Response of soft tissues and abdominal organs of rabbits and rats to implanting albucid-containing cross-linked polyurethane composite

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Background: *Craniofacial injuries represent 29% of all trauma cases, and patients need to have their affected orbit, orbital adnexa, and periorbital area surgically reconstructed or restored. However, the outcomes of these procedures depend also on the quality of implant materials. Previously, we have developed a polymer material made of cross-linked polyurethane (PU) and containing a biologically active substance, albucid; it seems to be a promising implant material.*

Purpose: *To investigate experimentally the response of soft tissues and abdominal organs of animals (rabbits and rats) to implanting the albucid-containing cross-linked PU composite.*

Material and Methods: *Assessment of soft tissue response to implantation of the synthetic polymer material. The skin response to implanting the albucid-containing cross-linked PU composite was assessed through the intracutaneous injection of the extract of the test material in rabbits. The soft tissue response was assessed through subcutaneous implantation of cross-linked PU composites in Wistar rats. The response of abdominal organs to implanting the cross-linked PU composite was assessed through the intraperitoneal injection (20 ml per kg body weight) of the extract of the test material in Wistar rats.*

Conclusion: *The intracutaneous injection of the extract of the test material caused neither erythema nor edema in rabbits, and the test sample of the albucid-containing cross-linked PU composite was considered non-irritating, since a difference between the average scores for the test extract and control extract (i.e., a value of the primary irritation index) was of 0 to 0.4 points. Over the period of observation of potential acute systemic toxicity, no animal injected with the extract of the test material (the albucid-containing cross-linked PU composite) displayed higher biologic response than animals injected with the control extract, and the test sample of the albucid-containing cross-linked PU composite conformed to the requirements of tests for systemic toxicity. This study demonstrated a natural process for a foreign body residing in the body (gradual foreign body separation from the surrounding tissues due to formation of a connective tissue capsule) after implantation of the samples of cross-linked PU composites in animal bodies. The cellular responses were (1) typical for a living body response to the presence of a foreign body at the site of implant placement and (2) characteristic for aseptic inflammation. The test samples produced moderate irritation when placed into the animal's body.*

Ключові слова:
сітчастий поліуретан, альбуцид, імплантація, реконструктивні операції на органі зору, гостра системна токсичність, локальні ефекти

Introduction

Craniofacial injuries represent 29% of all trauma cases [1, 2], and are primarily caused by anthropogenic and criminal-related ocular and orbital trauma [3]. In this connection, and after surgery for eye cancer, more and more patients need to have their affected orbit, orbital adnexa, and periorbital area surgically reconstructed or restored [4].

The ocular surgeon has to use implant materials to replace soft tissue and bone structures during restorative and reconstructive surgeries. Presently, a variety of biological and synthetic [5-10] implant materials are available. Biointegrable implants have an advantage of encouraging ingrowth of recipient's tissue cells with a reliable implant placement.

A promising material for this purpose is a polymer material based on cross-linked polyurethane (PU) [11, 12] which contains the urethane groups (-NHCOO-) in the polymer chain, and the group is structurally close to the peptide group of proteins –CONH–, which facilitates effective use of this class of synthetic materials [13].

Our previous preliminary experimental study [6] has demonstrated that the cross-linked PU composites with immobilized biologically active substances are promising implant materials. Therefore, pre-clinical studies of the albucid-containing cross-linked PU composite should be conducted to (1) test it for potential acute systemic toxicity, (2) check whether it causes irritation of body tissues, and (3) examine the local effects of placing it into the body.

The purpose of the study was to investigate experimentally the response of soft tissues and abdominal organs of animals to implanting the albucid-containing cross-linked polyurethane composite.

Material and Methods

Biological evaluation of a potential implant material, albucid-containing cross-linked PU composite, was conducted at the Testing Laboratory (Department for Medical Polymers, Institute for Chemistry of High-Molecular Compounds) certified by the National Certification Agency of Ukraine (Certificate No. 20725 issued 25 June, 2019) in three rabbits (Oryctolagus cuniculus; weight, 3-3.2 kg) and 30 Wistar rats (weight, 195-225 g).

