Article

Matrix metalloproteinase expression during the healing process of liver injuries treated with different biological sealants and adhesives

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Abstract: ***Background****. Adhesives and sealants can be used to repair and preserve solid damaged organs. This study explores the activity of different matrix metalloproteinases (MMP) during the healing of liver injuries treated with two biological adhesives (Tachosil and GelitaSpon) and a new synthetic elastic cyanoacrylate (Adhflex®).* ***Methods.*** *Liver traumatic injuries were experimentally induced in 90 male Wistar rats using a Stiefel biopsy punch in the liver. Wound healing was evaluated 2, 6, and 18 days after injury by determining MMP1, 2, 8, 9, and 13 expression. The histopathological repair was assessed by hematoxylin-eosin, Masson's trichrome, and Periodic Acid Schiff (PAS) staining. The three sealants used supported complete healing of the liver lesions. Both histopathology and MMP findings indicate that the degradation process of Adhflex® is slower and produces a strong initial inflammatory reaction.* ***Results.*** *All the MMPs measured disclosed higher values at early stage of the healing process in animals treated with Adhflex® and Tachosil, being the expression of for MMP2 and MMP9 significantly higher in the Adhflex-treated group. Animals treated with Tachosil had significant higher values of MMP8 and MPP13 than the Adhflex-treated group. Animals treated with Adhflex® showed a maintained overexpression in all the MMPs tested even at the latest wound healing stages.* ***Conclusion.*** *Notably, this MMPs overexpression did not influence negatively the histological healing process of the hepatic injuries. Given that all hepatic trauma injuries should be considered emergencies, any easy-to-use and rapid sealant, such as Adhflex®, could be considered as a suitable treatment option.*

Keywords: Hepatic injury, sealants, metalloproteinases, inflammatory response, wound healing.

1. Introduction

Interest in the use of biological adhesives has increased since Dermabond, a cyanoacrylate specially design for clinical use, was ratified by the Food and Drug Administration (FDA) in 1998. A variety of

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biological sealants and adhesive have been applied to control bleeding in cases of hepatic injuries [1,2,3] and different endoscopic biopsies of solid organs. [4,5]

The liver possesses a remarkable ability to self-repair and regenerate after injury[6]. Liver injury and regeneration have both been linked to complex extracellular matrix (ECM)-related pathways. While normal degradation of ECM components such as collagen or fibrin is an important feature of tissue repair and remodeling, irregular ECM turnover contributes to a variety of liver diseases[7]. Matrix metalloproteinases (MMPs) are the primary enzymes implicated in ECM degradation during wound healing; they also facilitate cell migration, deposition of new components in the ECM, and the development of regenerating tissue. [8]

Matrix metalloproteinases not only remodel the ECM, but they also regulate different immune responses. In addition, MMPs also play significant roles in diverse pathological processes such as cancer, multiple sclerosis and some skin disorders. [8] Matrix metalloproteinase activity is regulated by a combination of transcriptional control (e.g., by Interleukin-1 and TNF-∝), the presence or absence of factors required to transform proenzymes into their active forms, and the direct activity of MMP inhibitors (TIMPs). [9-11]

The clinical utility of measuring MMPs continues to expand thanks to advances in new, more-objective detection methods. [12] The asessment of MMPs, which is currently facilitating deeper molecular knowledge of the mechanisms of wound healing, therefore warrants the improvement of new therapeutic approaches. A recent investigation found that elevated MMPs in wound fluids from patients with acute traumatic injuries predicted both impaired healing and dehiscence of surgically closed wounds. [13] Some of the MMP-attributed roles in acute and chronic liver injury have been described, emphasizing the need for further experimentation to better understand their functions both in physiological conditions and during hepatic disease progression. [14]

Due to technological improvements, the use of tissue adhesives and glues for tissue approximation and hemostasis in surgery has increased. [1] Fibrin sealants are commonly used in liver surgery, although their effectiveness in routine clinical practice remains controversial. Tisseel/Tissucol and Tachosil yielded the greatest adhesion to liver cross-sections in a canine model of hepatectomy. [2] These results may enable the optimal choice of fibrin sealants for this procedure in clinical practice. [1] In addition, a multicentre, randomized clinical trial assessed the safety and effectiveness of a fibrin sealant patch in treating parenchymal bleeding. [2] This clinical trial confirmed that the fibrin sealant was safe and highly effective at controlling parenchymal bleeding following hepatectomy, regardless of the type of resection. [2]

This study focused on investigating the activity of MMPs during the healing of liver injuries treated with biological adhesives (Tachosil®, GelitaSpon® and Adhflex® (synthetic elastic cyanoacrylate). Histopathological changes were also monitored during hepatic injury healing and related to MMP activity. The clinical utility of measuring MMP expression during the healing of penetrating liver injuries may provide new insights into the repair processes of these lesions and shed light on how liver tissues react to these biological and synthetic adhesives. The superior adhesiveness and clotting speed of Adhflex® compared with conventional treatments in other organs suggests that Adhflex® could be considered a useful sealant substance. [14,15]

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2. Results

Matrix metalloproteinase expression

Of the MMPs included in the Mosaic ELISA MMP Panel, only MMP1, MMP2, MMP8, MMP9 and MMP13 were sufficiently expressed to be quantifiable (**Fig 1**) (**Table 1**).

