Research article

IN VIVO INVESTIGATION OF SOFT TISSUE RESPONSE OF NOVEL SILVER/POLY(VINYL ALCOHOL)/ GRAPHENE AND SILVER/POLY(VINYL ALCOHOL)/CHITOSAN/GRAPHENE HYDROGELS AIMED FOR MEDICAL APPLICATIONS – THE FIRST EXPERIENCE

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In this paper, we have shown for the first time the soft tissue response of novel silver/poly(vinyl alcohol)/graphene (Ag/PVA/Gr) and silver/poly(vinyl alcohol)/chitosan/graphene (Ag/PVA/CHI/Gr) nanocomposite hydrogels aimed for medical applications. These novel hydrogels were produced by *in situ* electrochemical synthesis of silver nanoparticles in the polymer matrices as described in our previously published works. Both Ag/PVA/Gr and Ag/PVA/CHI/Gr, as well as controls Ag/PVA, Ag/PVA/CHI and commercial Suprasorb© hydrogel discs, were implanted in the subcutaneous tissue of rats. Implants with the surrounding tissue were dissected after post-implantation on days 7, 15, 30 and 60, and then processed for histological examination. The tissue irritation index (TIrI) score, according to ISO 10993-6, 2007, as well as the number of leukocytes in the peri-implant zone and connective tissue capsule thickness were examined. The results show that each TIrI score, the leukocyte number around the implanted materials and capsule thickness gradually decreased during the observation period. At the endpoint of follow-up, the Ag/PVA/CHI/Gr implant was surrounded with a thinner capsule, while both the TIrI score and the number of leukocytes of the peri-implant zone were greater compared to the Ag/PVA/Gr implant. Despite the observed differences, we can conclude that our *in vivo* experiment suggested that both novel hydrogels were biocompatible and suitable for medical use.

Key words: biocompatibility, nanocomposite hydrogels, rat, soft tissue response

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INTRODUCTION

Regenerative medicine is a domain of great interest for medical researchers. The successful application of a medical device or implant depends on the properties of the biomaterials used to fabricate the device or implant, as well as on various other factors, including the manufacturing and processing of the implant materials, and the specific application in which the device is used [1]. Synthetic or natural polymers are a common base of such materials, because of their resemblance to real tissue. Hydrogels based on natural or synthetic polymers and their blends, are generally used for biomedical purposes. Their biocompatibility, hydrophilicity, and processability enable a wide range of applications.

Poly(vinyl alcohol) (PVA) is a synthetic polymer, which is commonly used in the preparation of biocomposites for various applications, such as wound dressings [2,3] and scaffolds for tissue engineering [4-6] due to its biocompatibility and non-toxicity. Due to their exceptional hydrophilicity, with many –OH groups on the side chain, PVA hydrogels are able to swell in aqueous solutions. This is particularly important for wound dressing applications, as the wound surroundings need to be kept moist at all times, and the hydrogel with a high water content can provide moisture and facilitate the removal of the dressing without damaging the wound tissue [7]. In addition, hydrogels are well suitable for tissue engineering applications, as their structure resembles that of soft tissue, and the porous matrices provide the appropriate environment for cell adhesion and tissue ingrowth [8,9]. A potential use of toxic cross linkers could also be eliminated by preparation of PVA hydrogels with a simple freezing-thawing method [10,11].

Chitosan is a natural linear polysaccharide with a polycationic nature, comprised of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units. It is obtained by partial or complete deacetylation of chitin, most commonly derived from crustaceans or fungi [12]. Due to the excellent biocompatibility and biodegradability, as well as to its natural antibacterial properties, chitosan has been used in various branches of tissue engineering and biomedicine [12,13]. Some examples of chitosan applications include wound dressings [14-17], tissue engineering [18,19], scaffolds or coatings for bone tissue implants [20-22] and drug delivery [23-25].

As a result of their unique properties, such as the antimicrobial activity against many microorganisms, silver nanoparticles (AgNPs) are especially attractive for use in medical applications [26,27]. AgNPs, however, tend to agglomerate due to their high surface energy, which leads to weakening of their antibacterial properties. This is why polymer-based stabilizers are frequently applied in order to lower their surface energy and to immobilize AgNPs in the polymer matrix. PVA is a polymer with many –OH groups on its chain that can act as binding sites for AgNPs and thus contribute to their stability inside the hydrogel [28]. Chitosan contains both –OH and –NH$_2$ groups that allow binding to AgNPs and their further stabilization in the porous hydrogel matrix [29,30].

Graphene (Gr) is a recently discovered material that has found frequent usage in biomedical applications [27,28]. Graphene is a two-dimensional (2D) monolayer of carbon atoms, stacked in a honeycomb lattice and this unique structure provides it with exceptional properties, such as high mechanical strength and great electrical and thermal conductivity [31,32]. All these properties could provide reinforcement to the polymeric materials, so graphene is often included in a polymer matrix as a nano-filler. The overall mechanical properties of the hydrogel matrices were shown to be improved by even small amounts of graphene in PVA/Gr hydrogels [33-35]. There is also a recent interest in the potential antibacterial activity of graphene [36], which could be beneficial for wound dressing or soft tissue implant applications, as these properties could provide additional bactericidal effects together with AgNPs, especially in the early phases of application to prevent biofilm formation. However, the antibacterial properties and specifically the mechanism of action is still debatable and somewhat controversial and more research, both *in vitro* and *in vivo*,is needed in this area of graphene application.

