Original paper

Circulating biomarkers of nitrosative stress, protein glycoxidation and inflammation in maxillofacial surgery patients treated with titanium implants

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Dental and Medical Problems, ISSN 1644-387X (print), ISSN 2300-9020 (online) *Dent Med Probl*. 2025;62(2):225–236

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Funding sources
This work was supported by the Medical University of Bialystok, Poland (grant No. B.SUB.23.250; B.SUB.23.309).

Conflict of interest
None declared

Acknowledgements
None declared

Received on April 5, 2024
Reviewed on May 1, 2024
Accepted on May 18, 2024

Published online on January 31, 2025

Cite as
Antonowicz B, Borys J, Zalewska A, et al. Circulating biomarkers of nitrosative stress, protein glycoxidation and inflammation in maxillofacial surgery patients treated with titanium implants. *Dent Med Probl.* 2025;62(2):225–236. doi:10.17219/dmp/188863

DOI
10.17219/dmp/188863

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Abstract

Background. Titanium (Ti) biomaterials are widely used in the surgical management of maxillofacial trauma, in oncology and orthognathic surgery. Although Ti is considered highly biocompatible, adverse reactions at the implant site have been reported in numerous clinical studies. However, the influence of Ti mandibular implants on glutathione metabolism, nitrosative stress and systemic inflammation has not been investigated to date.

Objectives. The study aimed to evaluate the acute (short-term) effects of Ti mandibular implants on the circulating biomarkers of the antioxidant defense system, on oxidative and nitrosative stress, as well as the inflammatory response of the blood plasma/erythrocytes, in maxillofacial surgery patients compared to the control group.

Material and methods. The experimental group consisted of 40 patients with bilateral mandibular fractures, who received osteosynthesis treatment with the use of Ti-6Al-4V alloy miniplates and screws. The control group comprised 40 age- and gender-matched patients who were qualified for the surgical treatment of craniofacial defects through bimaxillary osteotomy.

Results. An increase in the activity of pro-oxidant enzymes (↑ nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), ↑ xanthine oxidase (XO)), impaired glutathione metabolism (↓ total glutathione, ↑ oxidized glutathione (GSSG), ↓ reduced glutathione (GSH), ↓ redox status), higher levels of oxidative stress (↓ total thiols, ↑ malondialdehyde (MDA), ↑ lipid hydroperoxides (LOOHs)), ↓ total antioxidant status (TAS)), carbonyl stress (↑ dityrosine, ↑ N-formylkynurenine) and nitrosative stress (↑ nitric oxide (NO), ↑ S-nitrosothiols, ↑ peroxynitrite, ↑ nitrotyrosine), as well as an intensified systemic inflammatory response (↑ interleukin (IL)-1β, ↑ IL-6), were observed in maxillofacial surgery patients.

Conclusions. Despite the fact that the study examined only the circulating biomarkers of redox balance and inflammation, the results suggest that a systemic inflammatory response can be triggered by local immune reactions. Systemic inflammation and oxidative stress may stem from an early adaptive immune response to foreign objects in the body. Although further research is required, the removal of the existing Ti mandibular implants should be considered.

Keywords: inflammation, blood, nitrosative stress, titanium implants, circulating biomarkers

Highlights

- Maxillofacial surgery patients treated with Ti-6Al-4V show increased levels of the circulating biomarkers of oxidative stress, nitrosative stress and inflammation.
- A systemic inflammatory response can be triggered by local immune responses.
- The removal of Ti-6Al-4V implants in the craniofacial region should be considered.

Introduction

Titanium (Ti) and its alloys are the most widely used surgical biomaterials for the treatment and replacement of tissues and organs. Titanium implants are known for their high biofunctionality, biocompatibility, excellent biomechanical properties (durability, hardness and wear resistance), and the absence of a thrombotic response.[1,2] However, adverse reactions to Ti implants have been reported in numerous clinical studies.[3–5] The Ti-based prosthetic replacements of long bone joints have been found to induce inflammation, allergic responses and toxicity.[6,7] Additionally, grayish discoloration of the peri-implant tissues has been reported.[8,9] Metallic debris can be phagocytized by the circulating immune cells, which are stimulated to release pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and IL-8, and free radicals.[10,11] Side effects have also been observed in patients with dental implants.[12–14] Recent research indicates that there are differences in the response to Ti implants between the peri-implant soft tissue and the periodontal tissue.[15,16] Reactive oxygen species (ROS) are formed on the implant surface as a direct product of corrosion at the cathode, and from the interaction between the titanium dioxide (TiO_2) layer and hydrogen peroxide (H_2O_2) generated by the activated macrophages.[17–19] In turn, the chronic sterile inflammation caused by Ti implants in the surrounding tissues can lead to bone loss and prosthetic loosening.[20–22]

Titanium biomaterials are also commonly used in the surgical management of maxillofacial trauma, in oncology and orthognathic surgery.[23] Yet, recent research has confirmed the presence of Ti in the tissues surrounding mandibular implants.[24,25] Our previous studies revealed dysfunctions in enzymatic and non-enzymatic antioxidant defense systems, as well as protein and lipid damage caused by heightened oxidative and nitrosative stress, mitochondrial dysfunction, an intensified inflammatory response, and apoptosis, in patients who received osteosynthesis treatment with the use of Ti miniplates and screws.[26–28] However, the effects of Ti mandibular fixations on glutathione metabolism, nitrosative stress and systemic inflammation has not been studied to date. Titanium particles have been identified not only in the peri-implant tissues, but also in distal organs, such as lymph nodes, lungs, spleen, and liver.[4,29,30] The Ti ions have also been detected in the blood plasma of patients with Ti mandibular implants.[30,31] These observations clearly indicate the need for further research to expand our understanding of maxillomandibular implant degradation and its impact on systemic homeostasis, especially in the context of the ongoing debate on Ti implant removal.[32] Local redox imbalance, oxidative and nitrosative stress, and the inflammatory response may disrupt regenerative processes throughout the body.