Liver MMPs	*Time*	*Untreated (n=4)*	*Adhf® (n=4)*	*GelSp® (n=4)*	*TachS® (n=4)*	Kruskal-Wallis test (p value)
MMP 1	*T1*	*0,80 ± 0,22*	*2,75 ± 0,39**	*0,35 ± 0,10*‡*	*2,71 ± 0,31*†*	**0.005**
	T2	*0,85 ± 0,09*	*0,73 ± 0,06*	*1,02 ± 0,10*	*1,45 ± 0,08*‡†*	**0.005**
	T3	*1,02 ± 0,16*	*3,05 ± 0,36**	*0,96 ± 0,18‡*	*1,20 ± 0,15‡*	**0.016**
	Kruskal-Wallis test (p value)	*0.234^{ns}*	*0.021*	*0.024*	*0.008*	
MMP 2	*T1*	*1,81 ± 0,11*	*3,29 ± 0,04**	*1,16 ± 0,11*‡*	*2,27 ± 0,06*‡†*	**0.003**
	T2	*1,45 ± 0,09*	*3,00 ± 0,14**	*2,12 ± 0,06*‡*	*4,04 ± 0,13*‡†*	**0.003**
	T3	*1,55 ± 0,50*	*2,72 ± 0,08**	*1,41 ± 0,07‡*	*1,33 ± 0,08‡*	**0.024**
	Kruskal-Wallis test (p value)	*0.124^{ns}*	*0.007*	*0.007*	*0.007*	
MMP 8	*T1*	*0,68 ± 0,09*	*1,13 ± 0,25**	*0,33 ± 0,05*‡*	*1,28 ± 0,05*†*	**0.005**
	T2	*0,91 ± 0,07*	*0,62 ± 0,08**	*0,59 ± 0,15**	*1,10 ± 0,08‡†*	**0.007**
	T3	*0,84 ± 0,11*	*1,44 ± 0,05**	*0,78 ± 0,10‡*	*1,10 ± 0,10*‡†*	**0.005**
	Kruskal-Wallis test (p value)	*0.076^{ns}*	*0.015*	*0.012*	*0.037*	
MMP 9	*T1*	*1,27 ± 0.08*	*1,51 ± 0,16**	*0,34 ± 0,04*‡*	*1,44 ± 0,05*†*	**0.005**
	T2	*0,91 ± 0,13*	*0,45 ± 0,14**	*0,55± 0,09*	*0,90 ± 0,16‡†*	**0.009**
	T3	*0,91 ± 0,13*	*1,56 ± 0,14**	*0,77 ± 0,11‡*	*0,87 ± 0,13‡*	**0.020**
	Kruskal-Wallis test (p value)	*0.025*	*0.024*	*0.010*	*0.024*	
MMP 13	*T1*	*1,42 ± 0,06*	*1,63 ± 0,16*	*0,39 ± 0,04*‡*	*1,99 ± 0,10*†*	**0.003**
	T2	*0,95 ± 0,17*	*1,51 ± 0,10**	*0,90 ± 0,09*‡*	*1,21 ± 0,10†*	**0.008**
	T3	*1,18 ± 0,08*	*3,33 ± 0,19**	*1,38 ± 0,11‡*	*1,82 ± 0,12*‡†*	**0.003**
	Kruskal-Wallis test p value)	*0.011*	*0.019*	*0.007*	*0.011*	

TABLE 1. MMP1, MMP2, MMP8, MMP9 and MMP13 metalloproteinase mean values (± standard deviation) (pg/mL) in the three healing times (T1: 2 days after injury; T2: 6 days after injury, and T3: 18 days after injury) and in each treatment group. Mann-Whitney U test: (*) $p<0.05$ compared with untreated animals; (‡) $p<0.05$ with compared with the Adhfex® group; (†) $p<0.05$ compared with the GelSp® group. The Kruskal-Wallis test revealed the statistical differences among the four groups during each healing time period. (ns): no significance.

MMP1: Both time since injury and treatment method influenced MMP1 expression in the three treated groups (Kruskal-Wallis test for time, $p< 0.05$; for treatment, $p<0.05$) (**Table 1**). Along the healing process (T1,T2 and T3), the untreated group did not exhibit any statistical significan

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difference in the MMP1 mesasurements. (**Table 1, Fig 1-D**). The highest mean MMP1 expression level was recorded in the Adhflex® group. The lowest mean MMP1 concentration in the treated group was recorded in the GelSp® group (**Table 1**)**.** The Adhflex-treated group had the highest MMP1 concentration at 2 and 8 days after injury, and the difference was statistically significant higher at 18 days (T3as compared with the other treatments (**Table 1**).