The aim of this study was to examine the impact of the incorporated graphene on the specific characteristics of Ag/PVA and Ag/PVA/CHI nanocomposite hydrogels, as well as to examine the local response of the subcutaneous tissue after implementation of novel Ag/PVA/Gr and Ag/PVA/CHI/Gr implants.

MATERIALS AND METHODS

Material

For the preparation and synthesis of nanocomposite hydrogels, the following chemicals were utilized: chitosan (CHI) powder (medium Mw, Sigma Aldrich, St. Louis, MO, USA), poly(vinyl alcohol) (PVA) powder (fully hydrolyzed, Mw = 70–100 kDa; Sigma Aldrich, St. Louis, MO, USA), graphene (Gr) nanopowder (Graphene Supermarket, Calverton, NY, USA), silver nitrate (M. P. Hemija, Belgrade, Serbia), potassium nitrate (Centrohem d.o.o., Stara Pazova, Serbia), glacial acetic acid (Betahem d.o.o., Belgrade, Serbia). Ultra-pure water (Milli-Q system, Millipore, Burlington, MA, USA) was used in all experiments.

Preparation of PVA, PVA/Gr, PVA/CHI and PVA/CHI/Gr hydrogels

PVA and PVA/Gr hydrogels were prepared according to the procedure published elsewhere [37-39]. PVA/CHI and PVA/CHI/Gr hydrogels were obtained following similar procedure [30].

Electrochemical synthesis of Ag/PVA, Ag/PVA/Gr, Ag/PVA/CHI and Ag/PVA/CHI/Gr nanocomposite hydrogels

For the synthesis of silver nanoparticles (AgNPs), the PVA, PVA/Gr, PVA/CHI and PVA/CHI/Gr hydrogels were cut into discs with an approximate diameter of

5 mm and width of 2 mm (appropriate dimensions for *in vivo* experiments). Before the electrochemical synthesis, the discs were immersed in 0.25 mM $AgNO_3$ swelling dispersions, with 0.1 M KNO_3 added to ensure better electrical conductivity. The containers with hydrogel discs in swelling dispersions were kept at 48 h in a dark place, to achieve the equilibrium swelling of the samples. *In situ* electrochemical synthesis of AgNPs was performed by electrochemical reduction of Ag^+ ions at constant voltage inside the PVA, PVA/Gr, PVA/CHI and PVA/CHI/Gr polymer matrices. A custom made glass electrochemical cell was used, with two horizontal Pt plates as working and counter electrodes, between which the hydrogel samples were placed in a sandwich-like setup. The MA 8903 Electro-Phoresis Power Supply (Iskra d.d., Ljubljana, Slovenia) was used for the electrochemical synthesis. The synthesis was carried out at 90 V with implementation time of 4 min, and the polarity of the electrodes was reversed every 1 min. Before and after every experiment, the electrodes were cleaned with HNO_3 (1:1) and distilled water.

In vivo experiment

Sterilized Ag/PVA, Ag/PVA/Gr, Ag/PVA/CHI and Ag/PVA/CHI/Gr nano-composite hydrogel discs, as well as commercial Suprasorb©, a calcium alginate dressing (Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany) were implanted in the subcutaneous tissue of 16 anesthetized adult females. A small skin incision on the rat back, at the level of the midline of the thoracic spinal column, was made to create five pockets in the subcutis of back lateral sides, which distanced 3 cm from the back midline. The pockets were directed cervically and lumbally, so the distance between implanted material areas was about 3 to 3.5 cm (Fig. 1A) the implants were introduced through the pockets. A surgical incision made on the right lateral side opposite of the Suprasorb© implantation represented the control area. The experiment lasted 60 days. Surgical implantation of hydrogels was done according to the permeation of the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade.
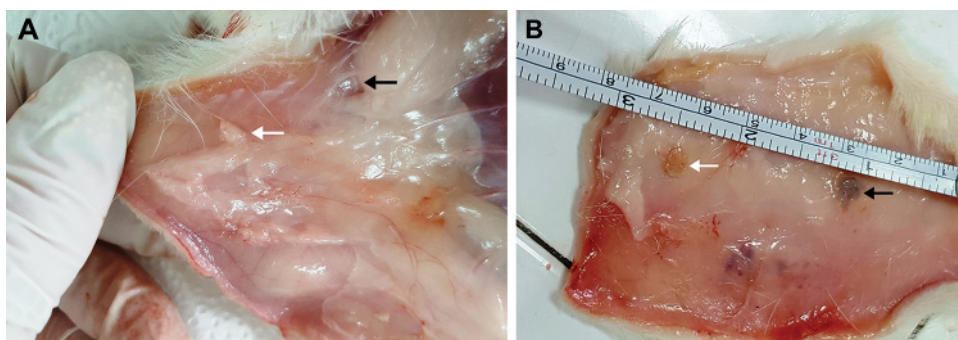

Figure 1. The appearance of Ag/PVA/Gr and Ag/PVA/CHI/Gr implants during surgical intervention, immediately after implantation **(A)** and at postimplantation day 60 **(B)**. Black and white arrows indicate Ag/PVA/Gr and Ag/PVA/CHI/Gr respectively, at both presented periods (A and B).

