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EVALUATION OF SOFT TISSUE REGENERATIVE PROCESSES AFTER SUBCUTANEOUS IMPLANTATION OF SILVER/ POLY(VINYL ALCOHOL) AND NOVEL SILVER/POLY(VINYL ALCOHOL)/GRAPHENE HYDROGELS IN AN ANIMAL MODEL

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A newly produced biomaterial is necessarily subject of standards, which are performed *in vivo* on animal models. For the evaluation of soft tissue regenerative possibilities after subcutaneous implantation of biomaterials – silver/poly(vinyl alcohol) (Ag/PVA) and novel silver/poly(vinyl alcohol)/graphene (Ag/PVA/Gr) provided for clinical use, sixteen rats were used, according to the instructions of international standards, ISO 10993-6, 2007. Histological sections were observed 7, 15, 30 and 60 days after grafting. These hydrogels were produced by *in situ* electrochemical synthesis of silver nanoparticles in the polymer matrices, which enabled obtaining completely safe and biocompatible materials, free from any additional toxic chemical reducing agents. Surgical implantation of hydrogels was done according to the permission of the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade. Immunohistochemical (IHC) studies included the assessment of smooth muscle expression actin in blood vessels (α-SMA), the expression of laminin and type I and type III collagen in the skin structures, and, the determination of cell proliferation marker expression (Ki-67) keratinocytes. The results were assessed in a semiquantitative manner. The data were analyzed in the statistical software package IBM SPSS 20. The conclusions indicated that Ag/PVA/Gr might be used as wound dressings to enhance the tissue healing potential and established faster integration and shorter retention in the tissue.

Key words: immunohistochemistry, nanocomposite biomaterials, rat, skin regeneration

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INTRODUCTION

Biomaterials such as hydrogels, mimicking the extracellular matrix (ECM) of tissues, may improve wound healing [1-3]. Poly(vinyl alcohol) (PVA), a synthetic polymer, whose *in vitro* biocompatibility has been shown in numerous studies, is often used in regenerative medicine [4-6]. The same is true for silver nanoparticles/PVA (AgNPs/PVA) composite materials [7]. Polymer-based wound dressings are the subject of many studies because there is a wide range of possibilities for their topical application, with the purpose of quickly restoring the skin defects and reducing the risk of infection and further injury [8-11]. In recent years, incorporation of graphene (Gr) in PVA hydrogels has been used in order to improve their mechanical strength and durability [12-15]. Furthermore, Gr has been reported to exhibit antibacterial activity, which could provide additional positive effects together with AgNPs [16]. Although Gr, as an inorganic component, is generally not considered to be very biodegradable. Recent studies have shown that Gr-based materials biodegradability is catalyzed by human eosinophil peroxidase [17]. Moreover, for various hydrogel applications, such as wound dressing and wound care, biodegradability might not be the key property, and it even might be considered a drawback, since a dressing needs to maintain its structure, elasticity and mechanical strength for long periods of time, in order to provide the proper support and protection to the wound during the healing process [18]. Gr and Gr oxide have often been used as fillers for wound dressings and drug delivery materials [19-21]. AgNPs with excellent antibacterial activity are often used as active component in biomaterials based on PVA hydrogels [22-26]. The silver/poly(vinyl alcohol) (Ag/PVA) and silver/poly(vinyl alcohol)/graphene (Ag/PVA/Gr) hydrogels have been fully characterized [26,27], and are intended for use in biomedical purposes, primarily as wound dressings [10]. The electrochemical method of *in situ* AgNPs synthesis directly inside the hydrogel matrix, stands out as a completely green process that does not make use of chemical reducing agents, such as NaBH4 that are toxic and difficult to remove from the dressing material. Therefore, these novel AgNPs-loaded hydrogels are completely non-toxic and biocompatible, as proven by our *in vitro* biological investigations that also indicate strong antibacterial activity [25]. Our presented data confirmed the biocompatibility of the novel Ag/PVA/Gr hydrogel after *in vivo* implantation [27].

Rodents are a very common animal model for the evaluation of the regeneration of skin defects and wounds [28-31]. There are evident differences in skin structure and the healing process between humans, other animals, and rats [29,32]. The regeneration of rat skin tissue is faster and more effective in comparison with many other species including humans. Skin healing time in rats is less than 5 days, mostly owing to skin contraction, while the same process takes about 12-14 days or longer in humans, depending on wound size [31,33].

In this study, we discuss some aspects of the repairing processes that occur during experimental trials in the rat soft tissue, following the interactions with the implanted

novel nanocomposite hydrogels. Several significant histological parameters of skin regenerative processes have been analyzed and evaluated in terms of speed and success of skin recovery.

MATERIALS AND METHODS

Materials

The following chemicals were utilized for nanocomposite hydrogels preparation: poly (vinyl alcohol) powder (fully hydrolyzed, *M*w = 70–100 kDa; Sigma Aldrich, USA), silver nitrate (M. P. Hemija, Serbia), potassium nitrate (Centrohem, Serbia), graphene nanopowder (Graphene Super-market, USA). Ultra-pure water was obtained from the Milli-Q system (Millipore, USA) and was used in all experiments.