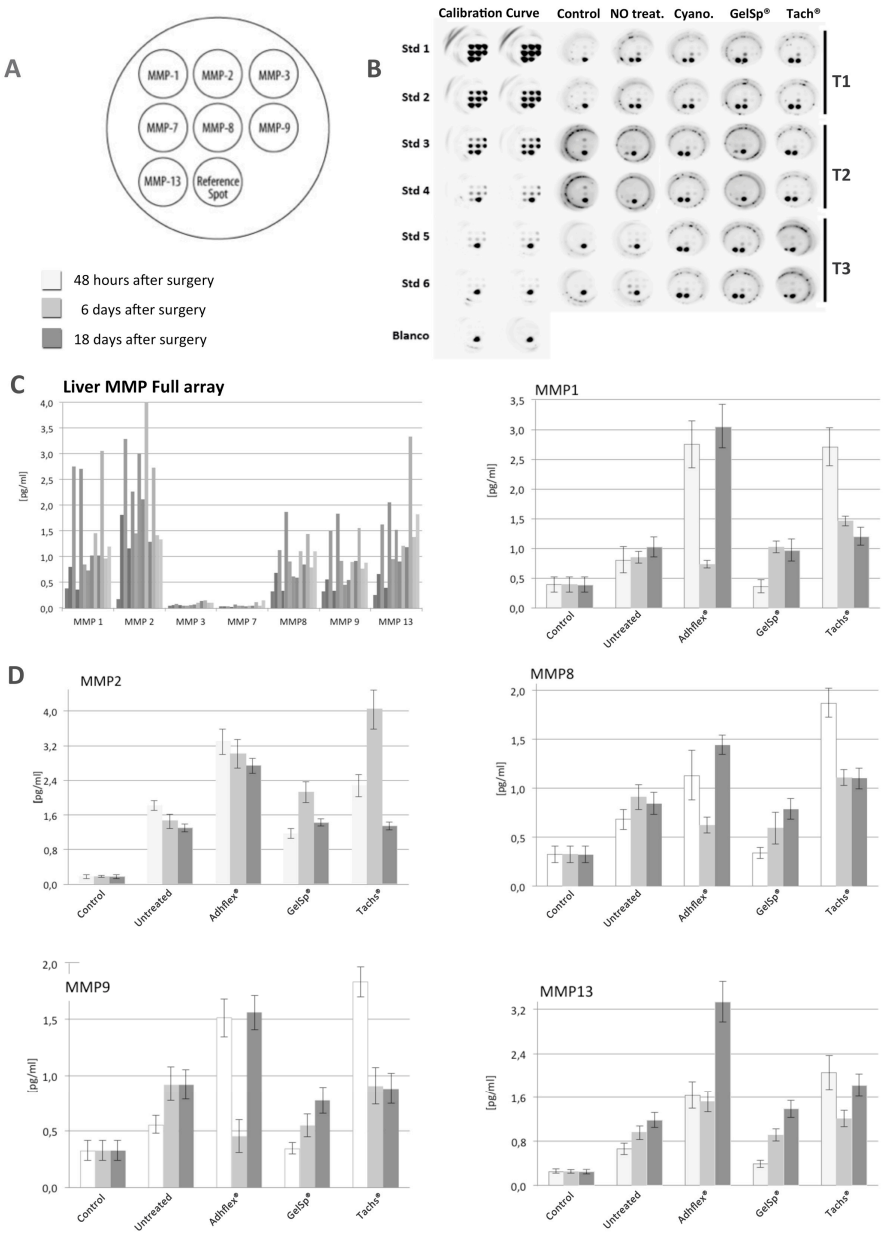

FIGURE 1. Metalloproteinase expression in sham-control, untreated and treated (Adhflex, GelitaSpon and TachoSil) kidneys. (A) Chart showing the most expressive metalloproteinase; (B) ELISA MMP panel (R&D Systems) plates of the rat metalloproteinase in all groups; (C) Individual graphs for the most expressed metalloproteinases in all groups (control, untreated, and Adhflex, GelitaSpon and TachoSil treated) tested at 2, 6 and 18 days post-injury.

MMP2: Except for the untreated group, we found significant differences in MMP2 expression between the 2, 6 and 18 days post-injury sampling times (Kruskal-Wallis test, $p<0.01$) (**Table 1).** MMP2 expression was also the highest 6 days post-injury and the

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lowest 18 days post-injury (**Table 1**) (**Fig 1**). The Tachosil-treated group had the highest concentration of MMP2 6 days after injury, and the difference was statistically higher that the concentrations exhibited by the other treatments (**Table 1**). Except for the Adhflex-treated animals, 18 days after injury all of the other groups restored normal expression of MMP2 compared with the control group.

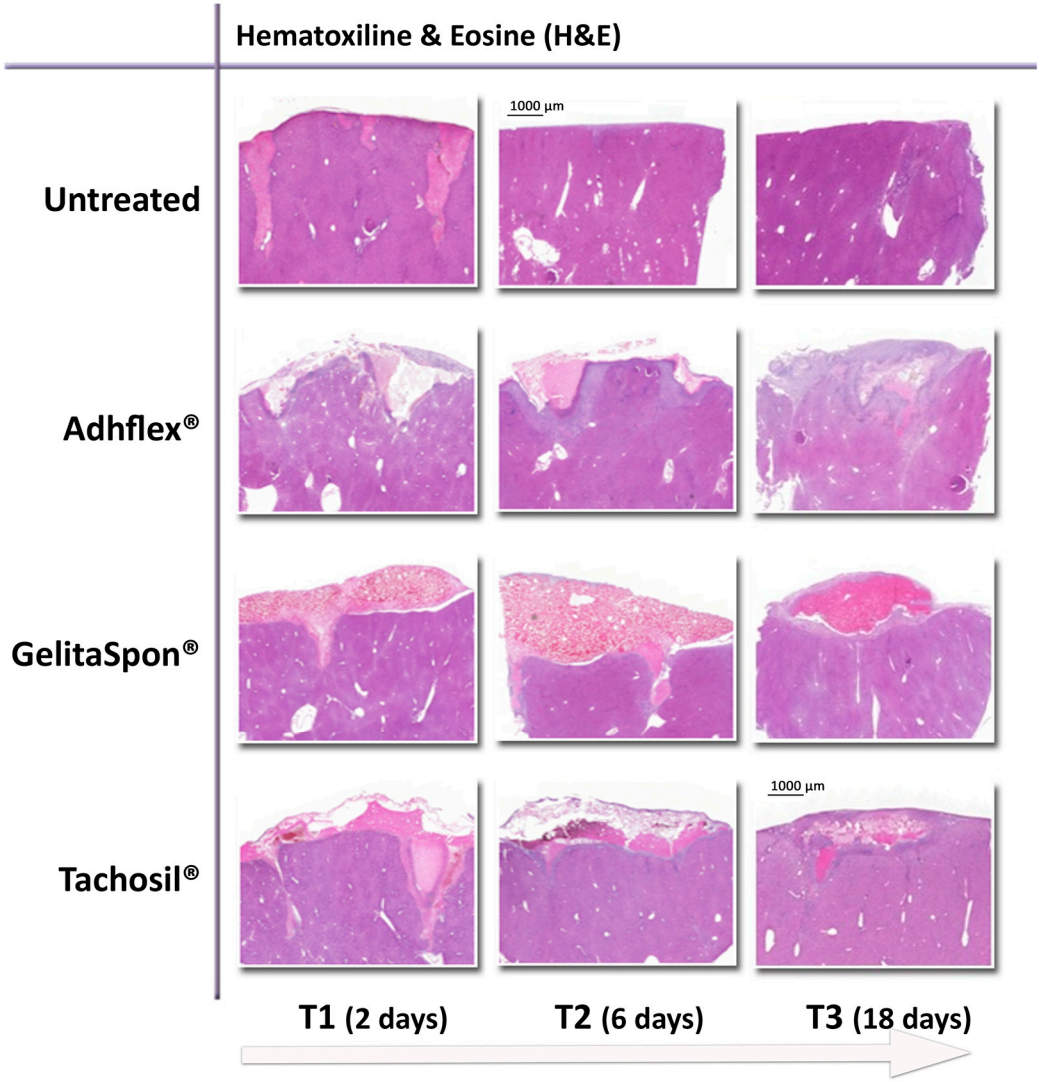

FIGURE 2 . **Hematoxiline & Eosine** stein (H&E) Panel presenting the evolution of untreated and treated injuries (Adhflex, GelitaSpon, and TachoSil) in the liver groups.