Therefore, the aim of the present study was to evaluate the acute (short-term) effects of Ti mandibular fixations on the circulating biomarkers of the antioxidant defense system, on oxidative and nitrosative stress, and the inflammatory response of the blood plasma/erythrocytes in maxillofacial surgery patients compared to the control group. We assessed the activity of the main pro-oxidant enzymes (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and xanthine oxidase (XO)), the ROS scavenging capacity in the total antioxidant status (TAS) assay and the concentration of the main intracellular antioxidant, glutathione. In addition, we measured the concentrations of the most commonly assessed lipid peroxidation products (malondialdehyde (MDA) and lipid hydroperoxides (LOOHs)), and pro-inflammatory and anti-inflammatory cytokines (IL-1β, IL-6, IL-8, and IL-10).

Material and methods

Patients

The study involved 80 patients treated at the Department of Maxillofacial and Plastic Surgery of the Medical University in Bialystok, Poland. The study was approved by the Bioethics Committee of the Medical University in Bialystok (approval No. R-I-002/2/3/2-16). Patients in both the experimental and control groups were informed about the purpose of the study, the type and method of surgical treatment, and potential complications. All participants provided voluntary written informed consent to participate in the experiment.

The experimental group consisted of 40 patients (25 men and 15 women), aged 22–34 years, with bilateral mandibular fractures, who received osteosynthesis treatment with the use of Ti-6Al-4V alloy miniplates and screws (ChM Lewickie Sp. z o.o., Juchnowiec Kościelny, Poland). Bilateral mandibular corpus fractures were treated by fixation with 2 five-hole Ti miniplates and 4 screws per plate, with 1 patient receiving 4 miniplates and

16 screws. At the time of sustaining mandibular trauma, none of these patients had additional bone fractures or other organ injuries.

The control group comprised 40 patients (25 men and 15 women), aged 22–34 years, who were qualified for the surgical treatment of craniofacial defects through bimaxillary osteotomy.

The following exclusion criteria were applied: other bone fractures; a brain injury or other organ injuries; the presence of any Ti implants (dental, orthodontic, prosthetic, joint prostheses); chronic systemic or localized diseases; oral diseases, such as periodontitis; the inflammatory response triggered by periodontal disease (stomatitis, tonsillitis); the use of medications, supplements, psychoactive substances, or narcotics within 3 months before the commencement of the study; alcohol consumption; smoking or chewing tobacco; an unhealthy body mass index (BMI); abnormal results of biochemical analyses – blood morphology test, blood clotting parameters (prothrombin time (PT), activated partial thromboplastin time (APTT) and the international normalized ratio (INR)), the levels of electrolytes (sodium (Na) and potassium (K)), blood glucose, creatinine, urea, and liver enzymes (aspartate transaminase (AST) and alanine transaminase (ALT)).

In the experimental group, Ti miniplates and screws were removed at 3–4 months after surgery. No complications were observed during the process of fracture healing in any of the patients in the period between surgery and implant removal. There were no signs of inflammation, reddening, swelling, abscesses, allergic reactions, or implant exposure at the peri-implant site. The surgical procedures were performed by the same experienced maxillofacial surgeon (J.B.) in both groups.

Blood samples

In the experimental group, blood samples were collected 3–4 months after mandibular osteosynthesis, specifically on the day of miniplate and screw removal. In the control group, blood samples were collected before the bimaxillary osteotomy procedures.

Blood samples from both the experimental and control groups were obtained following overnight fasting and stored at 4°C. Plasma samples were separated via centrifugation at 3,000 rpm for 15 min and were subsequently stored at −80°C until biochemical analyses. Erythrocytes were rinsed 3 times with cold 0.9% NaCl solution and hemolyzed by the addition of a 9-fold volume of cold phosphate-buffered saline (PBS) (50 mM, pH 7.4).[33]

Biochemical analyses

The blood samples were analyzed to determine the activity of pro-oxidant enzymes, glutathione metabolism/TAS, the concentrations of protein and lipid oxidation products, glycoxidation products, nitrosative stress biomarkers, pro-inflammatory and anti-inflammatory cytokines, and total protein concentration.

All reagents for redox assays were supplied by either Sigma-Aldrich Chemie (Taufkirchen, Germany) or Sigma-Aldrich, Inc. (St. Louis, USA), unless stated otherwise. Absorbance and fluorescence measurements were conducted using the Infinite® M200 PRO Multimode Microplate Reader (Tecan Group, Männedorf, Switzerland). All biochemical analyses were performed in duplicate, and the results were standardized to milligram [mg] of total protein.

Pro-oxidant enzymes

The activity of erythrocyte NOX (EC 1.6.3.1) was determined by chemiluminescence, with lucigenin as the luminophore.[34] The rate of superoxide radical anion formation in the presence of NOX was measured. Enzymatic activity was defined as the quantity of the enzyme required to catalyze the synthesis of 1 nM of the superoxide radical anion per minute. In addition, the activity of erythrocyte XO (EC 1.17.3.2) was measured according to the method of Prajda and Weber.[35] This involved measuring uric acid formation from xanthine and quantifying the increase in absorbance at 290 nm. The activity of XO was defined as the amount of the enzyme required to release 1 µmol of uric acid per minute. The results were expressed in mU/mg of protein.

