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Angiogenesis Inhibiting Crosslinked Albumin Hydrogel as a New Approach for Adhesion Prevention

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Authors' contributions

This work was carried out in collaboration between both authors. Author OE designed the study, approved the protocol, managed the literature searches and wrote the manuscript. Author FC executed the surgery and managed the analyses of the study. Both authors read, corrected and approved the final manuscript.

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ABSTRACT

Aims: Two crosslinked albumin hydrogels with angiogenetic inhibition activities were tested *in situ* for their potential to prevent adhesions in rabbits.
Methodology: The albumin hydrogels – one viscous and another non-viscous - were applied with a 4:1 double chamber syringe onto a traumatized rabbit abdominal wall. This allowed testing anti-adhesion properties and biocompatibility with respect to their wound healing effects. Seprafilm® was used as a negative control; the positive control remained untreated.
Results: The study indicated good biocompatibility properties for both hydrogels and Seprafilm®, such as the absence of cytotoxic effects, missing signs of systemic toxicity as well as undisturbed wound healing. However, while Seprafilm® was near to complete resorption, some remnants of the hydrogels could still be seen after 21 days. Additionally, slight signs of inflammation, especially in the spleen were observed after the intra-abdominal implantation of 1.3 ml hydrogel per kg body

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weight possibly due to beginning phagocytosis. Nevertheless, both hydrogels as well as Seprafilm® displayed good anti-adhesive effects in the abdominal wall model. The trend for the hydrogels to have lower and less tense adhesions compared to the untreated control and even Seprafilm® is in part explained by the angiogenetic inhibition effect of the hydrogels.

Conclusion: The crosslinked albumin hydrogels and the Seprafilm® showed good anti-adhesive efficacy. These promising results of crosslinked albumin hydrogels may even lead to further application forms such as non-viscous sprays which would increase fast, easy and secure handling of this effective device.

Keywords: Adhesion prevention; angiogenesis inhibition; crosslinked albumin hydrogel; rabbit abdominal wall model.

1. INTRODUCTION

Post-operative peritoneal adhesions present a challenging problem for surgeons and patients. Following abdominal and gynecological surgery, these abnormal fibrous strings develop in response to the trauma of the peritoneal tissue [1-4]. Those strings often get neovascularized causing intestinal obstruction, reduced fertility and pelvic pain [5-7]. Moreover, patients who undergo abdominal operations remain at risk for future development of adhesions [8-14]. The aim of adhesion prevention strategies is to reduce their incidence, severity and extent, while allowing normal healing to occur without increased risk of infection [15-17]. Multiple agents have been developed to decrease the formation of adhesions with varying degrees of success. One approach is to disrupt the inflammatory cascade and the fibrin-forming process, the other is the so far most successful approach separating damaged peritoneal surfaces with artificial barrier materials – which may take the form of a membrane, a gel or a slowly absorbed liquid [18-25]. However, there has been considerable disagreement as to which method or agent is most effective [26] and their usability and preference might be depending on the surgeon as well. Although, the severity of adhesions depend on the vascularization of the fibrous bands which get stronger and more pronounced during the time and remain as an abnormal organ disturbance or undesired tissue connection [27]. Therefore, inhibition of this vascularization might be a good target to minimize adhesions as well.

In this study, a standardized rabbit abdominal wall model [28,29] was used to test the adhesion prevention efficacy of two components polymerizing to a hydrogel *in situ*. This hydrogel separates not only physically the injured peritoneal tissue but inhibits the angiogenesis as well [30,31]. While inhibiting the formation of vascularization it is expected that the strength and occurrence of adhesion strings are rarer, less dominant and be easier to be degraded physiologically as determined macroscopically and microscopically.