Electrochemical synthesis, physico-chemical characterization and *in vitro* biocompatibility examination

In situ electrochemical synthesis process of silver-doped poly(vinyl alcohol)/graphene composite hydrogels, their physico-chemical and thermal properties have been described in our previous work [26]. *In vitro* biological investigation, i.e. examination of the cytotoxicity and antibacterial activity of these hydrogels has been established [22,23].

In vivo experiment

Animals and experimental protocol

Sterilized Ag/PVA, Ag/PVA/Gr nanocomposite hydrogel discs, as well as commercial Suprasorb®, a calcium alginate dressing (Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany) were implanted in the subcutis of 16 anesthetized adult female rats. Surgical hydrogel implantation was performed in accordance with the international standard ISO 10993-6 2007. The surgical procedure was approved by the Ethical Commitee of the Faculty of Veterinary Medicine, University of Belgrade (no. 01-488, 22.07.2016.). The surgical procedure was performed under general injection anesthesia, by intraperitoneal administration of 75 mg/kg ketamine hydrochloride (Ketamidor 10%, 100mg/ml, RICHTER PHARMA AG, Austria) and 10 mg/kg xylazine (Xylased 2%, BIOVETA, Czech Republic). Before the implantation, butorphanol, 1mg/kg (Nembutal, OAK) was administered subcutaneously for analgaesia. After that, the operative site was shaved and disinfected with a solution of povidone-iodide. During the preparation and operation procedure, the experimental animals were under constant monitoring by using a veterinary monitor (Votem v7 Patient-monitor, Korea). The animals were sacrificed by an intraperitoneal injection of pentobarbitone solution, 160mg/kg (Euthasol 400mg/ml, Produlab BV, The Netherlands). The experimental materials were retrieved from the subcutaneous tissue 7, 15, 30 and 60 days post-implantation (p.i.d.).

Tissue processing for immunohistochemistry

Tissue sections were used to determine the α-SMA (α-smooth muscle actin antigen) (ab124964) (Abcam, UK), laminin (ab11575) (Abcam, UK), type I (ab34710) (Abcam, UK) and type III collagen (ab6310) (Abcam, UK), Ki-67 (cell proliferation marker) (ab16667) (Abcam, UK) expression in the skin above the implantation zone, capsule and pericapsular area. Visualization of these markers was performed using a highly sensitive and specific two-stage indirect immunohistochemical (IHC) technique, where secondary antibodies labeled with horseradish peroxidase are directly bound to dextran polymers (Thermo Scientific UltraVision LP Detection System/HRP Chromero & DAB TL-060-HD, Lab Vision, USA).

Semiquantitative evaluation of α-SMA, laminin, type I and III collagen expression

Alpha-SMA IHC staining was used for blood vessels visualization, since the antibody for this intermediate filament stains smooth muscle cells in the tunica media of all blood vessels, including the microvascular bed and pericytes. A semiquantitative method was used to assess the degree of vascularization of the capsule and pericapsular zone. The method used is a points-based system, starting from 1 going up to 4 pluses, where blood vessels occupy up to 10%, 30%, 31-50% and over 50% of the observed surface, respectively. The expression of laminin was assessed semiquantitatively in the structural elements of the skin and subcutaneous tissue. Type I and III collagen expression was performed in the connective tissue of the dermis, hypodermis and cutaneous muscle. All samples were assessed in a semiquantitative manner (1+, mild; 2+, moderate; and 3+, strong).

Estimation of Ki-67 positive keratinocytes

In the epidermis, proliferating keratinocytes (Ki-67$^+$) of the basal layer were counted and expressed as the total number of Ki67$^+$ cells per 100 basal keratinocytes. Cells were counted in five representative areas of each sample, using an ocular mesh of 0.250 mm^2, magnification 40x.

All of these measurements were performed on a standard Olympus CX31 microscope and processed with CellSens Entry morphometric measurement software (Olympus, Tokyo, Japan). Histological samples were recorded with an Olympus UC50 digital camera.

Statistical analysis

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

RESULTS

Expression of α-SMA in blood vessels, the capsule and pericapsular zone vascularization degree

The vascularization degree of the connective tissue capsule and the pericapsular zone was high during all observed time periods (7th, 15th, 30th and 60th p.i.d.), after Ag/PVA and Ag/PVA/Gr implantation (Figure 1A–H). Blood vessels in these experimental groups occupied 30% to 50% of the capsule zone and 10% to 30% of the surrounding connective tissue located in the pericapsular zone, in all observed time periods (Table 1). In the case of Suprasorb®, on the 15th, 30th and 60th p.i.d. the capsule was less vascularized compared to the 7th p.i.d. where the blood vessels occupied from 10% to 30% of the capsule zone (Figure 1I–L). Regarding the degree of vascularization of the pericapsular connective tissue zone, it was similar to the values obtained from

