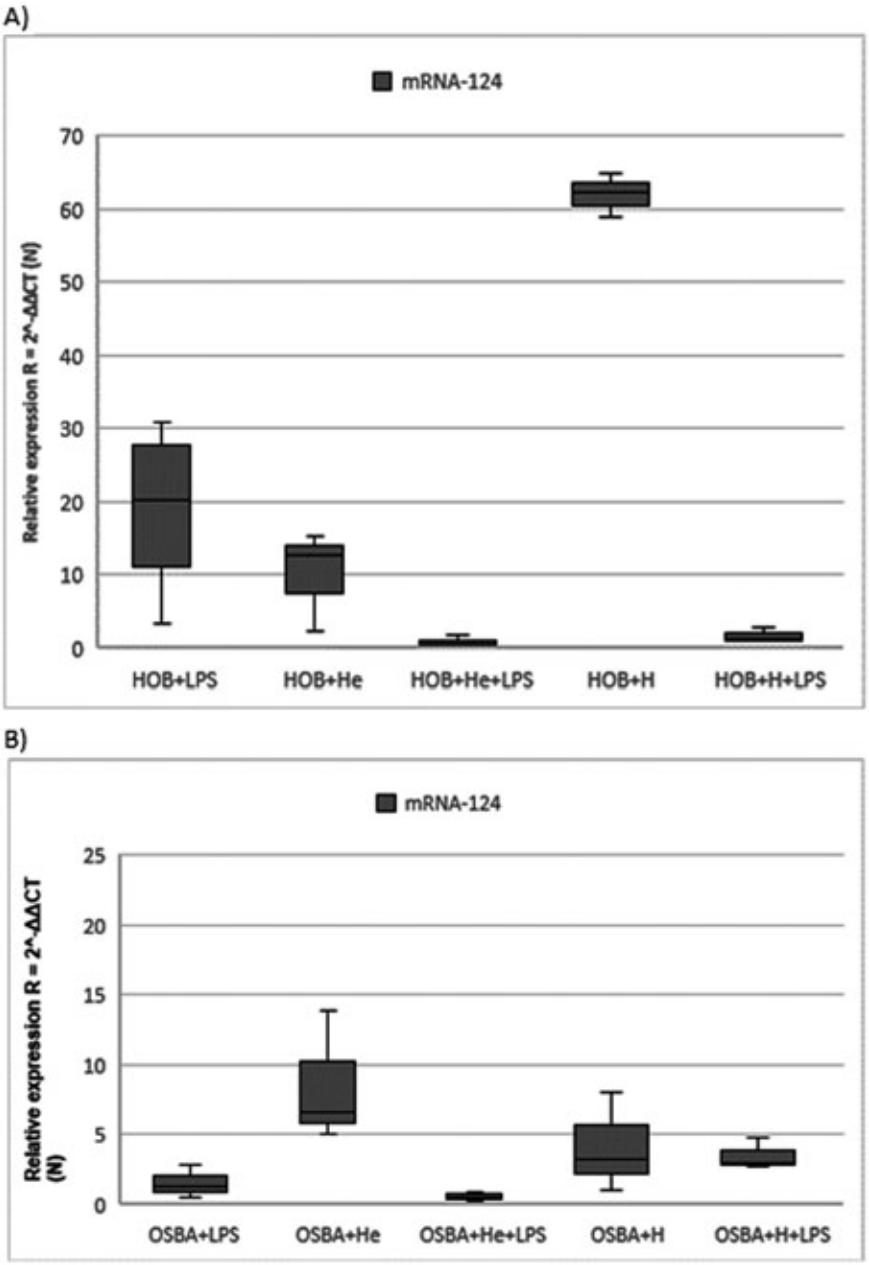

Fig. 3. Comparison of relative expressions of miR-124 in presence of LPS and/or metal alloys A) in human physiological osteoblasts (HOB); B) in human osteoarthritic osteoblasts (OSBA)

Footnote: HOB – human physiological osteoblasts, OSBA – human osteoarthritic osteoblasts, H – hydrogen treated alloy, He – helium treated alloy treated alloy, LPS – bacterial lipopolysaccharide from *E. coli*

In case of physiological osteoblasts, comparing medium in LPS to a combination of alloy and LPS (hydrogen/LPS or helium/LPS), the combination caused significantly lower expression in miR-124 suggesting interference of LPS and alloys in both, hydrogen and helium cases.

In conclusion, our results strongly suggest that miR-124 overexpression is caused by inflammation and NiTi alloys. Considering that inflammation is a natural response of a body to any harm, and a joint replacement

surgery causes this response, we should further research on the potential effect of miR-124 overexpression and its impact on the cells. Overexpression of miR-124 causes decreased expression of osteoblastic markers Dlx5, Dlx3, Dlx2 and decreases the activity of alkaline phosphatase and therefore the mineralization of a bone matrix (13). Furthermore, our results suggest, that comparing hydrogen treated samples with LPS to helium treated samples with LPS, there is no significant difference between the type of surface treatment of alloy.

CONCLUSIONS

- NiTi alloys with hydrogen and helium surface treatment cause miR-124 overexpression in human physiological and osteoarthritic osteoblasts.
- The highest overexpression was observed in NiTi alloys with hydrogen surface treatment.
- Our results suggest the existence of "anti-inflammatory preconditioning" in osteoblasts, as physiological osteoblasts overexpress a significantly higher amount of miR-124 than osteoarthritic osteoblasts as a response to LPS.
- The combination of NiTi alloy and LPS has no effect on miR-124 expression in physiological osteoblasts.

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