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Cold atmospheric plasma in combination with mechanical treatment improves osteoblast growth on biofilm covered titanium discs

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ABSTRACT

Treatment of implants with peri-implantitis is often unsuccessful, because an instrumented implant surface and residual microbial biofilm impedes re-osseointegration. The application of cold atmospheric plasma (CAP) could be a simple and effective strategy to overcome the inherent problems of peri-implantitis treatment. CAP is able to destroy and eliminate bacterial biofilms. Additionally, it increases the wettability of titanium, which supports cellular attachment. In this study, the behaviour of osteoblasts on titanium discs was analysed after treatment of bacterial biofilms with CAP, brushing, or a combination of both.

A human plaque biofilm was cultured on titanium discs. Treatment with a brush (BR), 1% oxygen/argon CAP (PL), or brushing combined with CAP (BR + PL) was used to eliminate the biofilm. Discs without biofilm (C), autoclaved biofilm (AUTO) and untreated biofilm (BIO) served as controls. Subsequently, human osteoblastic cell growth (MG-63) was observed after 1 and 24 h.

Biofilm remnants on BR and PL impaired osteoblastic cell development, whereas the BR + PL provided an increased area of osteoblastic cells. A five-day cell growth was only detectable on BR + PL treated discs.

The combination of established brushing and CAP application may be a promising strategy to treat peri-implantitis.

1. Introduction

In dental implantology, the successful treatment of peri-implant lesions still is one of the biggest challenges. Peri-implantitis is a known and increasing problem in dentistry. The biofilm, which is located on the implant, causes inflammation of the tissue surrounding the implant. The consequence is a circumferential bone loss, which may compromise the longevity of an implant. A meta-analysis based on 11 studies showed a mean prevalence of peri-implantitis of 22% and a positive relationship between insertion time and prevalence [1]. Due to the missing predictability of any established therapeutic regimen, there still is no generally accepted implant preserving golden standard. Peri-implantitis affects about 10% of implants and 20% of patients during a 5–10 year observation period after implant placement [2] and it seems to be the main biologic cause for long-term implant failure [3]. The primary etiologic factors for peri-implantitis are bacterial biofilms on the implant surface [4]. Thus the cornerstone of any peri-implantitis therapy comprises mechanical removal of the biofilm [5], which should not change the micromorphology of the implant surface [6]. Mechanical *in vitro* and *in vivo* treatments with an air powder abrasion device or with a titanium brush left around 10% bacterial residues on rough sand blasted titanium surfaces [6,7].

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Besides these bacterial residues, the physico-chemical properties of the implant surface seemed to influence resolution of alveolar bone craters around implants [8]. After installation of pristine, virgin implants into extraction sockets, large gaps between the newly installed implant and the socket wall became completely filled and osseointegration occurred [9]. In contrast to this, bone craters caused by microbially induced peri-implantitis did not heal after mechanical treatment of exposed implant surfaces. Neither mechanical nor chemical antimicrobial treatment alone lead to predictable re-osseointegration of initially microbially exposed implant surfaces [10]. To promote re-osseointegration it was postulated that additionally, the contaminated implant surface has to be "rejuvenated". However, to do so there is no defined procedure available, which is described precisely by Lindhe [10], who invented the term "rejuvenation".

Previous studies of our group [11,12] indicated that cold atmospheric plasma (CAP) treatment has the potential to change physico-chemical properties of implant surfaces in a direction positively supporting re-osseointegration. Generally, CAP is a quasi electrically neutral, highly ionized gas composed of ions, electrons, excited atoms and molecules, as well as chemically reactive and neutral particles, and it emits vacuum ultraviolet and ultraviolet irradiation [13]. Plasma was able to clean titanium surfaces [14] and to decrease the water contact angle by increased surface hydrophilicity [11,12,15]. Because of increased hydrophilicity the tissue adhesion and spreading of osteoblastic cells were improved after plasma treatment [11]. Furthermore, animal and human experiments demonstrated that in principle hydrophilic titanium surfaces improved early stage of wound healing of soft and hard tissues compared to non-treated titanium (contact angle >138°) [16,17]. Plasma induced hydrophilic surfaces showed the same results in beagle and rabbit studies [18,19].

Depending on the magnitude of energy output, CAP could inactivate and destroy bacteria or remove biofilms completely [20–22]. The CAP device used in our previous experiments was able to kill bacteria on the one hand [22] and to promote osteoblast adhesion and spreading on the other hand [11]. To mimic a clinical situation in an *in vitro* experiment, first biofilms have to be removed and then osteoblasts should be grown on the initially microbially exposed surface. Until now limited information is available concerning cellular behaviour on initially microbially contaminated and consecutively CAP cleaned surfaces.

In this study, we hypothesized that the combination of a mechanical with a CAP-based treatment regime on biofilm contaminated titanium implants would be able to completely decontaminate and at the same time "rejuvenate" the surfaces of these implants thus inducing beneficial effects for consecutive cell spreading. To prove this hypothesis, we investigated the cellular behaviour of osteoblastic cells on initially microbially contaminated titanium surfaces after the following three treatment regimens: conventional mechanical treatment, CAP application, and a combination of mechanical treatment with CAP application.

2. Material and methods

2.1. Titanium discs

For experiments, sandblasted-etched titanium discs (Straumann, Freiburg, Germany; grade 4, diameter 15 mm, thickness 1 mm, $R_a = 3.2 \pm 0.02$ μm) were used.