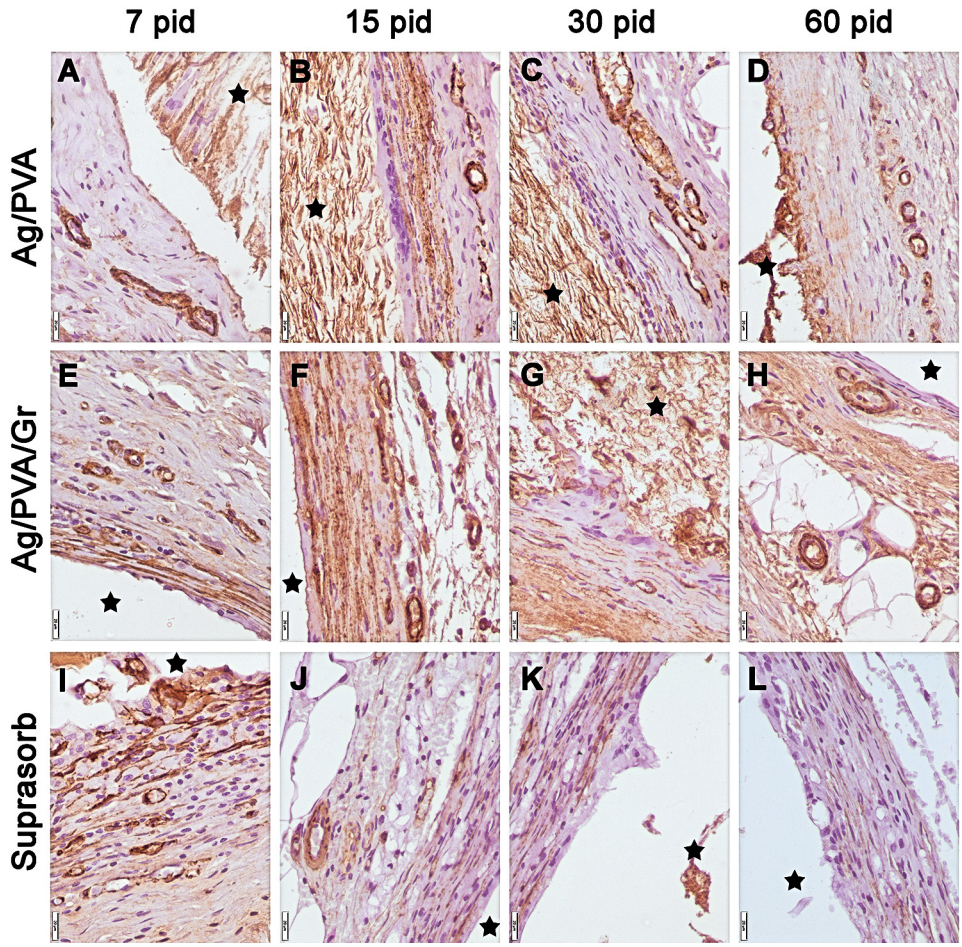

Figure 1. The capsule and pericapsular zone vascularization of Ag/PVA, Ag/PVA/Gr and Suprasorb® (star) through the observed follow-up periods. bar:20 μm

the other implants (Ag/PVA and Ag/PVA/Gr) (Table 1). IHC data showed increased smooth muscle actin expression in the blood vessels of the skin and periimplant zone, which indicates moderate to high degree of neovascularization.

Table 1. Vascularization of the capsule and the connective tissue around the capsule

Postimplantation day (p.i.d.)	Semiquantitative assessment of the vascularization degree		
	Ag/PVA	Ag/PVA/Gr	Suprasorb®
7 p.i.d.			
capsule	+++	+++	+++
pericapsular zone	++	++	++
15 p.i.d.			
capsule	+++	+++	++
pericapsular zone	++	++	++
30 p.i.d.			
capsule	+++	+++	++
pericapsular zone	++	++	++
60 p.i.d.			
capsule	+++	+++	++
pericapsular zone	++	++	++

blood vessels occupy up to 10% of observed surface (+); blood vessels occupy up to 30% of observed surface (++); blood vessels occupy 31% - 50% of observed surface (+++); blood vessels occupy over 50% of observed surface (++++)

Expression of laminin in the skin and periimplant zone after hydrogels implantation

Laminin in intact skin is expressed by epidermal cells and their derivatives - sebaceous gland epithelial cells and hair follicles, in the subcutis, in the connective tissue surrounding adipocytes and in the cutaneous muscle cells. The expression of laminin in the blood vessels of the subcutis and cutaneous muscle cells is similar to the expression in the blood vessels of the dermis. In addition, this expression in the skin after subcutaneous implantation of Ag/PVA and Ag/PVA/Gr, on the 7th and 15th p.i.d. was very similar to the observations in intact skin (Figure 2A, B; Table 2). The expression level decreased on the 30th and 60th p.i.d. for both hydrogels, and were higher than the samples of the intact skin control by about 40% (Table 2). However, the pattern of laminin expression during the follow-up p.i.d. somewhat differed in the case of the subcutaneously implanted Suprasorb®. The degree of laminin expression on the 15th, 30th and 60th p.i.d. was far higher than on the 7th p.i.d., and was higher than the expression in the samples of intact skin (Figure 2C; Table 2). Regarding Ag/PVA and Ag/PVA/Gr implants, the laminin expression in the capsule and pericapsular zone were moderate on the 7th and 15th p.i.d (Figure 2D, E) and weak on the 30th and 60th p.i.d. (Table 2). In contrast, laminin expression in the connective tissue capsule and periimplant zone surrounding Suprasorb® was significantly higher compared to the other observed hydrogels on the 15th, 30th and 60th p.i.d. (Figure 2F, Table 2). Laminin

was most strongly expressed in these histological zones on the15th and 30th p.i.d. after Suprasorb® implantation (Figure 2F, Table 2).

