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In vitro proinflammatory gene expression changes in human whole blood after contact with plasma-treated implant surfaces

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ABSTRACT

Background: The aim of this *in vitro* study was to identify changes in gene expression of proinflammatory cytokines in human whole blood after contact with titanium implant surfaces after plasma treatment.
Materials and methods: Grade 4 titanium dental implants were conditioned with low-pressure plasma (LPP) and atmospheric-pressure plasma (APP) and submerged in human whole blood *in vitro*. Unconditioned implants and blood samples without implants served as control and negative control groups, respectively. Sampling was performed at 1, 8, and 24 h. Changes in mRNA expression levels of interleukin 1-beta (*IL1-β*) and tumor necrosis factor-alpha (*TNF-α*) were assessed using RT-qPCR.
Results: In the control group, significant increases in *IL1-β* and *TNF-α* expression were observed. Significant decreases in the expression of *IL1-β* and *TNF-α* were identified in blood with implants after plasma treatment.
Conclusion: Differences in gene expression of proinflammatory cytokines after incubation of plasma-conditioned titanium implants can be assessed using human whole blood. The results of the present study indicate that plasma treatment (APP and LPP) of titanium dental implants leads to downregulation of proinflammatory cytokine gene expression, which might be beneficial in early osseointegration.

1. Introduction

Tissue repair after implantation is due to host responses. Host immune responses are determined by the physico-chemical nature of the implant surface (Franz et al., 2011). For this reason, many researchers have attempted to modify the surface properties and thus optimize tissue responses to dental implants (Junker et al., 2009). Surface treatment with technically produced plasma as the fourth state of matter is a promising approach for the surface modification of titanium dental implants (Metelmann et al., 2018). This leads to an increase in the rate of accumulation of osteoblasts (Swart et al., 1992) and an improvement in cell adhesion of fibroblasts in the first eight hours following implant placement (Canullo et al., 2013). Animal experiments have demonstrated fewer chromosomal aberrations and cell nucleus deformations in plasma-conditioned surfaces than with untreated titanium surfaces (Tavares et al., 2009). Chair-side plasma conditioning of titanium implants has been reported to result in 300% higher bone-to-implant contact (BIC) and a 30% improved bone area fraction occupancy (BAFO) rate compared to untreated implant surfaces in a canine model (Coelho et al., 2012). The signaling processes that cause the abovementioned cellular effects have not yet been fully elucidated. It is undisputed that contact between the implanted biomaterial and human tissue constituents leads to the formation of mediators that generate an inflammatory reaction (Urbanski et al., 2017; Bielemann et al., 2018).

The first response to the insertion of an implant is an inflammatory reaction that initiates the early phase of osseointegration. One characteristic of the inflammatory processes is the

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activity of proinflammatory cytokines such as interleukin 1-beta (*IL1-β*) and tumor necrosis factor-alpha (*TNF-α*) (Urbanski et al., 2017; Bielemann et al., 2018). A key mediator of the inflammatory process is *IL1-β*, which is significantly involved in the reparative phase by either affecting cell function or by shedding additional cytokines and growth factors (Clark, 1996; Dirschnabel et al., 2011). Chronic and excessive inflammatory reactions are considered to be high risk factors and may lead to the loss of implants by inhibiting osseointegration (Williams, 2008). The expression of *TNF-α* in the early phase of osseointegration may have a direct impact on the subsequent success of implantation, as demonstrated by the increased expression of *TNF-α* in the early and middle phases of osseointegration, which could predict later implant-related complications (Slotte et al., 2012). Additionally, association between late implant failures (Kanagaraja et al., 1996; Thor et al., 2007; Montes et al., 2009; Linderback et al., 2010; Dirschnabel et al., 2011; Jacobi-Gresser et al., 2013) and peri-implantitis with peri-implant bone loss (Shimpuku et al., 2003a, 2003b; Laine et al., 2006) with regard to *IL1-β* polymorphisms has been described.

