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(71) Applicants: **FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD** [ES/ES]; Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES). **SERVICIO ANDALUZ DE SALUD** [ES/ES]; Avda. de la Constitucion, 18, 41071 Sevilla (ES).

(72) Inventors: **CAMPOS CUERVA, Rafael**; FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD, Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES). **FERNANDEZ MUÑOZ, Beatriz**; FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD, Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES). **GÓMEZ BRAVO, Miguel Angel**; FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD, Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES). **CEPEDA FRANCO, Carmen**; FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD, Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES). **MUNTANE RELAT, Jordi**; FUNDACIÓN PÚBLICA ANDALUZA PROGRESO Y SALUD, Avenida Américo Vespucio, nº 15, Edificio S2. Parque Científico y, Tecnológico Cartuja, 41092 Sevilla (ES).

(74) Agent: **HOFFMANN EITLE S.L.U.**; Paseo de la Castellana 140, 28046 Madrid (ES).

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(54) Title: HEMOSTATIC EFFICACY OF A NANOSTRUCTURED FIBRIN AGAROSE HYDROGEL

(57) Abstract: The present invention provides for nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (NFAH) or non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (FAH), as hemostatic agents designed for use as an adjunct or primary treatment in moderate intraoperative hemorrhage and in trauma. These hydrogelscan be applied topically to the wound either on the skin in a laparatomy or as non- invasive manner in surgical procedures. Its nanostructure technology generates an adhesive stable fibrin clot required for hemostasis. The attachment properties of the hydrogel, as well as the rapid formation of a fibrin clot, ensures that a strong stable fibrin clot is formed shortly afterapplication.

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Hemostatic efficacy of a nanostructured fibrin agarose hydrogel

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the application as an adhesive sealant component and hemostatic agent of nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels (NFAH). Such nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels (NFAH), may be use to stop bleeding or seal tissue *in vivo* with and without compression. This invention is particularly related to a need of incorporating a fibrin sealant in a viscous gel-like product that can resist the flow of blood in a wound in order to seal tissue and control vascular, epidermal, bone, or internal hemorrhage.

BACKGROUND OF THE INVENTION

Uncontrolled hemorrhage continues to be an important concern in surgical interventions and is responsible for higher rates of mortality, morbidity and longer hospital stays (Brustia, Granger, & Scatton, 2016). Injury of blood vessels triggers different mechanisms in order to establish a complex process called hemostasis. Hemostasis is an interruption of the escape of blood by either natural or artificial means.

Hepatic resection usually requires a perioperative transfusion due to intraoperative blood loss in patients. There are several surgical techniques used to reduce bleeding (Berrevoet & De Hemptinne, 2007). These methods however have a transitory or incomplete effect. This in turn requires a topical haemostatic agent to prevent postoperative blood loss (Moggia et al., 2016)(Eeson & Karanicolas, 2016).

Different hemostatic agents approved by Food and Drug Administration (FDA) are used in multiple surgical disciplines to reach hemostasis. Among these, fibrin sealant derived from human plasma has become a thriving hemostatic agent due to its capacity to imitate and promote the final step of coagulation cascade to assist hemostasis(MOSESSON, SIEBENLIST, & MEH, 2006)(Mankad & Codispoti, n.d.). It also reduces potential rejection and undesirable effects, and is the only FDA clinical material approved as hemostats, sealants and adhesives (Spotnitz, 2014). Despite advantages in the field, time to hemostasis, applicability and postoperative transfusion can further be improved.

A recent nanostructured fibrin and type VII agarose hydrogel (NFAH) was developed as biomaterial to use in multiple biocompatible models (Carriel et al., 2017). This tissue-like substitute is mainly

composed of human plasma and shows highly flexible and elastic properties as well as mechanical strength (MOSESSON et al., 2006; Scionti et al., 2014).

This invention focuses on the hemostatic effectiveness of agarose hydrogels, preferably type VII agarose hydrogels (NFAH), and illustrates such effectiveness in an *in vivo* model. To this purpose, NFAH was compared with two commercially available hemostatic products used in multiple surgical disciplines: Hemopatch, a polyethylene glycol-coated collagen pad and Tachosil, a fibrinogen and thrombin-coated collagen pad.

SUMMARY OF THE INVENTION

The present invention lies within the domain of biological tissue sealants and hemostats, which are biodegradable and nontoxic, intended for therapeutic use, for example, as an adjunct to hemostasis in laparotomy or laparoscopic surgery, in orthopedic surgery, trauma (spleen laceration or hepatic surgery), and large-bed wounds.