Antioxidant barrier

Total glutathione concentration was evaluated using the colorimetric method based on the enzymatic reaction between a chemically reduced form of NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and glutathione reductase.[36] Absorbance was measured at a wavelength of 412 nm. The results were expressed in µmol/mg of total protein. To determine the concentration of oxidized glutathione (GSSG), the samples were thawed, neutralized to pH 6–7 with 1 M triethanolamine hydrochloride, and then incubated with 2-vinylpyridine to inhibit glutathione oxidation. Subsequently, the concentration of GSSG was measured colorimetrically, employing a method similar to that used for total glutathione determination,[36] and calculated using a calibration curve for GSSG solutions. The results were expressed in µmol/mg of total protein. The concentration of reduced glutathione (GSH) was calculated as the difference between total glutathione and GSSG concentration. The results were expressed in µmol/mg of total protein. The redox status was calculated as the ratio of $[GSH]^2$ to [GSSG].[37]

The total antioxidant status was determined with a colorimetric assay, using a commercial kit (Randox Laboratories, Crumlin, UK). The samples were incubated at 37°C with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), peroxidase (metmyoglobin) and H_2O_2 until the

formation of the $ABTS^{\bullet+}$ radical. The antioxidants present in the sample suppressed color production to a degree proportional to their concentration. The measurements were conducted at a wavelength of 660 nm.

Oxidative stress

The concentration of carbonyl groups (PC) was measured colorimetrically based on the reaction with 2,4-dinitrophenylhydrazine (DNPH).[38] The increase in the absorbance of the resulting hydrazone was measured at 360 nm. The concentration of PC was calculated using the molar absorption coefficient (ε) for DNPH, which was determined to be 22,000 $M^{-1}cm^{-1}$. The results were expressed in nmol/mg of protein.

Total thiol concentration was estimated colorimetrically according to Ellman's method.[39] This involved the utilization of DTNB, which was reduced to 2-nitro-5-thiobenzoic acid in the presence of thiol groups. The results were expressed in µmol/mg of protein.

The concentration of MDA was determined with a colorimetric assay, using thiobarbituric acid (TBA).[40] Malondialdehyde reacts with TBA to produce a colored adduct, with maximum absorbance at 535 nm. The concentration of MDA was measured in duplicate and expressed in mmol/mg of total protein.

The concentration of LOOHs was measured colorimetrically. In this approach, LOOH reacts with a ferrous iron to form a ferric iron, which then reacts with 3,32-bis[N,N-bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein disodium salt (the xylenol orange reagent) to form a chromogen (the XOF complex).[40] The absorbance of XOF was measured at 560 nm. The results were expressed in µmol/mg of protein.

Glycoxidation products

The concentration of advanced glycation end-products (AGEs) was determined using a fluorescence-based assay according to the method described by Kalousová et al.[41] In this protocol, the fluorescence of furoyl-furanyl imidazole (FFI), carboxymethyl-lysine (CML), pyrraline, and pentosidine was measured at excitation and emission wavelengths of 350 nm and 440 nm, respectively. To determine the concentration of AGEs, the plasma samples were diluted 1:5 (v/v) in PBS (0.02 M, pH 7.0) and thoroughly mixed. Subsequently, a 200-microliter aliquot of the diluted sample was transferred to a 96-well microplate for fluorescence measurements.[42] The concentration of AGEs was determined in duplicate and expressed in arbitrary fluorescence units (AFU) per milligram of total protein.

To determine the content of amino acids modified during glycoxidation reactions (dityrosine, kynurenine, N-formylkynurenine, and tryptophan), the plasma samples were diluted 1:10 (v/v) in 0.1 M sulfuric acid and thoroughly mixed. A 200-microliter aliquot of the diluted sample was transferred to a 96-well microplate. Fluorescence was measured at various wavelength pairs: 330/415 nm (dityrosine), 365/480 nm (kynurenine), 325/434 nm (N-formylkynurenine), and 95/340 nm (tryptophan).[42,43] The content of amino acids modified by glycoxidation reactions was expressed in AFU/mg of total protein. All measurements were conducted in duplicate.

Nitrosative stress

The concentration of nitric oxide (NO) was determined with a colorimetric assay, as described by Grisham et al.[44] In this approach, nitrate (NO_3^-) reacts with sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride to produce a colored product, with maximum absorbance at 490 nm. The concentration of NO was calculated using a calibration curve for sodium nitrate ($NaNO_3$). The analyzed parameter was measured in duplicate and expressed in µmol/mg of total protein.

The concentration of S-nitrosothiols was determined with a colorimetric assay according to the protocol proposed by Wink et al.[45] In this approach, S-nitrosothiols present in the sample are quantified using the Griess reagent, following the reaction with the mercury ions (Hg^{2+}). The resulting compound exhibits maximum absorbance at 490 nm. The concentration of S-nitrosothiols was calculated using ε = 11,500 $M^{-1}cm^{-1}$. The analyzed parameter was measured in duplicate and expressed in µmol/mg of total protein.

The concentration of peroxynitrite was determined via a fluorometric assay by measuring the nitrosylation of phenol.[46] The reaction between peroxynitrite and phenol produces S-nitrophenol, which exhibits maximum absorbance at 490 nm (excitation) and 530 nm (emission). The concentration of peroxynitrite was calculated using ε = 1,670 $M^{-1}cm^{-1}$. The analyzed parameter was measured in duplicate and expressed in µmol/mg of total protein.

The concentration of nitrotyrosine was quantified using the Nitrotyrosine ELISA commercial kit (Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions. The results were expressed in nmol/mg of protein.

Pro-inflammatory and anti-inflammatory cytokines

The concentrations of IL-1β, IL-6, IL-8, and IL-10 were determined using commercial ELISA kits (EIAab Science, Wuhan, China) according to the manufacturer's instructions. Absorbance was measured at 405 nm, and the results were expressed in pg/mL.

