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Expression of metalloproteinase 2 (MMP-2) and metalloproteinase 9 (MMP-9) in THP-1 macrophages cultured with three-dimensional titanium mini-plate systems used for surgical treatment of condylar fractures

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Osteosynthesis with the use of three-dimensional (3D) titanium mini-plate systems in the treatment of condylar fractures is a technique commonly used by maxillofacial surgeons. It is increasingly often mentioned in the literature, especially in the context of bone regeneration. The break in tissue continuity associated with this technique causes activation of pro-inflammatory responses mediated by matrix metalloproteinases MMP-2 and MMP-9, enzymes which are also involved in the subsequent bone remodelling. This study showed that the use of 3D titanium mini-plates did not alter the expression of these enzymes in THP-1 macrophages.

Key words: three-dimensional (3D) titanium mini-plate systems, condylar fracture; inflammation, macrophages, metalloproteinase 2 (MMP-2), metalloproteinase 9 (MMP-9)

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Abbreviations: 3D titanium mini-plates, three-dimensional titanium mini-plates; CAMs, cell adhesion molecules; ECM, extracellular matrix; MMP-2, metalloproteinase 2; MMP-9, metalloproteinase 9; TIMPs, tissue inhibitors of matrix metalloproteinases

INTRODUCTION

Three-dimensional (3D) titanium mini-plate systems were first presented by M. Farmand in 1992 and are still treated as relatively new (Farmand & Dupoirieux 1992; Kinra *et al.,* 2017; de Oliveira *et al.,* 2018). The three-dimensionality is understood as their ability to make the stabilized bone elements resistant to twisting, shearing, and bending (de Oliveira *et al.,* 2018). This increased stabilization leads to a reduced infection rate following surgery (Sikora *et al.,* 2018). They also present improved ease of application when compared to the other implants, and, therefore, a shorter duration of surgery (Farmand & Dupoirieux, 1992).

Implantation of a 3D plate helps to avoid the risks associated with screw loosening in cases of implantation of a single plate, and eliminates the difficulties associated with fixing two plates to the operating field with at least six screws (Cortelazzi *et al.,* 2015; Hakim *et al.,* 2014; Kozakiewicz & Swiniarski, 2014; Sikora *et al.,* 2016).

Given the above, 3D titanium mini-plates are increasingly popular products used in the treatment of maxillofacial trauma to stabilize condylar fractures (Motamedi *et al.,* 2014). They ensure segment stability, facilitate recovery of the operated areas, and increase patient comfort (De Melo *et al.,* 2012). There are several different types of 3D plates used for osteosynthesis of mandibular condylar fractures (Cortelazzi *et al.,* 2015; Hakim *et al.,* 2014; Kozakiewicz & Swiniarski, 2014; Sikora *et al.,* 2016). In recent years, the technique of 3D plate usage has been significantly improved by the new technologies, such as computer-aided design/computer-aided manufacture (CAD/CAM) of wafers and three-dimensional (3D) printing (Cornelius *et al.,* 2015; Sugahara *et al.,* 2018). However, the most important condition determining the effectiveness of this type of treatment is the elimination or reduction of the inflammation that results from the activation of the immune system. It is equally important that the applied 3D titanium mini-plates do not interfere with natural regenerative processes following a break in tissue continuity.

Physiological and pathological bone metabolism (e.g. in osteoporosis) (Boyce *et al.,* 2009; Paiva & Granjeiro, 2017) significantly depends on the proteolysis of the extracellular matrix (ECM) by osteoclasts, large multi-nuclear cells of the myeloid line which attach themselves with pseudopodia to the sites intended for resorption (Datta *et al.,* 2008; Witten & Huysseune, 2009). The reconstruction of the bone matrix depends on matrix metalloproteinases (MMPs), enzymes involved in bone remodelling, and synthesized by osteoclasts and other cells, such as fibroblasts and macrophages (Cawston & Wilson, 2006; Lamort *et al.,* 2016).

MMPs are proteins belonging to the family of enzymes including gelatinases, collagenases, stromelysins, matrilysins, secretory MMPs, archetypal MMPs and membrane type I and II MMPs (Nagase *et al.,* 2006; Paiva & Granjeiro, 2017). MMPs are classified as extracellular enzymes, among which more than 20 subtypes have been identified so far, each encoded by a different gene. Metalloproteinases are assigned to one group of enzymes due to homologous sequences and thus similar structure and enzymatic activity (Lohi *et al.,* 2001; Woessner & Nagase, 2000). Most of them are excreted out of the cells, some are membrane-bound; they are regarded as endopeptidases and require zinc ions for their enzymatic activity (Woessner & Nagase, 2000).