MMP8: Like MMP1, both time since injury and treatment method influenced MMP8 expression. Significant differences in MMP8 expression between 2, 6 and 18 days post-injury were detected in the three treated groups ($p<0.05$) but not in the untreated group (**Table 1**) (**Fig 1-D**). The MMP8 concentration was lowest in the GelitaSpon (T1) group and significantly higher in the Tachs®-treated group (**Table 1**) **(Fig 1**).

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MMP9: MMP9 exhibited an expression pattern nearly identical to that of MMP8. Time since injury influenced MMP9 expression in all of the treated groups but not in untreated animals (**Table 1**). At the T3 time period, there were no differences among the treated and untreated groups. MMP9 expression was lower in the GelitaSpon-treated group than in the untreated group. As in the other MMPs, MMP9 expression was highest in the Adhflex-treated group, and the difference was statistically significant compared with the other treatments (**Table 1**) (**Fig 1**).

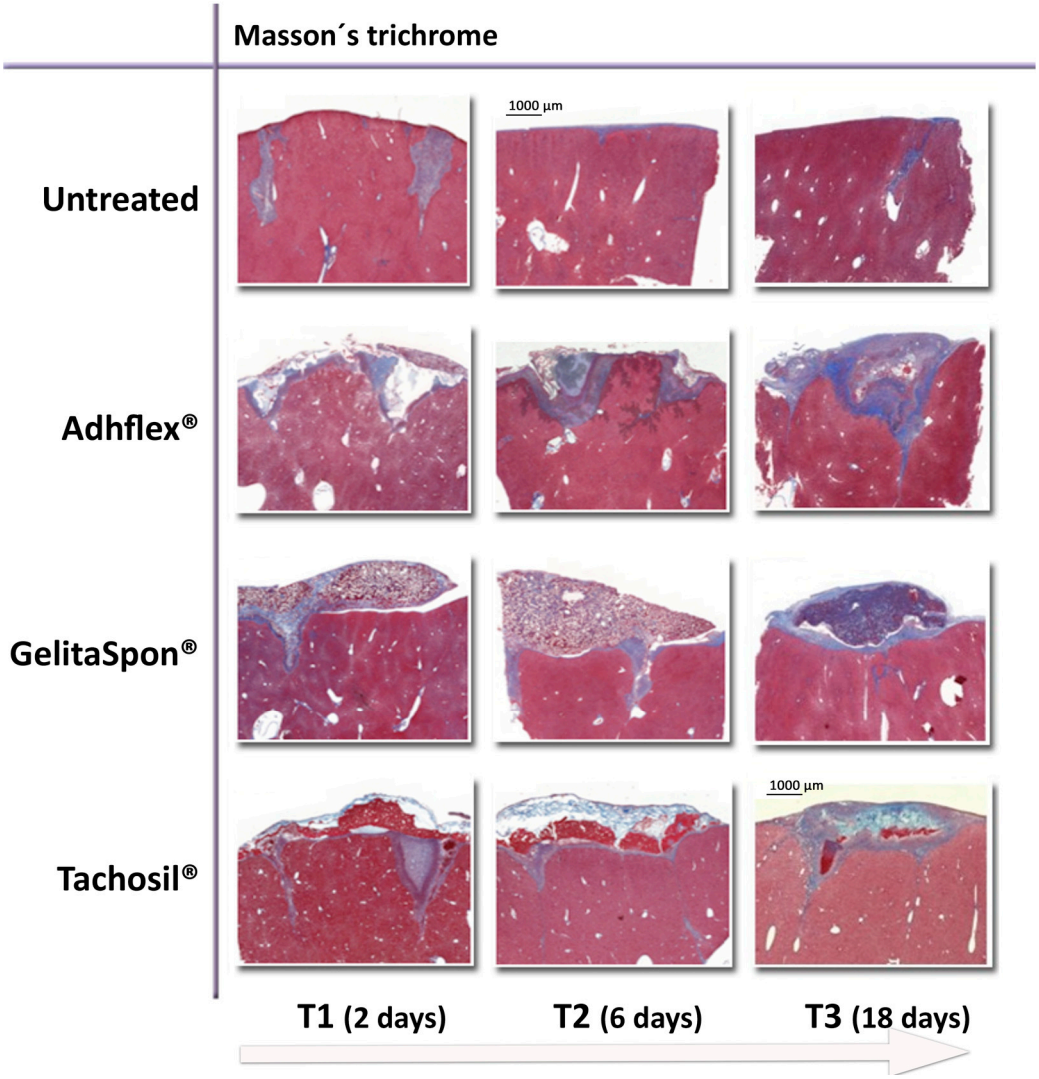

FIGURE 3. Masson's trichrome staining was used to visualize collagen and reticular fibers. Adhflex® generates an inflammatory reaction around the adhesive included in the wound (deep blue mark at the bottom of the lesions (T2 and T3).

MMP13: The expression of MMP13 followed a pattern similar to that of MMP8 and MMP9. Time since injury influenced MMP9 expression in all of the treated groups but not in the untreated animals (**Table 1**). There were significant differences in MMP13 expression between the untreated group and all three treated groups. The Adhflex-treated group exhibited the strongest MMP13 expression, which was significantly stronger than that of the other groups, parrtucularly at the T3 stage (**Table 1**) (**Fig 1**).

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Histological study. **Figures 2–6** show tissue sections stained with hematoxylin and eosin, Masson's trichrome, Periodic Acid Schiff PAS, CD31 and CD68 immunohistochemical marker. Each staining made it possible to observe a different feature of the healing lesions. For all of the tests in the untreated liver lesions (two days - T1), the wounds produced by the Stiefel biopsy punch were well defined, and a dark strip compatible with granulation tissue, comprising inflammatory cells and fibroblasts, was observed. The necrotic tissue had completely disappeared 18 days post-injury, and the wound edges were fully in contact. A column of dark violet connective tissue could be observed corresponding to the injury scar. The appearance of the parenchyma surrounding the scar was normal.