2. MATERIALS AND METHODS

2.1 Gel Development

The albumin hydrogels were synthesized according to Scholz et al. [30]: Component A: Human albumin (Sigma-Aldrich) was modified with maleimide (Sigma-Aldrich) and purified by size-exclusion chromatography from intermediates. A 3.7mM activated human maleimide-albumin with or without 0.5% hyaluronic acid (HA from Visiol, TRB Chemedica AG, Munich, Germany) in PBS was used in a 4:1 double chamber syringe (Medmix Systems AG, Rotkreuz, Switzerland). HA was used to increase viscosity as a measure to keep the gel securily in place. The crosslinking component B was a 15 mM thio-polyethylene glycol (Rapp Polymere GmbH, Tübingen, Germany) in PBS. Gelation was executed within approx. 2 minutes via Michael reaction of the maleimide-group to a SH-group of the albumin passing A and B through a mixing coil (Fig. 1). Biocompatibility of the hydrogel was proven according to Benz et al. [31]. Stability of the frozen compounds A and B at -80℃ were given for 2 years; unfrozen reagents were used within 24 hours.

2.2 Gel Preparation Prior Application

Albumin Hydrogel – viscous (AH-V): Preparation of the AH-V (component A) and the appendant cross-linking agent (component B) was performed in a ratio 4:1. Following 20-minute-defined defrosting process of component A and B from minus 80℃ to plus 38℃, the components were transferred into a double chamber syringe (DCS) having a chamber of 4 ml (viscous

component A) and of 1 ml (component B). While transferring both components, it had to be ensured that both solutions were free of air bubbles within the DCS. Due to the HA within the viscous component A, bubble-free loading of the DCS was aggravated.

Albumin Hydrogel – non-viscous (AH-N): The preparation, ratio and defrosting of the AH-N was identical to the viscous hydrogel. However, loading the DCS with 4 ml of the non-viscous component A (without HA) was easier due to the bubble free solution which was based on the missing HA.

For abdominal administration, the DCS was attached to a spiral coil having a broad slot applicator of 5 mm width (Fig. 1). Furthermore, homogenous mixture of the components was ensured by initial submission of gel prior to utilization within the abdomen. Both, DCS and spiral coil with broad slot applicator were provided as gamma sterilized materials.

2.3 Surgical Procedure and Macroscopical Evaluation

For the experiment, 36 female SPF New Zealand White rabbits were used having a body weight ranging from 3.4 to 4.0 kg. The study was approved by the German regional authority of Brandenburg (2347-A-4-10-2014) in compliance with the principle for animal care. Following an acclimatization period of at least 5 days, animals were appointed to 4 different groups comprising 9 animals each at random in order to assess the efficiency of adhesion prophylaxis. Group 1 and 2 were treated with the AH-V and AH-N respectively whereas Seprafilm® Mini Site (Genzyme Biosurgery, Framingham, MA, USA) was used as reference material in group 3. Animals of group 4 remained untreated and served as positive native control (Table 1).

Macroscopic examination was conducted after 21 postoperative days on all animals. Throughout the experiment the animals were caged individually having free access to a pelleted complete diet as well as to drinking water at all times. Enrichment was provided using hay bricks and plastic rings. The study took place in animal rooms provided with filtered air at a temperature of 20 C ± 3, with relative humidity being at least 30% and not exceeding 70% as well as with air changes of 10 times/hour. The room was illuminated to give a cycle of 12 hours light and 12 hours darkness. For surgery, animals were put under general anaesthesia using ketamine (40 mg/kg) and xylazine (6 mg/kg) intramuscularly. Perioperative, Butorphanol (0.5 mg/kg s. c.) was applied for analgesia. Following shave, cleaning and disinfection of the abdomen, midline laparotomy and eventration of the cecum was performed. Peritoneum viscerale of the cecum was bluntly traumatized using sterile gauze until punctual to areal serosal bleeding occurred. The abdominal wall defect of a size of approximately 3 cm x 4 cm was created by extirpation of the Fascia transversalis and scarification of the rectus sheath as well as the Musculus rectus abdominis. The traumatization is presented in Fig. 2.