By surface modification of implants, such as by photofunctionalization or plasma treatment conditioning, the immune responses can be modulated; inflammatory responses can be reduced, thereby improving osseointegration (Williams, 2008). The presence of proinflammatory cytokines allows us to evaluate the effect of the surface modification of implants (Urbanski et al., 2017). A study performed using an animal model, in which the activity of proinflammatory cytokines was measured, reported that surface modification by photofunctionalization leads to a significantly reduced early inflammatory response (Harmankaya et al., 2012). To the best of our knowledge, the extent of influence of plasma treatment of implant surfaces on expression of proinflammatory cytokines in humans is unclear. Whole blood stimulation is a well-documented method that mimics the natural environment and can be used to evaluate the effects of surface modifications of titanium surfaces on the activation of immune cells and secretion of cytokines (Thurm and Halsey, 2005; Harder et al., 2012b).

Therefore, the aim of the present study was to investigate the influence of plasma conditioning of titanium dental implants on the gene expression of proinflammatory cytokines (*IL1-β* and *TNF-α*) in human whole blood *in vitro* by means of real-time, reverse transcription polymerase chain reaction (RT-qPCR). For this purpose, two different plasma generation methods at low and atmospheric pressure were used to demonstrate the possible effects of the type of plasma used on the immune response.

2. Materials and methods

The test set-up used in the present study was based on a previous investigation, which described that whole blood mimics the natural environment and was confirmed to be a suitable means to study cell activation and cytokine production *in vitro* in order to obtain reproducible results (Thurm and Halsey, 2005). Previous studies conducted by our group reported that direct exposure of titanium surfaces to human whole blood showed comparable increases in gene expression of proinflammatory cytokines (Harder et al., 2012a, 2012b).

2.1. Titanium Implants

In the present study, two-part dental implants were used as specimens (Camlog SCREW-LINE Promote plus; Camlog Biotechnologies, Wimsheim, Germany). The implants were made of grade 4 titanium with an abrasive-blasted acid-etched surface. The outer length of the implant body was 9 mm, and the external diameter was 4.3 mm. Ten implants each were used in the two test groups, and another ten implants served as a control group.

2.2. Blood samples

A total of 65 ml of whole blood was obtained by venipuncture from a healthy male Caucasian subject (age 39 years). The blood was aspirated using a Butterfly BD Valu Set (Sarstedt, Numbrecht, Germany). The withdrawn blood was collected in an EDTA tube (Sarstedt) and immediately divided into 30 portions of 2 ml each. The portions were placed in 15 ml pyrogen-free Falcon reaction tubes (Becton Dickinson, Franklin Lakes, USA), and the remaining blood was discarded.

2.3. Experimental setup and plasma treatment

First, the implant holders were degreased and cleaned with isopropanol. The implants were removed individually from the sterile packaging and placed into a holder using a receiving instrument to avoid contamination. For treatment with atmospheric-pressure plasma (APP), five groups of two implants each were formed, and the conditioning process was commenced after the loaded holders were placed in the plasma jet. For low-pressure plasma (LPP) treatment, ten implants were placed in the holder and loaded into the chamber of the plasma system.

Control implants were removed from the outer packaging, as described above. The implants were then incubated in the blood-filled reaction vessels immediately after removal from the outer packaging under constant agitation on a mini-tumble rotation table (WT 17; Biometra, Göttingen, Germany) at 50 rpm. This apparatus was placed inside a water-jacketed incubator (Thermo Scientific, Waltham, USA) at 37 °C and 5% CO_2.

2.4. APP treatment

A plasma jet (FG 5001 Plasmatreat GmbH, Steinhagen, Germany) was used to generate the APP (Kewitz et al., 2015). The following discharge parameters were used: voltage: 300 V, frequency: 19 kHz, gas flow: 30 slm (nitrogen), pulse cycle time (PCT): 100%, power: 800 W, The distance between the nozzle outlet of the plasma jet and the implant was 4 mm, and the velocity of the moving substrate (implant) beneath the plasma plume was 4 m/s and the resulting treatment duration was always set to one minute.

2.5. LPP treatment

A plasma system (Femto PCCE Zahntechnik, Diener electronics GmbH + Co. KG, Ebhausen, Germany) was used to generate LPP. The following parameters were used: argon gas saturation: 100%, power: 200 W, gas pressure: 50 Pa, treatment time: 15 min.