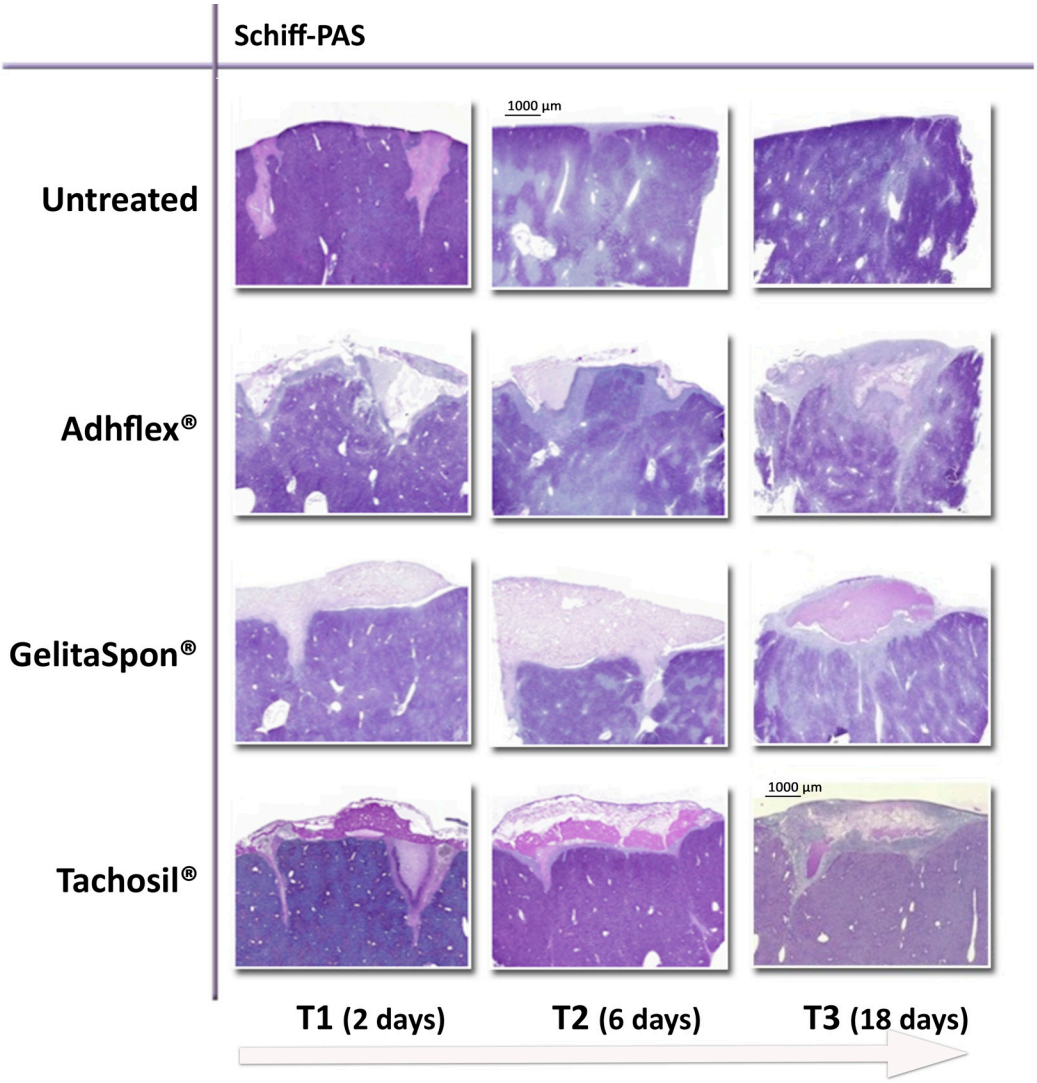

FIGURE 4. Periodic Acid Schiff (PAS) staining was used to identify glycogen and mucin. The color contrast of the stains reveals well each part of the lesions very well and the biomaterials used for wound sealing.

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By studying all of the stains, the evolution of lesions and the behavior of the sealants that we used could be clearly assessed. In the untreated group at 18 days (T3), the lesions were fully healed. In contrast, the wounds that were treated exhibited traces of the biomaterial adhered at the bottom of the lesions at 18 days. Additional comments are included in the figure captions (**Figs 2–6**).