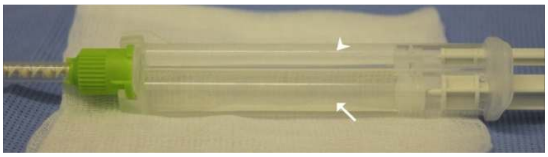

Fig. 1. Preparation of the Albumin Hydrogel prior to application

Double chamber 4:1 syringe being filled with Albumin Hydrogel (→, with or without HA) and the cross-linking agent (➤)

Subsequently, the abdominal wall defect was treated with 5 ml of the albumin hydrogel (viscous or non-viscous) in a meandering manner, with 4 cm x 5 cm Seprafilm® Mini Site. The positive control remained untreated. An overlapping application of the albumin hydrogel and Seprafilm® Mini Site was ensured. Following application, the cecum was put back into the abdomen and the laparotomy site was closed in tree layers with continuous sutures (Safil®, B.Braun, sizes USP 3/0 and 4/0). In addition, intracutaneous sutures were secured by skin bonding (Histoacryl®, B.Braun). In order to support recovery, animals were intravenously supported with physiologic saline and Amynin (Merial). Moreover, Butorphanol was applied for analgesia for a period of approximately 4 days. After 21 postoperative days, animals were euthanized after general anaesthesia using Embutramid (T61, Intervet) intravenously. For further histological examination, samples of the abdominal wall defect including remnants of applied devices or adhering organs were taken from the first three animals of each group. Samples were put up, fixed in 4% buffered formalin for further embedding, sectioning and staining in Hematoxyline/Eosin. The application sites of the other animals of the different groups were evaluated according to the following criteria: adhesions versus free of adhesions, areal extension of adhesions (%) and scoring adhesion according to Zühlke et al. [32].

Table 1. Test group arrangement

Name of group	Applied device	Animal numbers	Time of implantation post operationem
Group 1	Albumin Hydrogel – viscous (with hyaluronic acid) AH-V	9	21 days
Group 2	Albumin Hydrogel – non-viscous (without hyaluronic acid) AH-N	9	21 days
Group 3	Seprafilm® Mini Site (negative control)	9	21 days
Group 4	untreated (positive control)	9	21 days

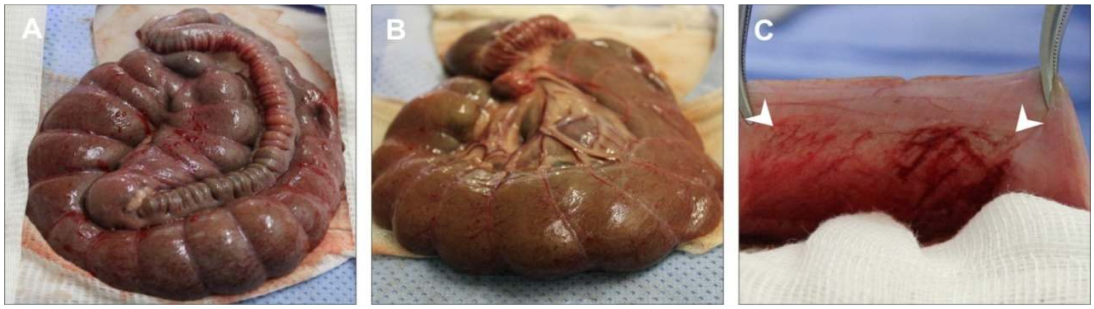

Fig. 2. Application site. (A and B) Blunt traumatization of the cecum, (C) traumatization site of the abdominal wall 3 cm x 4 cm (➤)

Abdominal cavity and organs were entirely examined in all 36 animals.

3. RESULTS

3.1 Surgical and Macroscopical Outcome

After defrosting, the AH-V was characterized as mildly opaque, clear and viscous solution having a vast number of air bubbles. The AH-N displayed a transparent to slightly opaque, clear and liquid appearance without bubbles. Furthermore, the defrosted cross-linking agent was described as rather liquid, transparent and clear. Meandering and areal application of both albumin hydrogels onto the abdominal wall defect was well performable with a broad slot applicator. The AH-V displayed a topping-like and shiny appearance on the abdominal wall whereas a part of the gel accumulated under the defect site in the depth of the abdomen (Fig. 3).

However, the AH-N drained off the abdominal wall defect immediately after application accumulating in the depth of the abdomen. But even after having been drained off instantly, the application site displayed a shiny surface (Fig. 4).

On the opposite, Seprafilm® MINI Site was applied dry directly onto the abdominal wall defect. Seprafilm® MINI Site was characterized by prompt adhering onto the underlying tissue being visible as a shiny layer henceforth. The untreated control group served as a measure for the adhesion area in comparison to the other groups (Fig. 5).