All animals survived the surgery. No wound healing complications were observed after the surgery and during the whole experiment. The experimental materials were retrieved from the rats after post-implantation day (p.i.d.) 7, 15, 30 and 60, and there were thus four subgroups of animals corresponding to the different periods, i.e. each subgroup consisted of four animals. After the endpoint of the above mentioned experimental periods, the animals were sacrificed and implants with the surrounding tissue were dissected, fixed, and processed by conventional procedures for histological examination. At each end point of the experiment, including postoperative day 60, the distance between implants corresponded to that of implantation time, and we did not notice that implant come close to each other or were joining (Fig. 1B).

Histological examination

Tissue processing for light microscopy

Tissue paraplast blocks were serially sectioned stained with Hematoxylin-Eosin (H&E) and Van Gieson staining protocols, and then were evaluated under a light microscope. However, for histomorphometry, only sections with the largest length and width diameters of the implanted discs were collected.

Morphological examination

In order to estimate the processes of tissue response, regeneration and the tissue-implant interface, the obtained slides were examined semiquantitatively by following histological reactions: epithelial alterations, relative number of inflammatory cells, vascular congestion, edema, and the presence and connective tissue capsule thickness. All these parameters were presented as the tissue irritation index (TIrI) score, according to the ISO 10993-6, 2007. The TIrI was as follows: the value of 1 to 5, 6 to 10, 11 to 15, and 16 to 20 indicates minimal, mild, moderate and severe tissue responses, respectively.

Histomorphometry

Morphometric methods included: 1) counting the number of inflammatory cells in the peri-implanted area and 2) measuring the capsule thickness. The capsule thickness was measured with an ocular micrometer, at x 100 magnification. The total capsule thickness from four sides (upper-lower-right-left) was measured, and the mean value of these measurements was obtained. The number of inflammatory cells of the peri-implant area was counted in five representative zones of each specimen, by using ocular grids which consist of a square limiting test area of 0,250 mm^2, at magnification of 40x. The results were expressed as the number of inflammatory cells per mm^2 of profile area. All sections were examined using standard Olympus BX-41 microscope and archived with Olympus XC50 digital camera, using the CellSens Entry software (Olympus, Tokyo, Japan).

Statistical analysis

The data were analyzed in the statistical software package IBM SPSS 20. Analysis of variance for repeated measurements was performed within the general linear model. All results are expressed as mean ± SE. The minimum level of statistical significance was set at $p < 0.05$.

RESULTS

Soft tissue response – general observation during the post-implantation period for all implanted hydrogels

Total irritation index score

Semiquantitative evaluation of the local soft tissue reaction (i.e. TIrI score) to the subcutaneously implanted Ag/PVA, Ag/PVA/Gr, Ag/PVA/CHI, Ag/PVA/CHI/Gr and Suprasorb© hydrogels, as well as tissue reaction at pseudooperated regions (incision skin) is shown in Fig. 2.
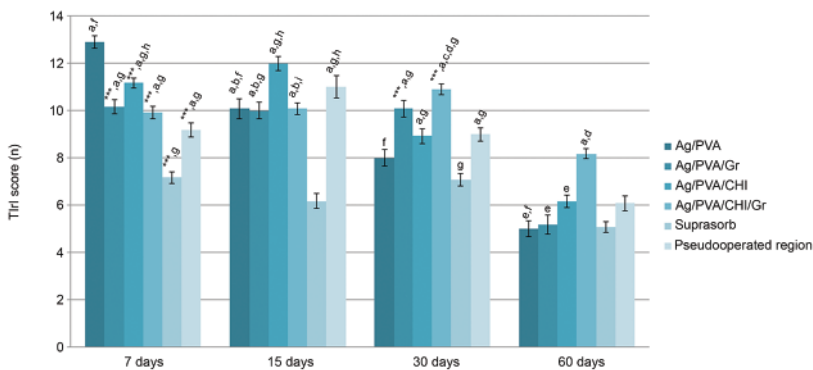

Figure 2. TIrI score for all examined implanted biomaterials and control area of skin incision. ***$p < 0.001$ relative to Ag/PVA; a – $p < 0.001$ relative to Suprasorb; b – $p < 0.01$ relative to Ag/PVA/CHI; c – $p < 0.001$ relative to Ag/PVA/CHI; d – $p < 0.001$ relative to Pseudooperated region; e – $p < 0.001$ relative to Ag/PVA/CHI/Gr; f – $p < 0.001$ relative to all other observed periods; g – $p < 0.001$ relative to 60 days; h – $p < 0.001$ relative to 30 days; i – $p < 0.01$ relative to 60 days.