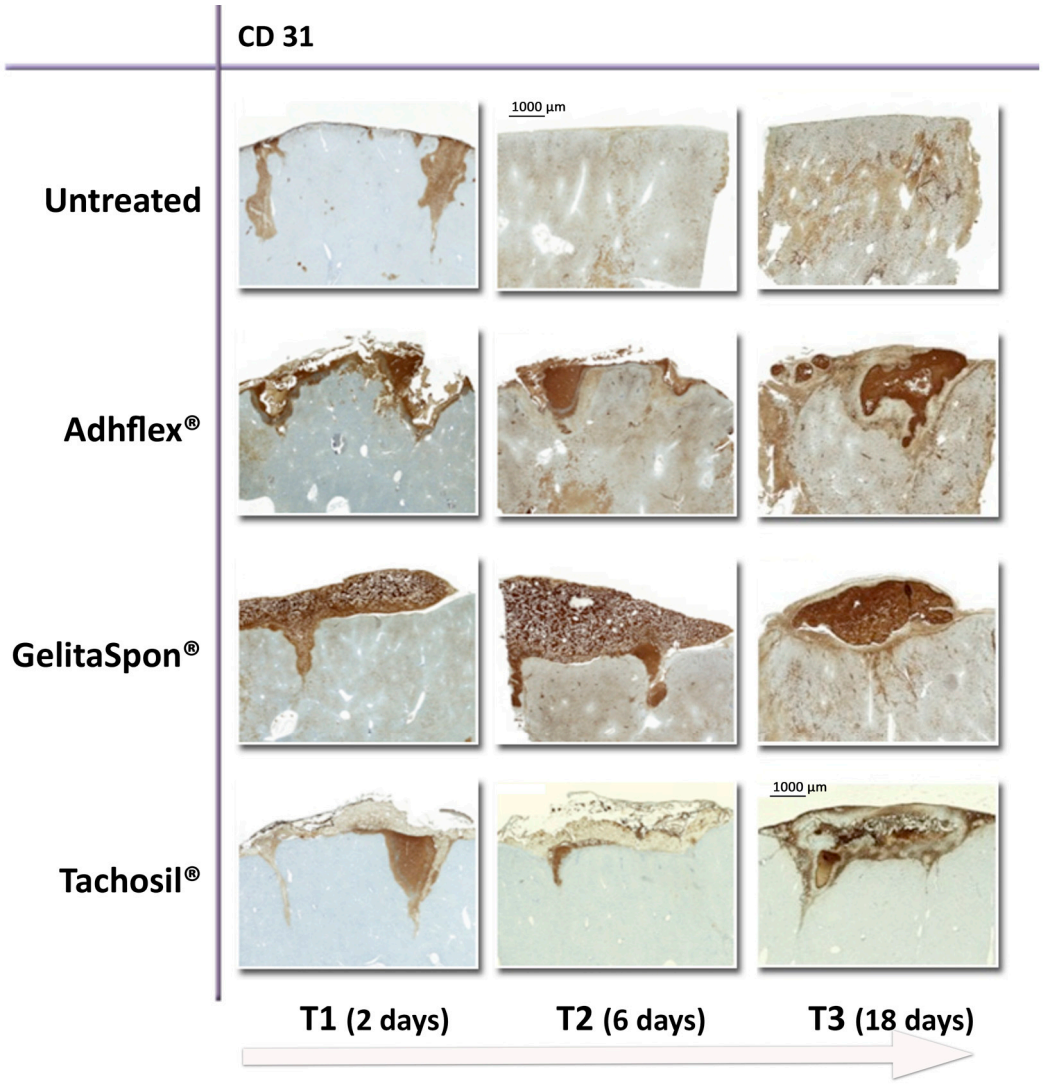

FIGURE 5. CD31 immunohistochemical marker is selective for endothelial cells associated with vascular neoformation. This marked wound shows reveals the injuries produced by the punch in the untreated group at 2 days staining (T2). The contact of the materials used to treat the lesion is very well defined in this staining.

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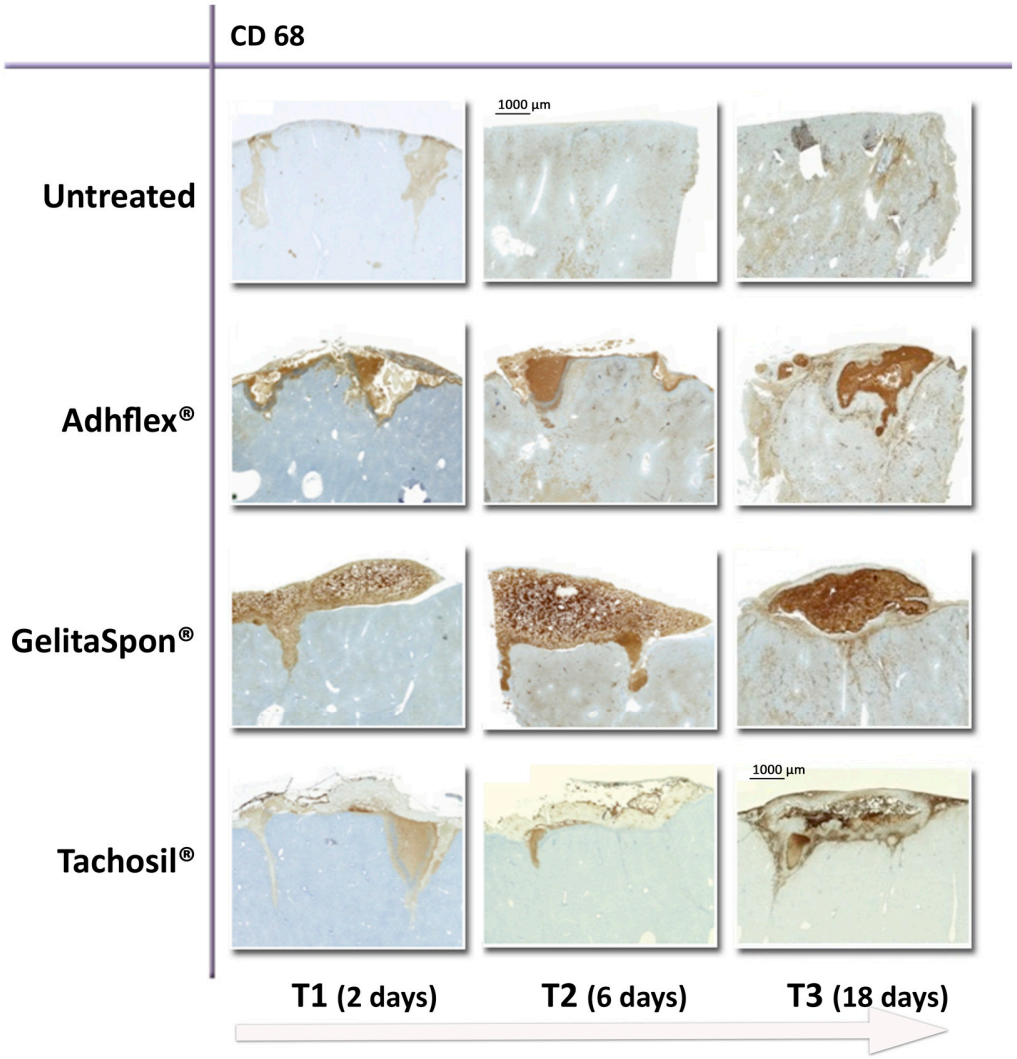

FIGURE 6. CD68 marker detects the cytoplasm of mast cells and histiocytes, which are the macrophages present in the post-trauma inflammatory process. As in the CD31 immunohistochemical marker, the panel images show very well the contact of the sealants employed and the evolution of the wound healing.

3. Discussion

This study describes the changes in MMP expression after application of three surgical sealants (TachoSil, GelitaSpon, Adhflex) in an experimental penetrating hepatic injury. As has been described for renal injuries[14], the histological healing process of hepatic lesions in response to the three biomaterials was comparable. However, MMP expression varied depending on the sealant used. All of the MMPs exhibited higher expression at early stages of the healing process (2 days) in animals treated with Adhflex and Tachosil. Differences between these two sealants were statistically significant only for MMP2 and MMP9, and expression was higher in the Adhflex-treated group. In the case of MMP8 and MPP13, animals treated with Tachosil had significant higher values of these MMPs than the Adhflex-treated group. Apart from the overexpression of MMPs at early stages of wound healing, animals treated with Adflex exhibited a maintained overexpression in all of the MMPs tested, even at the latest wound healing stages (18 days post-injury). Notably, this

MMP overexpression did not influence negatively the histological healing process of the hepatic injuries.