2.6. Incubation

After completion of each conditioning process, the conditioned implants were removed using the substrate holder and introduced into the reaction vessel filled with 2 ml whole blood. Each implant was completely immersed in blood in the reaction vessel. The vessel was sealed immediately after insertion of the implants. Subsequently, the 10 reaction vessels were placed on a mini-tumble rotation table (Biometra, Göttingen, Germany) at 50 rpm, and this apparatus was placed inside a water-jacketed incubator (Thermo Scientific, Waltham, USA) at 37 °C and 5% CO_2. Consideration was given to the staggering effect caused by the maximum of five implants per conditioning process, when the start of incubation was recorded ($t = 0$).

2.7. Sampling

Blood samples (200 μl) were collected from each group after 1, 8, and 24 h. Sub-aliquots of 2 × 100 μl each were mixed with 800 μl of Prisure (Promolgene, Berlin, Germany) and stored at −80 °C for RNA extraction.

2.8. RNA extraction

After thawing the blood samples and incubation at room temperature (21 °C) for 5 min, RNA was extracted using the method described by Chomczynski and Sacchi (2006). The resulting RNA pellet was dissolved in 30 μl of diethylpyrocarbonate-treated water (Promolgene, Berlin, Germany). RNA concentration was measured using a Nanodrop 1000 spectrophotometer (Peqlab, Erlangen, Germany), and extract quality was determined using a Screentape Lab 901 System (Peqlab). The remaining RNA was stored at −20 °C until cDNA synthesis was performed.

2.9. cDNA synthesis

After thawing, aliquots of 2 × 200 ng total RNA from each blood sample was reverse transcribed into cDNA using a cDNA synthesis kit (Promolgene) that conformed to the original method described by Sambrook et al (Green and Sambrook, 2012). This procedure was performed in a Thermocycler I instrument (Biometra, Göttingen, Germany) according to the manufacturer's protocol using the oligo-dT-V primer provided. The reaction had the following temperature profile: (1) the primer and RNA were incubated at 65 °C for 5 min, (2) the samples were immediately placed on ice, and a reaction mixture containing reverse transcriptase enzyme, buffer, and dNTPs was added, (3) the samples were incubated for 60 min at 37 °C, (4) a denaturation step of 10 min at 72 °C. The resultant cDNAs corresponding to the original two aliquots of RNA were pooled, and the remaining enzyme, buffer, and dNTPs were removed using the spin columns and buffers provided with the cDNA synthesis kit. The remaining, purified cDNAs were each brought to a total volume of 100 μl and stored at −20 °C for subsequent analysis.

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

IL1-β and *TNF-α* expression levels were analyzed by RT-qPCR using a Rotorgene 3000 platform (Corbett, LTF, Wasserburg, Germany). Additionally, the following housekeeping genes were analyzed: beta-2-microglobulin (*β2M*), 18S ribosomal RNA (*18SrRNA*), and beta actin (*ACTB*). To this end, 2.5 μl of the cDNA (equivalent to 10 ng of RNA) was used per analysis, and the total reaction volume was 25 μl. Each sample was run in duplicate. Gene-specific primers, as well as a SYBR green-based qPCR mix, were purchased from Promolgene. The cycle threshold (C_t) was determined manually.

2.11. Data analysis and evaluation

The RT-qPCR data were analyzed by the $\Delta\Delta C_t$ method normalized to the mean C_t values of the following housekeeping genes: *ACTB, 18S rRNA* and *β2M* (Pfaffl, 2001). Two-fold changes in gene expression (both decreases and increases) were considered statistically significant.

The delta-C_t values for the blood samples of the control group at time "0" (prior to implant incubation) were set as "1", and the -fold changes that occurred over time were calculated using the following equations:

1. In cases of decreased gene expression: ($1/2^{\wedge}\Delta C_t$ at time "0") − (ΔC_t at time of incubation)
2. In cases of increased gene expression: ($2^{\wedge}\Delta C_t$ at time "0") − (ΔC_t at time of incubation)

The delta-C_t values obtained for the blood samples incubated with control (naïve) implants at any time were set as "1," and -fold changes that occurred over time were calculated using the following equations:

1. In cases of decreased gene expression: ($1/2^{\wedge}\Delta C_t$ of naive implant) − (ΔC_t of treated implant)
2. In cases of increased gene expression: ($2^{\wedge}\Delta C_t$ of naive implant) − (ΔC_t of treated implant)

To separate the plasma conditioning-related effects from the effects of the implant itself, gene expression in the test group was defined in relation to the corresponding gene expression in the control group. Therefore, the reference value was the C_t value of the control group at time points 1, 8, and 24 h.

