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The connective tissue response to Ti, NiCr and AgPd alloys

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Abstract: The aim of the study was to compare the connective tissue response of Lewis rats to Ti, NiCr and AgPd alloys. It was found that implants were covered by collagen-rich, well vascularized capsules. Titanium was covered by the thinnest capsule (57±20 μm) and AgPd alloy was covered by the thickest capsule (239±50 μm). The PCNA+ cell prevalence in the capsules was lower for titanium than for AgPd and NiCr. Mast cells formed a gradient to a depth of 1200 μm only for titanium implants. Cells with brown to black silver granules in the cytoplasm were observed close to AgPd implants. The results suggest that titanium implants induce a weaker connective tissue response than implants made from NiCr and AgPd alloys.

Key words: TiN coating, connective tissue, proliferation, mast cells, dental alloys.

Introduction

Dental alloys (*i.e.* AgPd, NiCr) and pure metals (*i.e.* titanium) have applications mainly in prostodontic restorations, but also in endosseous implants and endoprosthesis (*i.e.* titanium) or diagnostic equipment such as endoscopes. The anti-corrosion properties of metals have an influence not only on their functional properties and the clinical performance of prosthetic reconstructions, but also on biocompatibility [1,2]. The quality of the corrosion products, their type and their distribution in the human body will affect the degree of their toxicity [3]. Corrosion products pose a danger to the patient's health due to the occurrence of hypersensitivity reactions to metal ions released from implants [4]. Primary allergies to prosthetic implants are very rare; different sources are mainly responsible for allergic reactions.

The influence of implanted materials is evaluated by routine histopathological tests; the presence of inflammatory infiltrate cells, such as neutrophils or macrophages, is recorded while investigating the connective tissue cells surrounding the capsule [5-7]. Subcutaneous implants made of NiCr alloys induce inflammation after 7 days from implantation, and the Ni content reaches a concentration of 4 μg/g. After this period, pure nickel causes heavy inflammation with necrosis, with the nickel concentration up to 48 μg/g [8]. Research on the subcutis of guinea pigs sensitized to $PdCl_2$ and then implanted with AgPd, CuAu and PdAu implants showed that each examined implant was surrounded by a connective tissue capsule with adjoining mastocytes [9]. Titanium plates (c.p.) implanted within the peritoneal cavity were surrounded by a connective tissue capsule in which titanium ions were found [10].

Research on the influence of many ions (*e.g.* Ag, Co, Nr, Pd, Mo) at a range of concentrations from 0.003 to 10 nM/L, on a range of fibroblast culture models including human gum fibroblasts, showed inhibition of DNA synthesis in these cells [3]. Histopathological research of subcutaneous implants in rats (examined after 15-60 days) showed that grafts made from NiCr evoke the strongest histological responses in animals, while alloys with the highest gold and palladium content cause the weakest response [11]. According to a study by Schedle *et al.*, it was found that the histamine released due to the toxic influence of silver was dependant on the concentration of human mastocytes [12]. However, the contribution of mastocytes in the healing process, including their density in the tissue surrounding implants, has not yet been systematically investigated [13].

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The present study was undertaken to determine the least reactive material implanted into the connective tissue of rats. The study measured the density and the fiber composition of the connective tissue capsule, the cell proliferation of the capsule and surrounding connective tissue and the density and the distribution of mastocytes within it.

Materials and methods

Alloy samples. Commonly-used metal alloys were examined in this study: Heraenium NA (NiCr) (Heraus-Kulzer) and Spall (WT-52) (AgPd) produced by the National Mint and titanium (c.p.). These were used to make the samples, which were cast in the shape of rounded plates (8 × 8 × l mm). They were cast in the laboratory according to standard procedure from new, industrial alloys. These samples were drilled with two 1 mm holes for sutures to stabilize them in the tissue, and then they were polished mechanically. Five samples were made of the Heraenium alloy, 5 samples of the Spall alloy and 5 samples of titanium. They were cleaned, dried and sterilized.