ECM components are the main substrates for metalloproteinases, and a structural change of the matrix is the effect of MMP-induced catalysis. MMPs also have other functions, such as regulation of cell activity or participa-

tion in inflammatory reactions. Many peptides released during partial ECM proteolysis may affect cell activity, but metalloproteinases, in addition to ECM digestion, are also responsible for the catalysis of other biologically active substances, such as growth factors or cell adhesion molecules (CAMs) (McCawley & Matrisian, 2001). In this way, MMPs play an important role in the regulation of processes such as cell differentiation and migration, regulation of growth factors, angiogenesis and development of inflammation. MMPs also influence cell survival or apoptosis and intercellular communication (Parks *et al.,* 2004; Sternlicht & Werb, 2001). Metalloproteinases are synthesized in an inactive form and gain catalytic capacity by cleaving a specific part of a precursor enzyme (zymogen) (Yong *et al.,* 1998). Activated metalloproteinases are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs), which bind them non-covalently. So far, four types of TIMP have been identified (TIMP1-4) (Dzwonek *et al.,* 2004). Regulation of the expression and activity of metalloproteinases is an extremely complex process associated with their potential ability to cause massive degradation of tissues (Overall & Lopez-Otin, 2002).

The activity and expression of MMPs are controlled mainly at three levels: (1) through the action of TIMP (serum inhibition of MMPs *via* α-2-macroglobulin is also possible); (2) through activation of the proenzyme – initially MMPs are synthesized as inactive zymogens – and (3) through regulation at the gene transcription level (in many cases metalloproteinases can only be expressed through cell activation or simulation). The expression of MMP genes can be stimulated by increased concentrations of growth factors, phorbol esters, oncogene products, and cell–to–cell and cell–to–ECM interactions (Wright & Harding, 2009). In addition to these three main mechanisms, there are also additional mechanisms regulating the activity of the selected MMP, such as internalization, post-transcriptional modifications (glycosylation in the case of MMP-9), storage (e.g. MMP-9) in granules of neutrophil granulocytes, as well as the compartmentalization and availability of substrates (Yong, 2005).

There is a number of factors activating MMPs (including plasmin, thrombin, interaction of different MMPs) which stimulate inactive proenzyme to autocatalysis, consisting of breaking the bond between the rest of the cysteine and the zinc atom within the enzyme, as a result of which the enzyme gains the full catalytic capacity (Overall, 2002).

The gelatinases examined in this study, i.e. MMP-2 and MMP-9, have a specific structure. They contain the N-terminal signal peptide removed immediately after synthesis in the endoplasmic reticulum, and three characteristic tandem repeats in the catalytic domain, thanks to which these enzymes can bind to elastin and collagen, and then carry out their proteolytic decomposition (Sternlicht & Werb, 2001). Pro-MMP-2 forms a complex of membrane metalloproteinase MT1-MMP (MMP14) and TIMP2, which enables activation of this membrane enzyme on the cell surface and effective proteolysis. Pro-MMP-2 can also be bound via the α2β1 integrin (Dumin *et al.,* 2001). Similarly, the active form of MMP-9 is combined with hyaluronate receptor CD44. Some MMPs can also bind to the cell surface via heparin sulphate chains of membrane proteoglycans, thus catalyzing proteolytic reactions on the cell surface (Yu *et al.,* 2002).

Inflammation is a local or systemic reaction of the body to factors that damaged cells and tissues. In other words, it is an unspecific response to homeostasis imbalance (Nowak, 2010). The main effects of the inflammatory reaction include increased blood flow at the site of the irritant's action, higher permeability of capillaries and inflow of leukocytes to the site of damage or infection (Jin *et al.,* 2009). The inflammatory response can also be divided based on the mechanism of reaction into cellular and humoral response. The key cells involved in the cellular response are neutrophilic granulocytes, monocytes, lymphocytes, basophils, eosinophils and platelets belonging to the protein-cellular system. An important role is also played by macrophages, mast cells and fibroblasts (belonging to the pool of connective tissue cells) and endothelial cells. Leukocytes are the first to migrate to the damaged tissue. Then, as a result of the action of certain factors, they are activated, which is followed by their firm adhesion (Kelly *et al.,* 2007). During activation they begin to produce a number of pro-inflammatory substances which aim at eliminating the potential threat to the body. These include interleukins (IL-1 alpha/beta, IL-6, IL-8) and TNF-α. Monocytes and granulocytes move from the circulation to the damaged tissues where they transform into phagocytes, i.e. macrophages and microphages, respectively. Then, they phagocytise either microorganisms or apoptotic corpuscles, depending on the aetiology of the inflammation (Kelly *et al.,* 2007).

After eliminating the inflammatory factor, inflammation is cancelled via two mechanisms. The first is the decomposition of the pro-inflammatory factors and discontinuation of the production of new ones (e.g. lowering the activity of kinases involved in the synthesis of pro-inflammatory kinins). The second is the appearance of substances with anti-inflammatory effects. These include TGF-beta, IL-10 and IL-4, which belong to the group of cytokines, as well as derivatives of polyunsaturated fatty acids such as resolvins, protectins, maresins and lipoxins, which seem to have gained significance in inflammation research in recent years (Nowak, 2010).