Table 2. Semiquantitative assessment of laminin expression in the structural elements of the skin, capsule and pericapsular zone

Hydrogel/ observed structure	**Laminin expression in structural elements of the skin, capsule and pericapsular zone**			
	Ag/PVA	**Ag/PVA/Gr**	**Suprasorb®**	**Intact skin**
	15 th post implantation day			
1. Epidermis	+++	+++	+++	+++
2. Dermis				
Sebaceus gland	++	++	++/+++	++
Hair follicle	++/+++	++	++/+++	++
M. arrector pili	++/+++	++	++	++
blood vessels	++	++	+/++	+
stroma	0	0	0	0
fibroblasts	+	+	1	+
3. Hypodermis	+	+	+	+
blood vessels	++	++	++	+
4. *M. cutaneus*	++	+	++	++
Total score (1+2+3+4), max. 30	18.0/30	15.0/30	19.0/30	15.0/30
	30 th post implantation day			
1. Epidermis	++/+++	+++	+++	+
2. Dermis				
Sebaceus gland	++	++	+++	+/++
Hair follicle	+/++	+	+++	+
M. arrector pili	+	++	+++	+
blood vessels	+	+	++	+
stroma	0	0	0	0
fibroblasts	+	+	+	+
3. Hypodermis	+	+	+	+
blood vessels	+	+	++	+
4. *M. cutaneus*	++	++	++/+++	+
Total score (1+2+3+4), max. 30	14.0/30	14.0/30	23.0/30	9.5/30

Hydrogel/ observed structure	**Ag/PVA**	**Ag/PVA/Gr**	**Suprasorb®**
	15 th post implantation day		
5. Capsule	++	++	+++
6. Pericapsular zone	++	++	+++
Total score (5+6), max 6	4/6	4/6	6/6
	30 th post implantation day		
5. Capsule	+	+	+++
6. Pericapsular zone	+	+	+++
Total score (5+6), max 6	2/6	2/6	6/6

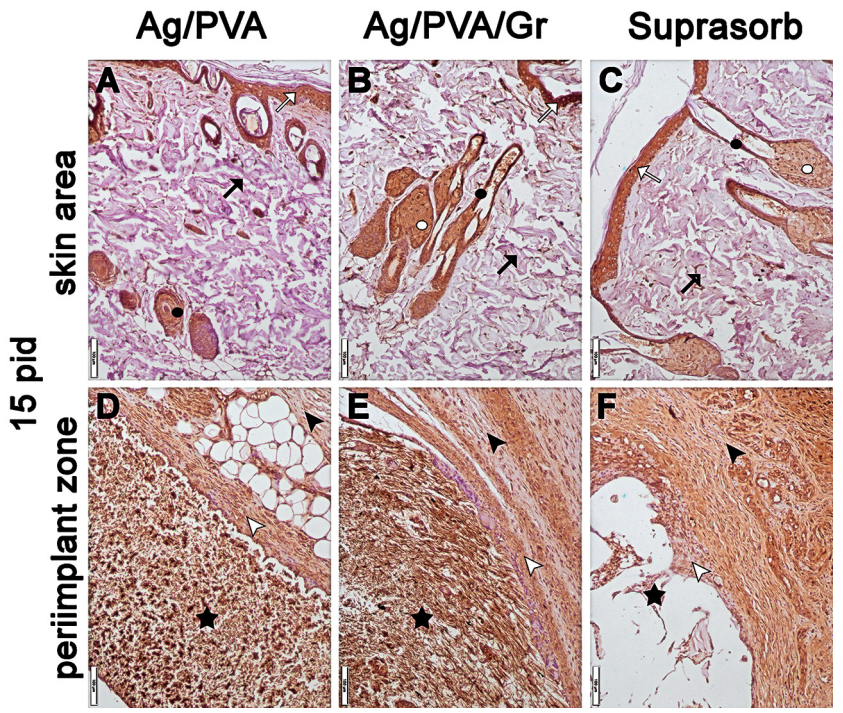

Figure 2. Laminin expression in structural elements of the skin (white arrow - epidermis, black arrow - dermis, black dot - hair follicle, white dot - sebaceus gland) and subcutaneous tissue **(A, B, C)**; Connective tissue capsule (white arrow head) and periimplant connective tissue (black arrow head) **(D, E, F)** after the application of different hydrogels (star) on the 15th p.i.d. bar:100 μm

Expression of type I and type III collagen in the skin and periimplant zone after application of hydrogels