Before performing one-way analysis of variance (ANOVA), the data were tested for normal distribution by the Kolmogorov–Smirnov test. Statistical analyses (ANOVA followed by Bonferroni post hoc test) were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA), and data were presented as mean ± standard deviation of relative fold changes; P values of 0.05 or less were considered statistically significant.

3. Results

Gene expression was compared over time between the test and control groups. Both the control and test implants regulated multiple targets in the blood samples (Figs. 1 and 2).

3.1. Results for IL1-β

At time 0, there were no significant changes in gene expression observed in *IL1-β* in either the test or control groups.

After 1 h, the control group exhibited no changes for *IL1-β*. In the test groups, a decrease in gene expression was observed after 1 h for APP and LPP test implants, but only the decrease for LPP was considered to be significant ($P < 0.05$).

After 8 h, gene expression was significantly increased in the control group ($P < 0.0$), while APP and LPP test groups showed a significant decrease in gene expression ($P < 0.0$).

After 24 h, a significant decrease in gene expression was observed in the control group ($P < 0.001$), whereas there was a significant increase ($P < 0.05$) in the APP and LPP test groups.

3.2. Results for TNF-α

At time 0, no changes in gene expression were observed in either the control or test groups.

After 1 h, there was a significant increase in *TNF-α* gene expression observed in the control group ($P < 0.001$), while no significant increase was observed in the APP test group, and a significant decrease was observed in the LPP test group ($P < 0.05$).

After 8 h, gene expression in the control group remained at a significantly increased level ($P < 0.001$), whereas for the APP test group, a significant decrease was observed ($P < 0.05$), and in the LPP test group, the decrease in gene expression was significant ($P < 0.05$).