A number of reports showed that implantation of biomaterials may cause a release of proinflammatory factors such as cytokines and chemokines and cause activation of the complement system, which adversely affects the host-implant interaction (Refai *et al.,* 2004, Campoccia *et al.,* 2006, Fukano *et al.,* 2006, Suwarsa *et al.,* 2017, Wang *et al.,* 2018, Pettersson *et al.,* 2017). However, in our previous paper (Sikora *et al.,* 2017) 3D titanium mini-plates seemed to be a good alternative to traditional plates, not only due to their desirable technical parameters but also because they did not stimulate the cyclooxygenase-dependent production of prostanoids (prostaglandin E2 and thromboxane B2) in THP-1 monocytes/macrophages, thus limiting the development of inflammation.

Considering a large number of procedures conducted by maxillofacial surgeons using 3D titanium mini-plates in condylar fractures, it seems reasonable to examine whether the plates affect the expression of metalloproteinases involved in the modulation of inflammation and regenerative processes in the bones and soft tissues. The results of such research may improve the assessment of the risk of complications associated with this technique.

MATERIALS AND METHODS

Reagents. THP-1 monocytic cells were obtained from American Type Culture Collection (ATCC, Rockville, USA). RPMI medium, glutamine, and antibiotics (penicillin and streptomycin), phosphate buffered saline (PBS),

Table 1. Detailed manufacturer data on the plates and screws used for testing.

	Element [% weight]								
	Fe	O	N	C	H	Al	V	other	Ti
Ti grade 2 (ASTMF 67:2000)	0.3	0.25	0.03	0.08	0.0125				rest
Ti6Al4V ELI grade5 (ASTM F136:2002; ISO5832-3) (TAV)	0.25	0.13	0.05	0.08	0.012	5.5–6.5	3.5–4.5		rest
Ti6Al7Nb (ASTM F1295; ISO:5832-11) (TAN)	0.25	0.20	0.05	0.08	0.009	5.5–6.5		Nb 6.5–7.5 Ta max. 0.5	rest

phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Aldrich (Poznań, Poland). Fetal bovine serum was purchased from Gibco (Gibco, Paisley, UK). Metalloproteinase 2 (MMP-2) EIA Kit and metalloproteinase 9 (MMP-9) EIA Kit were purchased from Cayman, USA; Micro BCA Protein Assay kit was purchased from Thermo Scientific, USA.

3D condylar titanium plates. Sets consisting of 3D titanium condylar plates and dedicated screws were obtained from three manufacturers. Plates obtained from DePuySynthes (manufacturer no. 1) were made of pure titanium TiCP – DIN ISO 5832-2, with screws made of titanium alloy (TAN) – DIN 5832-11. Plates manufactured by KLS Martin (manufacturer no. 2) were made of pure titanium – DIN-ISO 5832-2, DIN 17850 and ASTM F 67, with screws of titanium alloy (Ti-6Al-4V) – DIN ISO 5832-3, DIN 17851 and ASTM F 136a. Plates provided by Medartis (manufacturer no. 3) were made of pure titanium ASTM 67, ISO 5832-2, with screws made of titanium alloy ASTM F136, ISO 5832-2. Detailed manufacturer data on the plates and screws used for testing are provided in Table 1. Data on 3D condylar titanium plates and dedicated screws, along with the assigned symbols, are presented in Table 2.

Cell culture and treatment. The experiments were conducted using human macrophages derived from THP-1 cells. Cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma, St. Louis, MO), supplemented with 100 IU/ml penicillin and 10 μg/ml streptomycin (Life Technologies, Inc., GrandIsland, NY) in the presence of 10% heat-inactivated fetal bovine serum (FBS, LifeTechnologies). The cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 and the media were refreshed every 48 hours.

Table 2. 3D condylar titanium plates and dedicated screws.

Item	Manufacturer	Catalog number	Symbol
Plate	SYNTHES	04.503.834	S1
Plate	SYNTHES	04.503.833	S2
Screw	SYNTHES	04.503.406.01C	S3
Screw	KLS MARTIN	25-872-09	C1
Plate	KLS MARTIN	25-283-05	C2
Plate	KLS MARTIN	25-285-05	C3
Screw	KLS MARTIN	25-882-09	C4
Screw	MEDARTIS	M-5240.06	M1
Screw	MEDARTIS	M-5245.06	M2
Plate	MEDARTIS	M-4318	M3
Plate	MEDARTIS	M-4852	M4
Plate	MEDARTIS	M-4894	M5
Plate	MEDARTIS	M-4658	M6

Before the experiment, THP-1 cells were plated onto culture flasks at an initial density of 2.5×10^5 cells/well (Corning, Cambridge, MA). The differentiation of THP-1 cells into macrophages was achieved by administration of 100 nM PMA for 24 h (28).