In one aspect, the present invention relates to biocompatible adhesive nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels (NFAH), which are bio-reabsorbable and nontoxic, for surgical or therapeutic use. It also relates to a single-part application containing a nanostructured fibrin and a agarose hydrogel, preferably a type VII agarose hydrogel (NFAH), which can be released in a given site to produce a stable fibrin clot.

In vivo studies shown herein illustrate that nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels (NFAH), are an excellent general hemostatic agent candidate for use as adjunct and primary treatment in moderate bleeding. The agent is durable, easy to store, poses minimal risk, requires little training to use, and is highly effective against bleeding.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Characterization of isolated rat fibroblast by collagenase-base technique: (A) Rat fibroblast in culture after isolation; (B) Expression of vimentin was determined by immunostaining analysis in rat fibroblast cultured; HaCat, an immortal keratinocyte cell line was used a negative control; (C) Karyotype analysis of single cell-dissociated culture.

FIG. 2. Time to hemostasis after treatment application. NFAH had superior hemostatic effectiveness relative to Hemopatch and Tachosil. (A): Hemostatic effect in 0.5 cm hepatic resection. (B):Hemostatic effect in 1.5 cm hepatic resection.

FIG. 3. Comparation of hemostasis in Hemopatch and c-NFAH treatments. Similar results were obtained in term of time to hemostasis for different lengths of liver resection.

FIG. 4. Sequential application of NFAH in hepatic resection. NFAH had the easiest applicability of treatment groups. 1-3) Hepatic resection and wound exposition. 4-6) NFAH application and final positioning.

FIG. 5. Bar graph showing the incidence of hematoma. Results showed no presence of hematoma in any cellularized or acellularized NFAH. A) Percentage of animal in 0,5 cm hepatic resection. B) Percentage of animal in 1,5 cm hepatic resection.

FIG. 6. Percentage of grade of adhesion. NFAH showed lower adhesion than other treatments in hepatic resection 24 hours after application. The grade of adhesion was determined by next ranking: No adhesion (separate anatomical structures); Thin adhesions (adhesion separable by gravity); Thick adhesions (adhesion no separable by gravity).

FIG. 7. NFAH showed no presense of hematoma and lower adhesion than other treatments in hepatic resection 24 hours after application. (A): Hemopatch causing high grade of adhesion. (B): NFAH showing no adhesion. (C): Presence of hematoma in Hemopatch treatment. (D): No presence of hematoma in NFAH treatments.

FIG. 8. NFAH showed neither rebleeding nor migration in hepatic resections 24 hours after application.

FIG. 9. Inflammatory factors in animals with 0,5 cm hepatic resection after 24 hours. There were no significant differences of C-reactive protein and IL-1β between cellularized NFAH and Hemopatch treatments.

FIG. 10. Inflammatory factors in animals with 1,5 cm hepatic resection after 24 hours. (A) A-NFAH treatment showed no significant difference in levels of CRP inflammatory factor than Hemopach and Tachosil treatments. (B) Hemopatch treatment caused higher levels of IL-1β inflammatory factors than a-NFAHs treatments. (C) A-NFAH showed no significant difference of TNF-α inflammatory level in respect to either Hemopatch or Tachosil.

FIG. 11. Examples of hemorrhage, inflammation and necrosis classification.

FIG. 12. Bar graphs showing percentage of hemorrhage, inflammation and necrosis in 0,5 and 1,5 cm hepatic resections after treatment with hemostatic agents. Each sample was classified in absent, mild, moderate or intense (see methods for classification). (A-C): Histological analysis of 0,5 cm hepatic resection; (D-F): Histological analysis of 1,5 cm hepatic resection.

FIG. 13. Generation of nanostructured fibrin-agarose hydrogel (NFAH)

DETAILED DESCRIPTION

In the present invention, we investigated the hemostatic success of a cellularized NFAH (c-NFAH) in partial hepatectomy in rats. The findings showed that c-NFAH has greater hemostatic success than commercially available hemostatic agent Hemopatch. Such promising results prompted us to extend the experiments increasing the length of hepatic section and including other hemostatic agents. To this end, TachoSil was incorporated to the study owing to its similar composition in respect to the NFAH under investigation. An acellularized NFAH (a-NFAH) was also included in order to check if lower time of hemostasis was mainly due to NFAH composition rather than by the presence of cells. One more time, results showed NFAH have lower time to hemostasis than Hemopatch. Moreover, this effectiveness of NFAH in reaching hemostasis was also significantly superior to Tachosil. In this study, Hemopatch was also found to have a greater success than Tachosil in achieving immediate hemostasis in hepatic surgical model.

In addition, although as shown in the examples, hemostatic pads need a long time of local compression in order to reach hemostasis, NFAH placement on sectinated liver was enough to act as hemostatic agent. This different application procedure confers a great advantage to NFAH avoiding mechanical compressions that cause hematomas.