The methodologies as per DSTU ISO 10993-10:2004 (ISO 10993-10:1995, IDT) "Biological evaluation of medical devices – Part 10: Tests for irritation and skin sensitization", DSTU EN ISO 10993-11:2015 (EN ISO 10993-11:2009, IDT; ISO 10993-11:2006, IDT) "Biological evaluation of medical devices – Part 11. Tests for systemic toxicity", and DSTU EN ISO 10993-6:2015 (EN ISO 10993-6:2009, IDT; ISO 10993-6:2007, IDT) "Biological evaluation of medical devices – Part 6: Tests for Local Effects after Implantation" were employed taking into consideration the area and method of application of the developed polymer material.

The test materials (PU, albucid-containing cross-linked PU composite) were implanted under general anesthesia in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from the European Treaty Series [14].

In the current study, we evaluated the albucid-containing cross-linked PU composite (Fig. 1) for irritation and skin sensitization, potential acute systemic toxicity, and soft tissue response after implantation in experimental animals.

At the first stage of the study, we examined animal skin response to the implanted albucid-containing cross-linked PU composite. Three adult male rabbits (Oryctolagus cuniculus; weight, 3-3.2 kg) whose skin had no signs of irritation or trauma were used for the evaluation. Cross-linked PU composite extracts with 0.9% sodium chloride solution or with sesame oil were injected intracutaneously into the rabbits. The intracutaneous injection test in rabbits is specified in the current ISO testing standards and has been used to evaluate biomaterial extracts.

The animals were housed individually in metal cages. Each cage was labeled with a card that indicated the sex and identification number of the animal and the date of test. The animal room was maintained on a 12-hr light/dark cycle. The target temperature range was 20-22°C, and the humidity was 60%. The animals were fed standard, commercial laboratory animal diets, and water ad libitum. There are no known contaminants in the diet or water which at the levels detected would be expected to interfere with the purpose, conduct or outcome of the study.

The albucid-containing cross-linked PU composite was used to obtain extracts. Test samples were placed into laboratory glassware of a size appropriate for extraction. The glassware was charged with the tested material and a sufficient amount of the extraction medium (0.9% sodium chloride solution or sesame oil) to disperse the material with gentle stirring (about 20 cm3 of the extraction medium per 2 g of the tested material). Extraction was conducted at 37 °C for 72 hours. Thereafter, the resultant extract was stirred, decanted into clean glassware and stored at room temperature for up to 24 hours before being tested. The extraction media without test material were prepared as controls in the same way as and simultaneously with the extracts of the test material.

A day before testing, hair was clipped at the back of an animal, with enough space left on the lateral sides of the back for injecting the extracts. Immediately before testing, each animal was identified properly and weighted. A 0.2 ml dose of the test article extract prepared in a polar (0.9% sodium chloride) solution was injected intracutaneously into five sites spaced 2 cm apart on one side of the back of each rabbit. The smallest needle appropriate to the viscosity of the test material was used for intracutaneous administration. A 0.2 ml dose of the control extract prepared in a polar solution was injected in the same way into five sites spaced on the same side of the back of each rabbit. Similarly, 0.2 ml of the non-polar solvent control was (sesame oil) injected on five sites of the contralateral side of each rabbit.

The injected sites were examined at 24, 48 and 72 hours for evidence of tissue reaction such as erythema or edema. Observations were scored according to the Classification System for Scoring Skin Reactions (Table 1).