Firstly, there was not strong tissue response to different implanted materials in any of the observed periods, and necrosis of surrounded peri-implant tissue was not observed. Secondly, the results show that the TIrI scores, after the type of hydrogels applied, in each observation period were between the minimal and mild value limits, except for the p.i.d. 7 in the case of implanted Ag/PVA, the p.i.d. 7 and 15 in the case of implanted Ag/PVA/CHI, and p.i.d. 30 in the case of implanted Ag/PVA/CHI/Gr, when registering moderate tissue reactions. Thirdly, at the end of the observed period, the tissue response to the implanted materials was minimal or mild. Finally,

at the end of the observation period, the smallest TIrI was recorded in the case of implanted Ag/PVA and Ag/PVA/Gr hydrogels, and these values did not differ from the control values. Ag/PVA/CHI/Gr was the only material which had significantly higher values of TIrI scores at the end of the observation period compared to the implanted materials, including Suprasorb© or the control pseudooperated region.

Leukocyte infiltration

The leukocyte number around the implanted materials was the highest at p.i.d. 7 compared to all other periods (p<0.001) and gradually decreased during the observation period, with a certain deviation in the case of implanted Ag/PVA/CHI/Gr. In addition, in all observed periods, there were significantly higher leukocyte numbers around each of the implanted materials compared to the implanted Suprasorb© (p<0.01), with the exception on p.i.d. 60, when no significant differences were recorded between the Suprasorb© and Ag/PVA hydrogels. In addition, it can be noted that implantations of the hydrogels in each observed period caused a lower inflammatory response in comparison to incision surgery (p<0.01), with the exception of Ag/PVA/CHI/Gr implant at p.i.d. 60, when the number of leukocytes was significantly higher (p<0.001) (Fig. 3).

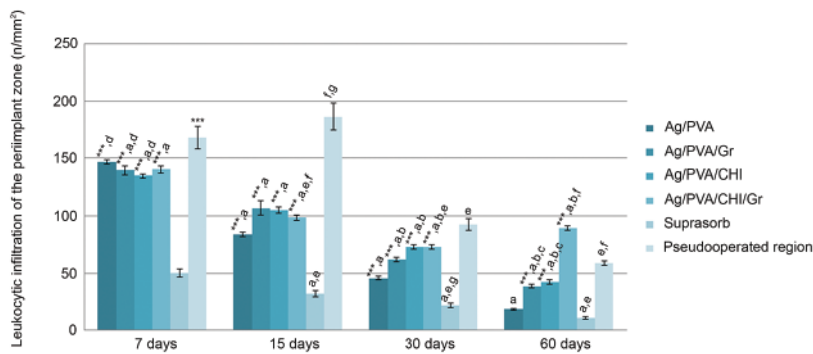**Figure 3.** Leukocytic infiltration of the peri-implant zone for all examined biomaterials and control area of skin incision. ***p<0.001 relative to Suprasorb; a – p<0.001 relative to Pseudooperated region; b – p<0.001 relative to Ag/PVA; c – p<0.001 relative to Ag/PVA/CHI/Gr; d – p<0.001 relative to all other observed periods; e – p<0.001 relative to 7 days; f – p<0.001 relative to 30 days; g – p<0.05 relative to 60 days.

Connective tissue capsule

During the first weeks after surgery, the site of implantation was embedded with a connective tissue capsule in a similar way for each of the implanted materials, and thus isolated from the surrounding tissues. A progressive increase of connective tissue capsule thickness around each implanted material was observed from day 7 to day 30, but after that the thickness of the capsule decreased over time and finally, at p.i.d. 60, it reached the mean dimensions between 33±2.39 and 70±2.68 mm for implanted Suprasorb© and Ag/PVA/Gr, respectively. The capsule thickness in all examined

periods after implantation was the highest in the case of implanted Ag/PVA/Gr. During the observation period, the thinnest capsules around Ag/PVA/CHI were at the 30th day after surgery, as well as surrounding Ag/PVA/CHI and Suprasorb© on the 60th day after surgery (Fig. 4).
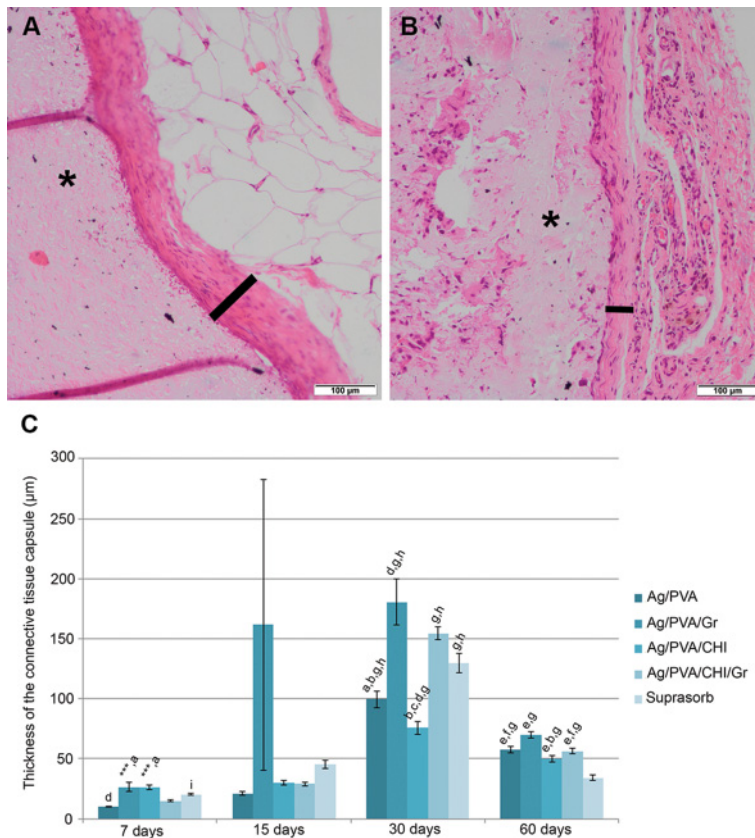