Plate-Induced Inflammatory Reaction in Macrophages. THP-1 macrophages were cultured for 24 h and 48 h in RPMI medium with 10% FBS with the 3D condylar plates and screws used for surgical treatment of patients with condylar fractures. Plates and the suitable screws were obtained from three different manufacturers: (1) manufacturer no. 1 (S1, S2, S3); (2) manufacturer no. 2 (C1, C2, C3, C4); (3) manufacturer no. 3 (M1, M2, M3, M4, M5, M6); for details see Table 1. Control cells were incubated in RPMI medium with 10% FBS. After incubation, the cells were harvested by scraping and the pellets were obtained by centrifugation (800 x G, 10 min). Afterwards, cool PBS was added to the pellets and the samples were stored at -80°C until further analyses. The measurement of protein concentration was conducted using Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). The remaining supernatants were placed in new tubes and stored at –80°C until further analyses, i.e. the extraction and measurement of MMP-2 and MMP-9 expression by ELISA method.

The determination of MMP-2 concentration. MMP-2 expression was determined with a commercially available quantitative ELISA test (Thermo Fisher Scientific, MMP-2 Human ELISA Kit, KHC3081) according to the manufacturer's instructions.

The determination of MMP-9 concentration. MMP-9 expression was determined with a commercially available quantitative ELISA test (Thermo Fisher Scientific, MMP-9 Human ELISA Kit, BMS2016-2) according to the manufacturer's instructions.

Protein concentration. Protein concentration was measured with MicroBCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) using a spectrophotometer (UVM340, ASYS).

Immunocytochemical staining. The immunohistochemistry was performed using specific primary antibodies, mouse anti-MMP 2 and mouse anti-MMP 9 (overnight incubation at 4°C) and a secondary antibody conjugated with fluorochrome: anti-mouse IgG-FITC (incubation for 45 min at room temperature). The nuclei of cells were stained with DAPI. Image acquisition was performed with a fluorescent microscope using filters: 38 HE GFP for green fluorescence and 49 DAPI for blue fluorescence.

Statistical analysis. Statistical analysis of the obtained results was conducted using Statistica 10 software (Statsoft, Poland). The results were expressed as arithmetical means ± standard deviation (S.D.). The distribution of variables was evaluated using the Shapiro-Wilk W-test.

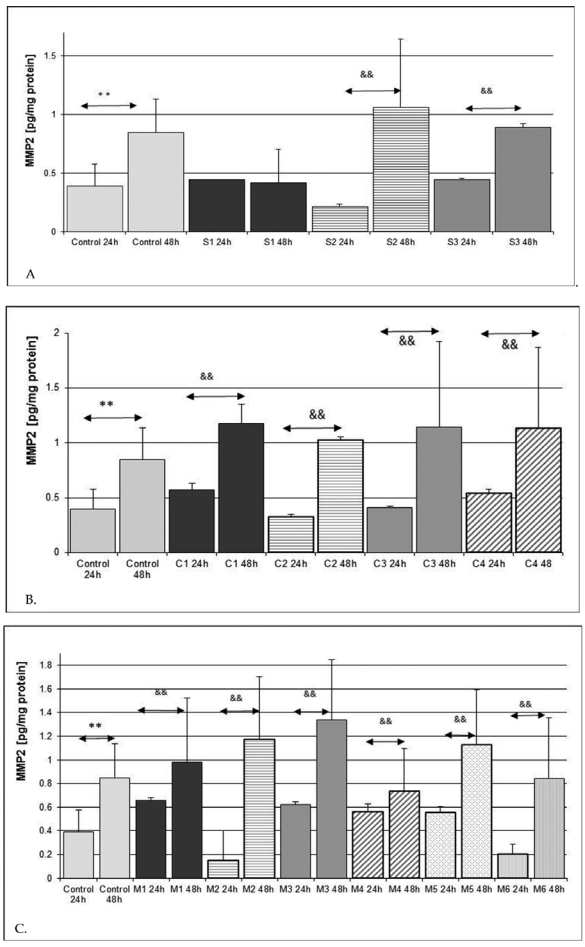

Figure 1. Expression of metalloproteinase 2 (MMP-2) in culture supernatants in macrophages cultured with various 3D plates used for the surgical treatment of condylar fractures.
Macrophages were cultured for 24 h and 48 h in RPMI medium with 10% FBS with various plates: (**A**) SYNTHES (S1, S2, S3); (**B**) MARTIN (C1, C2, C3, C4); (**C**) MEDARTIS (M1, M2, M3, M4, M5, M6); for details see Table 1. After incubation, cells were harvested by scraping and MMP-2 concentration was measured by ELISA method. Experiments were conducted as six separate assays (each assay in three replicates). *Statistically significant differences in comparison to Control 24 h ($p \leq 0.05$); **Statistically significant differences in comparison to adjacent plate ($p \leq 0.05$). Control cells were incubated in RPMI medium with 10% FBS.