Twenty healthy young adult male Wistar rats (weight, 200-250 g) whose skin had no signs of irritation or trauma were used for the evaluation of soft tissue response. The animals were housed individually in metal cages. Each cage was labeled with a card that indicated the sex and identification number of the animal and the date of test. The animal room was maintained on a 12-hr light/dark cycle. The target temperature range was 20-22°C, and the humidity was 60%. The animals were fed standard, commercial laboratory animal diets. The test materials

(PU, albucid-containing cross-linked PU composite) were implanted under general anesthesia in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from the European Treaty Series [14]. Surgical procedures on animals were done under aseptic conditions. The hair was removed from the surgical site and the operative field was prepared with chlorhexidine. Thereafter, and a polymer sample implant (sized 10.0x5.0x5.0 mm) was placed subcutaneously in the back of each animal without additional suture fixation to exclude the influence of suturing on wound healing. The wound was closed with sterile sutures. Rats were euthanized at days 7, 14 and 30 postimplantation. Implanted polymer samples were collected with surrounding connective tissue, and fixed in 10% buffered formalin solution. This was followed by processing, embedding in paraffin, sectioning to 10-12 μm, mounting and hematoxylin and eosin staining in a routine manner [15, 16]. Images were taken with a Primo Star (Carl Zeiss Microimaging, Oberkochen, Germany) microscope using a Canon Powershot A640 camera with a Soligor tele adapter tube for Canon A610/ A620. Histological evaluation of local effects of implants is specified in the current ISO standards and used for the assessment of the local effects after implantation.

Ten Wistar rats (weight, 195-225 g) were used to assess the response of abdominal organs of animals to implanting albucid-containing cross-linked polyurethane composites in accordance with the current ISO standards. The animals were housed individually in metal cages. Each cage was labeled with a card that indicated the sex and identification number of the animal and the date of test. The animal room was maintained on a 12-hr light/ dark cycle. The target temperature range was 20-22°C, and the humidity was 60%. The animals were fed standard, commercial laboratory animal diets. Extracts of the test material (albucid-containing cross-linked PU composite) were prepared in the same way as for evaluation of irritation reactions. Animals were examined for reaction to implantation immediately after the extract of the test material was injected intraperitoneally and at days 1, 2 and 3 after injection. Observations included appearance, movement and behavior patterns, skin and hair condition, and ocular mucous membranes. Longitudinal fasting weight measurements were taken immediately before the extract of the test material was injected intraperitoneally and in the morning on days 1, 2 and 3 after injection. The extract of the test material (20 ml per kg body weight) was injected intraperitoneally in animals of the main group using a disposable sterile syringe. Similarly, 0.9% sodium chloride solution was injected in animals of the control group. After the experiment was completed, the experimental animals were euthanized, and a histopathological study was conducted to assess macroscopically visible changes in the shape, dimensions, color, volume, and other characteristics of the internal organs compared to controls.

Results

Irritation tests

Observations of tissue reaction (erythema and/or edema) to the extract of albucid-containing cross-linked PU composite prepared in 0.9% sodium chloride solution in rabbits were scored according to the Classification System for Scoring Skin Reactions (Table 1). All sites of injection appeared normal immediately after injection, and all the rabbits appeared normal over the assessment period.

The primary irritation index for each animal was determined. The erythema and edema scores obtained at 24, 48 and 72 h were added together and divided by the total number of observations to obtain average scores for the test extract and control sites. A difference between the average scores for the test article extract and control extract (i.e., primary irritation index) of less than or equal to 1 was considered non-irritating (Table 2).

Extracts of test materials prepared in 0.9% sodium chloride solution caused neither erythema nor edema after intracutaneous injection test in rabbits. The erythema and edema scores for extracts of test materials prepared in sesame oil were not larger than those for pure sesame oil after intracutaneous injection in rabbits.

The test sample of the albucid-containing cross-linked PU composite was considered non-irritating, since a difference between the average scores for the test extract and control extract was of 0 to 0.4 points and conformed to the requirements of DSTU ISO 10993-10:2004 “Biological evaluation of medical devices – Part 10: Tests for irritation and skin sensitization” (ISO 10993-10:1995, IDT).