Total protein concentration

Total protein concentration was determined with a colorimetric assay, using the commercial Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA),

based on bicinchoninic acid (BCA). Bicinchoninic acid reacts with the copper ions (Cu^{2+}) to form a stable complex, with maximum absorption at 562 nm.[47] Total protein concentration was read from a calibration curve for bovine serum albumin (BSA), and expressed in µg/mL.

Statistical analysis

The statistical analysis was conducted using the GraphPad Prism 9.5.1 software (GraphPad Software, La Jolla, USA). The normality of data distribution was checked with the Shapiro–Wilk test. The mean values for the experimental and control groups were compared using Student's *t* test at a significance level of $p \leq 0.05$. All data was presented as mean and standard deviation (*M* ±*SD*). The number of subjects in each group was determined based on our previous experiment ($n = 15$), with the aim of achieving a test power of 0.8 (ClinCalc sample size calculator; https://clincalc.com/stats/samplesize.aspx).

Results

Routine laboratory tests

The results of the routine laboratory tests are shown in Table 1. In both the control and experimental groups, the values of all biomarkers were within the reference ranges. However, the levels of white blood cells (WBC), potassium ions (K^+) and C-reactive protein (CRP) were significantly higher in the experimental group as compared to the control group, while the hemoglobin (HGB), hematocrit (HCT) and platelet count (PLT) values were significantly lower.

Pro-oxidant enzymes

The activity of erythrocyte NOX (+19.12%; $p = 0.024$) and XO (+14.71%; $p = 0.045$) was significantly higher in maxillofacial surgery patients than in the control group (Fig. 1).

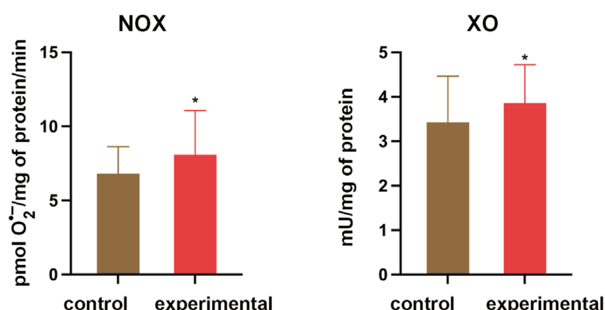

Fig. 1. Activity of pro-oxidant enzymes in the erythrocytes of control and experimental groups

NOX – nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; XO – xanthine oxidase; *$p < 0.05$.

Table 1. Routine laboratory tests in the control and experimental group

Parameter	Control group (*n* = 40)	Experimental group (*n* = 40)	*p*-value	Reference value
WBC [×10³/µL]	5.53 ±0.75	6.23 ±1.04	0.001*	4.0–10.0
RBC [×10⁶/µL]	4.71 ±0.52	4.80 ±0.39	0.356	4.5–6.0
HGB [g/dL]	15.24 ±0.92	14.63 ±1.10	0.009*	14.0–18.0
HCT [%]	47.87 ±3.58	46.16 ±3.68	0.038*	40.0–54.0
MCV [fL]	85.64 ±4.30	85.38 ±3.72	0.773	80.0–94.0
MCHC [g/dL]	34.25 ±1.85	34.15 ±1.55	0.789	31.0–37.0
PLT [×10⁶/µL]	260.40 ±36.90	232.70 ±53.67	0.009*	130.0–350.0
PT [s]	13.48 ±1.43	13.73 ±1.26	0.410	12.0–16.0
APTT [s]	29.50 ±4.37	28.97 ±3.48	0.550	26.0–40.0
INR	0.98 ±0.10	0.99 ±0.11	0.627	0.8–1.2
Na^+ [mmol/L]	139.20 ±2.55	139.00 ±2.85	0.680	136.0–145.0
K^+ [mmol/L]	4.14 ±0.46	4.40 ±0.56	0.029*	3.5–5.1
Glucose [mg/dL]	90.05 ±5.68	88.42 ±6.78	0.246	70.0–99.0
Creatinine [mg/dL]	0.96 ±0.15	0.90 ±0.12	0.053	0.7–1.2
Urea [mg/dL]	25.23 ±5.55	26.09 ±6.72	0.532	10.0–50.0
AST [U/L]	26.55 ±5.09	27.53 ±6.08	0.439	5.0–34.0
ALT [U/L]	28.98 ±6.16	28.30 ±8.92	0.695	0.0–55.0
CRP [mg/L]	1.61 ±0.55	3.07 ±1.33	0.0001*	0.0–10.0

Data presented as mean ± standard deviation (*M* ±*SD*).
WBC – white blood cells; RBC – red blood cells; HGB – hemoglobin; HCT – hematocrit; MCV – mean corpuscular volume; MCHC – mean corpuscular hemoglobin concentration; PLT – platelet count; PT – prothrombin time; APTT – activated partial thromboplastin time; INR – international normalized ratio; Na^+ – sodium ions; K^+ – potassium ions; AST – aspartate transaminase; ALT – alanine transaminase; CRP – C-reactive protein; * statistically significant.

Antioxidant barrier

The total glutathione (–44.73%; $p < 0.0001$) and GSH (–44.73%; $p < 0.0001$) plasma levels were significantly lower in the experimental group than in the control group. The plasma concentration of GSSG was significantly higher in the experimental group than in the control group (+33.33%; $p < 0.0001$). Maxillofacial surgery patients exhibited significantly lower plasma TAS values in comparison with the control group (–9.80%; $p = 0.007$).

Furthermore, the redox status was significantly lower in the experimental group relative to the controls (−73.02%; $p < 0.0001$) (Fig. 2).