Also, NFAH treatment groups, unlike Hemopatch and Tachosil, showed no incidences of hematoma in any hepatic resection size. These results are probably due to the lower time to hemostasis of NFAH treatments and its easier applicability avoiding the hematoma formation.

Also, the low grade of adhesion produced by NFAH pads compared to hemostatic agents Hemopatch and Tachosil is also a remarkable aspect. This attribute can be an important advantage to its use in any surgical application.

Moreover, postoperative re-bleeding were found in any treatment group as expected and the only migrated pad found in Tachosil treatment group was probably due to an involuntary change of position during the suture.

On the other hand, no significant differences were observed in CRP and IL-1β levels between c-NFAH and Hemopatch in 0.5 cm hepatic resections. However, these results were not reproducible in 1.5 cm resections. In 1.5 cm hepatic resections, there was not an only treatment with the lowest levels in all inflammatory factors analyzed. Firstly, lowest CRP level was obtained with Tachosil treatment, but a-NFAH showed no significant differences with Hemopatch; secondly, Tachosil produced the lowest IL-1β level with no significant differences with NFAH treatments; and finally, Hemopatch group had the lowest TNF-α levels with no significant differences with a-NFAH.

Although results showed higher level of CRP in c-NFAH with respect to a-NFAH, probably due to the presence of cells that caused higher immunity response, IL-1β and TNF-α levels were not significantly different.

In conclusion, a-NFAH treatment had at least no higher inflammatory levels with respect to commercially available hemostatic agents included in this study. These findings indicate that a-NFAH is a safe product with no higher inflammatory response. In general, NFAH treatments showed better histological results than commercially hemostatic agents under study. In 1.5 cm hepatic resections, the best hemorrhage, inflammation and necrosis results were obtained by a NFAH treatment. Only c-NFAH had higher presence of neutrophil granulocytes and necrosis in 0.5 cm hepatic resection.

On the basis of the above results, NFAH (a-NFAH as well as c-NFAH) is described in the present invention, for the first time, as a hemostatic agent and tissue sealant, for use as an adjunct to hemostasis or primary treatment in surgical procedures and in treatment of traumatic wounds. Such results can be extrapolated to agarose hydrogels in general for use as an adjunct to hemostasis or primary treatment in surgical procedures and in treatment of traumatic wounds. It is noted, that agarose is thermo-reversible and can be modified to melt and gel at a variety of temperatures. This allows the gel to be used in myriad applications across fields as diverse as the food industry, molecular biology, cell biology, and tissue engineering. Agarose hydrogels are preferably understood in the context of the present invention as thermosensitive hydrogels that have a melting temperature at around 40ºC and a gelling temperature at approximately 32ºC, making this hydrogel suitable for *in vitro* and *in vivo* applications. Because of this thermal gelation properties, agarose has the added advantage or requiring no additional crosslinking step.

It is noted that NFAH can be made as described in any of the protocols reported in the prior art (Alaminos, M., Sanchez-Quevedo, M.C., Munoz-Avila, J.I., Serrano,D., Medialdea, S., Carreras, I., Campos, A., 2006. Constructionof a complete rabbit cornea substitute using a fibrin–agarosescaffold. Invest. Ophth. Vis. Sci. 47, 3311–3317; Carriel V, Alaminos M, Garzon Iet al . 2014a; Tissue engineering of the peripheral ner-vous system.Expert Rev Neurother14 : 301– 318; Carriel V, Garrido-Gomez J, Hernandez-Cortes Pet al . 2013; Combination of fibrin– agarose hydrogels and adipose-derived mesenchymal stem cells for pe-ripheral nerve regeneration. J Neural Eng 10 : 026022; Carriel V, Garzon I, Jimenez JMet al .2012; Epithelial and stromal developmental patterns in a novel substitute of the human skin generated withfibrin– agarose bioma-terials.Cells Tissues Organs196:1– 12; Garzon I, Martin-Piedra MA, Alfonso-Rodriguez Cet al . 2014; Generation of a biomimetic human artifi cial cornea model using Wharton' s jelly mesenchymal stem cells.Invest Ophthalmol Vis Sci55 : 4073– 4083; Scionti G, Moral M, Toledano Met al .2013; Effect of the hydration on the biomechani-cal properties in afibrin– agarose tissue-like model.J Biomed Mater Res A102 (8): 2573– 2582) or as described in the examples illustrated in the present invention.