Tests for Local Effects after Implantation

The test samples implanted did not cause aggression or changes in behavior in the animals that received them. Daily visual evaluation of the epithelial response at the site of surgery showed that the wound healed within 3 days after surgery without signs of inflammation. No hematomas, edemas, scarring, degenerative changes, tumors, tissue necrosis or other apparent abnormalities was found by macroscopic morphological examination. The implanted materials were palpable through the skin throughout the period of experiment, and their shape and location did not change throughout the period of implantation. At all examination time points, an implanted sample macroscopically appeared to be surrounded by the connective tissue which closely adhered to the surface of the sample, with no difference in color or structure between this tissue and those located further from the site of implantation.

During histological analysis, attention was focused on inflammatory signs in the implant-tissue interface area.

At day 7 after implantation, a well-shaped and rather mature connective tissue capsule with variations in cell content along its length was observed around the polyurethane samples containing no albucid. Thus, some sites of the capsule showed elongated and spindle-shaped fibroblasts within bundles of mature collagen fibers (рис.

2). At some other sites, the capsule was less mature, and the most common cells were polymorphonuclear neutrophils. In addition, there was apparent monocyte/macrophage infiltration on some sites of the capsule, which indicated increased phagocytic activity of macrophages. Moreover, numerous mid-size blood vessels were characteristic for the capsule. Most blood vessels were engorged and dilated, indicating problems with microcirculation, and individual vessels showed stasis and thrombosis.

At day 14 after surgery, a rather thick and well-shaped connective tissue capsule was observed around the polyurethane samples containing no albucid (Fig. 3). Similarly to day 7, the most common cells of the capsule were polymorphonuclear neutrophils and macrophages, which indicated an increased phagocytic activity of these cells. In addition, there were ingrowths of connective tissue into the porous PU sample at some locations (Fig. 4). A thin and mature connective tissue capsule was observed around the PU samples at some locations; the capsule was made up of collagen fiber bundles separated by spindle-shaped fibroblasts. Moreover, at this time point, there was evidence of normalized microcirculation in blood vessels which were few in number.

At day 14 after surgery, a rather thick and well-shaped connective tissue capsule with variations in cell content along its length was observed around the albucid-containing polyurethane samples. At some locations of the capsule, there were collagen fiber bundles separated by spindle-shaped fibroblasts and oriented along the surface of the sample. At other locations, residual infiltration with round cells (mostly, polymorphonuclear neutrophils and macrophages, and, rarely, lymphocytes) was observed (Fig. 5). In addition, there was uneven ingrowth of connective tissue into the porous albucid-containing PU sample at some locations, in a way similar to the PU sample containing no albucid at this time point. Moreover, there was evidence of normal microcirculation in blood vessels which were few in number.

At day 30, the connective tissue capsule surrounding the PU sample containing no albucid appeared significantly thickened and denser than at the previous time point due to active fibroblast synthesis of collagen and other components of the extracellular matrix (Fig. 6). In addition, the capsule showed variations in cell content along its length. At some sites of the capsule, the most common cells were young fibroblasts and spindle-shaped fibroblasts which were observed within bundles of mature collagen fibers. Marked infiltration of white blood cells and low infiltration of macrophages were characteristic for other sites and the internal layer of the capsule. Blood vessels were few in number, and individual vessels showed insignificant problems with microcirculation resulting in stasis and thrombosis.

At day 30, the connective tissue capsule around the albucid-containing PU sample appeared rather mature and denser than at the previous time point. In addition, the capsule appeared significantly thicker than at the previous time point due to active fibroblast proliferation and active production of collagen and other components of the extracellular matrix by fibroblasts (Fig. 7). The capsule was mostly composed of mature spindle-shaped fibroblasts entrapped within collagen bands and oriented along the surface of the sample. It was also composed of numerous macrophages, indicating their increased phagocytic activity. Blood vessels were few in number, and individual vessels were engorged and dilated.

At day 90, the connective tissue capsule around the PU sample containing no albucid appeared thin and mature and showed no variations in cell content along its length. The capsule was composed of collagen fiber bundles separated by wavy-pattern fibroblasts (Fig. 8); in addition, it still appeared rather dense due to increased fibroblast production of collagen and extracellular matrix components. At some sites of the capsule, there were small accumulations of macrophages with increased phagocytic activity. Blood vessels with normal microcirculation were few in number at this time point.