Collagens type I and III were expressed in the connective tissue of the dermis, hypodermis and cutaneous muscle in the intact control skin and the skin after implantation of all three hydrogels. The highest expression was detected in the dermis and such an expression pattern remained unchanged throughout the follow-up period (Table 3). The expression of type I and III collagen was similar in both areas: the capsule and periimplant zone (Figure 3A–F). Regarding Ag/PVA and Ag/PVA/Gr hydrogels, the capsule and the pericapsular subcutaneous connective tissue in the pericapsular zone had mild to moderate expression of type I and III collagen on the 15th, 30th and 60th p.i.d., while the collagen expression after Suprasorb® implantation was weaker (Table 3). Slightly increased expression was observed around the capsule in the case of Ag/PVA/Gr on the 7th p.i.d. compared to collagen expression in the case of Ag/PVA and Suprasorb® (Table 3).

Table 3. Semiquantitative assessment of collagen type I and III expression in the structural elements of the skin, capsule and pericapsular zone

Hydrogel/ observed structure C.T. – connective tissue	**Coll type I and Coll type III expression in structural elements of the skin, capsule and pericapsular zone**			
	Ag/PVA	**Ag/PVA/Gr**	**Suprasorb®**	**Intact skin**
	7 th post implantation day			
C.T. of the dermis	++	++	++	++
C.T. of the hypodermis	+	+	+	+
C.T. of the M. cutaneus	+	+	+	+
Total score (max. 9)	4/9	4/9	4/9	4/9
	15 th post implantation day			
C.T. of the dermis	++	++	++	++
C.T. of the hypodermis	+	+	+	+
C.T. of the M. cutaneus	+	+	+	+
Total score (max. 9)	4/9	4/9	4/9	4/9
	30 th post implantation day			
C.T. of the dermis	++	++	++	++
C.T. of the hypodermis	+	+	+	+
C.T. of the M. cutaneus	+	+	+	+
Total score (max. 9)	4/9	4/9	4/9	4/9
	60 th post implantation day			
C.T. of the dermis	++	++	++	++
C.T. of the hypodermis	+	+	+	+
C.T. of the M. cutaneus	+	+	+	+
Total score (max. 9)	4/9	4/9	4/9	4/9
Hydrogel/ observed structure	**Ag/PVA**	**Ag/PVA/Gr**		**Suprasorb®**
	7 th post implantation day			
Capsule	+	+		+
Pericapsular zone	+	+		+
	15 th post implantation day			
Capsule	+/++	+/++		+
Pericapsular zone	++	++		+
	30 th post implantation day			
Capsule	+/++	+/++		+
Pericapsular zone	++	++		+
	60 th post implantation day			
Capsule	+/++	+/++		+
Pericapsular zone	++	++		+