Matrix metalloproteinases are a family of proteases using zinc-dependent catalysis to break down ECM glycoprotein, enabling cell movement and tissue reorganization.[20] At the present time, there is strong evidence that MMPs play key roles in the healing process, especially during the inflammatory and proliferative phase. [10-13] Therefore, the sampling times used here were established according to the inflammatory, proliferative and maturation phases of injury healing. [21] Most MMPs act simultaneously, at times even sharing the same substrates, with the activity of one MMP often leading to the activation of others. For this reason, here we drew comparisons between groups of MMPs organized into defined subfamilies**.** [22]

Although MMP involvement in pathology is more than simple excessive matrix degradation or an imbalance between MMPs and their specific TIMPs, MMP inhibition may be of therapeutic benefit, so synthetic MMPs inhibitors had been developed and are currently being clinically tested. [23] In liver tissue, MMPs and their specific inhibitors (TIMPs) play a pivotal role in both fibrogenesis and fibrolysis. [24]

Of the MMPs tested, only the collagenases (MMP1, MMP8 and MMP13) are capable of breaking the fibrillar collagen triple helix. We found that the collagenases had differing expression profiles. MMP1 expression was higher in the Adhflex- and Tachosil-treated groups than in the untreated group. The highest MMP1 expression was recorded for the Adhflex-treated group 18 days post-injury (**Table 1** & **Fig 1-C**). According to the histopathology findings, the high MMP1 values coincide with a persistence of biomaterial in the lesion (**Figs 3–6**). Therefore, the increased expression of MMP1 might be related to the persistent inflammatory reaction induced by Adhflex® at that healing time. In fact, when an injury becomes chronic, as in dermal ulcers caused by burns, the MMP1 concentration remains high after the first week of healing. [25] In chronic skin ulcers, prolonged MMP1 activity can have a critical effect on the re-epithelialization of tissues.[26]

Gelatinases (MMP2 and MMP9) play an important role in the formation and maturation of granulation tissue during wound healing. [27] Both MMP2 and MMP9 have been reported to act synergistically with collagenases**.** [28] Once the collagenases have cleaved the collagen triple helix, the gelatinases begin degrading the Type I, II and III collagen fibers.

MMP2 has been shown to retard fibroblast differentiation during healing[29] . Therefore, the control of MMP2 activity could act as a means of preventing hypertrophic scarring. Various authors have described increased gelatinases expression following traumatic injury. [30,31] Nessler [28] measured MMP2 expression levels in patients with healing wounds (1, 7 and 25 days post-injury) and detected the highest levels 7 days post-injury, which is consistent with other studies, which typically found that MMP expression peaked at between 5 and 7 days post-injury, which coincides with the completion of the inflammatory phase and the formation of granulation tissue. [32] These data are in accordance with the current findings in which MMP2 was signficantly higher in samples taken 6 days after injury, particularly in the Tachosil-treated animals.

While MMP2 expression is important during the remodelling phase, the gelatinolytic activity of MMP9 appears to be higher in early wound healing. This finding is in

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accordance with our findings of MMP9 peak values during the early wound healing stage in the control and the GelSp-treated groups. However, in the Adhflex® and Tachs® groups, MMP9 activity remained high 18 days after injury. The finding that MMP9 expression peaked between 2 and 6 days post-injury may be associated with the normal healing process. The histological findings indicate that Adhflex® and Tachs® treatments yield stronger inflammatory reactions on liver tissue throughout the healing process and possibly contribute to the increased expression of MMP9 (**Figs 3–6** in the Adhflex®- and Tach®-treated samples),

The predominant role of MMP8 in ECM turnover, modulation of inflammatory responses and other physiological processes is well documented. [33] MMP8 is stored in the granules of neutrophils and is released during the first few hours after injury. Its activity can be extended to the end of the inflammatory phase. [33-35] In this study, MMP8 expression peaked 2 days post-injury in animals treated with Tachosil and 18 days post-injury in animals treated with Adhflex®. In the GelSp-treated groups, MMP8 values were below the reference controls during the entire healing process. The MMP8 expression profiles described here are consistent with a normal healing process.

MMP13 expression was high at all stages of the healing process post-injury and was highest in the Adhflex-treated group 18 days after injury. MMP13 expression was initially higher in the Tachosil-treated group. The GelSp sealant did not induce any effect on MMP13 expression. Several studies have indicated that MMP13 activity during the early stages of healing is beneficial and related to the formation of the three-dimensional collagen matrix, as well as modification of fibroblast morphology and viability. [30,36] MMP13 also acts on the activity of myofibroblasts and angiogenesis, particularly during the formation of granulation tissue**.** [31] Despite these beneficial effects, high MMP13 expression has also been documented in numerous chronic skin disorders, as well as in other chronic diseases, such as rheumatoid osteoarthritis, where it leads to a destruction of the collagen matrix. [36] The stronger expression of MMP13 in the Adhflex®-treated samples at 18 days may be the consequence of the remaining cyanoacrylate at the bottom of the wounds (**Figs 3-5)**.

Regarding the histopathology analyses, the healing progression was similar across all of the study groups. Application of each of the sealants produced a marked coagulated hematoma at the affected area. Over time, granulation tissue could be observed around the necrotic area, with widely dispersed inflammatory infiltration. The scar tissue initially covered a relatively large surface area in the days following the lesion. However, after 18 days the size of the scar area was significantly reduced (**Fig 3**). Eighteen days post-injury, the necrotic tissue had been eliminated in almost all samples and had been replaced by a strip of connective tissue. This tissue formed a scar and showed signs of contraction that indicated maturation of the scarred tissue. The healing progress was similar regardless of the sealant used.

4. Materials and Methods

Design: The experimental design and animal welfare procedures were approved by the Animal Welfare Committee (Research Ethical Committee for Animals Studies) of the Valencia Regional Government (ref. number: 2015/VSC/PEA/00097), in compliance with

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applicable legislation (Royal Decree 53/2013) and FDA recommendations related to animal welfare in experimentation.

Using an experimental rat model, hepatic injuries were made on the anterior aspect using a Stiefel Biopsy Punch (8 mm diameter, 3 mm depth), followed by local treatment with either TachoSil®, GelitaSpon® or Adhflex®. An untreated injured group was also included. Wound healing was evaluated 2, 6 and 18 days post-injury.

