Journal of Dentistry 66 (2017) 91–101

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

Full length article

The effect of metal ions released from different dental implant-abutment couples on osteoblast function and secretion of bone resorbing mediators☆

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ARTICLE INFO

Keywords:
Dental implants
Peri-implant bone loss
Platform-switching
Titanium
Corrosion
Ion release

ABSTRACT

Objectives: The etiology of the reduced marginal bone loss observed around platform-switched implant-abutment connections is not clear but could be related to the release of variable amounts of corrosion products. The present study evaluated the effect of different concentrations of metal ions released from different implant abutment couples on osteoblastic cell viability, apoptosis and expression of genes related to bone resorption.

Methods: Osteoblastic cells were exposed to five conditions of culture media prepared containing metal ions (titanium, aluminum, vanadium, cobalt, chromium and molybdenum) in different concentrations representing the amounts released from platform-matched and platform-switched implant-abutment couples as a result of an earlier accelerated corrosion experiment. Cell viability was evaluated over 21 days using the Alamar Blue assay. Induction of apoptosis was measured after 24 h of exposure using flow cytometry. Expression of interleukin-6, interleukin-8, cyclooxygenase-2, caspase-8, osteoprotegerin and receptor activator of nuclear factor kappa-B ligand (RANKL) by osteoblastic cells were analysed after exposure for 1, 3 and 21 days using real-time quantitative polymerase chain reaction assay

Results: Metal ions in concentrations representing the platform-matched groups led to a reduction in cell viability ($P < 0.01$) up to 7 days of exposure. Stimulated cells showed higher rates of early apoptosis ($P < 0.01$) compared to non-treated cells. Metal ions up-regulated the expression of interleukin-6, interleukin-8, cyclooxygenase-2 and RANKL in a dose dependent manner after 1 day of exposure ($P < 0.05$). The up-regulation was more pronounced in the groups containing the corrosion products of platform-matched implant-abutment couples.

Conclusion: Osteoblastic cell viability, apoptosis, and regulation of bone resorbing mediators were significantly altered in the presence of metal ions. The change in cytokine levels expressed was directly proportional to the metal ion concentration.

Clinical significance: The observed biological responses to decreased amounts of metal ions released from platform-switched implant-abutment couples compared to platform-matched couples may partly explain the positive radiographic findings in respect to crestal bone level when utilising the "platform-switching" concept, which highlights the possible role of corrosion products in the mediation of crestal bone loss around dental implants

1. Introduction

Dental implants have been widely used for the replacement of missing teeth in fully and partially edentulous patients. According to the American Academy of Implant Dentistry, 3 million people in the United States have dental implants and that number is growing by 500,000 a year [1]. The use of endosseous dental implants was initiated by the discovery that these implants could be anchored in the jawbone with direct bone contact [2,3]. In 1991, Zarb and Albrektsson described the osseointegration phenomena as "a process in which a clinically asymptomatic rigid fixation of alloplastic material is achieved and maintained in bone during functional loading" [4] For proper osseointegration, several factors must be controlled [5,6], including biocompatibility of the implant material, design and surface of the implant, the condition of the tissues in the implant site, the surgical techniques, and loading procedures [5]. Biocompatibility of an implant

☆ Work submitted in partial fulfillment of the PhD degree of GOA.

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http://dx.doi.org/10.1016/j.jdent.2017.08.002
Received 13 March 2017; Received in revised form 30 June 2017; Accepted 2 August 2017

material is closely related to its susceptibility to corrosion [7]. Therefore, titanium (Ti) has been the material of choice for dental implants due to its superior corrosion resistance behaviour and desirable mechanical properties [8,9].

An important parameter in the long-term success of dental implants is the stability of the peri-implant bone. Previous literature has showed that alterations in the connection geometry of the dental implant-abutment interface, such as platform-switching, may lead to a decrease in peri-implant bone loss that occurs through time [10,11]. Platform-switching is defined as a protocol that includes smaller diameter restorative components that have been placed onto larger diameter implant restorative platforms – the outer edge of the implant-abutment interface is horizontally repositioned inwardly and away from the outer edge of the implant platform [10]. Nevertheless, the etiology for this difference is still questioned. A recent study [12] demonstrated an increase in the amount of metal ions released through accelerated corrosion from platform-matched compared to platform-switched implant-abutment couples.

The role of implant corrosion products in peri-prosthetic osteolysis has been extensively demonstrated in the orthopedic literature [13,14]. This phenomenon may occur as corrosion and wear products can influence the metabolic pathways of various cells including macrophages, lymphocytes, fibroblasts, osteoclasts, and osteoblasts [13,14]. Osteoblasts exposed to cobalt (Co) and chromium (Cr) ions undergo a dose dependent reduction in proliferation [15]. Titanium (Ti) ions at concentrations of 10 ppm or higher for 24 h were found to be toxic [16]. Additional past studies have demonstrated that nontoxic concentrations of metal ions influence the differentiation and function of osteoblastic cells *in vitro* [17,18].

Metal ions/particles may also stimulate osteoblasts to produce pro-inflammatory mediators that contribute to the overall inflammatory process involved in peri-prosthetic osteolysis [13,19–25]. It has been shown that cobalt ions stimulate increased prostaglandin E2 (PGE2) secretion in primary human osteoblasts [26]. This was preceded by up-regulated cyclooxygenase COX-1 and COX-2 gene expression [19,26,27]. Secretion of interleukins 6 and 8 (IL-6 and IL-8) by osteoblasts in response to Ti and other experimentally derived wear particles/ions has also been previously reported [28–30]. Receptor activator of nuclear factor kappa-B ligand (RANKL) is another important protein in peri-prosthetic osteolysis and acts by stimulating osteoclastogenesis [19]. Osteoprotegerin (OPG) is an inhibitor of RANKL. Mine et al. revealed that Ti ions enhanced the expression of RANKL in osteoblast-like cells, suggesting that Ti ions may have adverse effects on bone remodelling at the interface of dental implants and tissues [31].

Although several investigations have documented the potential toxicity and the ability of metal ions/particles to stimulate cytokine production in cultured cell systems [32–34], little is known regarding cell apoptosis, or programmed cell death [35]. It has been suggested that biocompatibility testing should include assessment of apoptosis [36] which is featured by the stimulation of cysteine proteases called caspases. An *in vitro* study [37] showed that Ti particles could induce apoptosis in osteoblasts which may lead to suppressed bone formation.

Although the orthopedic literature is replete with studies regarding the influence of corrosion products on the peri-prostheic tissues and cells [13–37], the dental literature, however, contains little information about the direct interaction between metal ions released from dental implants and osteoblasts from peri-implant tissues. This interaction may provide important insights into the pathogenesis of the observed marginal bone resorption around dental implants [38,39]. In highly corrosive environments, such as the oral cavity, metals, including those of the implant and abutment materials, are prone to degradation [40,41]. The combination of an acidic medium, due to inflammation, presence of acidogenic bacteria, fluorides or food intake, and the micromotion, resulting from occlusal forces, can lead to disruption of the oxide layer protecting the titanium surface [12,40–43]. A recent study [12] demonstrated that Ti implants connected to platform-matched abutments released significantly larger amounts of corrosion elements compared to implants connected to platform-switched abutments, following an accelerated corrosion process. The authors' hypothesis was that this difference in corrosion may be significant on a cell metabolic level in order to change the peri-implant bone homeostasis [12].