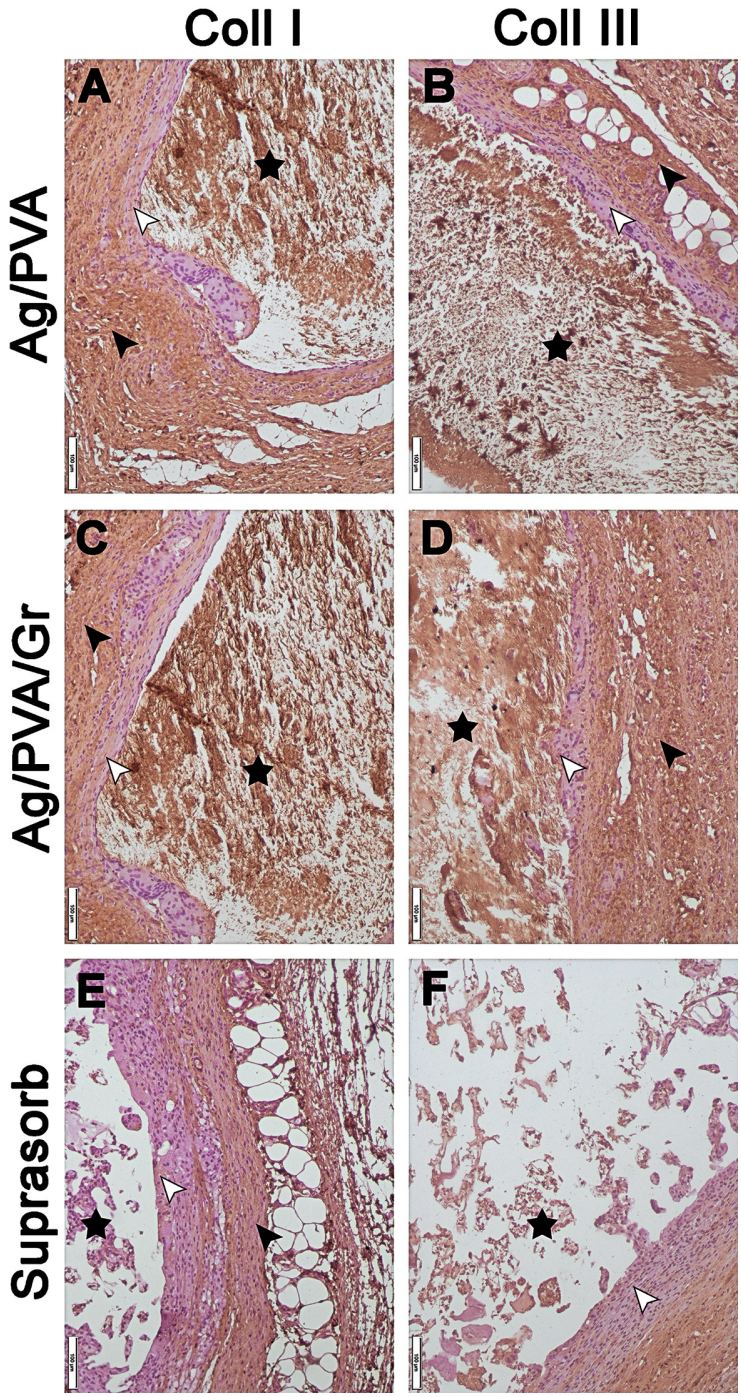

Figure 3. Coll type I and Coll type III expression in connective tissue capsule (white arrow head) and subcutaneous periimplant connective tissue (black arrow head) after the application of different hydrogels (star) on the 15th p.i.d. bar:100 μm

Proliferation of keratinocytes in the skin and periimplant zone after subcutaneous implantation of hydrogels

IHC determination of Ki67+ expression was used to calculate the proliferation index of epithelial cells in the skin (Figure 4D). The keratinocytes number in the epidermis above the implantation zone of the two examined hydrogels was highest on the 7th p.i.d. (Figure 4E). On the 15th p.i.d. these values were significantly higher in the case of Ag/PVA and Ag/PVA/Gr hydrogels in relation to Suprasorb® (Figure 4 E). Numerous populations of keratinocytes with a high mitotic index were observed in the basal layer of the epithelium (Figure 4 A, B, C, E). That number gradually decreased in the case of Ag/PVA and Ag/PVA/Gr implants, from the 30th to the 60th p.i.d. (Figure 4E). The commercial Suprasorb® stimulated proliferation on the 60th p.i.d. (Figure 4E). When comparing Ag/PVA and Ag/PVA/Gr hydrogels, higher values were observed in the case of Ag/PVA in relation to Ag/PVA/Gr, both on the 15th and 30th p.i.d. (Figure 4A, B, E).

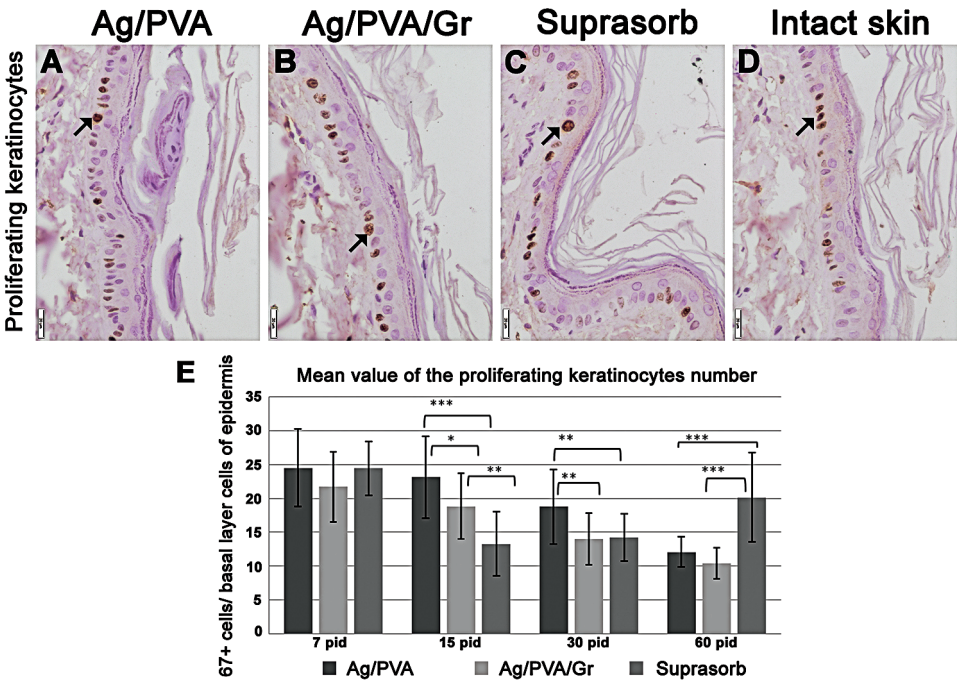

Figure 4. Proliferating keratinocytes (black arrow) of epidermis basal layer after subcutaneous implantation of Ag/PVA **(A)**, Ag/PVA/Gr **(B)**, Suprasorb® **(C)** and control intact skin sample **(D)** at the 15th p.i.d. bar:20 μm **E** - Mean values of the proliferating keratinocytes number in the basal layer epidermis after subcutaneous implantation of various hydrogels (Ag/PVA - 7p.i.d.:30p.i.d., p=0.004; 7p.i.d.:60p.i.d., p<0.001; 15p.i.d.:30p.i.d., p=0.048; 15p.i.d.:60p.i.d., p<0.001; 30p.i.d.:60p.i.d., p=0.001. Ag/PVA/Gr - 7p.i.d.:30p.i.d., p<0.001; 7p.i.d.:60p.i.d., p<0.001; 15p.i.d.:30p.i.d., p=0.003; 15p.i.d.:60p.i.d., p<0.001; 30p.i.d.:60p.i.d., p=0.049. Suprasorb® - 7p.i.d.:15p.i.d., p<0.001; 7p.i.d.:30p.i.d., p<0.001; 7p.i.d.:60p.i.d., p=0.039; 15p.i.d.:60p.i.d., p<0.001; 30p.i.d.:60p.i.d., p=0.002.

