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ORIGINAL ARTICLE

CONTENTS

A nickel implant induces cell death through autophagy in the connective tissue capsule in an experimental model

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ABSTRACT

Aim: To identify cellular autophagy markers around nickel-containing implant as evidence of metal hypersensitivity reactions in an animal model.

Materials and Methods: Rats were sensitized to nickel using a modified model involving the administration of $NiSO_4$ with adjuvants. Subsequently, nickel plate implants (Ni content at 98.9%) were placed subfascially in the rats. Five months after implantation, the capsule morphology and autophagy were examined through the immunohistochemical detection of Beclin1 and GRP78. Implants tissue capsules without previous $NiSO_4$ exposition were considered as control.

Results: A high immunoreactions to GRP78 were observed in the implant capsule wall, with Beclin1-positive cells primarily noted at the interface with the implant. GRP78 and Beclin1 were significantly higher (p=0.01) expressed in cases with adjuvants, serving as a model for provoking an acute tissue response to implant.

Conclusions: In addition to inflammation and necrosis, cell death in the connective tissue capsule wall occurs through autophagy. Autophagy, mediated by Beclin1, is prominent at the implant interface and is closely associated with GRP78, which is strongly expressed throughout the capsule thickness, indicating significant oxidative stress in the cells surrounding the nickel plate implant.

KEY WORDS: adverse reaction to metal implant, nickel, capsule, autophagy, cell death

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INTRODUCTION

The choice of specific metals or alloys depends on their intended use, but it is important to note that high concentrations of metals can disrupt normal physiological functions. This, in turn, can lead to the development of both local and systemic hypersensitivity reactions.

Hypersensitivity reactions (HSR) are one of the most complex issues in orthopedic and trauma practice, leading to extensive discussions among specialists. These reactions are a function of the adaptive immune system and, according to Gell and Coombs classification, are divided into four types: Type I, Type II (antibody-mediated), Type III, and Type IV (cell-mediated, delayed). Most hypersensitivity reactions associated with orthopedic implants are classified as Type IV, which are delayed-type hypersensitivity reactions [1].

The first documented case of a hypersensitivity reaction to metal (HRM) caused by an orthopedic implant was published in 1966 by Foussereau and Laugier [2]. They described a case of eczematous dermatitis, which sparked significant interest in the medical community. Following this publication, many similar cases have been reported, highlighting the relevance of the issue.

The spectrum of local and systemic reactions associated with metal implants is now collectively referred to as "adverse reaction to metal debris" (ARMD). These include elevated metal ion levels in blood or serum, metallosis (accumulation of metal debris in periprosthetic tissues), adverse local tissue reactions (ALTR), pseudotumors, and osteolysis.

Currently, there is no unified view on the causes of such reactions, highlighting the need for further investigation into the molecular mechanisms of tissue damage around orthopedic implants. It is known that implanted metal components undergo some degree of corrosion, leading to the release of metal ions into the surrounding tissues [3, 4].

These ions can have harmful effects on the structure of bone tissue and bone marrow [5]. Cadosch et al. described the growth and differentiation of osteoclast

precursor cells on the surfaces of stainless steel, titanium, and aluminum implants. They demonstrated that mature osteoclasts can induce corrosion of metal surfaces, leading to the release of metal ions [6-8]. However, metal corrosion can result from mechanisms beyond this, including processes such as crevice corrosion and fretting.

It is important to note that dynamic components of implants often contain particles that are primarily localized in periprosthetic tissues. The accumulation of metal particles in these tissues can lead to sustained antigen concentrations, which in turn may activate a local immune response and potentially cause implant rejection [5].

The inflammatory response of the body is directly related to the amount of metal particles present. These particles have a significant pro-inflammatory effect, activating osteoclasts through macrophage engulfment. Metal ions bind to proteins recognized by antigen-presenting cells [9]. These processes lead to osteolytic lesions around implants. The activity of osteoclasts in periprosthetic areas triggers the activation of their precursors and the release of cytokines, which promote their differentiation and activation [6, 10, 11].