At day 90, a thin and mature connective tissue capsule was seen both around albucid-containing PU samples. The capsule was composed of elongated and spindle-shaped fibroblasts entrapped within collagen bands and oriented along the surface of the sample (Fig. 9). Numerous fibroblasts were seen at some sites of the connective tissue surrounding the implant, which is consistent with their increased phagocytic activity. Blood vessels also increased in number, some of them being engorged and dilated.

Therefore, we assessed tissue response to short-time implantation of cross-linked PU composites containing and not containing albucid in animal bodies.

This study demonstrated a natural process for a foreign body residing in the body (gradual foreign body separation from the surrounding tissues due to formation of a connective tissue capsule) as early as the first time points after implantation of the samples of cross-linked PU composites containing and not containing albucid in animal bodies. The cellular responses were typical for a living body response to the presence of a foreign body at the site of implant placement. A gradual process of maturation of the connective tissue capsule was observed throughout a 90-day period of the experiment. Until day 30, the degree of maturity of the capsule was low, but the latter was well-shaped and appeared to completely separate the implanted samples from the adjacent connective tissue. In addition, apparent cellular responses were observed. Macrophages were the most common cell type in the capsule; they were actively involved in the phagocytosis of cellular metabolism products, and their activity was aimed at implementing complementary protective mechanisms. Some sites of the capsule were represented by the elongated and spindle-shaped fibroblasts that were actively producing collagen and other components of the extracellular matrix.

Therefore, test samples of cross-linked PU composites produced moderate irritation when placed into the animal's

body and comply to the requirements of DSTU EN ISO 10993-6:2015 (EN ISO 10993-6:2009, IDT; ISO 10993-6:2009, IDT) "Biological evaluation of medical devices – Part 6: Tests for Local Effects after Implantation".

Tests for acute systemic toxicity

We evaluated potential acute systemic toxicity of the albucid-containing cross-linked PU composite at 24, 48 and 72 hours after injection of extracts of the test material. It should be noted that the appearance, movement and behavior patterns, skin and hair condition, and ocular mucous membranes of the test animals were satisfactory and did not differ from those of the controls. Table 3 presents the results of this assessment.

The percentage change in the body weight of experimental animals after injection was within 10%. Our histopathological study on completion of the experiment found that intraperitoneal injection of the extract of the test material caused no macroscopically visible changes in the shape, dimensions, color, volume, and other characteristics of the internal organs compared to controls. There was no difference in the appearance of the site of injection, peritoneum and muscles of the peritoneal wall between experimental animals and controls.

Therefore, over the period of observation of potential acute systemic toxicity, (1) no animal injected with the extract of the test material (the albucid-containing cross-linked PU composite) displayed higher biologic response than animals injected with the control extract, and (2) the percentage change in the body weight of three or more experimental animals after injection was within 10%. This indicates that the test sample of the albucid-containing cross-linked PU composite conformed to the requirements of DSTU EN ISO 10993-11:2015 (EN ISO 10993-11:2009, IDT; ISO 10993-11:2009, IDT) "Biological evaluation of medical devices – Part 11. Tests for systemic toxicity".

To conclude, our tests, conducted in accordance with the international standards, showed that the developed albucid-containing cross-linked PU composites are safe and the use of the test materials may be recommended for limited clinical studies, in restorative and reconstructive procedures in ophthalmic and maxillofacial surgery.

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The authors certify that they have no conflicts of interest in the subject matter or materials discussed in this manuscript.

Fig. 1. Albucid-containing cross-linked polyurethane

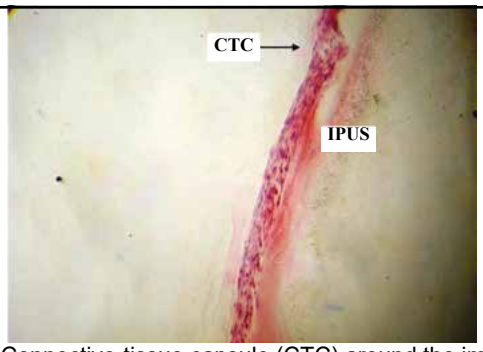

Fig. 2. Connective-tissue capsule (CTC) around the implanted PU sample (IPUS) at day 7 of the experiment. Hematoxylin and eosin staining. Magnification ×200.