Animals. The study was carried out on 20 male Lewis/Hann/Lodz rats (body weight of 280-300g at the beginning of the experiment), obtained from Department of Laboratory Animals, (Medical University of Lodz, Poland) under a protocol approved by the Faculty of Dentistry Ethics Committee, (Medical University of Lodz, Poland). The animals were kept in 12-hour cycle day-night behind a sanitary-hygienic barrier. They were given access to Murigran fodder (Akropol, Motycz) and tap water *ad libitum*. The animals were divided into four groups with 5 animals in each group. Group I was the reference group, later named as control. These animals were bred parallel with the experimental animals, there were no experiments carried out on them. Fifteen animals were divided into 3 groups, of 5 rats each, which had metal implants grafted under the skin of the neck: AgPd alloy plates (group II – SPALL), NiCr alloy (group III – HERAENIUM) and titanium (group IV – TYTAN). The surgery was carried out under hexobarbital anesthesia, in aseptic conditions in the operating room of the Department of Laboratory Animals, (Medical University of Lodz, Poland). After shaving the neck skin and sterilising the operating site, the skin was cut and "the pocket" was bluntly formed in the connective tissue where the implant was introduced. Dexon 6/0 sutures were introduced into the two holes made earlier in each plate and then fixed to the muscular layer, the knots were tied and the wound was closed with two stitches. All animals survived the surgery. During the first week after the surgery, the wounds healed swiftly. Twelve weeks after the plates were implanted, all animals were euthanized in a CO_2 chamber for 5 minutes.

After the implant was located, the surrounding capsule was incised on one side and then separated bluntly. The capsule, along with the fragment of the surrounding connective tissue, was closed in cassettes and fixed for 14 hours in 4% formalin (pH 7.4 temp. 4°C, in depolymerized PFA). After paraffin embedding, the tissue was cut into slices 4.2 µm thick and stained: a) staining with hematoxylin and eosin; b) Van Giesson staining of collagen fibers and Singha's method staining of reticulin fibers; c) hydroquinone (the developer) staining of silver grains in the cell cytoplasm around Spall alloy implants, and ammonium polysulphate staining of the grains around Heraenium alloy implants (to find nickel sulphate); d) Bensley's method staining of mastocytes with pinocyanol erythrosinate; e) anti-PCNA monoclonal serum (clon PC-10, Dako) and LSAB system (Dako) was used to stain nuclei cells containing Proliferating Cell Nuclear Antigen (PCNA). The peroxidase was stained with DAB*HCl with the addition of cobalt salt. The samples were examined quantitatively and qualitatively: a) the type of fibers forming the capsule , b) the presence of cells containing silver grains or nickel sulphate; c) the thickness of the connective tissue capsule surrounding the implants; d) the mastocyte distribution and density in the connective tissue adjoining the capsule, e) the PCNA+ cell prevalence in the capsule and in the connective tissue adjoining the capsule.

The capsule thickness was evaluated in a digital image analyzer (Multiscan Base 11.1). The evaluation of mastocyte distribution and thicknesses was performed by counting the mastocyte number within fields of 100 × 100 µm to a depth of 1200 µm (a total of 12 fields being sampled) in the connective tissue layer immediately adjoining the capsule. In each investigated animal, a minimum of 20 fields adjoining the capsule (2 mm) and 12 fields inside the connective tissue (1.2 mm) were counted. In the control group, due to the lack of a capsule, mastocyte thickness and distribution was determined in 100 successive fields (l00 × l00 μm^2) of connective tissue samples for each animal. A minimum of 500 successive PCNA+ cell nuclei were counted separately in the capsule and in the connective tissue adjoining the capsule.

The results were analyzed by a number of statistical methods: a) evaluation of the distribution of results (the Chi-square test of independence, Shapiro-Wilk W test for normality, the Kolmogorov-Smirnov test for equality of distributions, and skewness and kurtosis assessment); the confidence level for normally distributed results p>90%); b) the t test was used to verify a hypothesis on equality of means between two independent groups; the difference was deemed to be statistically significant when $p \leq 0.05$. An attempt was also made to evaluate the mastocyte gradient with linear regression equations.

Differences in mastocyte density and PCNA+ cell distribution were determined by comparing tissue from animals which had undergone surgery with tissue from the same locations in animals from the control group. As the capsule *in status nascendi* is not the physiological structure, and that, consequently, there was no control sample for the capsule adjoining the implant, it was arbitrarily accepted that the estimated results of PCNA+ cell prevalence would be compared with the results of the PCNA+ cells in the connective tissues in the control group.

Results

The evaluation of histological specimens showed that each implant was surrounded by a connective tissue capsule, in which collagen fibers and argyrophillic (reticulin) fibers dominated. The latter appeared considerably rarely and at variable frequencies. Numerous section profiles of blood vessels surrounded by short, irregular collagen fibers, were found in the connective tissue surrounding the capsule. Argyrophillic fibers surrounded blood vessels and appeared very rarely in the connective tissue between vessels.