Oxidative stress

The plasma levels of MDA (+12.70%; $p = 0.035$) and LOOHs (+20.00%; $p < 0.0001$) were significantly higher in maxillofacial surgery patients in comparison with the control group. Additionally, the plasma concentration of PC was higher in the experimental group patients (+9.68%). However, the difference between the groups was not statistically significant. The plasma levels of total thiols were significantly lower in the experimental group than in the control group (−8.11%; $p = 0.025$) (Fig. 3).

Glycoxidation products

The plasma levels of dityrosine (+60.71%; $p < 0.0001$) and N-formylkynurenine (+7.14%; $p = 0.012$) were significantly higher in maxillofacial surgery patients in comparison with the control group. No significant differences were observed in the plasma concentrations of AGEs, kynurenine or tryptophan between the control and experimental group subjects (Fig. 4).

Nitrosative stress

The experimental group patients exhibited significantly higher plasma concentrations of NO (+10.26%; $p = 0.001$), S-nitrosothiols (+20.34%; $p = 0.002$), peroxynitrite

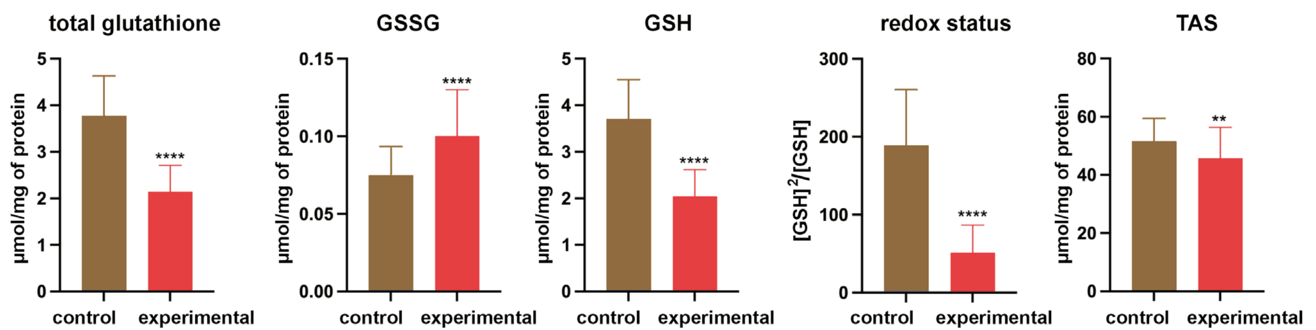

Fig. 2. Antioxidant barrier in the blood plasma of control and experimental groups

GSSG – oxidized glutathione; GSH – reduced glutathione; TAS – total antioxidant status; ** $p < 0.01$; **** $p < 0.0001$.

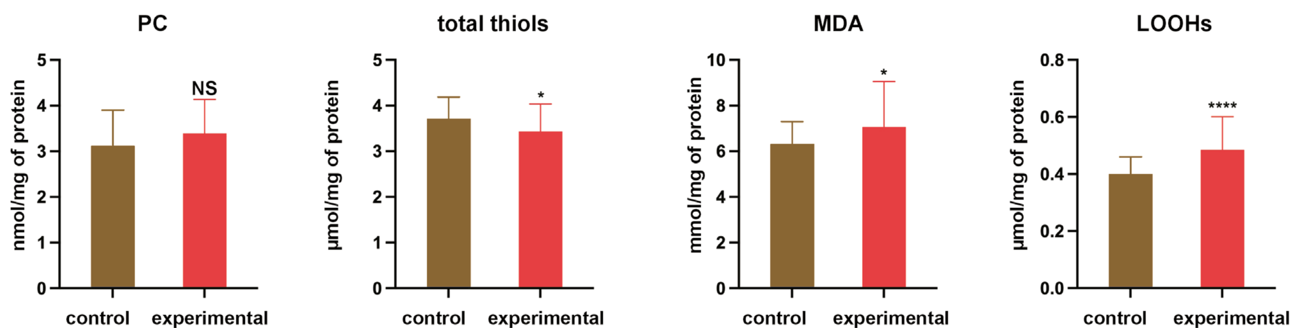

Fig. 3. Oxidative stress indicators in the blood plasma of control and experimental groups

PC – carbonyl groups; MDA – malondialdehyde; LOOHs – lipid hydroperoxides; * $p < 0.05$; **** $p < 0.0001$; NS – non-significant.

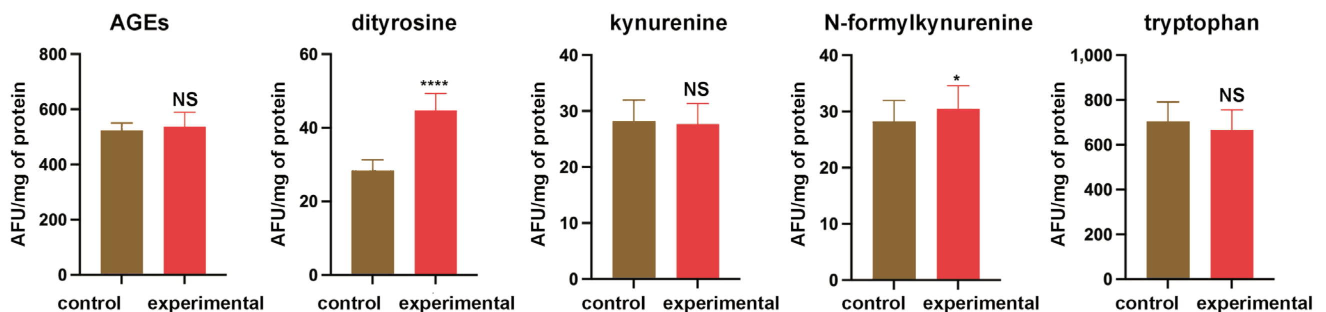

Fig. 4. Glycoxidation products in the blood plasma of control and experimental groups

AGEs – advanced glycation end-products; AFU – arbitrary fluorescence units; * $p < 0.05$; **** $p < 0.0001$; NS – non-significant.