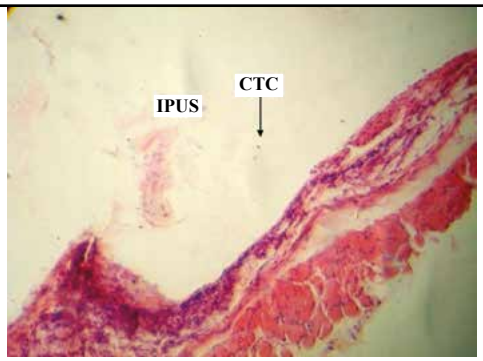

Fig. 3. Connective-tissue capsule (CTC) around the implanted albucid-containing PU sample (IPUS) at day 7 of the experiment. Hematoxylin and eosin staining. Magnification ×200.

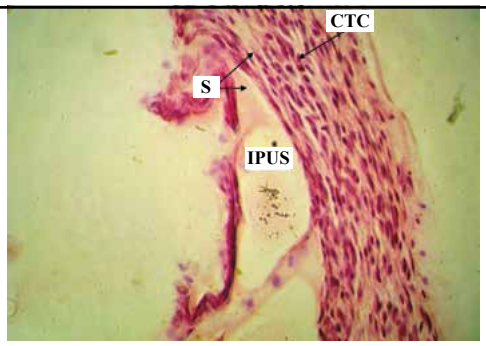

Fig. 4. Connective-tissue capsule (CTC) around the implanted PU sample (IPUS) and connective tissue strand (S) growing into the sample at day 14 of the experiment. Hematoxylin and eosin staining. Magnification ×400.

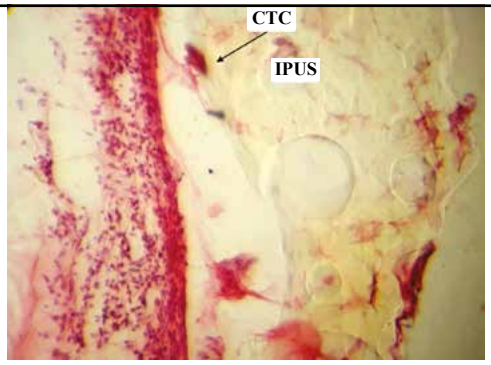

Fig. 5. Connective-tissue capsule (CTC) around the implanted albucid-containing PU sample (IPUS) at day 14 of the experiment. Hematoxylin and eosin staining. Magnification ×200.

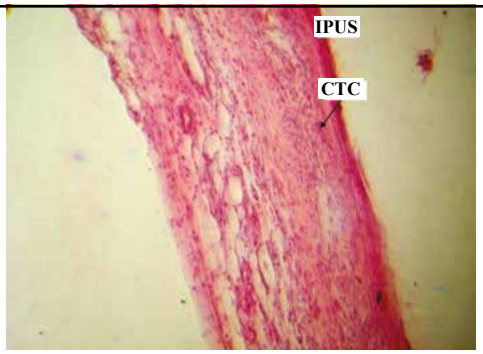

Fig. 6. Connective-tissue capsule (CTC) around the implanted PU sample (IPUS) at day 30 of the experiment. Hematoxylin and eosin staining. Magnification ×200.