Given the increasing frequency of joint replacement and the use of orthopedic implants, it is expected that the number of complications related to implant reactions will also rise [12-15]. This underscores the need for in-depth study of the molecular mechanisms of cellular damage around orthopedic implants. Such research will help to better understand cellular reactions to metals that may lead to rejection or adverse processes, such as allergic and inflammatory reactions. Understanding these mechanisms can aid in the development of diagnostic and prognostic tests for patients with metal implants.

As a continuation of our previous study [16], which demonstrated capsule growth around a nickel-containing implant and the occurrence of necrosis, this work focuses on cellular mechanisms of cell death, particularly autophagy.

Autophagy is a specific cellular response to damage, such as hypoxia and oxidative stress, aimed at preserving cell survival by removing damaged organelles [17]. However, it remains unclear whether autophagy is involved in regulating cellular reactions around a nickel-containing implant.

AIM

To investigate the cellular response associated with autophagy to a nickel-containing implant in an experimental model of metal hypersensitivity reactions.

MATERIALS AND METHODS

The study was conducted on 25 female Wistar rats, weighing 150-165 grams, aged 3 months at the start of the experiment. The animals were housed in the vivarium of the O. V. Palladin Institute of Biochemistry, NAS of Ukraine. The rats were randomly divided into 3 groups: Group 1 – Intact Group (IT), 6 rats that did not undergo any surgery; Group 2 – Control Group (K), 6 rats received incomplete Freund's adjuvant before implantation of a nickel-containing implant. They were pretreated with Freund's adjuvant with $NiSO_4$, followed by implantation of the nickel-containing implant; Group 3 – Experimental Group (E), 13 rats were pretreated with Freund's adjuvant with $NiSO_4$, followed by implantation of the nickel-containing implant. The immune response associated with nickel was modeled using a modified method described previously [18].

For this, the rats were intraperitoneally (i.p.) injected with 50 µl of NiSO4 solution (CAS 10101-97-0, Sigma-Aldrich, USA) at a concentration of 10 µmol/L in incomplete Freund's adjuvant (Sigma-Aldrich, USA). After 2 and 4 weeks, the rats received NiSO4 through intradermal (i.d.) injections of 50 µl (2 µmol/ml $NiSO_4$ in complete Freund's adjuvant, Sigma-Aldrich, USA) using 28G1/2 needles to restore the immune response.

The implantation of the nickel implant was performed after 6 weeks after last exposition of $NiSO_4$ under aseptic and antiseptic conditions, with general anesthesia using sodium thiopental (Kyivmedpreparat, Ukraine) at a dose of 50 mg/kg body weight, administered intraperitoneally.

The implants for the study were provided by LLC «ORTOSYNTEZ.» All samples were pre-screened by spectroscopy to assess the local elemental composition of the material, with Ni content at 98.9%. The rats underwent a dorsal incision along the vertebral line, and a subfascial pocket was created in the interscapular space to place the nickel-containing implant, measuring 6.0 x 4.0 x 1.0 mm. After the procedure, the wound was closed in layers with Prolene 3-0 sutures (Ethicon Inc, Johnson & Johnson, USA) and treated with the antiseptic Povidone-iodine (Betadine, Hungary). To prevent bacterial infections, the animals were administered the antibiotic Ceftriaxone (Arterium, Ukraine) at a dose of 20 mg/kg body weight, intraperitoneally. The animals were placed in individual cages (60x40x50 cm), and observations were conducted over a period of 5 months. No infections or other postoperative complications were detected.

Five months after implantation, the rats were euthanized using a lethal dose of sodium thiopental (i.p. 150-200 mg/kg) (Kyivmedpreparat, Ukraine). For histological analysis, tissue samples from the capsule around the implant were fixed in 10% neutral formalin. The samples were embedded in paraffin (Leica