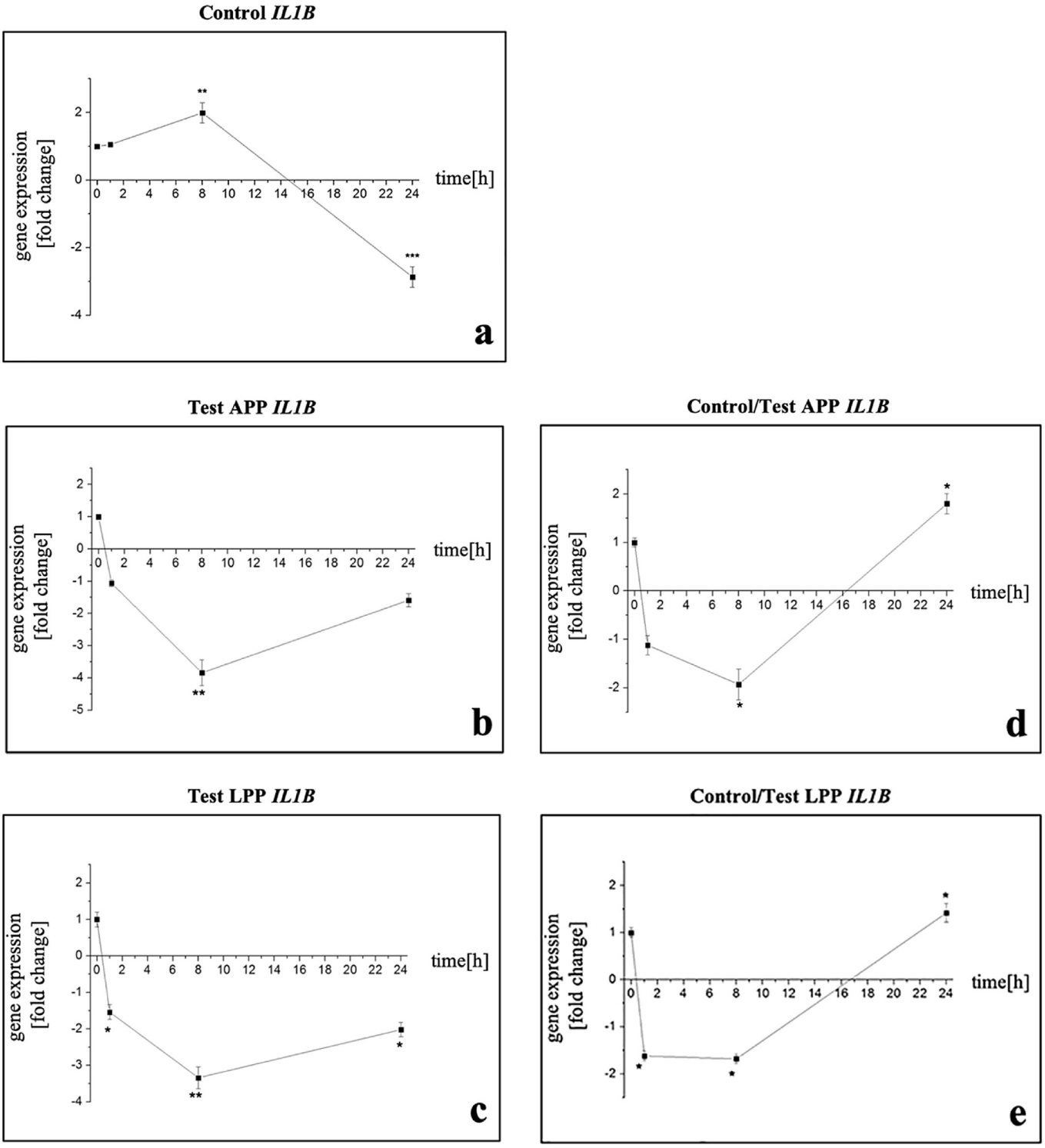

Fig. 1. Quantification of mRNA levels of interleukin-1 beta (***IL1-β***) is represented as -fold change in human whole blood. Line graphs display time-dependent immunostimulatory potential of untreated control (Control) implants (**a**) and test-implants (**Test**) after treatment with atmospheric-pressure plasma (**APP**) (**b**) and low-pressure plasma (**LPP**) (**c**) at 0, 1, 8 and 24 h. Separated plasma conditioning-related effects are displayed for APP (**d**) and LPP (**e**) test implants. Statistical significance is displayed by asterisks: *P < 0.05; ** = P < 0.0; *** = P < 0.001.

After 24 h, gene expression in the control group slightly decreased, but remained at a significantly increased level, while gene expression in both test groups became significantly increased (P < 0.001).

4. Discussion

The current study evaluated the effect of APP and LPP treatment on the expression of proinflammatory cytokines in an *in vitro*