Solitary, large cells with a regular nuclear contour, sometimes found in agglomerations that contained black or bronze grains in the cytoplasm, were only observed in specimens taken from the connective tissue immediately adjoining the capsule in animals with Spall alloy implants, after hydroquinone reduction of silver. Similar images were not observed in connective tissue cells (fibroblasts) obtained from animals with Heraenium alloy implants, after the reaction with ammonium polysulphate (Table 1 and 2).

The capsules surrounding the Spall alloy implants were the thickest (239.3±59.9 mm), while the capsules

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Table 1. The density of connective tissue capsule adjoining implants made of AgPd alloy (SPALL), NiCr (HERENIUM) and titanium (TYTAN)

Investigated group	PCNA positive cells percentage (mean±S.D.)	
	connective tissue	connective tissue capsule
Control	44.52±19.09	no cells were observed
SPALL	53.34±9.60	52.89:± 5.49
HERAENIUM	50.04±6.95	57.15±9.47
TYTAN	39.54±8.81	45.66±14.02

Table 2. The density of mastocytes (per 1000 mm^2) in the connective tissue of rats both in the control group and experimental groups with implants: AgPd (SPALL), NiCr (HERAENIUM) and titanium (TYTAN)

The distance from the capsule (mm)	Controls mean ±S.D.	SPALL mean ± S.D.	HERAENIUM mean ± S.D.	Titanium mean ± S.D.
Controls	4.56±1.71 connective tissue of control animals	1.91±1.41 connective tissue of animals with implant	3.15±2.18 connective tissue of animals with implant	3.18±1.79 connective tissue of animals with implant
100		5.14±2.29	3.31±2.37	6.65±4.32
200		5.51±3.42	3.99±2.66	5.05±3.37
300		4.73±2.78	4.52±3.84	6.32±4.26
400		3.53±2.41	3.95±3.58	5.06±2.18
500		4.19±2.61	4.24±4.12	4.64±2.86
600		3.51±2.15	3.44±3.37	2.44±2.07
700		4.54±2.75	4.98±3.33	2.81±2.91
800		3.06±2.15	4.60±2.63	2.45±1.64
900		4.28±2.13	4.74±1.83	2.60±1.62
1000		4.30±3.06	3.11±2.07	1.81±1.69
1100		4.08±3.00	3.24±1.65	2.47±2.72
1200		2.70±1.75	3.46±2.62	2.73±1.75

surrounding the Heraenium alloy implants were considerably thinner (170.9±69.6 mm). The thinnest capsules surrounded the titanium implants (57.44± 20.99 mm).

A change of MC density was observed in the connective tissue adhering to the capsule surrounding the implant; the MC number decreased as the distance from the implant increased. However, in the connective tissue around the titanium implant (Fig. 1), this reduction in MC density demonstrated a linear dependence, and can be described by the following formula:

$$ND_{MC} = 5.7 - 0.21 * \sqrt{D}$$

p=0.99; r=0.46; ND_{MC} – MC

D – distance from the connective tissue capsule around the implant.

In the case of Spall and Heraenium alloy implants, no such linear reduction was found.

In group II (SPALL) and III (HERAENIUM) this correlation was barely visible (SPALL in relation to distance: r=-0.20, $p<0.05$, and HERAENIUM in inverse relation to distance: r=-0.046, $p<0.1$). In the Heraenium alloy implant group, there was no normal distribution of MC thickness close to the capsule, and at 3 distances in the range 300-600 μm from the capsule. The lack of a normal distribution was not observed either in the control group or in the remaining groups. In comparison with the control group, each group, with the exception of Heraenium, displayed greater MC thickness close to the capsule. For Spall and titanium alloys, the MC prevalence close to the capsule was significantly higher than in the respective control.

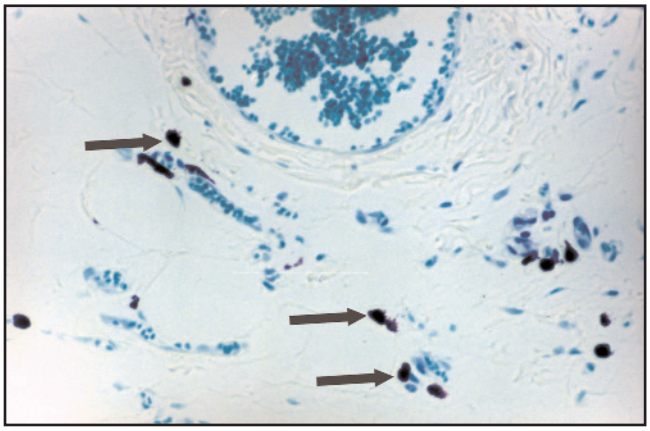

Fig. 1 MC in connective tissue surrounding the titanium implant, cells (some of them indicated with arrows) are arranged principally around small vessels (original magnification ×40; Bensley's method staining).