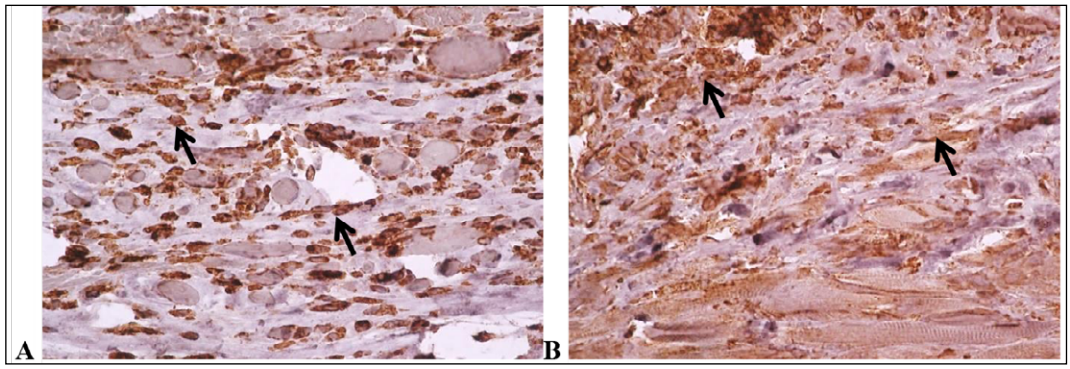

Fig. 1. Cells with GRP78-positive immunoreactivity along the outer contour of the capsule surrounding the implant in the control (A) and experimental (B) groups. Note: ← cells with positive reaction to GRP78. Immunohistochemical staining for GRP78. Magnification x400.

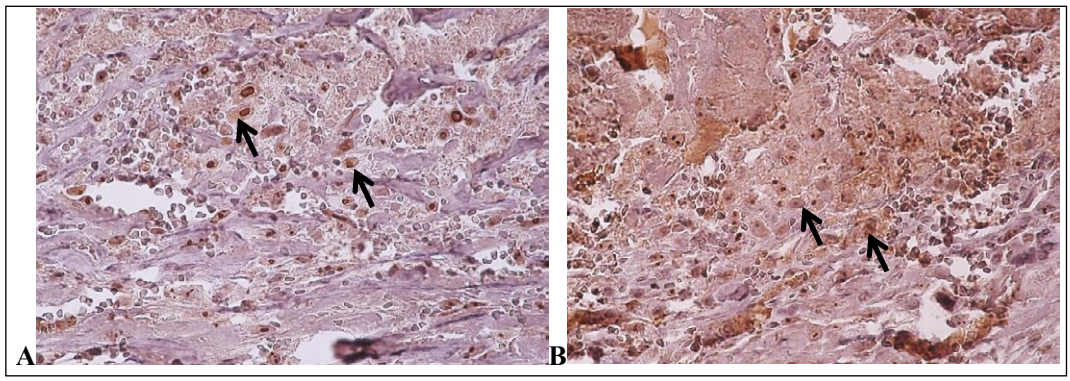

Fig. 2. Cells with Beclin1-positive immunoreactivity in the capsule wall around the implant in the control (A) and experimental (B) groups. Inner layer of the capsule. Note: ← cells with positive reaction to Beclin1. Immunohistochemical staining for Beclin1. Magnification x400.