Animals: Ninety male Wistar rats (body weight: 300–350 g) (Harlan Laboratories, Barcelona, Spain) were housed in a standard animal facility, with access to food and water both pre-operatively and postoperatively. Animal surveillance and care was conducted every 12 hours during the preoperative period and every 6 hours throughout the entire postoperative process (18 days). No animal became severely ill or died during the course of the experiment.

Experimental groups: The animals were divided into five groups: 1: sham non-injured (n = 3); 2: untreated (n = 6); 3: TachoSil-treated (n = 27); 4: GelitaSpon-treated (n = 27), and 5: Adhflex-treated (n = 27). In each treatment group, 5 rats were used for histological studies and 4 rats were used for for MMP expression during the three stages of the study: 2, 6 and 18 days post-injury (9 × 3 = 27 rats). Two of the untreated rats were used to study both histology and MMP expression in each stage of the study (2 × 3 = 6). The samples of control rats were divided into two groups to ensure at least two histological studies and two MMP determinations during each experimental period.

Biomaterials and adhesives used: Adhflex® (Bioadhesives Medtech Solutions s.l., Alicante, Spain) is a cyanoacrylate-based adhesive supplemented with acrylates to enhance elasticity, reduce stiffness and increase cohesive strength. Adhflex® has a greater polymer flexibility and lower polymerization temperature than other clinically used cyanoacrylates. TachoSil® (Takeda GmbH-Austria) is a haemostatic sponge containing human fibrinogen (5.5 mg per cm^2) and human thrombin (2.0 IU per cm^2). GelitaSpon® (Gelita MedicalGmbH-Germany) is a biodegradable, topical haemostat (absorbable, oxidized cellulose sponge). All three products possess adhesive and coagulant properties.

Anaesthesia: The animals were intraperitoneally anaesthetized with Ketamine (80 mg kg^{-1}) and Xylazine (10 mg kg^{-1}), and spontaneous breathing was maintained during a midline abdominal laparotomy in which the punch injury was created. Partial O_2 and CO_2 pressures were monitored transcutaneously, ensuring optimal haematosis throughout the surgical procedure. To minimize pain, the animals were treated with Buprenorphine (0.1 mg/kg every 12 h) during the first 48 hours after surgery.

Standardized treatments: Once the anaesthesia was completed, the abdominal cavity was exposed via a midline incision. Throughout a transperitoneal approach, the liver was located and carefully exposed by retracting the bowel loops. With direct vision, liver injuries were imposed on the anterior surface using a Stiefel biopsy punch. Lesions that were homogeneous in size (8 mm diameter) and depth (4 mm) were easily invoked by applying minimal pressure over the surface together with a slight twist. In the TachoSil® and GelitaSpon® groups, the wounds were covered with homogeneous circles of the biological adhesives. In the Adhflex® group, a single drop (21.3 ± 1.2 mg) was applied to each wound using a supplied applicator. After sealing the injury, the liver was observed for

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an additional 3 min to ensure hemorrhagic occlusion. Finally, 1 ml saline at 37ºC was injected into the abdomen and the incision was sutured in two layers.

Euthanasia: Nine animals from each of the treatment groups were euthanized by an intraperitoneal injection of sodium pentobarbital (lethal dose, 60 mg/kg) at 2, 6 and 18 days post-injury, and the samples were processed for histology and MMP determinations.

Healing evaluation:

1) Metalioproteinases expression: During healing, the activation and inhibition of different MMPs affects multiple processes. To test whether TachoSil®, GelitaSpon® or Adhflex® stimulated the local secretion of MMPs by the host hepatic cells, liver homogenates (60 µl of serum) were subjected to enzyme-linked immunosorbent assay (ELISA). We tested a panel of MMPs (Mosaic ELISA MMP Panel, R&D Systems).

Matrix metalloproteinases were quantified via chemiluminescence (according to the manufacturer's instructions). MMP-1, MMP-2, MMP-8, MMP-9 and MMP-13 levels were quantified. The panel and protocol have a sensitivity in the pg/ml range. The MMP levels were quantified by gel densitometry (Image J), using the mean of duplicate samples. Equal spot sizes were analysed per blot.

The Mosaic ELISA MMP Panel can detect up to seven different MMPs. However, because of limits of sensitivity, we restricted our analyses to only the most strongly expressed MMPs.

2) Histological Studies: Haematoxylin-Eosin staining (3 µm thick slices) was used for the study of the samples. Specific stains were used to observe histological changes produced in the development of hepatic lesions and the consequences of several treatments. Five liver tissue samples from each group were examined**.** Masson's trichrome staining protocol was used to visualize collagen and reticular fibers, highlighting the increased apposition of collagen at the time of healing. [16] Periodic Acid Schiff (PAS) staining was used to identify glycogen and mucin. [17] Five lever tissue samples from each group were examined. The CD31 immunohistochemical marker is selective for endothelial cells associated with vascular neoformation. [18] The CD68 marker detects a glycoprotein (approximately 110 kD) in the cytoplasm of mast cells and histiocytes, which are macrophages present in the post-trauma inflammatory process. [19]

Statistical analysis: SPSS Statistics (version 20.0, IBM, Armonk, NY, USA) was used for all of the statistical analyses. Fisher's Least Significant Difference (LSD) test was used to identify significant between-group differences ($p<0.05$) in the size of the gap between wound edges. Due to the small sample size of our rat cohort, we used the non-parametric Kruskal-Wallis test to compare differences in MMP expression among the groups. The Mann-Whitney U test was used to analyze differences in MMP expression between the untreated group and each treated group. *P*-values less than 0.05 were considered to be statistically significant. Normality and Levene's test were used to assess the equality of error variance for each variable.

5. Conclusions

In conclusion, the three sealants used in this study yielded complete healing of the liver lesions. Both the histopathology and MMP findings indicate that the degradation process of

Adhflex® was slower than the other biomatetrials, and produced a strong initial inflammatory reaction. However, at the end of the process, we noted complete healing of the lesions. Given that all hepatic trauma injuries should be considered emergencies, any easy-to-use and rapid sealant such as Adhflex® could be considered to be a suitable treatment option.

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