Particularly, and as shown in figure 13, for cellularized NFAH fabrication, human fibroblasts are previously thawed and momentarily kept in culture media in a specific number of cells. First, a Tube 1 is prepared adding human plasma, tranexamic acid and culture media with or without cells (for acellularized NFAH (a-NFAH) manufacturing, the volume of culture media added to the mix will be

the same but does not contains cells). Once tube 1 is ready, this is kept at 37ºC. Separately, a tube 2 is prepared by addition of calcium chloride ($CaCl_2$) and Phosphate-Buffered Saline (PBS). After this, a 2.2% agarose solution is warmed at 39-41ºC and added to a tube 2 and mixed. Immediately, tube 1 solution is added to tube 2, mixed and poured on a well. After 2 hours at room temperature, the fibrin-agarose hydrogel (FAH) obtained is kept with culture media in the incubator for at least 24 hours. Nanostructuration is the last step to obtain a nanostructured fibrin-agarose hydrogel (NFAH). This process consists in a compression and dehydratation of FAH. To this end, FAH is placed between nylon net filters. Blotting filter papers are then placed on nylon filters and nanostructuration is performed by application of a flat glass with a specific weight and surface.

Other types of agarose hydrogels different from those using type VII agarose might be prepared in a similar fashion as explained above.

Therefore a first aspect of the present invention refers to a composition for use in the control of bleeding with or without compression comprising nanostructured fibrin and an agarose hydrogel, preferably a type VII agarose hydrogel, (NFAH) or a non-nanostructured or a pre-nanostructured fibrin and an agarose hydrogel, preferably a type VII agarose hydrogel, (FAH). Preferably, this composition is intended for use as biological tissue sealants and hemostats, which are, more preferably, biodegradable and non-toxic. More preferably, this composition is intended for therapeutic use, for example, as an adjunct to hemostasis in laparotomy or laparoscopic surgery, in orthopedic surgery, trauma (spleen laceration or hepatic surgery), and large-bed wounds. Still more preferably, this composition is intended for use as a general hemostatic agent candidate for use as adjunct and/or primary treatment in moderate bleeding. The composition is durable, easy to store, poses minimal risk, requires little training to use, and is highly effective against bleeding.

In a preferred embodiment of the first aspect of the invention, the composition for use is preferably cellularized nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (c-NFAH) or non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (c-FAH).

In another preferred embodiment of the first aspect of the invention, the composition for use is preferably acellularized (without cells) nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (a-NFAH) or non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (a-FAH).

The composition for use according to the first aspect of the invention, wherein for the cellularized NFAH fabrication, human fibroblasts are preferably previously thawed and kept in culture media in a specific number of cells. Then, a Tube 1 is prepared by adding fibrin, preferably human plasma and tranexamic acid, and culture media with or without cells. Once tube 1 is ready, this is preferably kept at 37ºC. Separately, a tube 2 is prepared by addition of jellification agent such as calcium chloride (CaCl2) and Phosphate-Buffered Saline (PBS). After this, a 2.2% agarose solution

is warmed at 39-41ºC and added to a tube 2 and mixed. Preferably immediately, tube 1 solution is added to tube 2, mixed and poured on a well. After approximately 2 hours at room temperature, the fibrin-agarose hydrogel (FAH) obtained is kept with culture media in the incubator for at least 24 hours. Nanostructuration is the last step to obtain a nanostructured fibrin-agarose hydrogel (NFAH). This process consists in a compression and dehydratation of FAH. To this end, FAH is preferably placed between nylon net filters. Blotting filter papers are then placed on nylon filters and nanostructuration is performed by application of a flat glass with a specific weight and surface.

The composition for use according to the first aspect of the invention, wherein for acellularized NFAH (a-NFAH) manufacturing, the volume of culture media added to the mix will be the same as the one added for the cellularized NFAH but without cells.

The composition for use according to the first aspect of the invention, wherein for acellularized FAH or cellularized FAH manufacturing, the information is clearly derivable from the above paragraphs.

EXAMPLES

Materials and Methods

Hemostatic agents

Four different hemostatic agents were used in this study: two commercially available, Hemopatch® and Tachosil®, and two home-made NFAH, a cellular NFAH (c-NFAH) and an acellular NFAH (a-NFAH).