The aim of the present study was to investigate the effect of such differences in metal ion concentrations on cell viability, apoptosis, and inflammatory gene expression of human osteoblastic cells cultured within conditioned culture media containing the different concentrations of metal ions obtained from the earlier study [12]. The null hypothesis was that there would be no difference between groups regarding the aforementioned variables.

2. Materials and methods

2.1. Preparation of culture media containing metal ions

Five different conditions of culture media solutions were prepared containing different levels of metal ions obtained from the respective 5 groups of a recent study [12] that evaluated the levels of metal ions released from different implant abutment couples as a result of accelerated corrosion [44]. The metal ions corresponded to the following groups [12]: implants connected to platform-matched titanium (Ti6Al4V) abutments (TM), implants connected to platform-switched titanium (Ti6Al4V) abutments (TSW), implants connected to platform-matched cobalt-chrome (CoCr) abutments (CM), implants connected to platform-switched cobalt-chrome abutments (CSW) and unconnected titanium implants (UI). The amount of mismatch was 0.5 mm between the platform-switched and platform-matched abutments [12]. The concentrations of the measured elements [12] which were used in this study are presented in Table 1. To prepare culture medium containing these concentrations, single element standard solutions for ICP-MS for each measured element (titanium (Ti), vanadium (V), aluminum (Al), cobalt (Co), chromium (Cr) and molybdenum (Mo)) were utilized (TraceCERT®, Sigma-Aldrich Company Ltd.,Dorset, England). Each single standard solution of each element was sterilized by passing through 0.22 μm membrane filters (Millex, Merck Millipore Ltd., Germany) before diluting in culture medium (Clonetics™ OGM™ BulletKit™, Lonza, Walkersville, MD, USA). To reach the desired concentrations of the test solutions, the single element standard solutions were diluted with the serum-added culture medium, under pH monitoring, according to the method described by Taira et al. [45]. No visual precipitation was formed after adding the standard elements and the pH of the prepared solutions was measured immediately after preparation. Metal ion-free culture medium was used as a reference solution (REF) and served as the control group.

Table 1
Levels of metal ions (ppb) present in treated culture media solutions[12].

Test Groups	Code	Levels of Metal Ions (ppb)						
		Ti	Al	V	Co	Cr	Mo	Total
Unconnected implant	UI	998						998
Connected platform matched titanium alloy abutment (6 mm)	TM	1250	67	60				1377
Connected platform switched titanium alloy abutment (5 mm)	TSW	1080	57	36				1137
Connected platform matched cobalt-chrome abutment (6 mm)	CM	678			219	27	10	934
Connected platform switched cobalt-chrome abutment (5 mm)	CSW	623			122	11	6	762

2.2. Cells and cell cultures

Osteoblastic cells were purchased from Lonza (Clonetics™ Normal Human Osteoblast Cell System, NHOst, Lonza, Walkersville, MD, USA). Cells were cultured in monolayer in osteoblast basal medium (OBM™, Clonetics™ OGM™ BulletKit™, Lonza, Walkersville, MD, USA) containing 10% fetal bovine, 0.1% Gentamicin Sulfate/Amphotercin-B and 0.1% Ascorbic acid (OGM™ SingleQuot™, Lonza, Walkersville, MD, USA) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every two days. At 70–80% confluency, adherent cells were detached using 0.25% trypsin/EDTA solution (Trypsin EDTA, Gibco, Life Technologies, Thermo Fisher Scientific, NY, USA). Osteoblastic cells of passages 4–6 were used for the experiments. All experiments were performed three times.

2.3. Cell viability assay

For viability experiments, the osteoblastic cells were transferred to 24-well plates and were seeded in triplicate at a density of 3000 cells/well. The cells were allowed to attach for 24 h, then the metal ion-free medium was replaced by the respective metal ion-containing medium. Cells incubated with metal ion-free medium served as test controls. The cell viability assay was conducted at time points of 1, 4, 7, 10, 14 and 21 days. After 21 days, RNA was extracted for later gene expression analysis. Cell viability at each time point was determined using Alamar Blue™ (AB) bioassay (AbD Serotec, UK). Absorbance measurements were performed at 560 nm and 590 nm using a microplate reader (FLx800, BioTek Instruments Ltd, UK). Reduction of AB by cells was calculated as the percentage reduction from the blue oxidized form of AB to red reduced form according to the following equation:

$$\textit{Percentage reduction of alamarBlue} = \frac{\textit{Fl}590\,\textit{of test agent} - \textit{Fl}590\,\textit{untreated control}}{\textit{Fl}590\,\textit{of}\ 100\%\,\textit{reduced alamarBlue} - \textit{Fl}590\,\textit{untreated control}} \times 100$$

Where: Untreated control is a cell-free culture media subjected to similar incubation conditions as the test groups and control (REF)

Fl 590 = Fluorescent intensity at 590 nm emission (560 excitation)

The resultant AB reduction percentages represented the percentage of cell viability and was used in statistics for viability comparison between test groups and the metal ion-free control (REF).

2.4. Flow cytometric analysis of early apoptosis

For apoptosis experiments, the cells were transferred to 24-well plates and were seeded in triplicates at a density of 50 000 cells/well. The cells were allowed to attach for 24 h, then the metal ion-free medium was replaced by the respective metal ion-containing medium for a period of 24 h. Cells incubated with metal ion-free medium served as test controls. At the end of the 24 h exposure period, the cells were collected by centrifugation and washed twice with phosphate buffer solution (PBS, BioWhittaker, Lonza, Belgium). The cells were then re-suspended in Annexin Binding Buffer and aliquots (5 µL) of Phycoerythrin Annexin V and Propidium Iodide Staining Solution (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen™, BD Bioscience, UK) were added to each test tube following manufacturer instructions. The samples were placed into a fluorescence activated cell sorting (FACS) flow cytometer (EPICS XL®, Coulter Corporation, Florida, USA) for analysis. A minimum of 10,000 events in the target area was recorded for each sample.

2.5. Gene expression analysis

RNA levels of IL-6, IL-8, COX-2, Caspase-8, OPG and RANKL expressed by osteoblastic cells were analysed after incubation with metal ion-containing media for 24 h, 72 h and 21 days. Cells incubated with metal ion-free medium served as test controls. Cells were seeded onto 24-well plates in triplicates at different densities based on the exposure period to the metal ion-containing media. For the 24 h and 72 h exposure periods, cells were seeded at a density of 50 000 cells/well or 25 000 cells/well respectively. For the 21-day exposure period, RNA was extracted from the same cells that were initially seeded for the viability assay after conducting the 21 day time point viability analysis.

2.5.1. RNA extraction

After each exposure period, culture medium was removed from the wells, cells were washed twice with phosphate buffered saline solution (PBS, BioWhittaker, Lonza, Belgium) and were immediately lysed using the lysis buffer of an RNA extraction kit (RNeasy®Plus Mini Kit, QIAGEN GmbH, Hilden, Germany). Total RNA was extracted according to the protocol of the manufacturer. Quantity and purity of the RNA were determined by 260/280 nm absorbance measurements using TECAN plate reader (Infinite M200, TECAN, GmbH, Austria) The remaining RNA was stored at −80° C until complementary DNA (cDNA) synthesis was performed.

2.5.2. cDNA synthesis

RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, NY, USA) according to the manufacturer instructions in a final reaction volume of 20 µL. cDNA was synthesized with total RNA (100 ng) and was amplified by polymerase chain reaction (PCR) in a thermal cycler (PTC-100™ Programmable Thermal Controller, MJ Research Inc., MA, USA). Thermal cycling conditions were as follows: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C after which temperature gradually drops to 4 °C. The resulting cDNA was stored at −20° C until further analysis.