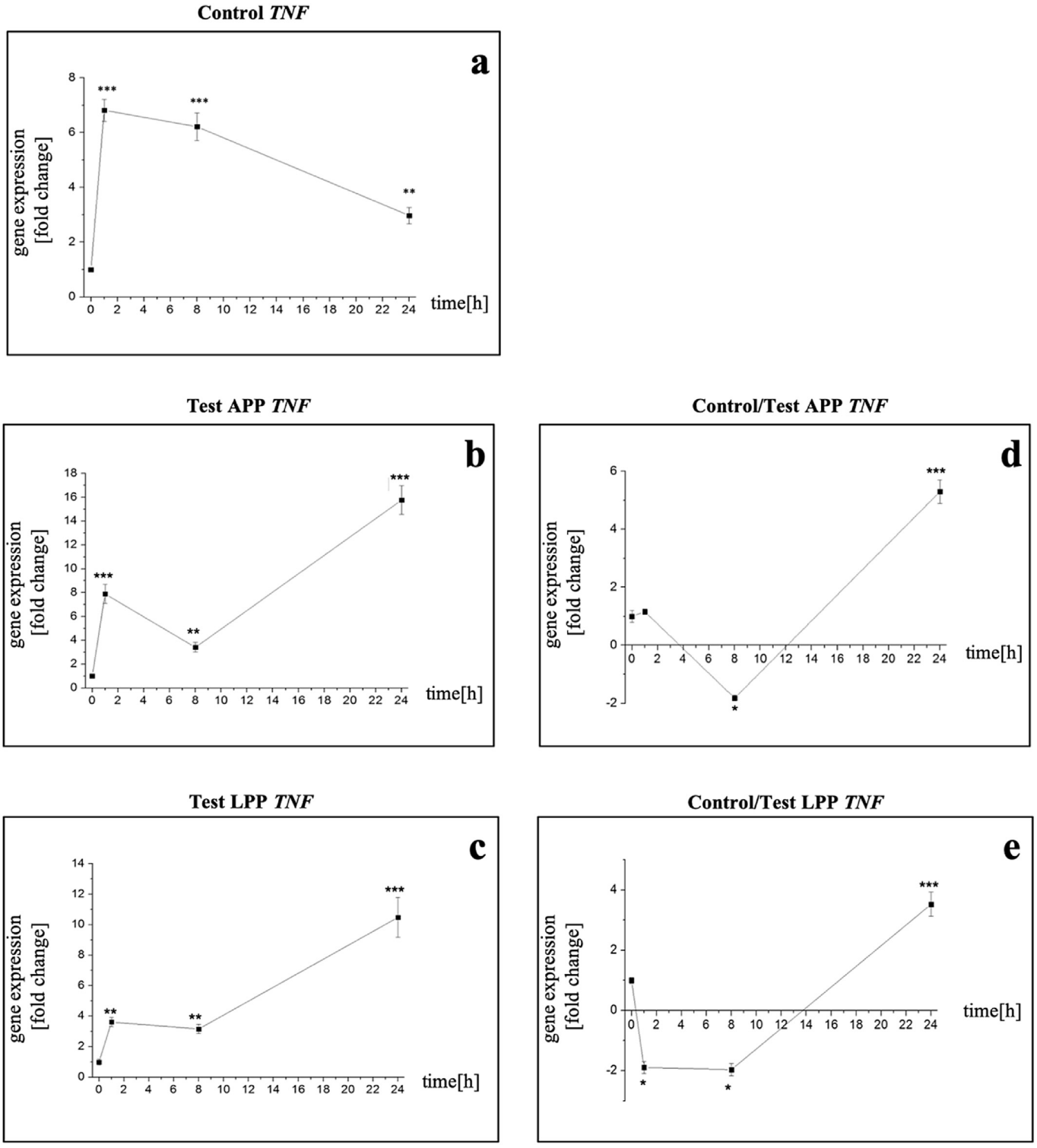

Fig. 2. Quantification of mRNA levels of tumor-necrosis factor (***TNF***) is represented as fold change in human whole blood. Line graphs display time-dependent immunostimulatory potential of untreated control (**Control**) implants (**a**) and test-implants (**Test**) after treatment with atmospheric-pressure plasma (**APP**) (**b**) and low-pressure plasma (**LPP**) (**c**) at 0, 1, 8 and 24 h. Separated plasma conditioning-related effects are displayed for APP (**d**) and LPP (**e**) test implants. Statistical significance is displayed by asterisks: *P < 0.05; ** = P < 0.0; *** = P < 0.001.

human whole blood model. The results of the present study show that APP and LPP treatment of titanium implants substantially reduced the expression of *IL1-β* and *TNF-α* in the initial contact period with human whole blood.

There are some limitations associated with this study. Since the immune reactivity of the blood stagnated after time without a supply of nutrients, the study period in this investigation had to be limited to 24 h. Furthermore, interindividual differences in the

response of the donor blood to coagulation could not be investigated in this study because the blood of only one donor was used. For further investigation with regard to coagulation behavior in particular, it will be necessary to use blood from at least three different donors.

The osseointegration of titanium dental implants is considered to be predictable, with a high probability of success (Moraschini et al., 2015). However, implant losses occur either in the early phase of osseointegration or through subsequent bony destruction of the bone-to-implant connection in the late functional phase (Bosshardt et al., 2017). Currently, the host-biomaterial relationship is at the center of all reactions around implants, since the presence of a foreign material (i.e. implants) stimulates greater immunological responses through activation of the complement system and macrophages, which is expected to control peri-implant events such as inflammation, healing, osseointegration, and long-term host responses (Trindade et al., 2018). Thus, the surface properties of a medical device for implantation are intended to modulate the foreign body reaction, especially during the early phase (Anderson et al., 2008; Bielemann et al., 2018). However, a sensitive equilibrium of this host response must be maintained in order to prevent excessive inflammatory reactions leading to implant rejection. In the case of titanium, the consequences of unbalanced immunoreactions have been established and are described as the ongoing stimulation of monocytes and macrophages, and the associated release of cytokines IL1-β and TNF-α in response to titanium particles. As a result, these proinflammatory cytokines (especially IL1-β and TNF-α) were reported to act as macrophage chemoattractants and to promote macrophage differentiation into osteoclasts responsible for bone resorption (Hamlet and Ivanovski, 2011; Alfarsi et al., 2014).