Postoperative infusion and analgesic therapy was conducted during the first four days after surgery. According to the clinical assessment, ongoing treatment was not necessary. For evaluation of adhesion prophylaxis efficiency, the areal extent of adhesions was documented for each abdominal wall defect. Furthermore, intensity of adhesions were scored according to Zühlke et al. [32]. Application of both albumin hydrogels prevented adhesion formation between the cecum and the abdominal wall in all cases. The missing HA in AH-N had no effect on the efficiency. In two cases being treated with Seprafilm® Mini Site, adhesion formation of Zühlke grade IV was noted. However, these adhesions were ongoing to the muscle suture leading to the assumption that adhesion prophylaxis was not successfully because this area had not been treated with Seprafilm®. With regard to the viscous/liquid character of the

albumin hydrogel, the agent was able to distribute onto a larger region of the abdominal wall due to movement of the bowel and the animal itself. However, 74% of the abdominal wall defect was affected from adhesions in the untreated positive control proving the suitability of the applied method (Table 2).

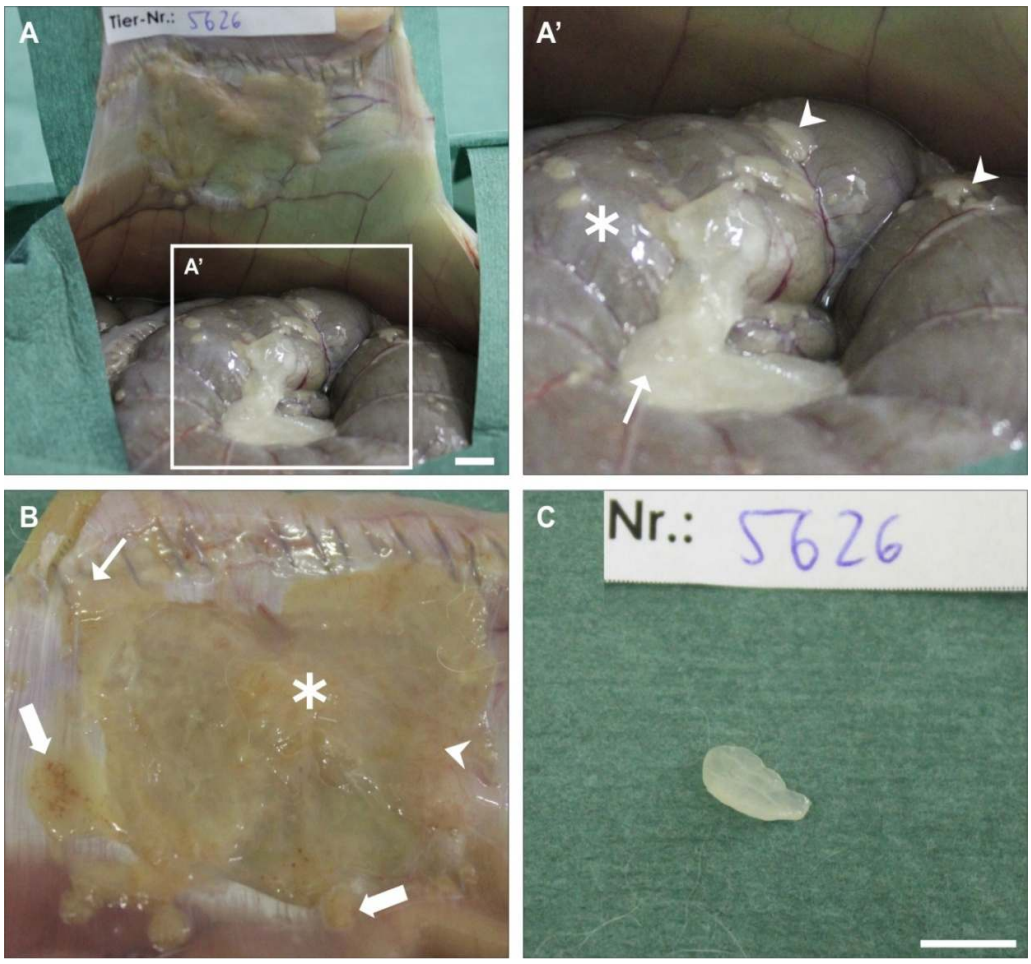

Fig. 3. Albumin Hydrogel – viscous (AH-V), macroscopic examination after 21 postoperative days