(+10.53%; $p = 0.006$), and nitrotyrosine (+40.00%; $p < 0.0001$) as compared to the control group subjects (Fig. 5).

Pro-inflammatory and anti-inflammatory cytokines

The blood plasma levels of IL-1β (+5.61%; $p = 0.005$) and IL-6 (+5.68%; $p = 0.009$) were found to be significantly higher in maxillofacial surgery patients as compared to the control group subjects. No significant differences in the concentrations of IL-8 or IL-10 were observed between the control and experimental groups (Fig. 6).

ROC analysis

The receiver operating characteristics (ROC) curve is a graphic representation of the relationship between the sensitivity and specificity of a test. The ROC curve is used to evaluate the diagnostic accuracy of a biomarker. The area under the curve (AUC) measures the capacity of a test to discriminate between correct and incorrect results. A higher AUC indicates greater diagnostic accuracy for a given biomarker.

Among the analyzed biomarkers, total glutathione, GSH, the redox status, and dityrosine were identified as having the greatest potential significance for clinical application. These parameters demonstrated the highest sensitivity (≥85%) and specificity (≥85%) in discriminating between patients treated with Ti implants and control group subjects (AUC of 0.946, 0.948, 0.954, and 1.000, respectively) (Table 2).

Discussion

This study is the first to evaluate the circulating biomarkers of inflammation, protein glycoxidation and nitrosative damage in maxillofacial surgery patients treated with Ti fixations. An increase in the activity of pro-oxidant enzymes (↑ NOX, ↑ XO), impaired glutathione metabolism (↓ total glutathione, ↑ GSSG, ↓ GSH, ↓ redox status), higher levels of oxidative stress (↓ total thiols, ↑ MDA, ↑ LOOHs, ↑ TAS), carbonyl stress (↑ dityrosine, ↑ N-formylkynurenine) and nitrosative stress (↑ NO, ↑ S-nitrosothiols, ↑ peroxynitrite, ↑ nitrotyrosine), as well as an intensified systemic inflammatory response (↑ IL-1β, ↑ IL-6), were observed in maxillofacial surgery patients.

Some researchers have proposed that Ti implants should be removed from the mandible due to potential long-term adverse effects.[32,48] In the 1970s, Ti miniplates and screws were routinely removed, even if no side effects were reported.[49,50] Currently, Ti mandibular fixations are removed only when they induce inflammatory changes, become exposed, hinder prosthetic treatment, or cause subjective discomfort in patients, such as excessive

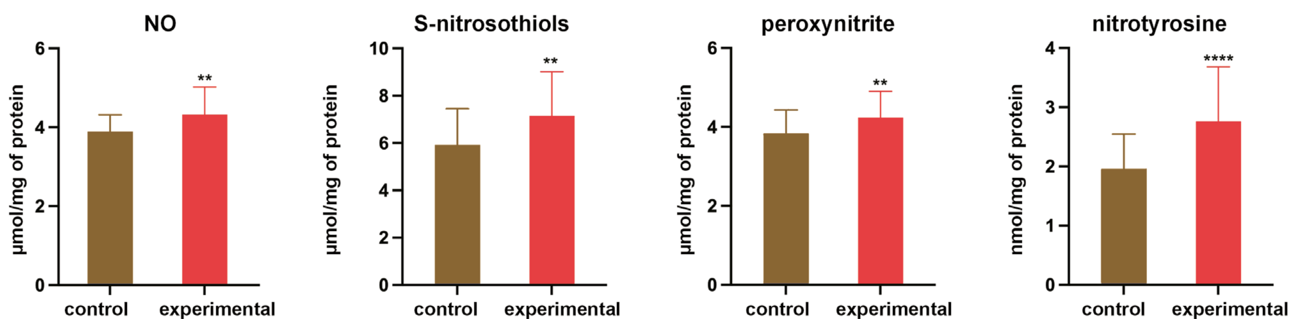

Fig. 5. Nitrosative stress indicators in the blood plasma of control and experimental groups

NO – nitric oxide; ** $p < 0.01$; **** $p < 0.0001$.

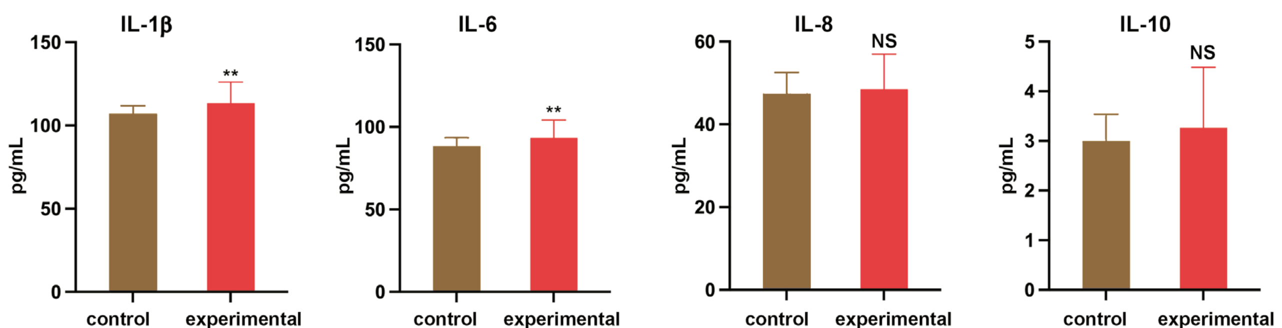

Fig. 6. Pro-inflammatory and anti-inflammatory cytokines in the blood plasma of control and experimental groups

IL – interleukin; ** $p < 0.01$; NS – non-significant.