Figure 4. Tissue capsule thickness (dash) around implanted **A** – Ag/PVA/Gr and **B** – Ag/PVA/CHI/Gr hydrogels (star) at postimplantation day 60. The pictures show that Ag/PVA/CHI/ Gr implant was surrounded with a thinner capsule than Ag/PVA/Gr implant. H&E, bar: 100 µm; **C** – Connective tissue capsule thickness for all implanted biomaterials. ***$p<0.001$ relative to Ag/PVA; a – $p<0.01$ relative to Ag/PVA/CHI/Gr; b – $p<0.001$ relative to Ag/PVA/Gr; c – $p<0.001$ relative to Ag/PVA/CHI/Gr; d – $p<0.05$ relative to Suprasorb; e – $p<0.001$ relative to Suprasorb; f – $p<0.01$ relative to Ag/PVA/Gr; g – $p<0.001$ relative to 7 days; h – $p<0.001$ relative to 60 days; i – $p<0.01$ relative to 60 days.

Both macrophages and giant cells were seen in the connective tissue capsule surrounding each implanted material in any examined period.

Tissue reaction in the case of implantations of Ag/PVA versus Ag/PVA/Gr

Ag/PVA implantation induced the strongest tissue response at p.i.d. 7 (TIrI 13±0.26), and it was significantly higher in relation to all observed periods, as well as with

comparison to all other implanted hydrogels or pseudooperated regions (p<0.001). However, after this, the tissue response to Ag/PVA progressively decreased up to p.i.d. 60, where TIrI score (5.00±0.35) had a minimal level and similar value to Suprasorb© and the pseudooperated region.

In contrast, monitoring of the tissue response dynamic during the Ag/PVA/Gr implantation demonstrated that this biomaterial caused induction a far smaller tissue response at p.i.d. 7 compared to implanted Ag/PVA. Also, the degree of tissue response to this biomaterial was unchanged during the first 30 days, but significantly higher (p<0.001) than the value observed at p.i.d. 60. Specifically, at p.i.d. 60, TIrI in the case of Ag/PVA/Gr hydrogels was similar to both Ag/PVA and Suprasorb© implantations as well as the control pseudooperated region (Fig. 2).

However, there was a higher leukocyte number in the peri-implant area surrounding Ag/PVA/Gr than Ag/PVA at both p.i.d. 30 and 60 (p<0,001) (Fig. 3). Similarly, throughout the monitoring period, there was a significantly thicker capsule surrounding the implanted Ag/PVA/Gr compared to Ag/PVA (p<0.001) (Fig. 4).

Tissue reaction in the case of implantations of Ag/PVA/CHI versus Ag/PVA/CHI/Gr

Ag/PVA/CHI implantation induced the strongest tissue response at p.i.d. 15, while in the case of Ag/PVA/CHI/Gr implantation this response was somewhat delayed and strongest at p.i.d. 30. After these observed peaks, there were significantly reduced tissue reactions at the end of the observed period for both implanted materials, and the degree of reduction was significant compared to each previous observation period (p<0.001). However, at p.i.d. 30 and 60, TIrI scores in the cases of implanted Ag/PVA/CHI/Gr were significantly higher in comparison with the control values, as well as in relation to implanted Ag/PVA/CHI (p<0.001) (Fig. 2).

Leukocyte infiltration of the peri-implant zone was gradually decreased during the entire monitoring period in the case of implanted Ag/PVA/CHI (p<0.001). However, in the case of implanted Ag/PVA/CHI/Gr there was an increase in leukocyte infiltration at p.i.d. 60. Also, although this increase was significant in relation to the previous study period (p<0.001), the leukocyte number was significantly lower compared to the highest values observed at p.i.d. 7 (p<0.001) (Fig. 3).