(A) *Overview application site,* ***(A`)*** *in detail: surface of cecum with grey-white depositions* ***(✱)****, mounted parts of the AH-V* ***(➤)*** *as well as free movable parts of the AH-V being associated with the cecum* ***(➞)****,* ***(B)*** *abdominal wall defect with AH-V being centrally elevated* ***(✱)****, AH-V on muscle suture* ***(➞)*** *and cranial as well as dorsal of the abdominal wall defect* ***(➡)****, vessel growth* ***(➤)****,* ***(C)*** *free floating part of AH-V being removed from the abdominal cavity. Scale bar 1 cm*

Table 2. Areal extension of adhesions on the transverse abdominal wall resection site (3 cm x 4 cm) connected with the cecum of the group treated with albumin hydrogel – viscous (AH-V), albumin hydrogel – non-viscous (AH-N), Seprafilm® Mini Site and untreated positive control

Group	Mean area of abdominal wall defect [%]		Adhesion strings to the muscle suture line
	Adhesion free	With adhesions	acc. Zühlke [32]
AH-V	100	0	1 x III
AH-N	100	0	-
Seprafilm®	97	3	2 x IV
untreated control	26	74	1 x II / 3 x IV

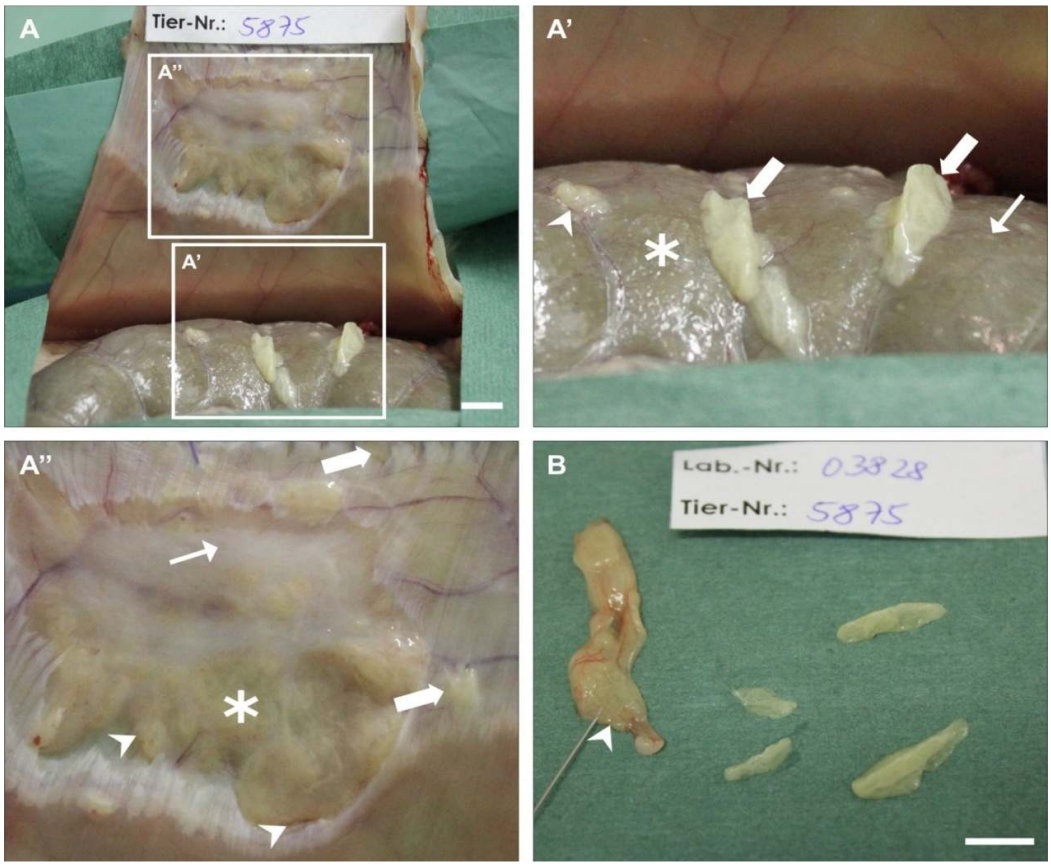

Fig. 4. Albumin Hydrogel – non-viscous (AH-N), macroscopic examination after 21 postoperative days