2.5.3. Real-time quantitative polymerase chain reaction (RT-qPCR) assays

For RT-qPCR, 5 µL of the abovementioned diluted cDNA was added to TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assay primer/probe mixes (TaqMan® Gene Expression Assays, Applied Biosystems™, Thermo Fisher Scientific, NY, USA) according to the manufacturer's instructions to achieve a final reaction volume of 25 µL. Gene expression was measured using primer–probe sets specific for human IL–6 (Hs00985639_m1), IL–8 (Hs00174103_m1), COX-2 (PTGS2) (Hs00153133_m1), Caspase 8 (Hs01018151_m1), RANKL (TNFSF11) (Hs00243522_m1), OPG (TNFRSF11B) (Hs00900358_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs03929097_g1) by means of RT-qPCR using 7300 Real Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, NY, USA). Gene specific primers and the TaqMan qPCR mastermix for FAM™ reporter dye were purchased from TaqMan® (TaqMan® Gene Expression Assays, Applied Biosystems™, Thermo Fisher Scientific, NY, USA). Each cell sample was assayed for each gene a minimum of three separate times in 96-well optical plates with primer concentrations of 0.8 mM. The PCR protocol consisted of: initiation at 1 cycle at 50 °C for 2 min and 1 cycle at 95 °C for 10 min, followed by amplification for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Ct data were collected via Sequence Detection Software 1.4 (7300 System SDS software RQ Study Application, Applied Biosystems). Gene expression was normalized to housekeeping gene (GAPDH) and expressed relative to the reference control group (REF) for each incubation time using the $2^{-\Delta\Delta Ct}$ method [46]. The ratio of RANKL/OPG was calculated by dividing the normalized fold expression of both genes within the same sample. Since the experiment was performed in triplicate and the PCR reactions were also performed in triplicate, there were 54 data points collected

2.6. Statistical analysis

All data were expressed as mean and standard deviation. For viability and apoptosis analysis, statistically significant differences were

tested by univariate analysis of variance (ANOVA) using SPSS version 22.0 (IBM SPSS Statistics, IBM, Tokyo, Japan) ($P < 0.05$). For gene expression analysis, statistically significant differences were tested by multivariate repeated measures (ANOVA) statistical model where all 54 data points were used to fit this model. Comparisons were performed between each test group and the control (REF) within each incubation period and between the platform-matched groups and the platform-switched groups within each abutment material and within each incubation period. Levene's test of homogeneity of variance was employed ($\alpha = 0.05$), following the assumption of equal variances. When equal variances were assumed ($P > 0.05$) the Bonferroni post hoc test was used to analyze significant differences between test groups. Whereas when equal variances were not assumed ($P < 0.05$) the Dunnett's T3 post hoc test was used to analyze significant differences between the test groups. To confirm statistically significant differences between the platform-matched groups and the platform-switched groups within each abutment material, *t*-test for two independent samples was used ($P < 0.05$).

3. Results

3.1. Effect of metal ions on cell viability

The percentages of reduction reaction from the blue oxidized form of AB to red reduced form over the 21-day time period, which represented the percentages of cell viability, are presented in Fig. 1. There was statistically significant lower cell viability in the TM ($P < 0.001$), TSW ($P < 0.001$) and CM ($P < 0.01$) groups compared to the control (REF) after 24 h of exposure as well as on day 4 ($P < 0.001$) and day 7 (TM and TSW ($P < 0.001$), CM ($P < 0.05$)). The platform-matched CoCr abutment group (CM) also showed lower cell viability compared with the platform-switched group of the same material (CSW) on day 4 ($P < 0.01$). On day 10, the TM was the only group that showed less cell viability when comparing with the control ($P < 0.05$). After 14 days of exposure, all test groups did not differ in their cell viability from the control ($P > 0.05$). However, on day 21, the CM and CSW groups had significant lower cell viability than the control ($P < 0.001$).

3.2. Effect of metal ions on early apoptosis

All groups of osteoblastic cells exposed to metal ion-containing media showed significantly higher percentage of apoptosis after 24 h compared to the control (UI, TM, CM, and CSW ($P < 0.001$), TSW ($P < 0.005$)) (Fig. 2). The percentage of apoptotic cells did not differ significantly between the different ion concentration groups ($P > 0.05$)

3.3. Effect of metal ions on gene expression

3.3.1. Interleukin-6 expression

Osteoblastic cells cultured with different metal ion types and concentrations released a higher amount of IL-6 compared to the non-stimulated reference group (REF) (UI, TM, TSW and CM ($P < 0.001$), CSW ($P < 0.05$)) (Fig. 3). This increase of IL-6 expression was directly proportional to the concentration of metal ions after 1 day of exposure. In particular, cells from the TM group increased the production of IL-6 by 3 fold in the first 24 h compared to the REF ($P < 0.001$) and the increase was also significantly higher compared to the osteoblastic cells of the TSW group ($P < 0.001$) (Fig. 3). Osteoblastic cells in the CM group also showed statistically higher expression of IL-6 compared to the CSW group after 24 h ($P < 0.005$). After 3 days of exposure, there was a significant tendency for cells incubated with metal ions released from implants connected to Ti alloy abutments (TM and TSW) to release more IL-6 compared to the REF group ($P < 0.001$). Moreover, after 3 days, osteoblasts from the TM group demonstrated higher IL-6 expression compared to cells from the TSW group ($P < 0.01$) (Fig. 3). A longer incubation time (21 days) caused higher IL-6 levels from osteoblastic cells in all experimental groups with an average expression 2-fold higher than the REF group ($P < 0.001$). This increase of IL-6 was already observed at the lowest metal ion concentration group (CSW) after 21 days of incubation (Fig. 3). No statistical difference was observed between CM and CSW groups for the 3 day and 21 day duration of the experiment.

3.3.2. Interleukin-8 expression

In the first 24 h of exposure, IL-8 expression followed the same pattern that was seen with IL-6 release in which osteoblastic cells