Table 2. Diagnostic accuracy of the analyzed biomarkers

Category	Biomarker	AUC	*p*-value	Cut-off value	*CIs*	Sensitivity [%]	Specificity [%]
Pro-oxidant enzymes	NOX [pmol $O_2^{\cdot-}$/mg of protein/min]	0.647	0.028*	>7.082	0.5220–0.7718	60.0	62.5
	XO [mU/mg of protein]	0.629	0.046*	>3.736	0.5055–0.7532	62.5	65.0
Antioxidant barrier	total glutathione [µmol/mg of protein]	0.946	<0.0001*	<2.876	0.9014–0.9911	90.0	87.5
	GSSG [µmol/mg of protein]	0.755	<0.0001*	>0.083	0.6478–0.8622	67.5	67.5
	GSH [µmol/mg of protein]	0.948	<0.0001*	<2.783	0.9044–0.9919	90.0	87.5
	redox status $[GSH]^2/[GSSG]$	0.954	<0.0001*	<104.500	0.9150–0.9927	85.0	85.0
	TAS [µmol/mg of protein]	0.698	0.002*	<48.780	0.5805–0.8157	72.5	70.0
Oxidative stress	PC [nmol/mg of protein]	0.598	0.133	>3.220	0.4727–0.7223	52.5	55.0
	total thiols [µmol/mg of protein]	0.637	0.035*	<3.501	0.5131–0.7606	65.0	62.5
	MDA [mmol/mg of protein]	0.591	0.160	>6.534	0.4641–0.7184	57.5	55.0
	LOOHs [µmol/mg of protein]	0.753	<0.0001*	>0.437	0.6422–0.8628	67.5	70.0
Glycoxidation products	AGEs [AFU/mg of protein]	0.617	0.080	>528.600	0.4872–0.7403	57.5	55.0
	dityrosine [AFU/mg of protein]	1.000	<0.0001*	>35.350	1.0000–1.0000	97.5	100.0
	kynurenine [AFU/mg of protein]	0.539	0.545	<28.090	0.4122–0.6666	55.0	52.5
	N-formylkynurenine [AFU/mg of protein]	0.649	0.022*	>28.490	0.5284–0.7691	57.5	55.0
	tryptophan [AFU/mg of protein]	0.616	0.075	<680.900	0.4916–0.7396	55.0	57.5
Nitrosative stress	NO [µmol/mg of protein]	0.696	0.003*	>4.051	0.5780–0.8145	67.5	65.0
	S-nitrosothiols [µmol/mg of protein]	0.728	0.0004*	>6.298	0.6155–0.8408	70.0	67.5
	peroxynitrite [µmol/mg of protein]	0.691	0.003*	>4.019	0.5731–0.8094	67.5	65.0
	nitrotyrosine [nmol/mg of protein]	0.768	<0.0001*	>2.340	0.6621–0.8729	72.5	75.0
Pro-inflammatory and anti-inflammatory cytokines	IL-1β [pg/mL]	0.670	0.009*	>109.200	0.5414–0.7986	62.5	65.0
	IL-6 [pg/mL]	0.676	0.007*	>90.200	0.5557–0.7968	62.5	65.0
	IL-8 [pg/mL]	0.543	0.513	>47.540	0.4130–0.6720	50.0	47.5
	IL-10 [pg/mL]	0.546	0.476	>3.059	0.4132–0.6793	52.5	55.0

AUC – area under the curve; *CI* – confidence interval; * statistically significant.

sensitivity to cold at the implant site or discomfort upon palpation.[11,49,51] In the present study, Ti mandibular fixations were removed to eliminate the future risk of adverse reactions to a foreign body. Most importantly, none of the patients experienced any complications during the healing process. Inflammatory changes, reddening, swelling, abscesses, allergic reactions, or implant exposure could have affected the concentrations of the evaluated biomarkers. However, no such changes were observed in the peri-implant area.

In healthy individuals, the mechanisms of homeostatic control enable adaptation to variable environmental conditions. These mechanisms neutralize external factors that can potentially damage tissues and organs, triggering a local immune response, such as inflammation or oxidative stress. The type and intensity of the adaptive response are determined by the type of a foreign threat and the duration of exposure to that factor. It is believed that the adaptive response triggered by the immune system in the presence of a foreign body can proceed in stages over an extended period of time.

In the present study, systemic oxidative and nitrosative stress, as well as inflammation, were observed in patients treated with Ti mandibular fixations. However, these effects were not observed in the control group subjects. Although only the circulating biomarkers of redox balance and inflammation were evaluated, it can be assumed that systemic changes were induced by inflammation and oxidative stress at the local level. In our previous study, grayish discoloration of the peri-implant tissues was observed in patients undergoing bimaxillary osteotomy, confirming that implant surgery results in metallosis.[11] Although metal particles and ions can be released during the surgical placement of miniplates and screws, metallosis is primarily caused by implant surface wear that affects the surrounding tissues.[8,26] Interestingly, severe tribocorrosion (material degradation caused by the combined effect of corrosion and wear) was observed during the surgical fixation of the mandible, which is the only movable bone of the skull.[27,52] Mechanical wear is intensified in the presence of hard metallic debris, which has abrasive properties and leads to secondary wear. The metallic debris (Ti particles and nanoparticles) released at the peri-implant site can be phagocytized by the circulating immune cells (neutrophils, monocytes) and macrophages, which become stimulated to release pro-inflammatory cytokines (IL-1, IL-6, IL-8) and free radicals.[10,17,53] This process has been observed to have adverse effects on cells and tissues. The induction of the inflammatory response and ROS overproduction lead to the activation of fibroblasts and osteoclasts, the stimulation of the osteolysis processes,[54,55] the inhibition of type I collagen synthesis by osteoblasts,[56] and the production of Ti(IV)-specific T cells, which aggravate inflammation.[57] Titanium particles and ions can also contribute to genome instability, which is implicated in the initiation of carcinogenesis.[58]