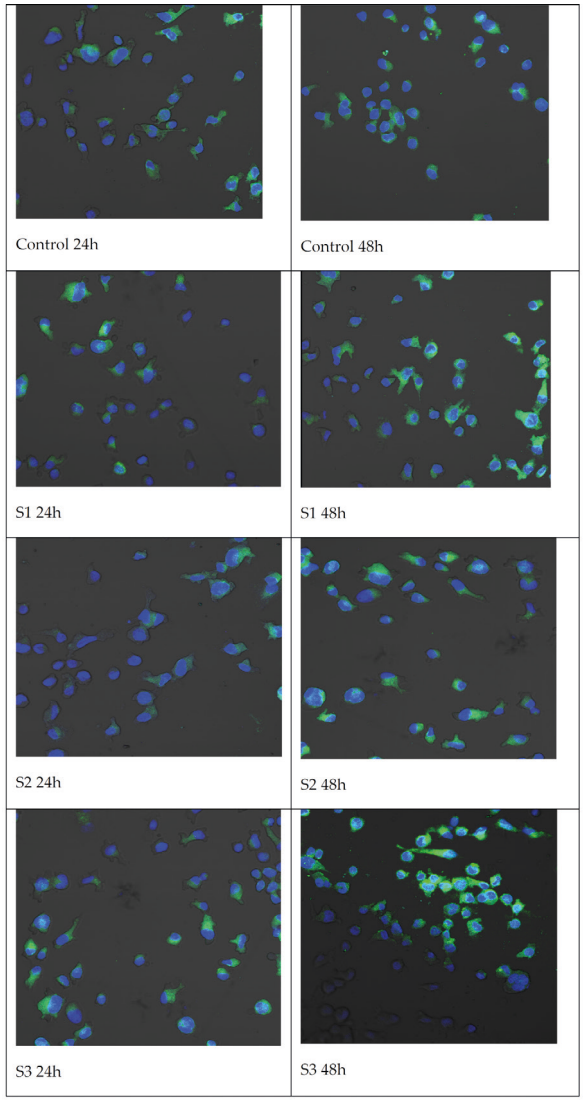

Figure 2. Detection of MMP-2 enzyme expression by fluorescence microscopy in macrophages cultured with various 3D plates used for the surgical treatment of condylar fractures.
Macrophages were cultured for 24 h and 48 h in RPMI medium with 10% FBS with various plates: (**A**) SYNTHES (S1, S2, S3); (**B**) MARTIN (C1, C2, C3, C4); (**C**) MEDARTIS (M1, M2, M3, M4, M5, M6); for details see Table 1. Experiments were conducted as six separate assays (each assay in three replicates). The immunohistochemistry was performed using specific primary antibody, mouse anti-MMP 2 (overnight incubation at 4°C) and secondary antibody conjugated with fluorochrome: anti-mouse IgG-FITC (incubation for 45 min at room temperature). The nuclei of cells were stained with DAPI. Imaging was performed with a fluorescent microscope using filters: 38 HE GFP for green fluorescence and 49 DAPI for blue fluorescence.

Nonparametric tests were used for further analysis because the distribution in most cases deviated from the normal distribution. The results were analyzed by Mann-Whitney U-test. The level of significance was established at $p < 0.05$.

RESULTS

Expression of MMP-2 in macrophages

The incubation time of macrophage culture significantly influenced the expression of MMP-2 in control conditions. Enzyme expression increased by more than 100% in Control 48 h *vs.* Control 24 h ($p \leq 0.0001$).

Incubation of macrophages with SYNTHES 3D plates (S)

The expression of MMP-2 significantly increased for S2 24 h *vs.* S2 48 h (increase by ca. 400%, $p \leq 0.0001$) and S3 24 h *vs.* S3 48 h (increase by ca. 100%, $p \leq 0.0001$). No statistically significant differences in enzyme expression were observed for S1 24 h *vs.* Control 24 h, S2 24 h *vs.* Control 24 h, and S3 24 h *vs.* Control 24 h.

In none of the observed cases MMP-2 expression in cultures of macrophages incubated with SYNTHES plates was statistically significantly higher than in the Control 48 h (Fig. 1). Visualization of MMP-2 staining in a confocal microscope confirmed an increase in MMP-2 protein expression in Control 24 *vs.* Control 48 macrophages. Similar observations were made in macrophages cultured in the presence of the plates S1 24 h, S2 24 h, S3 24 h in comparison to the respective cultures incubated for 48 h (Fig. 2).