The thickness of the capsule was significantly increased during the first 30 days after implantation (p<0.001; p.i.d. 30: p.i.d. day 7), and then was decreased at p.i.d. 60, in cases of both implanted Ag/PVA/CHI or Ag/PVA/CHI/Gr. Although the thickness of the capsule around the implanted Ag/PVA/CHI/Gr was significantly higher compared to the implanted Ag/PVA/CHI at p.i.d. 30 (p<0.001), this difference was lost at p.i.d. 60. However, the thickness of the capsules of both implanted materials remained significantly higher in relation to the capsule surrounding the implanted Suprasorb© (p<0.001), and significantly higher than the initial values at p.i.d. 7 (p<0.001) (Fig. 4).

Ag/PVA/Gr versus Ag/PVA/CHI/Gr - *in vivo* soft tissue response differences after implantation of these novel hydrogels

According to TIrI score, the tissue response was far more strong at p.i.d. 60 in the case of implanted Ag/PVA/CHI/Gr in comparison to implanted Ag/PVA/Gr (p<0.001) (Fig. 2). A similar situation exists with regard to the degree of leukocyte infiltration. (Fig. 3). However, the Ag/PVA/CHI/Gr implant was surrounded with a thinner capsule than Ag/PVA/Gr during all examined periods, and this difference is statistically significant at both the beginning and the end points of the follow-up period (p<0.01) (Fig. 4).

Degradation of implanted hydrogels

Macrophages penetrated all implanted hydrogels at the beginning of the monitoring period, and they were observed both in the periphery and the deeper zones of implanted materials during follow-up (Fig. 5). There are vacuoles with phagocytosed materials in the cytoplasm of these macrophages.
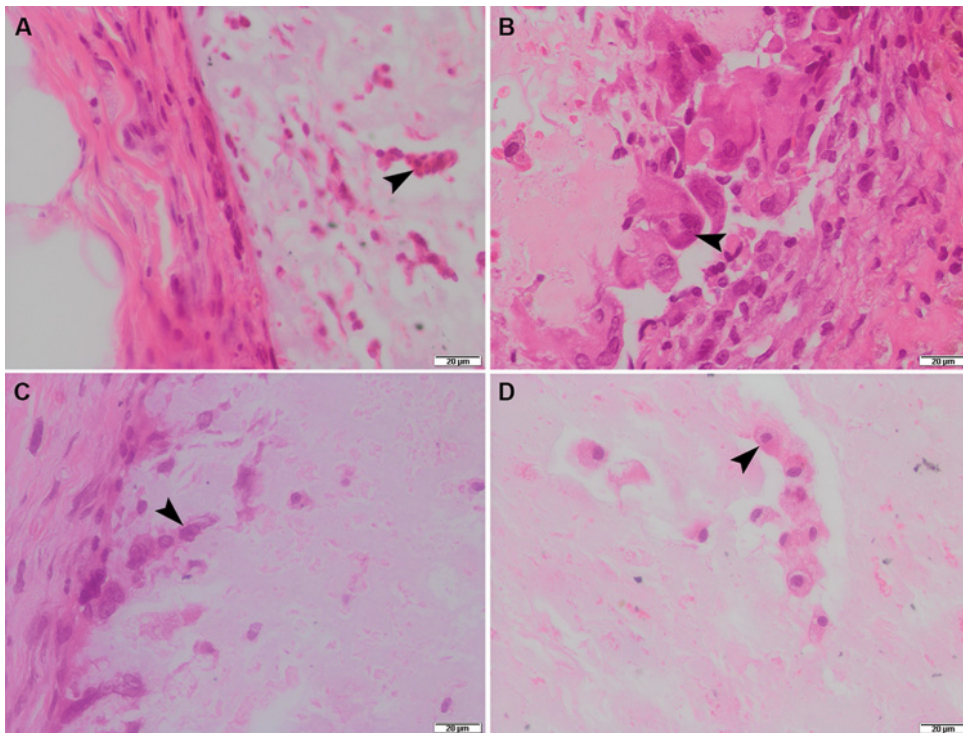

Figure 5. Implanted Ag/PVA/CHI/Gr resorption by giant cells or macrophages (arrows) activities during follow-up period. H&E, bar: 20 μm. **A** – 7 days, **B** – 15 days, **C** – 30 days, **D** – 60 days.

DISCUSSION

In this study, we investigated the biocompatibility of novel polymers based on PVA or PVA/CHI hydrogels with silver nanoparticles (AgNPs), in which graphene was incorporated to improve their properties. There are several studies of Ag/PVA/Gr [33,40−43], and Ag/PVA/CHI/Gr polymers [30,39,44], however, none provides information regarding their *in vivo* biocompatibility. For this reason, our *in vivo* investigations of novel Ag/PVA/Gr and Ag/PVA/CHI/Gr hydrogels with improved physical properties should be the basis for further preclinical and clinical research with the goal of their application in human and veterinary medicine.

Given the small amounts of the gelling agent and the high content of liquids (up to 99%), the hydrogels have a density close to water density, but possess the structural bonding of a solid substance. Polymeric hydrogels may be natural (including chitosan) or synthetic (including PVA) [45].