Hemopatch (Baxter AG, Vienna, Austria) is a collagen pad derived from bovine dermis with an active side coated with a protein reactive pentaerythritol polyethylene glycol ether tera-succinimidyl glutarate (TACHOSIL, 2010)

Tachosil (Takeda Austria Gmbh) is an equine collagen sponge with an active side coated with human derived fibrinogen (5.5mg/cm2) and thrombin (2.0 IU/cm2)
NFAH is a high-density fibrin-type VII agarose hydrogel with approximately 80% of hydratation and 50-60µm thickness

Cell culture

Fibroblasts used in this study were isolated from rat tissue. Skin rat biopsies were thoroughly washed in 1X phosphate buffered saline (PBS) with an antibiotic-antimycotic cocktail solution (100 U/ml penicillin G, 100µg/ml streptomycin, 20µg/ml gentamicin and 50µg/ml vancomycin) and fragmentized in pieces of approximately 1x1mm. Approximately, twenty pieces were transferred to a 50 mL conical centrifuge tube containing 5mL of preheated collagenase solution (2 mg/mL of

collagenase (SERVA Electrophoresis Gmbh) prepared in Dulbecco´s modified Eagle´s medium (DMEM). Samples were incubated 16 hours at 37ºC.

Digestion was neutralized by addition of 25 mL culture media (DMEM supplemented with 10% fetal bovine serum, 0,1 mM non-essential amino acid, 2 mM Glutamax, 100 U/ml penicillin G and 100µg/ml streptomycin). Finally, cells suspension was centrifuged, counted, plated in a 6-well plate and grown in a humidified incubator at 5% CO2 and 37° C.

Media was changed every 2-3 days until the culture reached 70-90% confluence. At this point, cells were subsequently harvested and expanded. Finally, cells were cryopreservated and kept in liquid nitrogen until generation of hydrogel.

Inmunofluorescence

Cells were seeded onto glass coverslips (VWR) in 24 well plates (Nunc) and incubated a humidified incubator at 5% CO_2 and 37° C. Once cell culture reaches confluence, coverslips were washed with PBS and fixed with 3.7% formaldehyde (Sigma) in PBS for 15 min at room temperature. After fixing, cells were washed 3 times with PBS, permeabilized with 0.05% Triton X-100 (Sigma) for 15 min at room temperature, and blocked with PBS containing 1 % BSA for 30 minutes at 37° C. Then, cells were separately incubated for 30 minutes at 37° C with the indicated dilution of the primary antibodies. All primary antibodies were diluted in PBS containing 0,1 % BSA. Once primary antibodies were incubated, coverslips were washed 3 times with PBS and incubated for 30 minutes at 37° C with secondary antibody. Nuclei were stained with a 1 µg/ml solution of 4`,6-diamino-2-phenilindole (DAPI; Life technologies). Then, coverslips with the stained cells were mounted with Prolong mounting reagent (Life technologies). Fluorescent microscopy was performed in a TiS microscope (Nikon).

Karyotyping

G-banding Karyotyping of the fibroblast line was performed by the Andalusian Public Health System Biobank following recommendations of the International Standing Committee on Human Cytogenetic Nomenclature (2013).

Generation of NFAH pad

The protocol is an adaptation of methods reported in previous reports (Alaminos et al., 2006; Carriel et al., 2012, 2013; Scionti et al., 2013; Garzon et al., 2014; Carriel et al., 2015). In order to obtain the volume of fibrin and agarose hydrogel (FAH) for a 6 well plate, a 30 ml mixture was prepared as follows: 25 ml of human plasma, 0.5 ml of tranexamic acid and 2 ml of culture media with or without human fibroblasts were placed into a Falcon tube (cellularized solution was prepared with 150.000 thawed cells per ml of final volume). Subsequently, a solution containing 1,8 ml calcium chloride 10%, 1,2 ml PBS and 2,2% melted type VII-agarose were added. After mixing,

5 ml of solution was place into each well and kept at 37ºC for 2 hours. Once gelation was completed, FAH was covered with culture media and kept at 37ªC for 24 hours until nanostructuration. If FAH contained cells, plates remained in the incubator for 7 days.

Nanostructuration is a compression and dehydration process. To this aim, FAH was placed between a couple of 10µm nylon net filter (Merck-Millipore) and compressed by extra thick western blotting filter paper (Thermo Scientific) under a flat glass surface of 500 gr for 1 minute and 40 seconds. A final NFAH is obtained with a high-density and approximately 80% of hydration.

Cell viability analysis

LIVE/DEAD ® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probe, Invitrogen) was used to determine cell viability in NFAH. The manufacturer's protocol was adapted to this aim. Briefly, 2x2cm pieces were cut and transferred to a Petri dish. Samples were washed three times with PBS. After incubation with solution T during 30min at room temperature, samples were washed three times and kept in PBS.

The number of live (green) and dead (red) cells was determined by fluorescence microscope. A minimum of four fields for each NFAH section were counted, with at least 100 cells visualized.

Animal Protocol and Hepatic resection

Wistar male rats with a weight of approximately 250gr were used for the study. Subcutaneous injection of 80mg/kg ketamine and 10mg/kg xylazine induced anesthesia that was maintained with isoflurane inhalation. Animals underwent longitudinal cut from 1 cm below the xiphoid in the craniocaudal direction and median lobe was exposure to proceed.