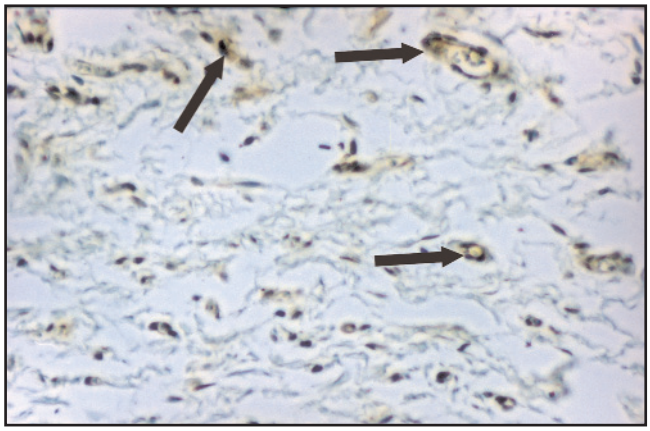

Fig. 2. Connective tissue surrounding the titanium implant, merely numerous MC with PCNA+ cells (some of them indicated with arrows) (original magnification ×40).

The evaluation of the PCNA+ cell prevalence in the investigated and control groups (Table 3) shows significant differences in the PCNA+ cell prevalence between only the capsule and the tissue surrounding the capsule. PCNA+ cell prevalence was found in half the evaluated cells in the investigated groups. Only in the TITANIUM group (Fig. 2) was the PCNA+ cell prevalence in the connective tissue capsule lower than in the SPALL and HERENIUM groups.

Discussion

The presence of the connective tissue capsule around implants is well documented in the literature and confirms the results of our study [6-9,11].

The capsule density around the investigated implants was diverse. It is important to remember during evaluation of capsule density that the average section angle for the space 2 and 3D equals 45°, so the actual capsule density should be divided by the root of 2 (1.415), which gives the capsule thickness of SP (SPALL), HE (HERENIUM) and Ti (TITANIUM) 169.25, 127.88 and 40.61 μm, respectively.

The connective tissue capsule surrounding the titanium implants was the thinnest and the proliferation was the lowest among evaluated capsules. The protein overcoat created on the surface of titanium implants is what changes the properties of the passivised surface [14] and this protein, fibronectine and collagen overcoat can be created on titanium implants [15]. The surface of implanted titanium is almost stoichiometrically coated by titanium oxide, which is subject to conversion by hydroxyl, acidic and alkaline groups. Phosphoric or calcium ions can link to these groups, followed by lipo- and glycoproteins, then proteoglycans, then collagen fibers and then finally, cells are absorbed on this surface [16]. No features of inflammation were observed after the implantation but it was suggested that released titanium particles can be phagocytosed, and then eliminated and surrounded by collagen fibers. Type I collagen dominates in the capsule that surrounded the titanium implants and type III collagen appears in a relatively smaller quantity [17]. Titanium clips, often used to protect vessels, are usually surrounded by a thick new net of vessels [18]. If small titanium particles are used in vitro (similar to those observed in ME between fibers), low particle concentrations stimulate fibroblast production, while high concentrations cause inhibition [19]. As our study shows, the weakest connective tissue reaction was to the titanium implant, and could be due to the inhibitory effect of titanium particles on the function of cells such as fibroblasts. There is no titanium presence in the blood after 3 years of titanium implant usage [20] nor any titanium presence in the brain, lungs, liver or spleen [20,21]. The concentration of all trace metals was low or very low [21]. The intraperitoneum injection of titanium particles did cause interferon release, while Ig production was inhibited in 8-12 days after its delivery. The release of cytokine from lymphocytes, T and B lymphocytes proliferation were also inhibited. These results indicate the immunosuppressive activity of titanium despite the lack of cytoxicity [22]. Inflammatory cells were not observed around implants for a radius of 5-8 mm.