The simple structure and unique properties of PVA polymers such as adhesion, strength, film formation, hydrophilicity but very low swelling capacity (due to their sensitivity to hydrogen bonding and over-crystallization), biocompatibility, safety and non-cancerous properties, make them desirable for specific biomedical applications [46]. To date, many uses of PVA hydrogels have been proposed in biomedicine, including their use as soft materials for building contact lenses [47], artificial cornea [48], artificial cartilage and meniscus [49,50], tendon and bone regeneration [51,52], hydrogel coating of the humeral head surface in shoulder joint prosthesis [53], for suppressing the formation of adhesions in peritonitis [54], vehicle for medicaments, including prolonged release of antibiotics in veterinary medicine [55,56], cell growth substrate [57], for prostate brachytherapy preparations [58], cardiovascular grafts [59], and other. It is necessary to look with great optimism at the results of the first clinical study with long-term follow-up (5-8 years) of the commercial PVA (Cortiva, RTI Surgical Inc., FL, USA) implant success in focal cartilage defects. These results showed that synthetic PVA hydrogel implants guarantee functional recovery of a knee joint in mid-life patients with focal lesions of the cartilage [60]. The first preliminary results of a clinical study on repairing the human joint surface metacarpal bone by implantation of PVA in patients with osteoarthritis were published [61]. Also, excellent clinical results five years after PVA hydrogel hemiarthroplasty of the first metatarso-phalangeal joint in advanced hallux rigidus were obtained [62].

However, physical properties of PVA hydrogels are generally reversible which disrupts their stability [45]. The viscosity, the degree of crystallization and pH sensitivity of PVA cryogels changes over time, which leads to poor mechanical properties. Also, in comparison with other hydrogels, PVA hydrogels weakly adsorbed the proteins resulting in low cell adhesion, which, in the opinion of some authors, makes them less effective in the treatment of cartilage defects by the matrix-associated autologous chondrocyte transplantation [63].

Therefore, many PVA copolimers have been synthesized up to now. PVA (50%) and silk fibroin (50%) copolymer hydrogel was successfully used in tissue engineering of auricular cartilage, so further preclinical research with this new biomaterial is expected [64]. Thus, important for the context of this paper is that PVA copolymers with graphene [40] chitosan [65, 66], graphene and chitosan [44] are also described. Initial studies of the biocompatibility of porous PVA/chitosan hydrogel composite designed for tissue engineering scaffold showed that this material has no toxicity, but after muscle implantation, there was a stronger inflammatory reaction on the 4th week, compared with porous PVA/nano-hydroxyapatite or porous PVA [67]. However, one recent study has shown that PVA/chitosan composite hydrogel can be used as a carrier for mesenchymal stem cells during focal cartilage defect therapy [65]. In novel nanocomposites based on chitosan-polyvinyl alcohol (CHI-PVA) and graphene oxide (GO), GO reinforced CHI-PVA engineering [40,44].

PVA is one of the most frequently and the oldest synthetic polymer employed as wound dressings. However, PVA hydrogel has inadequate elasticity, a stiff membrane, a relatively poor barrier property to bacterial penetration, sometimes poor mechanical stability and incomplete hydrophilic characteristics which restrict its use alone as a wound dressing polymeric membrane. Among the various hydrogels described in literature, hydrogels prepared using PVA blended with some natural polysaccharides and some other synthetic ones, including chitosan, graphene and Ag nanoparticles (AgNPs), are attractive and the most widespread route of membranes synthesis, because of the abundance of such polymers, easy for chemical derivatization or modification, and usually good biocompatible propreties [68]. However, these nanomaterials may potentially be risky for human health. Therefore, it is necessary to thoroughly examine their biocompatibility under *in vivo* conditions.

The introduction of AgNPs into polymer/copolymer hydrogels increases their antimicrobial activity, and the incorporation of graphene increases their mechanical properties. These materials are not biodegradable, so their use is recommended for surface wound treatment or as soft tissue implants. It was found that out of all metals with antimicrobial properties, silver had the most effective antibacterial action and lowest toxicity to animal cells [68]. However, the potential toxic effects of AgNPs on the circulatory, respiratory, central nervous system, liver and skin should not be forgotten. There is an increased trend for reduced usage of antibiotics in both humans and animals, as well as antibiotics in animal production. Numerous studies on the properties and possibilities of the application of the hybrid PVA/AgNPs material [55,69], proved its advantages for topical therapeutic use in the experimental creamy formula with silver, suitable for recovery of the microbial homeostasis in animals.

Graphene-based composite hydrogels, in which graphene is produced from graphene oxide, exhibit antibacterial activity particularly against Gram-negative bacteria [38]. Additionally, the presence of AgNPs in composite hydrogels with graphene accelerates the rate of wound healing in animal models [70]. Recently, the novel PVA/graphene oxide/starch/Ag polymer nanocomposite film was successfully produced for

biomedical use [43]. Incorporation of GO nanoparticles into PVA/starch/Ag films significantly increased the antimicrobial activity of films according to Gram-positive and negative bacteria. Contrary, some work suggests possible direct toxic effects of oxidized graphene to human osteoblasts [71] and respiratory system, i.e. exacerbation of allergic asthma [72]. However, a combination of GO with PVA reduces both the proliferation delay and the internal cell complexity alterations induced by GO on human osteoblasts, increases both cell viability and IL-6 levels, reducing both apoptosis and intracellular ROS content when compared to simple blending of both materials. The use of this strategy to modulate the biointerface reduces the toxic effects of graphene while preserving the reinforcement characteristics for application in tissue engineering scaffolds, and has enormous interest for polymer/graphene biomaterials development [71].