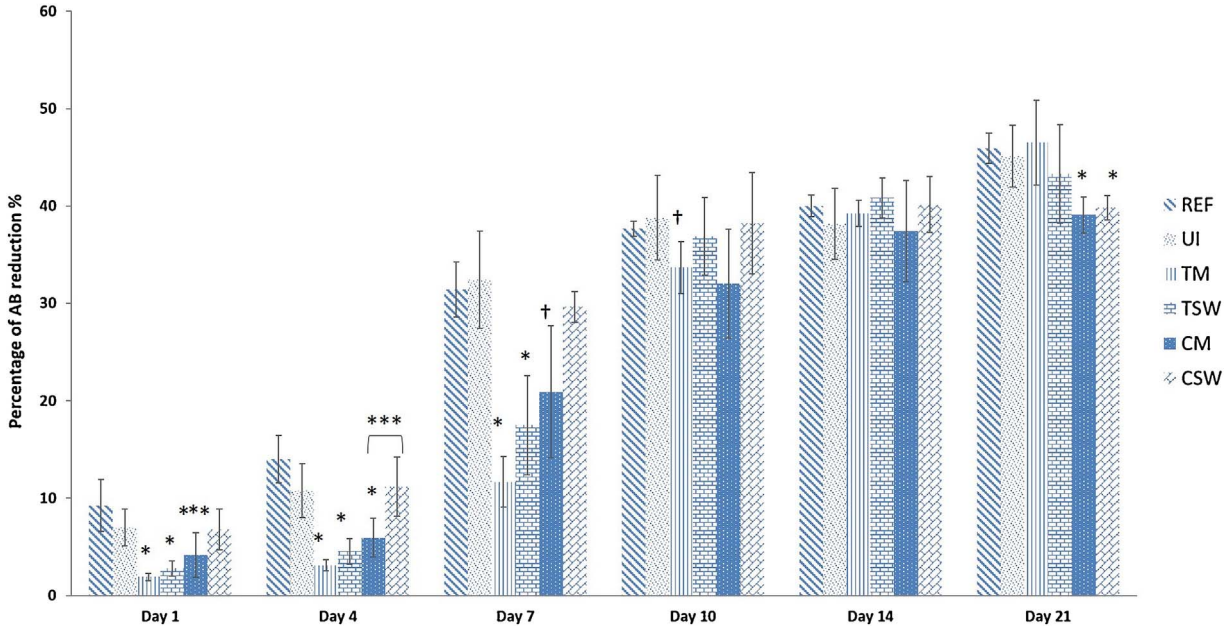

Fig. 1. Osteoblast reduction of Alamar Blue™ (%). Osteoblastic cells were treated with metal ion-containing culture media and the reduction in the Alamar Blue™ was measured over time points of 1, 4, 7, 10, 14 and 21 days of exposure. The results were expressed as mean percentage reduction (%) ± standard deviation $n = 9$ per group (*$P \leq 0.001$, ***$P < 0.01$, †$P < 0.05$, statistical differences with respect to the control REF, ⌐¬ statistical difference between platform-matched and platform-switched within each material).

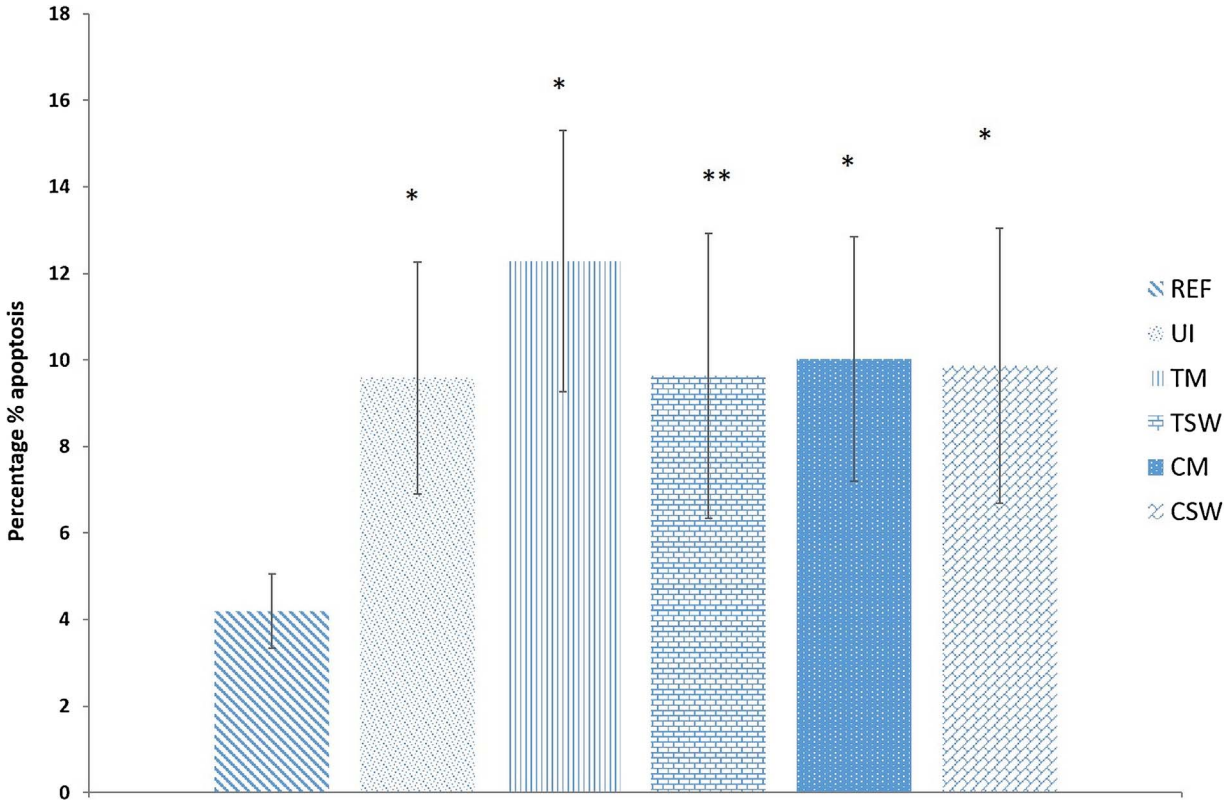

Fig. 2. Percentage of osteoblast apoptosis. Osteoblastic cells were treated with metal ion-containing culture media and the percentage of apoptotic cells was measured after 24 h of exposure. The results were expressed as mean percentage of apoptosis (%) ± standard deviation $n = 9$ per group (*$P \leq 0.001$, **$P < 0.005$, statistical differences with respect to the control REF).