Hepatic resections were performed in animals with a length of 0.5 or 1.5 cm.

All hemostatic agents applied were given a round shape of 24mm diameter and were applied on the wound surface by local compression. The time to hemostasis was defined as the arrest of free blood leakage after application of hemostatic agent.

Animals were euthanized one day after surgery to evaluate post-operative hemorrhage, presence of hematoma, migration of the hemostatic agent and intraabdominal adhesion adjacent to the wound. The grade of adhesion was determined by a score of 3 values (0: No adhesion; 1: thin adhesions separable by gravity; 2: thick adhesions no separable by gravity).

The liver was completely excised for liver section/total liver weight ratios calculation. Sections of hepatic tissue attached to the hemostatic agent were fixed in 4 % paraformaldehyde for a subsequent histological analysis.

The study was approved by the Research Ethics Committee (REC) of University Hospital Virgen Macarena and Virgen del Rocío.

ELISAS

Blood samples were collected with anticoagulant EDTA, centrifuged at 3000 rpm for 5 minutes and kept at -20ºC or -80ºC in small volumes to avoid repeated freeze-thaw cycles.

In order to measure the amount of inflammatory factors, ELISA kits were used following manufacturer's instructions: C Reactive Protein (CRP) (RayBio® Rat CRP ELISA Kit), Interleukin 1 beta (Rat IL-1β ELISA Kit- Elabscience®) y (Rat TNF-α ELISA Kit-CUSABIO®).

Microscopic analysis

After fixation with 4% paraformaldehyde, paraffin-embedded blocks of liver tissue were cut at 4 micrometres thickness and sections underwent Masson-Trichrome and hematoxylin-eosin staining (Hematoxylin solution-Sigma Aldrich GHS316-500ML; Alcoholic Eosin Y solution- Sigma Aldrich HT110116-500ML; Trichrome Stain (Masson) Kit-Sigma Aldrich HT15-1KT; and Weigert's iron hematoxylin solution HT1079-1set). Sectioned liver samples were analyzed in triplicate in different days.

Histological variables were studied by microscopic analysis and categorized as follow (Figure 11):

- Hemorrhage: Absent (groups of erythrocytes that come together without forming a free zone of hemorrhage); Mild (area of hemorrhage < 1 mm diameter); Moderate (area of hemorrhage 1-2,5 mm diameter and less than x 40 high power fields (hpf) of depth) ; and Intense (area of hemorrhage >2,5 mm diameter or more than x 40 high power fields of depth).
- Inflammation: Absent, Mild, Moderate and Intense. Classification was done based on the presence of neutrophil granulocytes per x40 high power field.
- Necrosis: Absent, Mild (0,1-0,4 hpf), Moderate (0,5-1,2 hpf) and Intense (>1,2 hpf).

Statistics

All statistical analyses were performed using GraphPad Prism 5.0 software.

Results

Fabrication of c-NFAH and a-NFAH

Rat fibroblasts were isolated from skin biopsies by collagenase-based technique. Isolated cells showed vimentin expression and normal karyotype (Figure 1) as expected for healthy proliferating fibroblasts.

Rat fibroblasts were used for production of c-NFAH (see experimental procedures for details).

NFAH showed increased hemostatic effect in a model of hepatic resection

This study initially started comparing the hemostatic effect of cellularized NFAH (c-NFAH) versus Hemopatch in a model of 0.5 cm hepatic resection. C-NFAH showed higher hemostasis effectiveness than Hemopatch in 0.5 cm resection ($p<0.05$) with a mean time to hemostasis equal to 2 sec (N=10, SEM=2, Range= 0-20) and 8.4 sec (N=10, SEM=2,6, Range= 5-20) respectively (Figure 2A).

Subsequently, the experiment was expanded to a 1.5 cm hepatic resection that included another two hemostatic agents, acellularized NFAH (a-NFAH) and Tachosil. In the case of 1.5 cm hepatic resection, a-NFAH had the lowest time to hemostasis in the group with 2.4 seconds (N=10, SEM=0.58, Range= 1-5), closed to c-NFAH with 2.9 seconds (N=10, SEM=1.72, Range= 1-7), both having significantly greater hemostatic success when compared to Hemopatch (9.8 seconds (N=15, SEM=1.02, Range= 3-30)) and Tachosil (11.2 seconds (N=10, SEM=0.49, Range= 3-17)) (Figure 2B).

Results showed similar time to hemostasis between 0,5 cm and 1,5 cm hepatic resection for c-NFAH and Hemopatch treatments (Figure 3).