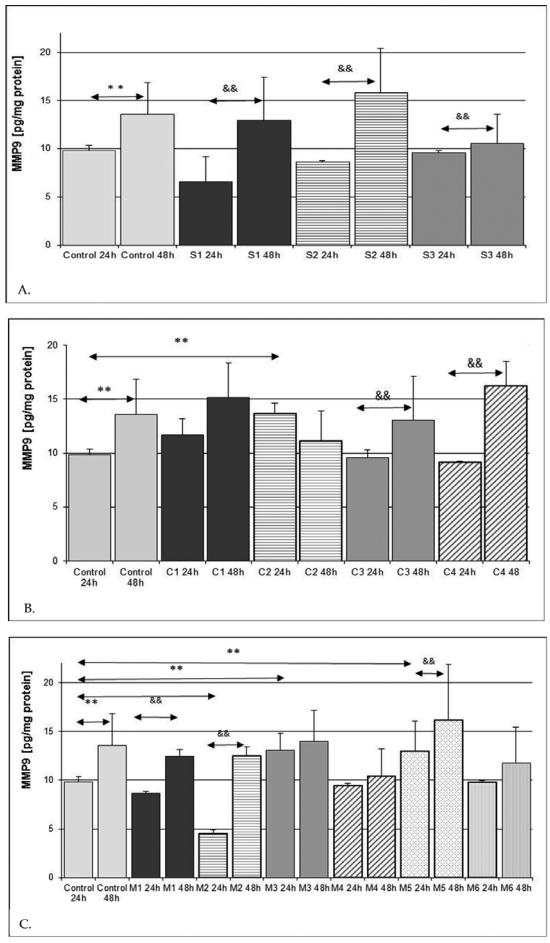

Figure 3. Expression of metalloproteinase 9 (MMP-9) in culture supernatants in macrophages cultured with various 3D plates used for surgical treatment of condylar fractures.
Macrophages were cultured for 24 h and 48 h in RPMI medium with 10% FBS with various plates: (**A**) SYNTHES (S1, S2, S3); (**B**) MARTIN (C1, C2, C3, C4); (**C**) MEDARTIS (M1, M2, M3, M4, M5, M6) for details see Table 1. After incubation, cells were harvested by scraping and MMP-9 concentration was measured by ELISA method. Experiments were conducted as six separate assays (each assay in three replicates). *Statistically significant differences in comparison to Control 24 h ($p \leq 0.05$); **Statistically significant differences in comparison to adjacent plate ($p \leq 0.05$). Control cells were incubated in RPMI medium with 10% FBS.

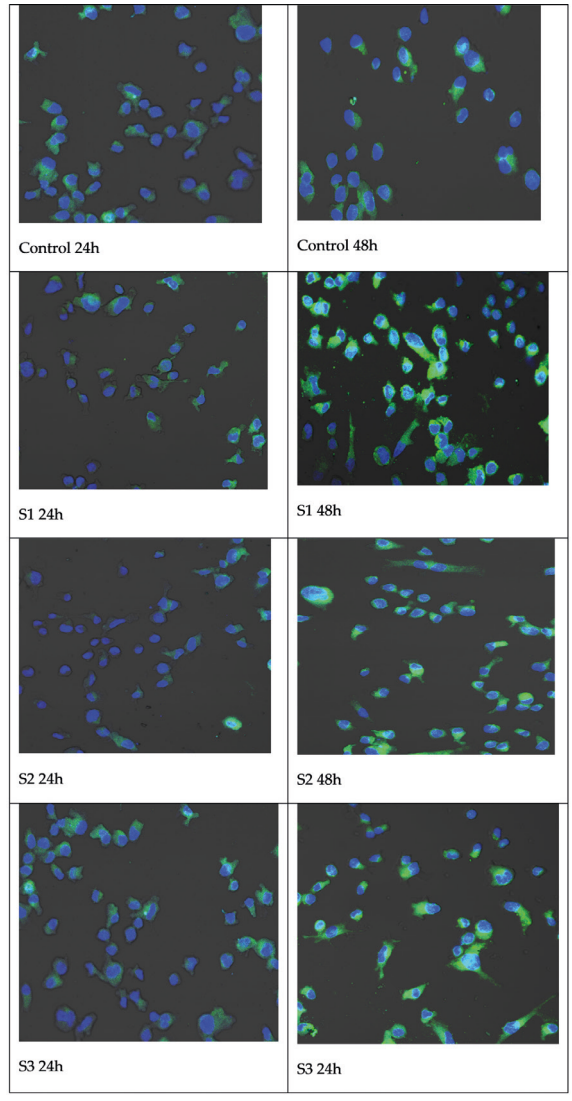

Figure 4. Detection of MMP-9 enzyme expression by fluorescence microscopy in macrophages cultured with various 3D plates used for surgical treatment of condylar fractures.
Macrophages were cultured for 24 h and 48 h in RPMI medium with 10% FBS with various plates: (**A**) SYNTHES (S1, S2, S3); (**B**) MARTIN (C1, C2, C3, C4); (**C**) MEDARTIS (M1, M2, M3, M4, M5, M6) for details see Table 1. Experiments were conducted as six separate assays (each assay in three replicates). The immunohistochemistry was performed using specific primary antibody, mouse anti-MMP 9 (overnight incubation at 4°C) and secondary antibody conjugated with fluorochrome: anti-mouse IgG-FITC (incubation for 45 min at room temperature). The nuclei of cells were DAPI stained. Imaging was performed with a fluorescent microscope using filters: 38 HE GFP for green fluorescence and 49 DAPI for blue fluorescence.