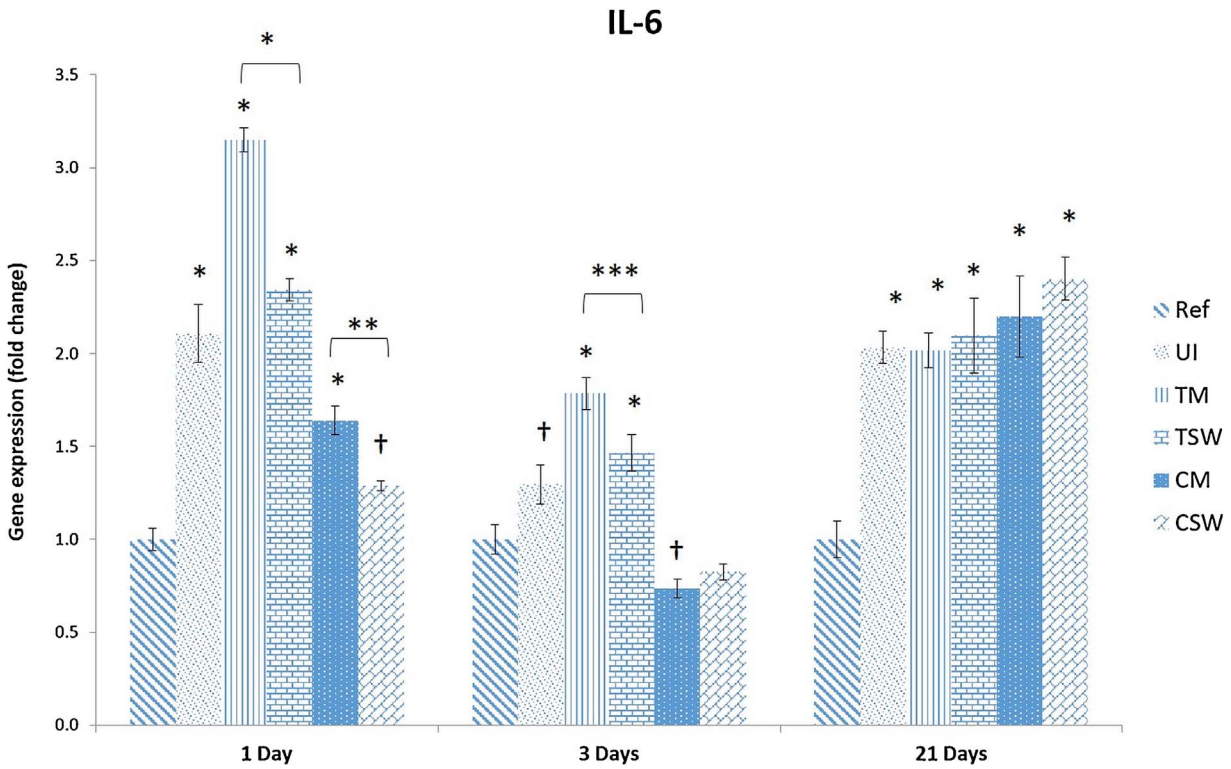

Fig. 3. Interleukin-6 expression by osteoblasts. Osteoblastic cells were treated with metal ion-containing culture media and the expression of IL-6 was measured over time points of 1, 3 and 21 days of exposure. The results were expressed as mean fold change ± standard deviation (*n=3 independent samples x 3 repeats)* (*$P \leq 0.001$, **$P < 0.005$, ***$P < 0.01$, †$P < 0.05$ statistical differences with respect to the control REF, ⌐¬ statistical difference between platform-matched and platform-switched within each material).

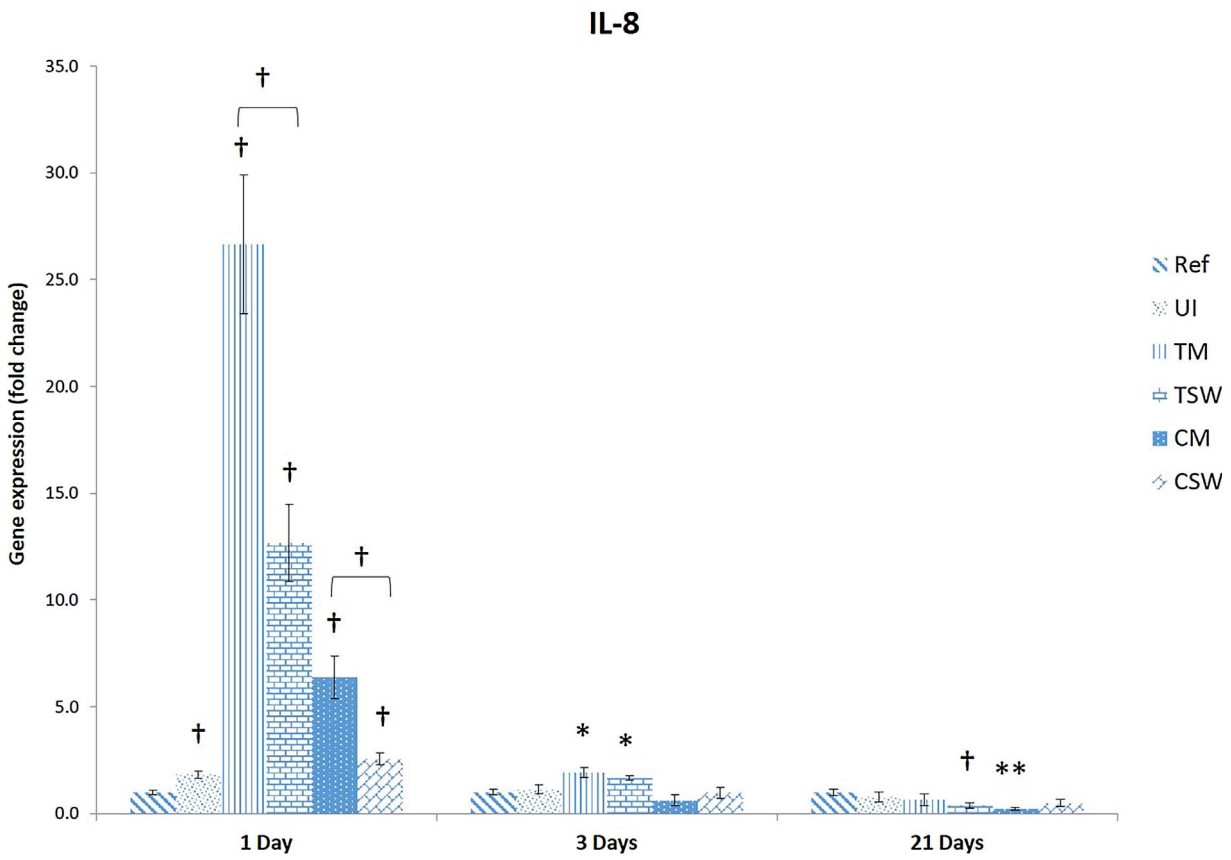

Fig. 4. Interlukin-8 expression by osteoblasts. Osteoblastic cells were treated with metal ion-containing culture media and the expression of IL-8 was measured over time points of 1, 3 and 21 days of exposure. The results were expressed as mean fold change ± standard deviation (*n = 3 independent samples x 3 repeats)* (*$P \leq 0.001$, **$P < 0.005$, †$P < 0.05$, statistical differences with respect to the control REF, ⌐¬ statistical difference between platform-matched and platform-switched within each material).

cultured with metal ions released a higher amount of IL-8 compared to the non-stimulated REF cells ($P < 0.05$) (Fig. 4). This increase of IL-8 expression was again directly proportional to the concentration of metal ions after 1 day of exposure. Cells in the TM group exhibited a major increase in IL-8 expression (25-fold, $P < 0.05$) compared to cells in the REF group, as well as compared to all other test groups including the platform-switched titanium abutment group (TSW) ($P < 0.05$). After 3 days of incubation, the TM and TSW groups continued to show higher IL-8 release compared to the control group ($P < 0.001$). However, the initial elevated expression of IL-8 inverted to a decreased expression after longer incubation (Fig. 4). This was significant for osteoblastic cells in the CM ($P < 0.005$) and TSW groups ($P < 0.05$) after 21 days compared to the reference (Fig. 4).

3.3.3. COX-2 expression

After 24 h of incubation, osteoblastic cells cultured with metal ions showed significant increase in expression of COX-2 in all test groups, except CSW (UI ($P < 0.001$), TM ($P < 0.01$), TSW and CM ($P < 0.05$), compared to the reference (REF)(Fig. 5). Although cells in the TM group showed the highest mean COX-2 expression among the test groups (~5 fold) it was not statistically significant from the TSW ($P > 0.05$) after 1 day. Osteoblastic cells in the CM group expressed statistically significant higher levels of COX-2 compared to cells of the CSW group ($P < 0.05$). After 3 days of incubation, there was a significant decrease in the expression of COX-2 in all test groups that represented connected implant-abutment couples (TM and CM ($P < 0.01$), TSW and CSW ($P < 0.05$)) (Fig. 5). Osteoblastic cells treated with metal ions from TM group showed down-regulation of COX-2 levels after 21 days of incubation ($P < 0.05$) compared to the REF (Fig. 5) and compared to the platform-switched group of the same material (TSW) ($P < 0.05$)

3.3.4. Caspase-8 expression

Caspase activity was not influenced by metal ions in the first 24 h of exposure ($P > 0.05$) (Fig. 6). However, after 3 days of incubation, there was a down regulation of Caspase-8 secretion from osteoblastic cells treated with metal ions from the platform-matched CoCr abutment group (CM) compared to the reference ($P < 0.05$) (Fig. 6). This decrease in Caspase-8 production was also evident in groups TM and CM ($P < 0.01$) after longer incubation time (21 days) when compared to the REF (Fig. 6)