The increased expression of *IL1-β* and *TNF-α* in response to titanium surfaces by human blood lymphocytes and monocytes has been demonstrated in previous reports (Moura et al., 2013; Thomas et al., 2013). These results should be tempered with caution in order to confirm the observations of the current study with regard to the non-plasma treated titanium surfaces of the implants in the control group. It should be noted, however, that the present study used human whole blood, which contains peripheral blood mononuclear cells (PBMCs) with probably the same responses but does not take into consideration only the reaction of the isolated cells, as in the study mentioned before. Increased expression of *IL1-β* and *TNF-α* in the early phase of osseointegration around titanium implants has been demonstrated in humans (Bielemann et al., 2018) and also in animal experiments (Preti et al., 2007; Harmankaya et al., 2012). An increase in the expression of the two genes was also observed in an *in vitro* study that used a human macrophage cell line (THP-1) and in the proteomic profile of platelets in contact with polished or micro-roughened, sandblasted, acid-etched (SLA)-titanium surfaces (Alfarsi et al., 2014). These findings might corroborate our own, as both cytokines were upregulated by the non-plasma treated surfaces of the control implants in the present study.

Plasma treatment produces a super-hydrophilic surface in addition to a number of other physico-chemical changes, as do ultrafine cleaning and surface activation, and is comparable to pretreatment with UV-light irradiation (Canullo et al., 2016; Henningsen et al., 2018a, 2018b). The aspect of super-hydrophilicity must be considered in further detail, as it allows certain comparability with other investigations as regards the observed reduction in expression of *IL1-β* and *TNF-α* in the present study. Data reporting reduced expression of *IL1-β* and *TNF-α* genes in connection with super-hydrophilic titanium surfaces after different surface modifications have been reported in various studies (Hamlet and Ivanovski, 2011; Hamlet et al., 2012; Harmankaya et al., 2012; Alfarsi et al., 2014; Anitua et al., 2015). With regard to the osseointegration process, studies have reported that the difference in the inflammatory response in two animal models led to improved osseointegration after a short observation period (Harmankaya et al., 2012; Anitua et al., 2015).

A possible explanation for the reduced gene expression of proinflammatory cytokines on super-hydrophilic titanium surfaces could be derived from the relationship between the capacity of blood coagulation and activating effects on the complement system. Titanium is considered to be a strong promoter of coagulation (Hong et al., 1999; Thor et al., 2007; Bax et al., 2014) and it has been reported that the pronounced thrombogenic properties of titanium might contribute to the favorable osseointegrating properties of the material (Hong et al., 1999). With regard to hydrophilic modifications of titanium implants, thrombogenic properties appear to be enhanced (Hong et al., 2013; Anitua et al., 2015). Results of a study observing the behavior of super-hydrophilic titanium surfaces after alkaline treatment and human whole blood exposure revealed significant reductions in the number of blood macrophages/monocytes compared to untreated titanium surfaces. This was accompanied by the formation of very different blood clots (Milleret et al., 2011). Interactions between the coagulation system as the basis of hemostasis and the complement system as the most important factor of innate immunity have been described previously; however, the exact molecular pathways involving interaction between both cascades remain unclear (Amara et al., 2008; Milleret et al., 2011; Oikonomopoulou et al., 2012). Titanium is considered to be a major activator of the complement system (Kanagaraja et al., 1996; Arvidsson et al., 2007; Linderback et al., 2010). A previous study demonstrated that super-hydrophilic titanium surfaces cause reduced activation of the complement system after UV light treatment, leading to lower gene expression of proinflammatory cytokines in peri-implant tissue (Harmankaya et al., 2012). The extent to which changes in surface properties (in particular, after plasma treatment) and their active agents (ions, electrons, reactive oxygen and nitrogen species (RONS)), as well as electric fields generated by non-thermal plasma exposure, contribute to the positive hematological and immunological effects *in vitro*, *in vivo,* and clinically, remains unclear and should be investigated in future studies.

5. Conclusion

To the best of our knowledge, the results of the present study are the first to suggest an association between plasma treatment of titanium implant surfaces and reduction in gene expression of proinflammatory cytokines during initial contact with human whole blood. Further investigations are necessary to understand the nature of the modulation of cytokine expression by plasma conditioning with regard to coagulation and complement activity.

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Conflicts of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcms.2019.05.004.

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