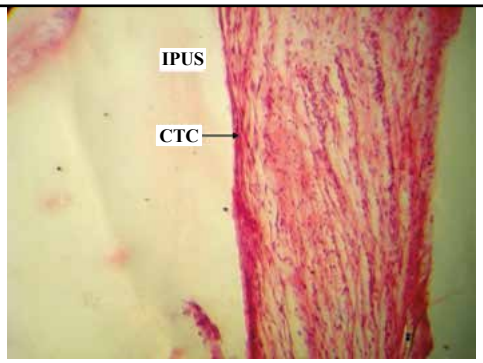

Fig. 7. Connective-tissue capsule (CTC) around the implanted albucid-containing PU sample (IPUS) at day 30 of the experiment. Hematoxylin and eosin staining. Magnification ×200

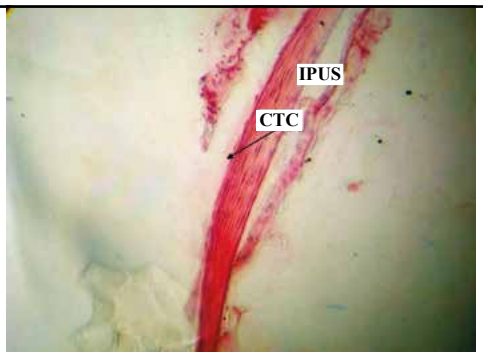

Fig. 8. Connective-tissue capsule (CTC) around the implanted PU sample (IPUS) at day 90 of the experiment. Hematoxylin and eosin staining. Magnification ×200.

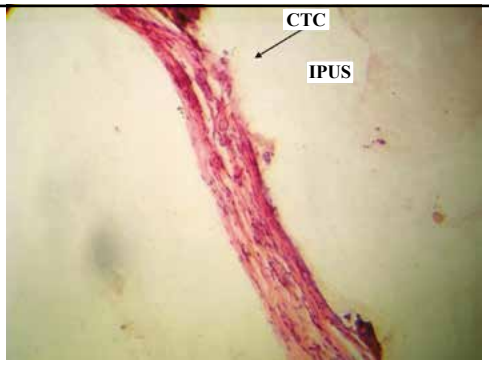

Fig. 9. Connective-tissue capsule (CTC) around the implanted albucid-containing PU sample (IPUS) at day 90 of the experiment. Hematoxylin and eosin staining. ×200.

Table 1. Classification System for Scoring Skin Reactions (edema and erythema) in rabbits

Erythema	Edema	Points
No erythema	No edema	0
Very slight (barely visible)	Very slight (barely visible) edema	1
Slight	Slight edema with well defined, elevated edges	2
Moderate	Moderate edema (1-mm elevation above surrounding skin surface)	3
Marked (dark red)	Marked edema (more than 1-mm elevation and extension beyond the site of exposure)	4

Table 2. Average skin reaction scores for the test and control groups in rabbits

Extract	Average score for the test group	Average score for the control group	Difference between the average scores of experimental and control groups	Requirements
0.9% NaCl solution	0	0	0	Primary irritation index within the range of 0 to 0.4
Sesame oil	1	1	0	Primary irritation index within the range of 0 to 0.4

Table 3. Rat body weight in the course of assessment for acute systemic toxicity

Control or test sample	Rat body weight before injection, g	Rat body weight 24 h after injection, g	Coefficient for change in rat body weight at 24 h, %	Rat body weight 48 h after injection, g	Coefficient for change in rat body weight at 48 h, %	Rat body weight 72 h after injection, g	Coefficient for change in rat body weight at 72 h, %
Control	210.8	221.3	4.7	223.5	5.7	225.9	6.7
	206.4	214.5	3.8	220.2	6.3	219.8	6.1
	217.8	227.2	4.1	229.6	5.1	230.5	5.5
	220.6	233.1	5.4	231.8	4.8	234.7	6.0
	214.4	226.2	5.2	228.4	6.1	230.8	7.1
Albucid-containing PU sample	198.8	215.4	7.7	216.5	8.2	218.1	8.8
	222.5	240.1	7.3	238.7	6.8	245.0	9.2
	215.9	236.3	8.6	238.2	9.4	239.1	9.7
	220.5	241.7	8.7	240.9	8.5	243.3	9.4
	217.6	235.2	7.5	238.8	8.9	240.2	9.4