(A) Overview application site, (A`) in detail: surface of cecum with grey-white depositions (✱), focal accumulations of AH-N (➞), associated (➤) and free moving within the abdomen (➡), (A``) in detail: abdominal wall defect with AH-N-topping dorsally having an irregular surface (✱), ventrally not elevated (➞), solitary parts of AH-N (➡), vessel growth (➤), (B) free parts of AH-N taken from the abdomen as well as associated within the Omentum majus (➤) Scale bar 1 cm

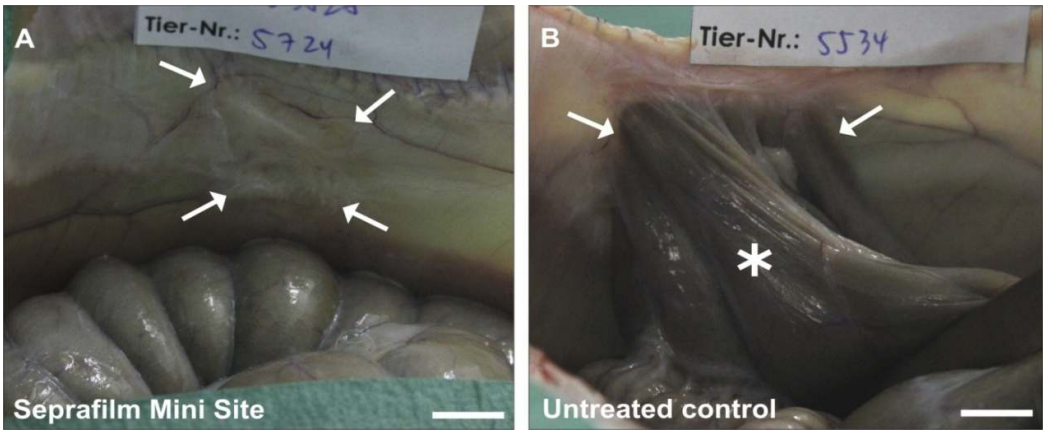

Fig. 5. Seprafilm® Mini Site and untreated control, macroscopic examination after 21 postoperative days

(A) Abdominal wall defect (➞) having been treated with Seprafilm® Mini Site, (B) untreated abdominal wall defect (➞) with adhesion to cecum (✱). Scale bar 1 cm

Table 3. Observations to the omentum majus, spleen and abdominal liquid formation
(wsf without specific findings)

Group	Omentum majus reactivity			Splenomegaly			Abdominal liquid			
	wsf	very low	low	wsf	very low	low	wsf	very low	low	moderate
AH-V	8	0	1	4	2	3	7	1	1	0
AH-N	1	4	4	4	3	2	6	1	2	0
Seprafilm®	6	3	0	8	1	0	4	1	3	1
untreated control	8	1	0	9	0	0	5	2	2	0

A more detailed macroscopical analysis of additional effects on the omentum and spleen are summarized in above Table 3 showing some slight reactions on the omentum majus as well as a weak splenomegaly for the test samples.

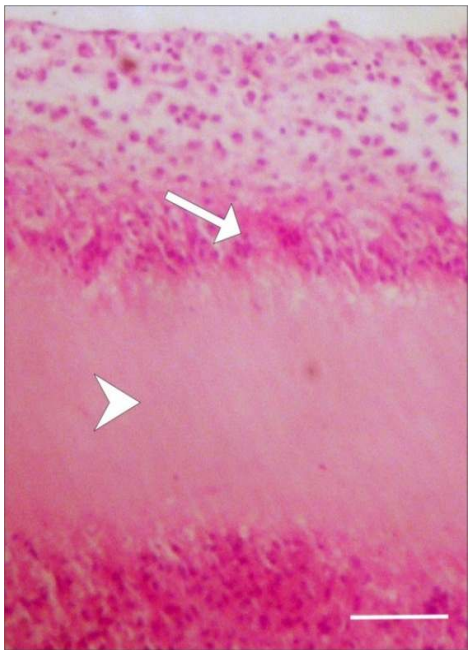

Fig. 6. Area of traumatized abdominal wall with mild to moderate active fibroplasia being cell-rich (→) around the slight eosinophilic albumin hydrogel (➤) with moderate infiltration of inflammation cells of various cell types (Scale bar 100 µm)

3.2 Microscopical Outcome

The histological evaluation after 21 days (Fig. 6) showed a good local tolerability for the albumin hydrogels and revealed only a mild to moderate active fibroplasia with the infiltration of phagocytotic macrophages and heterophilic granulocytes for both hydrogels supporting the biocompatibility results [30,31].