DISCUSSION

In this study, we analyzed the Ag/PVA and novel Ag/PVA/Gr hydrogels and the tissue response they evoke, by using IHC methods which enable visualization of the skin regenerative processes. Hydrogels can be applied to wounds, which can stimulate various processes important for skin regeneration [28]. In our study, for both tested hydrogels Ag/PVA and Ag/PVA/Gr, there was a solid vessel density in the wound bed, while in the case of the bioinert commercial Suprasorb® angiogenesis was not prominent, except on the 7th p.i.d. The expression of α-SMA in the tissue surrounding Ag/PVA and Ag/PVA/Gr hydrogels shows that blood vessels occupy 30% to 50% of the capsule zone and 10% to 30% of the pericapsular connective tissue, in all observed time periods, which indicates active angiogenesis necessary for healing. Damaged tissue reparation, stimulated by biomaterials has to follow the natural tissue healing processes, so the wound dressing has to fulfill many requirements [34-37]. Growth factors and cytokines are secreted at the site of injury in the recovering tissue, new blood vessels and extracellular matrix (ECM) are initially formed at the site of reparation in the following order: (1) migration and proliferation of fibroblasts at the site of the defect followed by collagen production; (2) ECM deposition and finally, (3) tissue remodeling [36,38,39].

Laminins and related ECM components, have essential roles in tissue homeostasis and during wound healing, they take part in the skin renewal process [39,40] Our research noted that after implantation of Ag/PVA and Ag/PVA/Gr, the distribution of laminin in the tissue was high in various skin cells, including epithelial cells, muscle and endothelial cells, as well as in the ECM of the dermis, and connective tissue capsules. These findings are consistent with literature data [41-43], although the results obtained in the implant zone cannot be compared with data of other authors, since there have not been *in vivo* studies with newly synthesized Ag/PVA/Gr so far. The increased expression of laminin in the case of Ag/PVA/Gr in all observed time periods, in relation to the intact skin control, is a consequence of its involvement in cell migration, adhesion and differentiation during active tissue remodeling induced by implanted material itself.

The ECM acts not only as a mechanical scaffold for the cells, but also as a bioactive and dynamic environment that mediates cellular functions [44,45]. Fibrillar collagen forms a large part of the connective tissue at the site of reparation [46]. Collagen synthesis begins 3 to 5 days after injury and lasts for several weeks, depending on the size of the wound [1]. The net accumulation of collagen does not depend only on increased synthesis, but also on reduced degradation [43]. Collagen is the most abundant component of the ECM, and is directly linked to cell differentiation processes [46]. According to the obtained results, at the end of the monitoring period (60th p.i.d.) a mild expression of type I and type III collagen, as well as a significant density of blood vessels were registered in the capsule following Ag/PVA and Ag/PVA/Gr implantation, indicating that the applied hydrogels led to a small degree of fibrosis and

significant neovascularization of the periimplant zone. Similar values were obtained for both hydrogels Ag/PVA and Ag/PVA/Gr. This data supports their biocompatibility, since the degree of biocompatibility is inversely proportional to collagen density in the connective tissue capsule, and directly proportional to angiogenesis [27]. Comparing intact skin control and the skin after implantation of all three hydrogels (Ag/PVA and Ag/PVA/Gr, Suprasorb®), the type I and III collagen were expressed in the connective tissue of the dermis, hypodermis and cutaneous muscle, with the highest expression being in the dermis. The capsule, pericapsular zone and subcutaneous connective tissue around Ag/PVA and Ag/PVA/Gr hydrogels had mild to moderate expression of type I and III collagen on the 15th, 30th and 60th p.i.d., while the collagen expression in the case of Suprasorb® was weaker.

Remodeling is the last phase in the wound healing process [45]. It is tightly regulated by degradation and synthesis, leading to normal skin regeneration [44]. Tissue regeneration begins by restoring the tissue damage, firstly by removing dead cells, then by proliferation of progenitor cells and lastly, functional restoration of the tissue [42]. Apoptosis is involved in the processes of wound healing and tissue regeneration, with the basic role of removing inflammatory cells and granulation tissue [47,48]. Skin stem cells also play an important role in wound healing and tissue regeneration [49]. Keratinocyte proliferation is high on the 7th p.i.d., although, highest keratinocytes proliferation values were observed in the case of the Ag/PVA implant on the 15th p.i.d. Both tested materials showed higher values in comparison with Suprasorb®. On the 30th and 60th day, these values gradually decreased, except for the significant leap in keratinocyte number in the case of Suprasorb® on the 60th p.i.d. probably owing to the high degradability of this material, which indirectly promotes signaling molecules that stimulate migration and proliferation of stem cells in the skin.