2.2. Cultivation of biofilms

The technique of the cultivation of biofilms is described in detail elsewhere [23]. In brief, the titanium discs were placed into 96-well microtitre plates (Techno Plastic Products AG, Trasadingen, Switzerland) and covered with 100 μl subgingival plaque, which was collected for every experiment from deep pockets of the same periodontally diseased volunteer. The subgingival plaque removal was approved by the ethics committee of the Ernst-Moritz-Arndt-University Greifswald, medical department (Registration number: BB 120/10). After this, the discs were completely covered by Dulbecco's Modified Eagle Medium (DMEM; Invitrogen GmbH, Karlsruhe, Germany), which contained 10% of foetal calf serum (FCS; PAA Laboratories, Pasching, Austria). The discs were incubated at 37 °C and the medium was removed and replaced every 24 h. After 7 d of cultivation, the medium was drawn off and the biofilm covered discs were washed with 0.9% sodium solution and transferred into a new, sterile micro-titre plate prior to the following treatment modalities.

2.3. Treatment modalities

Different treatment modalities were used to inactivate and to remove the biofilms on titanium surfaces. The biofilm-covered discs were treated with a nylon brush (BR), plasma (PL) or a combination of nylon brush and plasma (BR + PL). Untreated discs with vital biofilm (BIO) served as negative controls. Discs without biofilm (C) as well as with autoclaved biofilms (AUTO) served as positive controls.

BR: The cylindrical nylon brush (item number 9645, Komet Gebr. Brassler, Lemgo, Germany) was fixed in a dental hand sample (1000 rpm) with an application force of 30 g against the disc and continuous water-cooling. To standardize the treatment, the discs were secured on a computer driven 3 axes bench (Sirona Dental Systems, Bensheim, Germany) which was guided against the brush in a meandering motion with a velocity 1 mm/s for 120 s, each.

PL: To generate a CAP, a plasma jet (kINPen08, INP Greifswald, Germany) was used with an input power of 2–3 W [20]. A high frequency voltage of 1.82 MHz at 2–6 kV was coupled to a centred needle electrode. Argon (99%, ALPHAGAZ, Air Liquide, Düsseldorf, Germany) combined with 1% of oxygen (medical oxygen, 99.5%, Praxair Deutschland, Düsseldorf, Germany) was used as carrier gas with a gas flow of 5 slm (standard litre per minute) controlled by a flow controller (MKS Instruments, Munich, Germany). The length of the visible plasma plume was 7 mm.

The computer driven 3 axes bench directed the specimen below the plasma plume at nine spots on each disc (one in the centre and eight in the periphery) in meandering movement. Each spot was exposed for 60 s, resulting in a complete treatment time of 540 s per disc side. During treatment the distance from the nozzle of the CAP jet to the disc was 5 mm.

BR + PL: Combined treatment was performed as described above for every single treatment (BR and PL) in the sequence mechanical removal followed by CAP treatment.

AUTO: To assure sterility, the autoclaving procedure underwent the standard sterilization process as preset for dental instruments (duration of 45 min, 120 °C, 1 bar; Autoclave24, Melag, Berlin, Germany).

During biofilm cultivation, microorganisms could be grown on the opposite disc side too. Therefore, to avoid microbial regrowth from the opposite side by untreated microorganisms during the following cell culture tests, PL and BR treatments were carried out on both sides of the discs. Immediately after treatment, osteoblastic cells were seeded onto the top of the discs and grown for 1 and 24 h or 5 d as described in chapter 2.4.

2.4. Cell culture

Human osteoblastic cells (MG-63; ATCC, CRL-1427; LGC Promochem, Wesel, Germany) were cultured in DMEM with 10% FCS and 1% Gentamicin (Ratiopharm GmbH, Ulm, Germany) at 37 °C in a humidified atmosphere with 5% CO_2 and were split at 80% of confluence to get an adequate number of cells for the test. Cells were used up to passage 25.

After biofilm cultivation and treatment procedures, MG-63 cells were seeded onto the discs with a density of 22,700 cells per cm^2. To allow concomitant biofilm regrowth and osteoblast growth the treated discs were incubated in DMEM with 10% FCS without gentamicin for 1 and 24 h or 5 d at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. Scanning electron microscopy (SEM)

For the assessment of cell area, adhesion and morphology scanning electron microscopy (SEM) (DSM 960A, Carl Zeiss, Jena, Germany) with 10 kV and 3.3 A was performed. Therefore, MG-63 cells were fixed with 4% glutaraldehyde, dehydrated through a graded series of alcohol, dried in a critical point dryer (K 850, EMITECH, Taunusstein, Germany), and finally sputtered with a coater (SCD 004, BAL-TEC, Balzers, Lichtenstein).

Cell area: The cell area in the mean of a projected two-dimensional surface was measured on SEM micrographs (5 repetitions per instrumentation modality; 40 cells per disc) using ImageJ v1.38 (US National Institutes of Health, Bethesda, MD) and was expressed in μm^2. The inserted bar was used to set the scale in ImageJ using the software plug-in "Set Scale". Cells were circled by a frame using the software plug-in "Freehand Selections" and "Measure".

Cell number: To evaluate the adhesion of cells to the titanium discs, the cell number was counted with the software plug-in "Counter" of the software ImageJ. SEM images (magnification: ×200) were taken at five regions of interest (one in the centre and four in the periphery) of each disc.

Number of bacteria and cells: To determine the number of bacteria and cells after 5 d of cultivation after biofilm treatment, SEM images (magnification: ×500) were taken at nine points (one in the centre and eight in the periphery) of each disc and a rectangular grid (10 × 10 lines) was superimposed over the SEM images (using