4. DISCUSSION

Injuries to peritoneal mesothelial cells after surgery result in secretion of fibrin-rich exudates [2,3] and need a delicate balance between fibrinolysis and cellular growth to regulate the different wound healing stages (i. e. inflammation, cellular migration, proliferation, angiogenesis and tissue remodeling). If this balance is disturbed, adhesions occur and are frequently responsible for post-operative complications in abdominopelvic operations, being responsible for up to 6 % of all surgical readmissions [33]. Ellis et al. [12] identified adhesions in 93 % of patients who had already undergone surgery despite the fact even 10.4 % of the patients displayed adhesions without having had previous surgery [11]. The extents as well as the effects of intra-abdominal peritoneal adhesions show the necessity of the assignment of routinely performed adhesion prophylaxis. The commonly accepted membranes, hydrogels and liquids [25] are clearly based on the physical separation of the traumatized organ sections. Our hydrogel approach is in alignment with these physical barriers but inhibits the angiogenesis as well matching current research trying to prevent adhesions with pharmaceuticals, e.g. with pirfenidone, protein C or colchicine [27,34-38].

In the present study, the well established abdominal wall model using SPF Russian female albino rabbits [28,29] had to be slightly modified because the New Zealand White female rabbits were not as sensitive as the former one. The traumatization of the chosen race had to be adapted in terms of a prolonged time as well as of areal parietal treatment and the adhesions were clearly depending on the amount of bleeding of the cecum sides. A check of the bleeding pattern of the cecum sides of the positive control showed in 2 out of 9 rabbits only punctual and slight bleedings without any adhesions. This explains very well the rather low adhesion amount of 74% and shows that

adhesion formation might strongly dependent on race, bleeding effects or other patient-specific parameters. This might explain the sometimes divers results when comparing adhesion prophylaxis agents in different animal and clinical settings [25,26]. Nevertheless, the modified rabbit abdominal wall model was implemented with a 3 cm x 4 cm resection of the transverse abdominal wall muscle which was overlaid with 5 ml of hydrogel. In the untreated positive control group, adhesions covered 74% of the resected area while in AH-V, AH-N and Seprafilm® groups practically no adhesions were determined. The positive control group displayed very strong and tight strings with beginning vascularization; in contrast to rare vascularization of both albumin hydrogels where weak vascularization could only be detected at the border of the gel supporting the angiogenesis inhibiting effect of the albumin hydrogels [30,31]. Additionally, it should be mentioned that the anti-adhesion effect is not induced and enforced through the addition of HA into AH-V because of the excellent anti-adhesive effect of AH-N itself. While Seprafilm® was nearly completely absorbed, the hydrogels were still present causing a slight splenomegaly due to the remaining mass of the hydrogel and the beginning phagocytosis of the foreign material. This slight to moderate inflammation may be strongly reduced if less crosslinked albumin will be applied. Due to the liquidity of AH-N only a minor and shiny film left in place at the peritoneal defect still showing good anti-adhesive properties. These findings lead us to a pilot test with the liquid AH-N using a spray applicator creating a thin homogenous and less voluminous protection film. In a follow-up study the efficacy and physiological effect of the AH-N-film will be proven with such a spray applicator.

5. CONCLUSION

Both crosslinked albumin hydrogels and the Seprafilm® showed good anti-adhesive efficacy with a good biocompatibility. The usability and functionality of the crosslinked albumin hydrogel may be improved by applying the non-viscous crosslinked albumin hydrogel with a spray applicator.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was approved by the German regional authority of Brandenburg (2347-A-4-10-2014) in compliance with the principle for animal care.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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