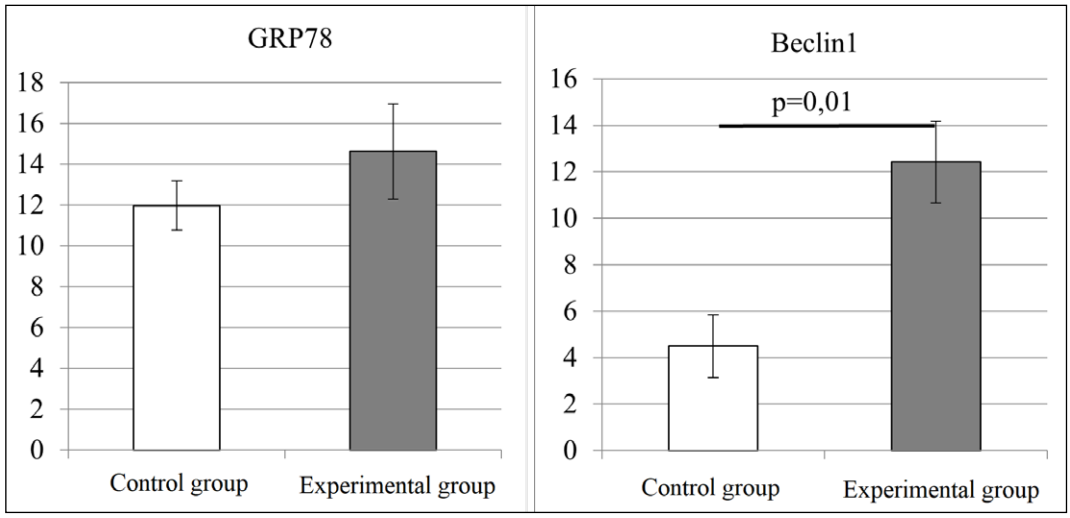

Fig. 3. Quantitative evaluation results of the specific area of immunopositive reaction to GRP78 and Beclin1.

Surgipath Paraplast Regular, Leica, USA). Sections of 4 μm thickness were prepared from paraffin blocks and mounted on Superfrost Plus slides (Thermo Scientific, Gerhard Menzel GmbH, Germany). Antigen retrieval and staining reactions were carried out according to the manufacturer's protocol for antibodies.

Primary antibodies against GRP78 (Invitrogen, PA5-34941, USA) and Beclin-1 (Invitrogen, PA5-20171, USA) were used. The primary antibodies were diluted at a ratio of 1:200. Visualization of the reaction product was performed using a detection system based on diaminobenzidine (EnVision FLEX; Dako, Glostrup, Denmark). The incubation time with primary antibodies was 60 minutes at 24°C, and the incubation time with secondary antibodies was 20 minutes at 24°C. Hematoxylin Gill I was used for staining cell nuclei.

Microscopic examination of the samples was performed using an Olympus BX51 microscope (Olympus, Japan), and microphotographs were obtained with a digital camera Olympus C3040ZOOM. Quantitative assessment of the immunohistochemical reactions was conducted using a densitometric method with ImageJ software.

Statistical data analysis was performed using the Mann-Whitney U test with Origin v.9.0 software. The data were presented as mean (M) ± standard error or mean (m). Differences between groups were considered statistically significant at $p < 0,05$.

RESULTS

Around the implants, a connective tissue capsule with a pronounced inflammatory infiltrate and necrotic material

was observed. On average, 35-45% of the capsule wall was composed of collagen fibers. Microscopically, the wall of the capsule in both the control and experimental groups appeared similar, though a significant cellular infiltrate with hemorrhagic infiltration was noted in the control group. Both groups exhibited multiple accumulations of hemosiderin, macrophages, and cellular detritus.

Immunohistochemical analysis assessed the intensity of GRP78 and Beclin1 synthesis by cells in the capsule wall surrounding the implants. This analysis allowed for the identification of some cellular responses in the capsule wall that could explain processes associated with cell death. GRP78 is associated with endoplasmic reticulum stress, while Beclin1 is linked to autophagy.

The immunohistochemical study revealed that the intensity of GRP78 staining indicated active cytoplasmic immune reactions in the cells of the capsule wall (Fig. 1). These cells were located among muscle fibers, around blood vessels, and between the fibrous elements of the capsule. Based solely on the positive reaction to GRP78, it is not possible to definitively determine the type or population of the cells. However, the majority of cells in the capsule wall exhibited active expression of GRP78. We observed a numerical, but non-significant increase in the density of GRP78-positive cells and specific area of immunoreaction (11,9±1,20 vs 14,6±2,33; p=0,15 via the Mann-Whitney U test). These results indicate that the intensity of processes in the capsule wall, hypothetically associated with cell damage involving the endoplasmic reticulum, was greater in the experimental group or did not differ between comparison groups.

The density of cells with positive immunoreactivity to Beclin1 depended on the distance from the capsule cavity. Thus, their density increased along the inner contour of the capsule and decreased towards the middle thickness of the capsule wall. Cells with positive immunoreactivity to Beclin1 were not detected outside the capsule (Fig. 2). These results indicate a distal dependence of autophagy development in the capsule cells around the implants. It can be inferred that cell death by autophagy occurred along the implant contour. According to densitometric analysis, the specific area of immunopositive reaction was significantly higher (4,49±1,34 vs 12,42±1,75; p=0,01 via the Mann-Whitney U test) in the experimental group compared to the control group (Figure 3). This suggests that autophagy occurs in the cells at the capsule's boundary with the implant, which is the molecular basis of their death. It appears that autophagy processes were significantly less pronounced regarding cellular reactions associated with GRP78, had a distal dependence on the implant, and were potentially more intense in the experimental group.