3.3.5. RANKL and OPG expression

The expression of RANKL, OPG, and the ratio of RANKL/OPG are presented in Fig. 7. RANKL expression was up-regulated in all test groups after 24 h of incubation when compared to the REF (UI and CM ($P < 0.05$) TM, TSW and CSW ($P < 0.005$)) (Fig. 7a) in a dose dependent manner. On the other hand, OPG production was not influenced in most experimental groups except for the TM group, where it was down regulated ($P < 0.001$) compared to the REF in the first 24 h of exposure (Fig. 7b) and compared to the platforms-witched titanium abutment group (TSW) ($P < 0.001$).These variable expressions led to high ratios of RANKL/OPG in all test groups after 24 h of incubation compared to the REF (UI and CM ($P < 0.001$), TM and TSW ($P < 0.05$) and CSW ($P < 0.005$)) (Fig. 7c) with the highest ratio observed in the osteoblastic cells of the TM group (22-fold) which was significantly higher compared to all the test groups including the platform-switched titanium abutment group (TSW) ($P < 0.001$). Moreover, the cell cultures of the CM group showed increased RANKL/OPG ratio compared to cultures in the CSW group ($P < 0.001$). After 3 days of incubation, the RANKL expression significantly decreased in the CM ($P \leq 0.001$) and CSW ($P \leq 0.05$) (Fig. 7a) when compared to the REF, while the TM group continued to show higher RANKL

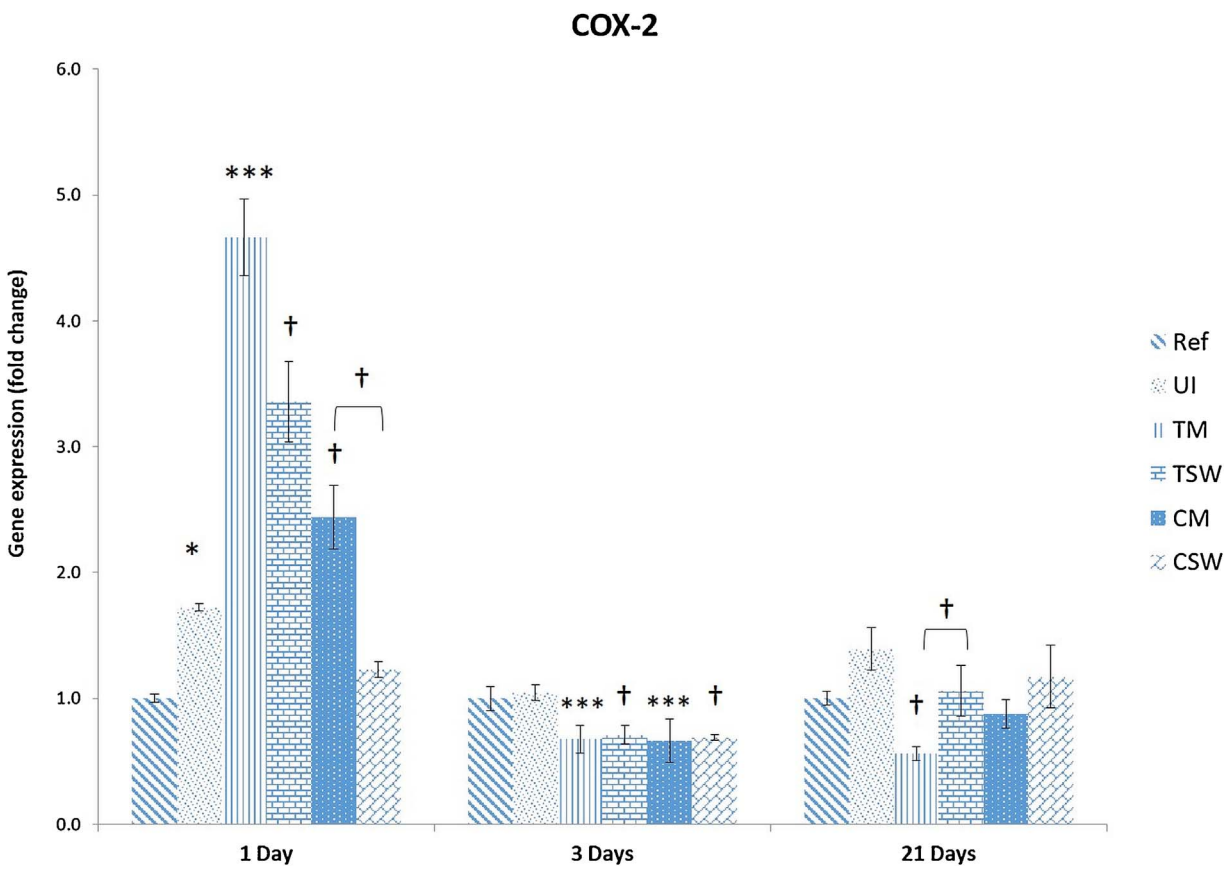

Fig. 5. COX-2 expression by osteoblasts. Osteoblastic cells were treated with metal ion-containing culture media and the expression of COX-2 was measured over time points of 1, 3 and 21 days of exposure. The results were expressed as mean fold change ± standard deviation (*n = 3 independent samples x 3 repeats)* (*$P \leq 0.001$, ***$P < 0.01$, †$P < 0.05$, statistical differences with respect to the control REF, ⌐¬ statistical difference between platform-matched and platform-switched within each material).

production compared to the control and to the platform-switched titanium abutment group (TSW) after 3 days of exposure ($P < 0.05$) (Fig. 7a). After longer incubation time (21 days), the ratio RANKL/OPG in almost all conditions (except for the UI and CSW) was significantly lower than the REF (TM ($P < 0.005$), TSW and CM) ($P < 0.001$) (Fig. 7c).

4. Discussion

The corrosion of implant materials *in vivo* is a type of material response to the host physiological environment [47]. At the same time, the host response towards implant materials or their corrosion products is manifested through different forms or types of response, of which, osseointegration is one type of a positive foreign body response to dental implants [5]. Marginal bone loss around oral implants, on the other hand, has been identified as a consequence of an aggravated foreign body response inevitable when placing foreign materials in bone [5]. The extent of foreign body response to the implant degradation products depends on the type, size, morphology and concentration of such products [48]. The present study, evaluated different biological responses of human osteoblastic cells to varying concentrations of metal ions, representing the amounts released from platform-matched and platform-switched implant abutment couples, as a result of accelerated implant corrosion [12]. Although the concentrations tested were not obtained from a clinical in situ study, they were physiologically relevant to the findings of He et al. [49] who recently demonstrated that the average content of Ti in the jaw bones of implant patients was 1940 μg/kg or ppb. Concentration of metal ions or particles has been reported to be directly proportional to the phagocytic response up to a saturation level [50]. Sun et al. [51] showed that the effects of metal ions (Ni, Co, Ti and V) on cell viability were a function of their concentrations. Cell viability in the present study was also influenced by the high concentrations of the metal ions in the TM, TSW, and CM groups, which caused a reduction in cell viability of osteoblastic cells exposed to these concentrations for up to one week of incubation. High concentrations of metal ions representing the platform-matched group TM continued to reduce cell viability for longer incubation periods. However, Ti ions alone in the UI group, though high, did not affect the cell viability. This finding is in agreement with several studies that showed that Ti ions in the concentration range of 1 to 9 ppm had no significant effects on the viability of osteoclast-like cells, osteoblast-like cells, epithelial-like cells or splenocytes [31,33]. Therefore, it could be suggested that the presence of a mixture of different types of metal ions and particles, as in the situation with tribochemical corrosion processes of alloys in the oral cavity, may be more cytotoxic to the cells in direct contact than exposure to a single type of metal ion or particle. Metal ions and particles both play a role by triggering different metabolic pathways [13,20]. Particulate wear debris are a source of metal ions since they have large surface area and they are prone to dissolution resulting in measurable increases different ions [15,52]. Therefore the increased levels of metal ions may play a role in the etiology of particle-induced osteolysis [52]. Haynes et al. [53] demonstrated that there is a difference in cellular response to different types of metal alloy wear particles that are of the same size. The authors [53] found that CoCr particles were likely to be more toxic than the Ti6Al4 V particles. Although the present study tested metals ions rather than particles, the results revealed similar tendency for the CoCr abutment groups (CM and CSW) being more cytotoxic after 21 days of incubation.