Incubation of macrophages with MARTIN plates (C)

Incubation with plates for 48 h caused an increase in the expression of MMP-2 in macrophages. This was found for C1 24 h *vs.* C1 48 h (increase by 40%, $p = 0.0012$); C2 24 h *vs.* C2 48 h (increase by 20%, $p \leq 0.0001$); C3 24 h *vs.* C3 48 h (increase by 35%, $p \leq 0.001$); C4 24 h *vs.* C4 48 h (increase by 34%, $p = 0.0022$). However, there was no statistically significant increase in the expression of the tested enzyme protein in macrophages incubated with any of the plates for 24 *vs.* Control 24 h. Extension of the incubation time to 48 h did not cause any significant increase in MMP-2 expression in macrophages for any of the MARTIN plates used as compared to macrophages incubated for 48 h under control conditions (Control 48 h).

Incubation of macrophages with MEDARTIS plates (M)

The incubation time significantly increased the expression of MMP-2 protein in macrophages incubated with plate M1 24 h as compared to M1 48 h (increase by about 33%, $p=0.001$), M2 24 h *vs.* M2 48 h (increase by about 87%, $p=0.002$), M2 24 h *vs.* M2 48 h (increase by ca. 87%, $p=0.002$); M3 24 h *vs.* M3 48 h (increase by ca. 116%, $p=0.0015$); M4 24 h *vs.* M4 48 h (increase by ca. 30%, $p=0.001$) and in the case of plate M5 24 h *vs.* M5 48 h (increase by approx. 103%, $p = 0.003$) and M6 24 h *vs.* M6 48 h (approx. 3-fold increase, $p = 0.005$). However, in the case of incubation with MEDARTIS plates no significantly higher concentration of MMP-2 was observed in macrophage cultures with the plates as compared to Control 48 h.

Expression of metalloproteinase 9 (MMP-9) in macrophages

Longer incubation time caused an increase in the expression of MMP-9 protein in Control 24 h compared to Control 48 h (by about 38%, $p \leq 0.001$).

Incubation of macrophages with SYNTHES plates (S)

Expression of MMP-9 protein increased in macrophages incubated with S1 24 h *vs.* S1 48 h (by 100%, $p \leq 0.0001$) and S2 24 h *vs.* S2 48 h (by 80%, $p \leq 0.0001$). Statistically significant differences were not observed in the enzyme expression for S1 24 h *vs.* Control 24, S2 24 h *vs.* Control 24 h, and S3 24 h *vs.* Control 24 h. When incubation time with SYNTHES plates was prolonged to 48 h for all plates studied, expression of MMP-9 in macrophage cultures was not significantly higher than in the Control 48 h (Fig. 3). Assessment of the enzyme protein expression carried out with a confocal microscope confirmed the increase of expression in Control 24 h *vs.* Control 48 h macrophages. An increase of MMP-9 protein expression was also observed in macrophages incubated with S1 24 h, S2 24 h, S3 24 h as compared to the respective cultures for 48 h incubation time (Fig. 4).

Incubation of macrophages with MARTIN plates (C)

Expression of MMP-9 protein increased in macrophages incubated with C2 24 h *vs.* Control 24 h (by about 19%, $p = 0.005$). An increase in protein expression was also observed for C3 24 h *vs.* C3 48 h (by about 37%, $p \leq 0.0022$); C4 24 h *vs.* C4 48 h (by about 77%, $p = 0.003$). Enzyme protein expression also increased in macrophages incubated with C2 24 h *vs.* Control 24 h. Extension of incubation time to 48 h did not cause any further statistically significant increase in MMP-9 expression in macrophages for any of the MARTIN plates when compared to Control 48 h.

Incubation of macrophages with MEDARTIS plates (M)

Expression of MMP-9 protein increased in macrophages incubated with M1 24 h *vs.* M1 48 h (by about 40%, $p = 0.001$); and M2 24 h *vs.* M2 48 h (by about 170%, $p = 0.0042$). MMP-9 expression decreased in macrophages incubated in M2 24 h *vs.* Control 24 h (by about 54%, $p = 0.0002$) and it decreased for M3 24 h plates *vs.* Control 24 h (by about 33%, $p = 0.0021$) and M5 24 h *vs.* Control 24 h (by about 32%, $p = 0.0032$). However, in the case of incubation with MEDARTIS plates no significantly higher concentration of MMP-9 was observed in macrophage cultures with plates as compared to Control 48 h.

DISCUSSION

The surgical treatment of condylar fractures is inevitably associated with breaks in the continuity of soft tissues, which lead to the excessive synthesis of pro-inflammatory cytokines (IL-1B, IL-6 and TNF-α). These cytokines initiate the development of inflammation, which may result in cell degeneration and damage to the surrounding tissues (Choo *et al.,* 2017; Sikora *et al.,* 2017). For this reason, it is important that the plates used by surgeons to stabilize the fracture do not additionally induce inflammatory processes, and at the same time do not disturb the regenerative process.