In the present study, only patients without systemic diseases, infections or complications during the process of fracture healing were included in the experimental group, thereby supporting the hypothesis that inflammation was induced by a local immune response in the peri-implant tissues. However, despite a significant increase in the plasma levels of IL-1β and IL-6 in the experimental group, the leukocyte count and other routinely assessed inflammatory biomarkers (e.g., CRP) remained within the reference range. Furthermore, no significant differences in the plasma levels of IL-8 and IL-10 were observed between the 2 groups. These observations indicate the presence of mild systemic inflammation in patients with Ti implants, possibly attributable to the reduced activity of the immune system cells engaged in a local immune response. Reactive oxygen species are primarily generated by immune cells, which could also explain the mild symptoms of systemic oxidative/carbonyl stress.[59] Despite a significant increase in lipid peroxidation biomarkers (MDA and LOOHs), the concentrations of PC, AGEs, kynurenine, and tryptophan did not differ significantly between the experimental group and the control group. Lipids are the primary targets of ROS activity in cells. The process of lipid peroxidation is a chain reaction that results in the synthesis of secondary radicals, which then initiate subsequent reactions.[60]

In patients treated with Ti implants, a reduction in the plasma concentrations of total glutathione and GSH was observed, along with an increase in the levels of GSSG. Similar observations were made in previous studies, suggesting that GSH plays an important role in a local immune response in the peri-implant tissues of the mandibular periosteum.[26,61] Glutathione acts as the first line of defense against toxic metals. This antioxidant enhances mitochondrial activity and decreases ROS production, thus protecting tissues from oxidative stress.[26,62] Thiol groups also play a similar role, which explains the observed decrease in plasma thiol concentrations in the experimental group. The ROC analysis revealed that the redox status, and the plasma total glutathione, GSH and dityrosine levels were the most sensitive and specific biomarkers for discriminating between patients treated with Ti mandibular implants and control group subjects. Although further research is needed to validate this observation, the abovementioned parameters could be considered potential biomarkers for evaluating the adverse effects of Ti fixation devices.

Special attention should be paid to the increase in the concentrations of the circulating biomarkers of nitrosative stress (NO, S-nitrosothiols, peroxynitrite, nitrotyrosine). Previous research demonstrated that nitrosative stress could be induced by the macrophages activated by the circulating Ti particles, thereby increasing the expression of inducible NO synthase (iNOS).[63] Inducible NOS not only triggers NO overproduction for peroxynitrite synthesis, but also increases the synthesis of several pro-inflammatory mediators (IL-1β, IL-6).[64] At the local level, increased production of peroxynitrite disrupts metabolic processes and bone remodeling in the peri-implant area.[65] Although iNOS expression in blood cells is mainly determined by endothelium-dependent vasoconstriction, it appears that the circulating Ti particles may also increase the bioavailability of cytotoxic NO at the systemic level.

Strengths and limitations

The study had both strengths and limitations. One of its greatest strengths was the composition of the experimental group, with the subjects carefully selected to ensure equivalence with the control group based on the participants' age, general health and surgical procedures (including the number and quality of osteosynthesis implants). With regard to the main circulating biomarkers of inflammation and oxidative/nitrosative stress, the patients were observed for only 3–4 months after surgery, which represents a limitation of the study. Another issue is the fact that in patients with large joint (hip or knee) prostheses, immune-mediated inflammatory responses, as well as oxidative/nitrosative stress, are likely to be intensified, which makes them easier to observe. Finally, it is not possible to definitively determine whether the observed changes were caused by the influence of Ti or the surgical procedure itself. Therefore, further studies on the long-term effects of Ti implants are required. The timing of exposure is a key factor contributing to the adverse effects of Ti fixations. In the future, the possibility of generalizing the results should be enhanced by planning multicenter studies, or including larger and more diverse patient populations.

Conclusions

The present study showed that Ti mandibular fixations caused systemic oxidative/nitrosative stress and inflammation, whereas no such changes were observed in the control group. Although only the circulating biomarkers of redox balance and inflammation were evaluated, it is probable that systemic changes were induced by local immune responses. Patients treated with Ti mandibular implants exhibited mild symptoms of systemic inflammation and oxidative stress. Titanium miniplates and screws were removed 3–4 months after surgery, once full bone healing had been achieved. It is possible that the observed changes resulted from an early adaptive immune response to a foreign object in the body. Therefore, long-term research is needed to confirm or reject the hypothesis that Ti mandibular implants induce chronic inflammation and oxidative stress in patients. Interestingly, in patients treated for mandibular fractures, inflammatory changes at the Ti-6A1-4V implant site were observed several months or several years after osteosynthesis, which could be due to individual characteristics and the presence of comorbidities.

Ethics approval and consent to participate

The experiment was conducted in accordance with the Declaration of Helsinki (1964). The study protocol was approved by the Bioethics Committee of the Medical University of Bialystok, Poland (approval No. R-I-002/3/2-16). All patients were informed about the purpose of the study and the type of planned examinations, and they agreed to participate in the experiment by signing informed consent forms.

Data availability

The datasets supporting the findings of the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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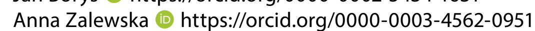
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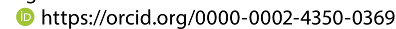
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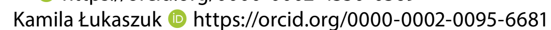
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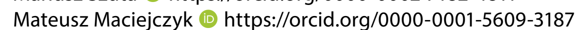
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