The current study found that the presence of metal ions in culture media induced programmed cell death, apoptosis, in human osteoblastic cells. This finding is in agreement with the results of earlier

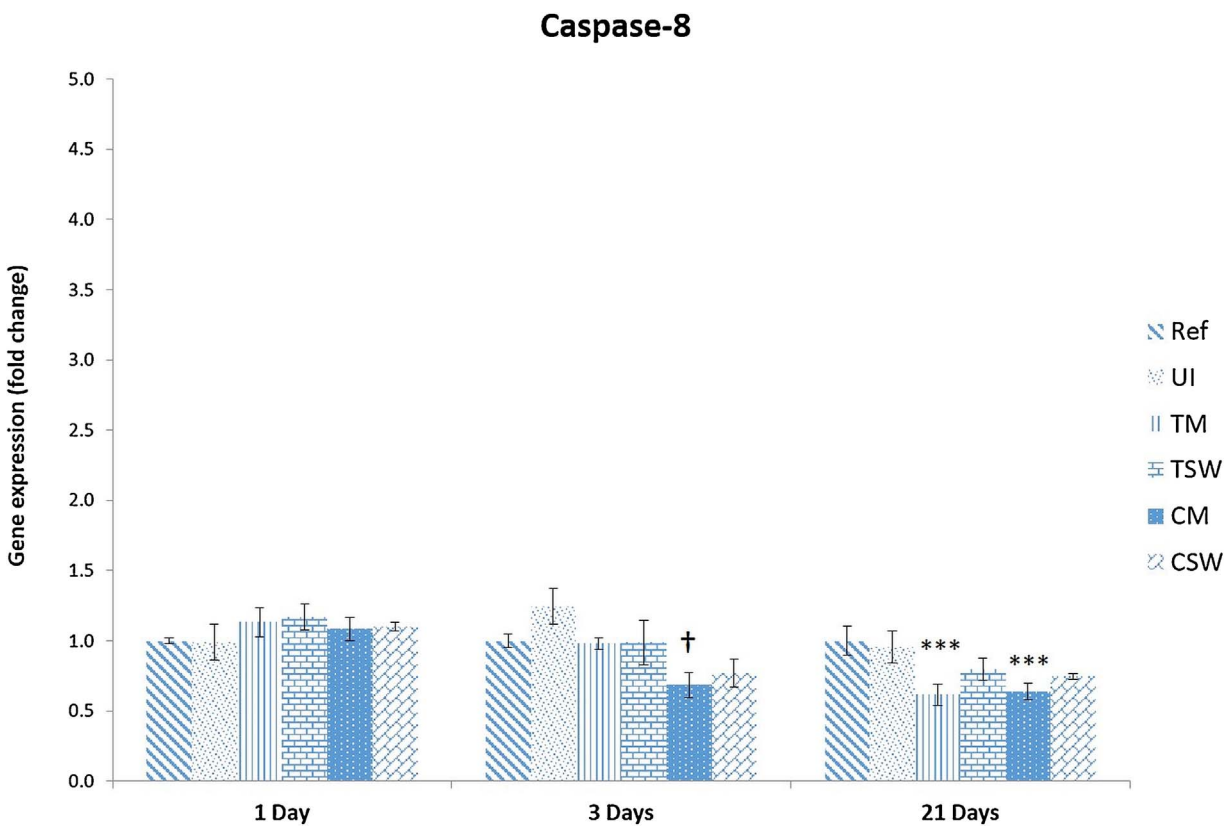

Fig. 6. Caspase-8 expression by osteoblasts. Osteoblastic cells were treated with metal ion-containing culture media and the expression of caspase-8 was measured over time points of 1, 3 and 21 days of exposure. The results were expressed as mean fold change ± standard deviation (*n = 3 independent samples x 3 repeats)* (***$P < 0.01$, †$P < 0.05$ statistical differences with respect to the control REF).

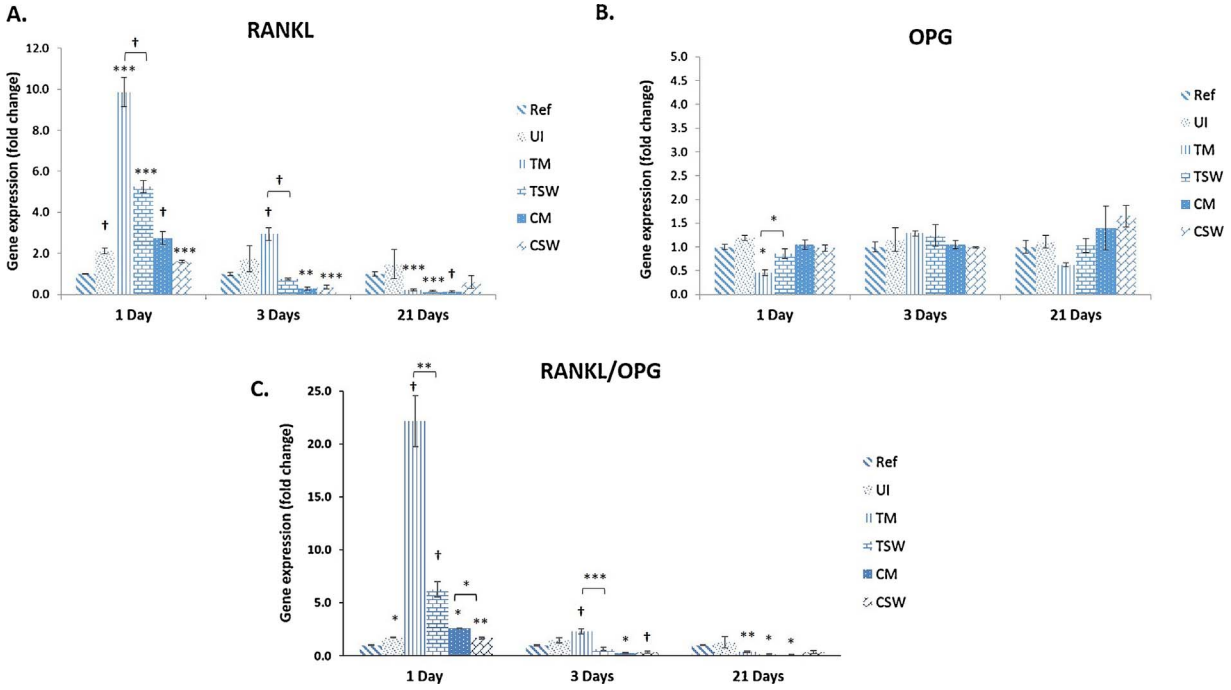

Fig. 7. Expression of RANKL, OPG and RANKL/OPG ratio by osteoblasts. Osteoblastic cells were treated with metal ion-containing culture media and the expression of RANKL and OPG was measured over time points of 1, 3 and 21 days of exposure. (A) RANKL expression by osteoblasts, (B) OPG expression by osteoblasts, and (C) ratio of RANKL expression to OPG expression. The results were expressed as mean fold change ± standard deviation (*n = 3 independent samples x 3 repeats)* (*$P \leq 0.001$, **$P < 0.005$, ***$P < 0.01$, †$P < 0.05$, statistical differences with respect to the control REF, ┌┐ statistical difference between platform-matched and platform-switched within each material).