NFAH showed easier applicability

Hemopatch and Tachosil were applied by manual compression as indicated by manufacturers. On the contrary, NFAHs were placed on the wound without applying any compression. This application procedure was enough to reach hemostasis with NFAH treatment (Figure 4).

NFAH showed lower adhesion and hematomas and no post-surgical rebleeding nor migration

No presence of hematoma was found in animals treated with c-NFAH in 0,5 cm hepatic resections. By contrast, Hemopatch showed a 40% (Figure 5 A).

In 1,5 cm hepatic resection, C-NFAH and a-NFAH showed no incidences of hematoma while Hemopatch and Tachosil had 73.3% and 40% respectively (Figure 5 B and 7C-D).

Undesired adhesion to tissues was estimated on the sacrifice. Results showed significant differences among the groups. C-NFAH and a-NFAH had similar results with most of the animal showing thin adhesion. However, Hemopatch and Tachosil showed more evident adhesion to adjacent organs (Table 1 and Figures 6 and 7A-B).

TABLE 1. Effectiveness of fibrin-agarose hydrogels in preventing peritoneal adhesions in rats

	a-NFAH	c-NFAH	Hemopatch®	Tachosil®
Score 2	0	1	11	5
Score 1	8	8	4	5
No adhesion	2	1	0	0
N	10	10	15	10

Table 1: Number of rats by treatment group and grade of adhesion. A-NFAH and c-NFAH showed similar lower adhesion relative to Hemopatch and Tachosil. The grade of adhesion was determined by a score of 3 values (No adhesion: separate anatomical structures; Score 1: thin adhesions separable by gravity; Score 2: thick adhesions no separable by gravity).

After 24 hours of hemostasis achievement, no presence of rebleeding was found in any treated group (Figure 8) and only one Tachosil pad seemed to migrate in a rat (probably was moved during suture).

NFAH showed no higher inflammatory response than Hemopatch and Tachosil

The levels of inflammatory factors for 0,5 cm and 1,5 cm hepatic resections are shown in Figure 9 and 10 respectively. A minimum of six animals were considered in each treated group.

NFAH treatments had better results in histological analysis for longer hepatic resections

Variables of microscopic analysis were categorized as previously indicated in materials and methods (Figure 11 shows examples of classification). Histological analysis of 0,5 cm hepatic resections showed lower presence of hemorrhage in c-NFAH treatment. However, inflammatory response and necrosis was higher than Hemopatch treatment (Figure 12 A-C). These results differ with 1,5 cm hepatic resections which showed lower inflammatory response and necrosis in NFAH treatments. Moreover, c-NFAH treatment had the lowest presence of hemorrhage (Figure 12 D-F).

CLAIMS

1. A composition for use in the control of bleeding with or without compression, whrein the composition comprises nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (NFAH) or non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (FAH).

2. The composition for use in the control of bleeding according to claim 1, wherein the composition is for use as biological tissue sealants and/or hemostats.

3. The composition for use in the control of bleeding according to claim 1, wherein the composition is for use as an adjunct to hemostasis in laparotomy or laparoscopic surgery, in orthopedic surgery, trauma (spleen laceration or hepatic surgery), or large-bed wounds.

4. The composition for use in the control of bleeding according to claim 1, wherein the composition is for use as adjunct and/or primary treatment in moderate bleeding.

5. The composition for use according to any of claims 1 to 4, wherein the composition is cellularized nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (c-NFAH).

6. The composition for use according to any of claims 1 to 4, wherein the composition is cellularized non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (c-FAH).

7. The composition for use according to any of claims 1 to 4, wherein the composition is acellularized (without cells) nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (a-NFAH).

8. The composition for use according to any of claims 1 to 4, wherein the composition is acellularized (without cells) non-nanostructured or pre-nanostructured fibrin and agarose hydrogels, preferably type VII agarose hydrogels, (a-FAH).