A slightly thicker connective tissue capsule was observed around NiCr implants. The nickel ion release that inhibits the synthesis of DNA, RNA, proteins, ATP and glucose-6-phosphate, was observed in surrounding tissues after 24-72 hours from NiCr alloy implantation [23]. The necrosis features were not

Table 3. The PCNA+ cell prevalence in the control connective tissue, in capsules and in the connective tissue of animals: control and experimental groups with implants: AgPd (SPALL), NiCr (HERAENIUM) and titanium (TYTAN)

Investigated group	PCNA positive cells percentage (mean±S.D.)	
	connective tissue	connective tissue capsule
Control	44.52±19.09	no cells were observed
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HERAENIUM	50.04±6.95	57.15±9.47
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found around NiCr implants and there were no inflammatory infiltrations in guinea pigs in literature [24]. The introduction of the pure, powdered nickel into the gum of a rat caused necrosis and lymphocyte infiltration in the nickel grain areas [25]. Beryllium is the most toxic component of NiCr alloys [26], which further intensifies the corrosion [27]. Morphological changes within nuclei and the accumulation of fat drops in the cytoplasm were found in fibroblasts cultured in nickel salts [28]. Nickel in culture possesses the ability to increase the production of IL-6 10-fold with slight cytotoxicity [29]. The same action in the composite culture (endotelium, monocytes, lymphocytes) induces Il-14 and TNF alpha production [30]. Mastocytes in culture possess the ability to accumulate nickel ions. Nickel salt solution added to MCs does not intensify histamine release (MC degranulation) [31]. The inhibition of fibroblast proliferation and fiber synthesis was not observed, although the reduction in MC density in the area adjoining the capsule was slight. The necrosis around NiCr implants was not seen after 12 weeks. It can be argued that the tissues' response to this implant is influenced either by the possible passivation mechanism or by nickel elimination from the area of the capsule through newly-formed blood vessels.

Although the thickest capsules are found around AgPd implants, Palladium ions have low toxicity [32], and the release of these ions was not observed in a study of the corrosion of AgPd alloys by lactic acid/NaCl [33]. Additionally, on the surface of AgPd alloys, an insoluble coating of AgCl is formed in vitro that can inhibit ion release, particularly Pd ions [34]. Silver ions in the form of nitrates in 0.5% aqueous solution introduced into the pleura of animals induce its overgrowth and the production of thin and thick collagen fibers [35]. It could be suggested that a similar mechanism operates on the implants studied in this paper, as ions are released in small quantities during passivation. The AgPd alloy inhibits the proliferation and the production of fibronectine in fibroblast cultures [36]. Palladium ions are able to inhibit hydroxyproline synthesis [37]. Silver in the cultures is cytotoxic [35]. The high number of MC cells adjoining the capsule do not cause toxic degranulation, however the histological images do not show local histaminemia [12].

The presence of numerous blood vessels in the capsule and the space adjoining the capsule, the considerable percentage of cells in the cycle (PCNA+) and the diversity of collagen fibers (thin and thick) and vascularization suggests that all the capsules were still growing after 12 weeks. Mastocyte aggregation accompanies all capsules.

Implanted biomaterials induce a histamine response in adjoining tissues which enables the passage of inflammatory cells [38]. The mastocyte cluster is localized in the area of angiogenesis. Human MCs release tryptase, which, in concentrations of 0.3-3 nM, stimulates the synthesis of mRNA procollagen [39]. Tryptase simultaneously intensifies fibroblast migration and proliferation without influencing (*intercellular*) matrix metalloproteinase [40]. Mastocytes contain the heparin linked with the basic fibroblast growth factor (bFGF) in granules, and upon heparin release, they simultaneously release bFGF, which intensifies fibroblast proliferation [41]. The growth of fibroblasts supported by MCs, followed by the production of fibers, can be combined with the other factors acting locally on PDGF. The heparin and glycosaminoglycans, along with their associated POOF, intensify the growth of fibroblasts in the lungs [42]. This data indicates that MC cells are engaged both in angiogenesis and the regulation of the growth of the capsule surrounding the implants. Assuming that these regulating mechanisms operate during the capsule's development around the implants, the thesis that titanium particles have an inhibiting influence on connective tissue capsule formation by mastocyte migration and growth, fiber formation and angiogensis, is legitimate, despite the crucial influence of mastocytes.

Conclusions

All investigated implants evoked local connective tissue response through the formation of a connective tissue capsule around the implant. The weakest connective tissue response was induced by the titanium implants and the strongest by AgPd alloy implants. Considering the high biocompatibility, the titanium implants are the most suitable for clinical application in dental surgery and prosthodonics.

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