DISCUSSION

The most common tissue reactions to a metal implant are inflammation and necrosis in the peri-implant tissue. Cellular responses such as proliferation, migration, and cell death occur through various mechanisms. Oxidative stress and peroxidation provide some insight into the cause-and-effect relationship in the development of cell necrosis. However, it turns out that cell death around the implant is a more complex phenomenon and can occur through atypical mechanisms, including cell autophagy. In this experiment, necrotized cells and cellular debris were found around the Ni-containing implant, directly at the interface between the formed connective tissue capsule and the implant. Importantly, among this mass of damaged cells, cells with pronounced Beclin1 expression were detected, which can be considered an immunohistochemical marker of autophagy initiation in these cells. Autophagy is known to be a self-degradation process in eukaryotic cells, involving specific rearrangements of the endoplasmic reticulum to capture and degrade cellular organelles [19]. Reactive changes in cells, which may be related to such responses, include GRP78 expression. This protein responds to oxidative stress and is associated with the endoplasmic reticulum. Studies show that reactive oxygen species (ROS) play a critical role in the initiation of autophagy [20]. Excessive activation of autophagy by oxidative stress can lead to the execution of cell death mechanisms. Increasingly, research indicates that various heavy metals, including Ni, can induce autophagy [20, 21].

There are reports that nickel (Ni) induces mitochondrial damage and mitophagy [22]. In this study, the pronounced reaction of cells expressing GRP78 in the capsule surrounding the implant indicates that the Ni-containing implant induces oxidative stress in peri-implant tissues. It became clear that autophagy occurs on the inner side of the capsule, while cells with a strong immunoreaction for GRP78 were observed throughout almost the entire thickness of the capsule. These results suggest that a certain critical level of oxidative stress around the implant triggers autophagy, mediated by Beclin1. The main conclusion is that autophagy, along with apoptosis and necrosis, and independently of these processes, can regulate the intensity of cell death around the implant with bioactive properties. While necrosis is considered an unregulated process resulting from critical changes and damage to cells, apoptosis and autophagy are regulated processes. Both are triggered by various pathways and conditions and both control cell survival or death. There is clearly a close connection between autophagy and apoptosis, which requires further in-depth study. In this study, we only state the fact of detecting autophagy and its topographical proximity to the Ni-containing implant, which further confirms the bioactivity and toxicity of Ni.

The limitation of this study lies in the restricted methods for detecting autophagy and other mechanisms of cell death. It is important to investigate the connection between autophagy and immune responses to establish a reliable link with reactions similar to allergies and metal hypersensitivity. Therefore, further research is needed to study the pathways and mechanisms of cell death associated with inflammation around Ni-containing implants.

CONCLUSIONS

Undesirable tissue reactions and complications to the Ni-containing implant include inflammation and cell death in the connective tissue capsule surrounding the implant. Autophagy is one of the pathways of cell death in the capsule wall, mediated by Beclin1 and closely linked to GRP78. The latter is a reliable marker of cellular response to damage, where autophagy represents contact-dependent cell death in relation to the implant.

PROSPECTS FOR FURTHER RESEARCH

As a potential approach, one of the likely ways to prevent the initiation of cell death, particularly delayed autophagy, could be coating the implant with a material that prevents cells from coming into contact with nickel or other bioactive metals of the implant.

COMPLIANCE WITH ETHICAL REQUIREMENTS

The experimental studies were conducted in compliance with the requirements for humane treatment of experimental animals in accordance with the Law of Ukraine «On the Protection of Animals from Cruelty» (No. 3447-IV dated February 21, 2006), adhering to the requirements of the European Parliament and Council (2010), and the European Convention «For the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes» (Strasbourg, March 18, 1986).

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

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