There are no published studies of *in vivo* biocompatibility of novel silver/poly(vinyl alcohol)/graphene and silver/poly(vinyl alcohol)/chitosan/graphene hydrogels aimed for medical applications. In this paper, we have shown for the first time the soft tissue response of novel silver–doped/PVA/Gr and PVA/CHI/Gr nanocomposite hydrogels aimed for medical applications. These novel hydrogels were produced by *in situ* electrochemical syntheses of silver nanoparticles in adequate matrixes as we described in our previously published works [30,37−39]. The results of our present study show that each of the TIrI scores, the leukocyte number around the implanted materials and capsule thickness were gradually decreased during 60 days follow-up. At the end-point of follow-up, Ag/PVA/CHI/Gr implant was surrounded with a thinner capsule, while both the TIrI scores and number of leukocytes of the peri-implant zone were greater compared to Ag/PVA/Gr implant, similar to the results of other investigations of porous PVA/chitosan hydrogels [67] or PVA/graphene oxide/starch/Ag polymer nanocomposite films [43]. Despite the observed differences, we can conclude that our *in vivo* experiment suggested that both novel hydrogels were biocompatible and potentially suitable for medical use.

CONCLUSION

The results of histological analyses of *in vivo* soft tissue applications of Ag/PVA/Gr and Ag/PVA/CHI/Gr hydrogel implants in rats indicated a satisfactory tissue response to these novel polymers. Together with our previous studies of their synthesis, and mechanical, thermal and biological *in vitro* properties, our present study indicated that both Ag/PVA/Gr and Ag/PVA/CHI/Gr are promising biomaterials for use in human and veterinary medicine.

Acknowledgments

Authors' contributions

TLB and VT were participated in the study design, performed the experimental work, an analyzed the data, and drafted the manuscript. VMS, KN and KYR carried otu the novel hydrogels synthesis, as well as supervised and facilitated in the overall write-up. DM, BBP, IM and ND participated in the study design and performed the experimental work, IM helped in prepared figures. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ODGOVOR MEKOG TKIVA NA IMPLANTACIJU NOVOSINTETISANIH SREBRO/POLIVINIL ALKOHOL/ GRAFEN I SREBRO/POLIVINIL ALKOHOL/HITOZAN/ GRAFEN HIDROGELOVA NAMENJENIH BIOMEDICINSKOJ UPOTREBI- PRVO ISKUSTVO

LUŽAJIĆ BOŽINOVSKI Tijana, MARKOVIĆ Danica, TODOROVIĆ Vera, PROKIĆ Bolka Bogomir, MILOŠEVIĆ Ivan, DRNDAREVIĆ Neda, NEŠOVIĆ Katarina, KYONG Yop Rhee, MIŠKOVIĆ-STANKOVIĆ Vesna

U ovom radu prvi put su prikazani rezultati *in vivo* ispitivanja rekcije mekog tkiva na nove nanokompozitne hidrogelove implantirane u potkožno tkivo pacova, koji su predviđeni za medicinsku primenu. Novi nanokompozitni hidrogelovi – srebro/poli(vinil alkohol)/grafen (Ag/PVA/Gr) i srebro/poli(vinil alkohol)/hitozan/grafen (Ag/PVA/CHI/Gr) su proizvedeni *in situ* elektrohemijskom sintezom nanočestica srebra u polimernim matricama, u obliku diskova, kao što je opisano u našim prethodno objavljenim radovima. Ag/PVA/Gr i Ag/PVA/CHI/Gr, kao i kontrolni Ag/ PVA, Ag/PVA/CHI i komercijalni Suprasorb©, implantirani su u potkožno tkivo pacova. Posle 7, 15, 30 i 60. dana od implantacije, izdvajani su implantati s okolnim tkivom, a zatim obrađeni za histološki pregled. Ispitivani su indeks iritacije tkiva (TIrI) prema ISO 10993-6, 2007, kao i broj leukocita u zoni neposredno oko implantata i debljina vezivnotkivne kapsule. Rezultati pokazuju da su se TIrI, broj leukocita oko implantiranih materijala i debljina kapsule postepeno smanjivali tokom perioda promatranja. Na kraju eksperimentalnog perioda, implantat od Ag/PVA/CHI/Gr imao je veću vrednost TIrI i veći broj leukocita u peri-implantnoj zoni, ali je bio okružen tanjom kapsulom, u poređenju sa implantatom od Ag/PVA/Gr. Međutim, i pored ovih razlika, možemo zaključiti da rezultati našeg *in vivo* eksperimenta sugerišu da su novi hidrogelovi biokompatibilni i prikladni za medicinsku upotrebu.