studies, using different cell types [35,54] which demonstrated that the exposure of macrophages to Co and Cr ions for a short period (24 h) mostly stimulated apoptosis. Pioletti et al. [36] showed that implant particles stimulated apoptosis, particularly in less mature osteoblasts. The high susceptibility to apoptosis of the less mature osteoblast could affect bone remodelling. If less mature osteoblasts undergo apoptosis *in vivo*, the quantity of mature osteoblasts available to synthesize new bone will also decrease, favoring the resorption process. Apoptosis is featured by cell shrinkage, surface blebbing, chromatin concentration and generation of apoptotic bodies [35]. However, microscopic evaluation was not performed in the present study. It would be interesting to correlate the flow cytometry measurements with microscopic analysis in future studies. In order to confirm the occurrence of apoptosis in the examined osteoblastic cells, the expression of caspase-8, a protein involved in apoptosis signaling, was measured but no correlation was found with the increase in the percentage of apoptotic cells. In contrast, Pioletti et al. demonstrated increased caspase activity up to the last exposure point of 72 h [36]. A possible explanation for this observed difference in results may be that in the present study, caspase-8 was evaluated while the previous study [36] had measured caspase-3. Both caspases are important mediators in apoptosis, and further studies should be performed in this area to clarify this discrepancy.

The results of this study also demonstrated that the presence of metal ions in the culture media of human osteoblastic cells induced the production of pro-inflammatory cytokines in a dose dependent manner. IL-6 is an important marker for chronic inflammatory processes and is assumed to cause osteoclast activation [29]. The results of this study demonstrated that osteoblastic cells from the cultures representing the platform-matched groups (TM) and (CM) demonstrated higher expression of IL-6 compared to cells in the platform-switched groups within the same material respectively (TSW) and (CSW). Increased secretion of IL-6 by stimulated osteoblastic cells in this study corroborates the earlier findings of Vermes et al. [21] who confirmed that IL-6 is present and continuously secreted by particle-stimulated cells in the peri-prosthetic space, and that its long-term *in vivo* autocrine and paracrine effects are critical in the pathogenesis of the peri-prosthetic osteolysis. The expression of IL-8, which acts as potent chemo-attractant of neutrophils and macrophages, was also directly proportional to the concentration of metal ions and was most marked for the cells in the platform-matched Ti6Al4 V abutment group (TM). However, the enhanced chemokine expression of IL-8 did not continue after longer incubation times which may be due to the fact that IL-8 is usually expressed immediately, as early as 1 h after exposure to a stimuli [34] and reaching maximal levels in 2 h of exposure [55] after which it starts to decline [34]. Other investigators have also demonstrated this decline of expression levels [29,34,56]. Up-regulation of IL-8 has been documented *in vivo* in patients with *peri*-implantitis [57]. Hence, by triggering IL-8, osteoblastic cells may attract more inflammatory cell infiltrates and contribute to their migration into the peri-implant tissue and thus enhancing the inflammatory reaction to dental implants and their corrosion products [40].

To assess the stimulation of PGE2 production, we examined if this occurs at a transcriptional level by using real time PCR to measure the expression of COX-2 enzyme, a key enzyme involved in prostaglandin synthesis [19]. The activation of COX-2 expression was associated with higher concentrations of metal ions ($>$ 765 ppb). The influence of abutment mismatch was mostly evident within the CoCr abutment groups. Queally et al. [26] also observed an increase in COX-2 secretion from osteoblasts exposed to Co and Cr ions which suggested that the cobalt ions increased PGE2 levels by inducing the COX enzyme at a transcriptional level [19,26]. PGE2 has been reported to induce osteolysis by stimulating osteoclasts, and increased osteoclast differentiation [19,27]

Additionally, the enhancement of expression of RANKL by osteoblastic cells following metal ions stimulation was also detected in the present study in a dose dependent manner. The RANKL/OPG ratio was higher in cell cultures of the platform-matched titanium alloy group (TM) compared to those in the platform-switched group (TSW). This tendency was also observed in the CoCr abutment groups. The alteration of the ratio of RANKL to OPG triggers the imbalance of bone metabolism, an important causative factor of pathologic bone resorption [31]. Therefore, the results of this study suggested that metal ions could alter cellular components in osteoblastic cells regulating osteoclast differentiation. Mine et al. [31] also demonstrated that Ti ions at 9 ppm altered the expression of RANKL and OPG mRNAs in osteoblast-like cells after 24 h of exposure [31,58]. However, the concentrations tested in that study were far higher than the concentrations investigated in the present study. Using more clinically relevant concentrations, which were also close to the concentrations used in the present study, Zijlstra et al. [59] recently showed that RANKL/OPG ratio was increased after 72 h of incubation of osteoblast cells with almost all Co and Cr concentrations tested (1–100 ppb) [59]. The observed elevation was also dependent on the ion dosages of Co and Cr. The exact mechanism underlying the down-regulated expression of RANKL and some other genes tested in the present study after longer incubation periods with metal ions is not clearly understood, however, one might suspect some cellular stress due to continuous exposure to the Alamar Blue dye for prolonged incubation periods [60]. Although the observed down regulation cannot be fully explained, it pointed out that metal ions altered the function of stimulated osteoblastic cells at a transcriptional level which could lead to changes in the normal fate of these cells and consequently affecting their ability for bone formation.

Within the limitations of the present *in vitro* study, the results indicated that cytokine and chemokine levels expressed by human osteoblastic cells were significantly altered in the presence of metal ions. This change was directly proportional to the metal ion concentration. Therefore, this study has demonstrated, for the first time, that the increased levels of metal ions released from corrosion of platform-matched compared to platform-switched implant abutment couples [12] resulted in an increase in the expression and secretion of cytokines and chemokines related to bone resorption from human osteoblastic cells mostly after 24 h of exposure to the metal ions. Changes in cytokine levels have a crucial effect on immune and inflammatory responses [33,61], and the outcome of bone loss may be attributable to the relative imbalance of these cytokines. Therefore, these observed *in vitro* results may be correlated to the *in vivo* positive radiographic findings [10,11] in respect to crestal bone levels when utilising the “platform-switching” concept, thereby, possibly providing a biologic plausibility to the proposed theory of the role of corrosion products in the mediation of crestal bone loss around dental implants.

Further investigations are needed, especially trying to simulate more closely the clinical situation where other modes of corrosion might also take place including fretting corrosion in which metal particles are released. The results of this study might also provide further insight into the possible role of corrosion products on the etiology of bone loss around dental implants.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

Special thanks to Mr. David Boniface from the Eastman Dental Institute for the help with statistical analysis. The study was funded by a scholarship from King Saud University, Kingdom of Saudi Arabia.

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