Various hydrogels described in literature are widely accepted in clinical procedures for wound dressings, including hydrogels based on a PVA copolymer with silver nanoparticles (AgNPs/PVA) [49-51]. The presence of AgNPs in composite hydrogels with Gr accelerates the rate of the wound healing process in animal models [52]. Various experiments have demonstrated that Gr-based biomaterials have excellent cytocompatibility, which could lead to an increase in early adhesion, spreading, proliferation, and remodeling of cytoskeletons of fibroblast cells [53]. Based on a relatively long follow–up periods (7, 15, 30, 60 days after implantation) of rats with subcutaneously implanted Ag/PVA and Ag/PVA/Gr, our results confirmed the biotolerance of the organism for these hydrogels [27]. The new Ag/PVA/Gr material synthesized by using nanotechnology techniques showed a wider connection area and a stronger implant-skin interface, potentiated by adhesive proteins that acted as a strong stimulus for signaling molecules and regenerative tissue response, thus speeding up repair processes and shortening the time necessary for tissue regeneration. Regarding the expertise of all *in vivo* biocompatibility assessments of new materials, it is crucial to choose the appropriate animal model. Another fact that must be taken into account, when interpreting results from these or similar studies, is the discrepancy in biological responses of organisms belonging to different species [54].

CONCLUSION

The evaluation of the tissue response and skin regeneration processes after Ag/PVA and Ag/PVA/Gr implantation indicates that these biotolerant and biocompatible hydrogels can absorb high volume of fluid and establish rapid solubility of nanomaterials in the tissue for a short period of time. Mild and weak reactions around the commercial Suprasorb® hydrogel were observed. We noted that both tested hydrogels contributed to the formation of a thin well-vascularized capsule with a moderate amount of collagen and the formation of pericapsular subcutaneous connective tissue. They consequently stimulated the invasion of immune and stem cells, which positively regulate regeneration processes in the skin. All these results demonstrate that Ag/PVA/Gr may be a prospective wound dressings for enhancing the wound healing process.

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Authors' contributions

TLB, VT, VMS and DM participated in the study design, analyzed the data, and drafted the manuscript. TLB carried out the experimental part of the study and made a substantial contribution to conception, design and analysis, acquisition and interpretation of data. IM and VG were responsible for animal care and welfare. TLB, NI and VT performed immunohiostochemical analyses. VMS and KN carried out the novel hydrogels synthesis, as well as, supervised and facilitated the overall write-up. BBP performed the surgical procedures, including the implantation and removal of the hydrogels. TLB, IM and VG performed the statistical analysis. DM, VT, VMS and JSF were involved in the final drafting of the manuscript and revised it critically for important intellectual content. All authors discussed the results and contributed to 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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PROCENA REGENERATIVNIH PROCESA MEKIH TKIVA NAKON SUPKUTANE IMPLANTACIJE SREBRO / POLI (VINIL ALKOHOL) I NOVOG SREBRO / POLI (VINIL ALKOHOL) / GRAFENSKOG HIDROGELA NA ANIMALNOM MODELU

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Bilo koji novoproizvedeni biomaterijal nužno podleže standardima, po čijim se protokolima sprovode *in vivo* istraživanja na životinjskim modelima. Za procenu mogućnosti regeneracije mekog tkiva nakon potkožne implantacije biomaterijala - srebra/poli(vinil alkohola) (Ag/PVA) i novog materijala srebra/poli(vinil alkohola)/grafena (Ag/PVA/Gr) predviđenog za kliničku upotrebu, šesnaest pacova je korišćeno, po uputstvima međunarodnog standarda, ISO 10993-6 2007. Histološki uzorci su sakupljeni 7, 15, 30 i 60 dana nakon implantacije. Ovi hidrogelovi su proizvedeni *in situ* elektrohemijskom sintezom nanočestica srebra u polimernim matricama, što je omogućilo dobijanje potpuno sigurnih i biokompatibilnih materijala, bez ikakvih dodatnih toksičnih hemijskih sredstava za redukciju. Hirurška implantacija hidrogela urađena je prema dozvoli Etičkog komiteta Fakulteta veterinarske medicine Univerziteta u Beogradu. Imunohistohemijske (IHC) studije uključivale su procenu ekspresije aktina glatkih mišića u krvnim sudovima (α-SMA), ekspresiju laminina i kolagena tipa I i III u strukturama kože i određivanje ekspresije markera proliferacije (Ki-67) keratinocita. Rezultati su procenjeni semikvantitativnom analizom. Podaci su analizirani u statističkom softverskom paketu IBM SPSS 20. Zaključci su ukazali da se Ag/PVA/Gr može koristiti kao obloga za rane kako bi se povećao potencijal zarastanja tkiva i uspostavila brža integracija i kraće zadržavanje u tkivu.