Surgical treatment of mandibular condyle fractures with the use of metal plates and the associated break in the continuity of tissues is a factor that can initiate the development of inflammation (Paiva & Granjeiro, 2017; Wagner, 1996; Yan *et al.,* 2016). The matrix metalloproteinases MMP-2 and MMP-9 that were tested in this study also contribute to inflammatory processes in tissues by interacting with chemokines. MMP-2 (similarly to MMP13 and MMP14, but not MMP7-9) removes the N-terminal amino group of chemotactic tetrapeptide of monocyte-chemotactic protein 3 (MCP3). Modified in this way, MCP3 becomes a chemokine receptor antagonist, leading to the extinction of the inflammatory reaction (McQuibban *et al.,* 2000). This is one of the mechanisms determining the chemokine-mediated contribution of MMP-2 to the inflammatory reactions.

A completely different effect occurs as a result of the activation of MMP-9 in the context of inflammatory reactions. MMP-9 removes the six amino acids from the N-terminus of interleukin-8 (IL-8), as a result of which the shortened form of IL-8 becomes a much stronger stimulus, causing an increased inflow of neutrophils (Van den Steen *et al.,* 2000). It can, therefore, be concluded that increased expression of this enzyme by activation by pro-inflammatory interleukins is characteristic for the initial stage of inflammation (Van den Steen *et al.,* 2000). Increased synthesis of MMP-2 is associated with the extinction of the inflammatory reaction (McQuibban *et al.,* 2000). In our study, the lack of significant changes in the expression of MMP-9 and MMP-2 enzymes in THP-1 macrophages incubated in the presence of the examined plates indicates the lack of additional induction of expression of these enzymes by the plates.

Not only cytokines and chemokines influence metalloproteinases. Interaction of MMPs with other molecules in places where the continuity of tissues has been interrupted is also extremely important. Damage resulting from the surgical intervention increases nitric oxide (NO) synthesis by endothelial cells (Berra-Romani *et al.,* 2013). NO activates MMP-9 through S-nitrosylation (Gu *et al.,* 2002), which may cause increased degradation of the extracellular matrix and promote pro-inflammatory processes associated with the activity of MMP-9. The fact that the plates tested by us did not increase the synthesis of the enzyme itself is another proof of their safety. It confirms the results of our earlier studies which showed that the plates do not initiate inflammation via the cyclooxygenase pathway (COXs) (Sikora *et al.,* 2017).

Metalloproteinases are also very important during bone remodelling and regeneration. They are expressed in bone cells, cartilage, inflammatory cells and endothelium. MMPs participate in the regeneration of bones after fractures or other damage mainly by coordinating the mechanism of reconstruction of target matrix proteins (Wang *et al.,* 2013).

Bone reconstruction is accompanied by temporary co-expression of certain enzymatic systems (MMP-2/MMP14, MMP-9/MMP13) (Colnot *et al.,* 2003; Lehmann *et al.,* 2005). Genetically modified mice without the expression of metalloproteinases (including MMP-2 and MMP-9) showed delayed wound healing, pseudo-orthosis, and even a lack of bone healing (Paiva & Granjeiro, 2017). Studies on bone regeneration in rodents showed that alveolar process reconstruction after tooth extraction, characterized by three separate stages (exudative, proliferative and generative), take place with the participation of MMP-2, MMP-9 and reversion-inducing cysteine-rich proteins with Kazal motifs (RECK), expressed at all these stages, mainly in osteoblasts, connective tissue and endothelial cells (Accorsi-Mendonca *et al.,* 2008). The implanted biomaterials, implants or

metal plates should either support the regenerative processes or not interfere with their natural course in the tissue. Demineralized (mainly collagen I) or inorganic bone (hydroxyapatite) implants require MMPs for biomaterial resorption or reconstruction of the surrounding tissue, where MMPs are secreted by macrophages surrounding the transplant (Accorsi-Mendonca *et al.,* 2008; Thais Accorsi-Mendonc *et al.,* 2005; Zambuzzi *et al.,* 2009). During the healing of extensive injuries, the printing of inorganic bone induces increased expression of MMP-2, MMP-9 and vascular endothelial growth factor (VEGF) (Rocha *et al.,* 2014). The results presented in this paper indicate the safety of 3D titanium mini-plates in the treatment of condylar fractures with regard to the expression of MMP-2 and MMP-9.

In this study, the presence of the plates in the cultures of THP-1 macrophages did not increase the expression of MMP-2 or MMP-9. Based on the results of this study, it can be concluded that 3D titanium mini-plates do not increase the inflammatory response in macrophages associated with the activity of the described enzymes. It can also be speculated that they do not affect bone regeneration processes in which MMP-2 and MMP-9 play significant roles.

CONCLUSIONS

The results presented in this paper confirm that 3D titanium mini-plates do not alter MMP-2 and MMP-9 protein expression in the cultured macrophages.

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Conflicts of interest

The authors declare no conflict of interest.

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