FIGURES

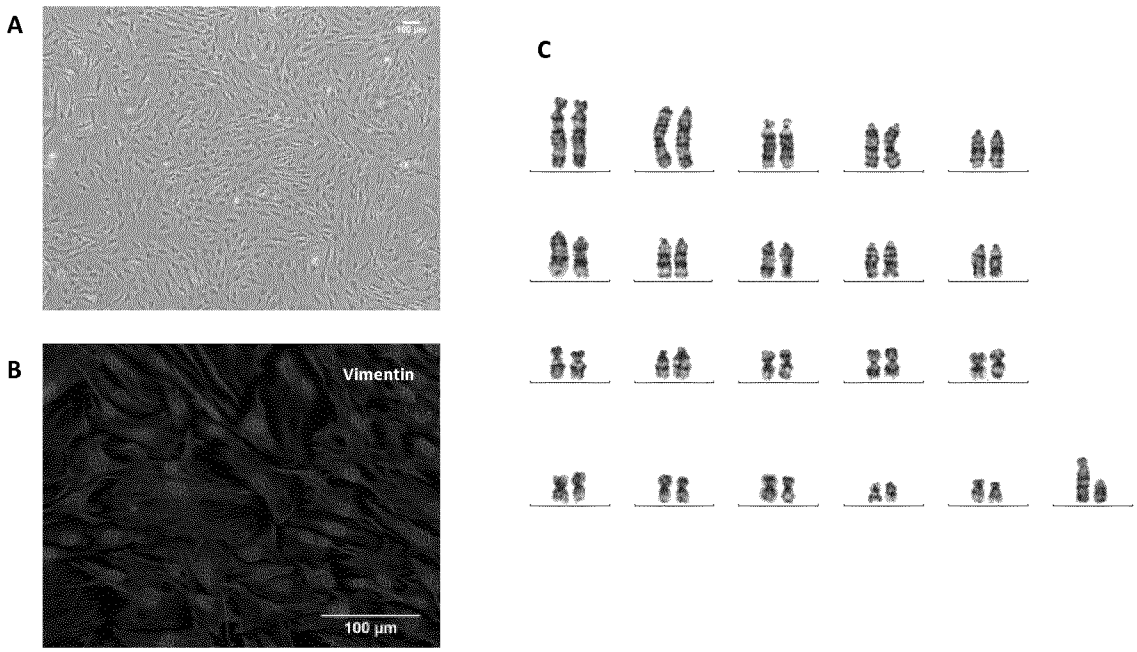


Fig. 1

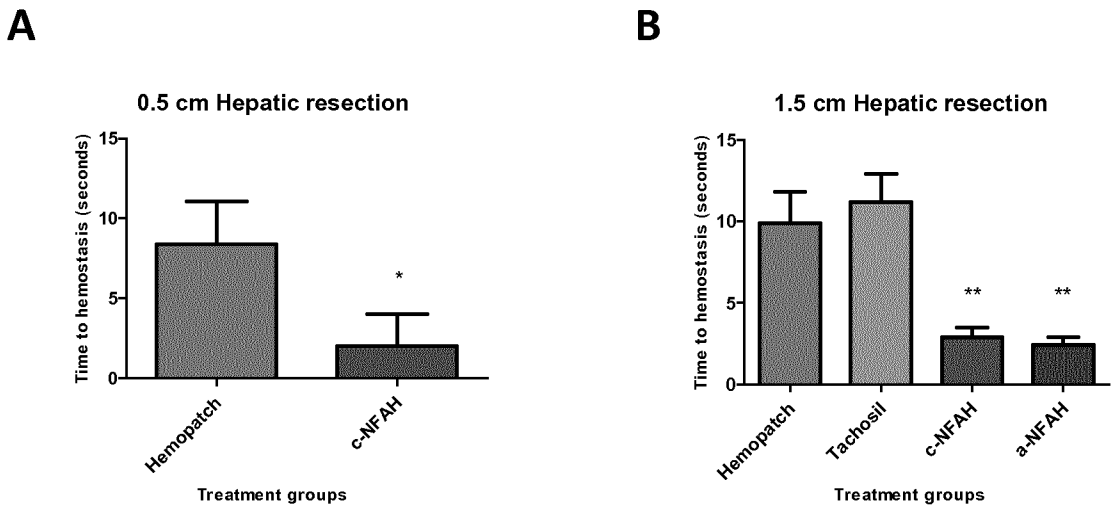


Fig. 2

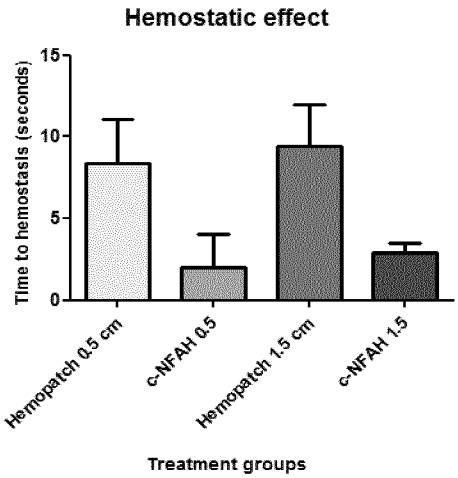

Hemostatic effect
Time to hemostasis (seconds)
15
10
5
0
Hemopatch 0.5 cm
c-NFAH 0.5
Hemopatch 1.5 cm
c-NFAH 1.5
Treatment groups

Fig. 3

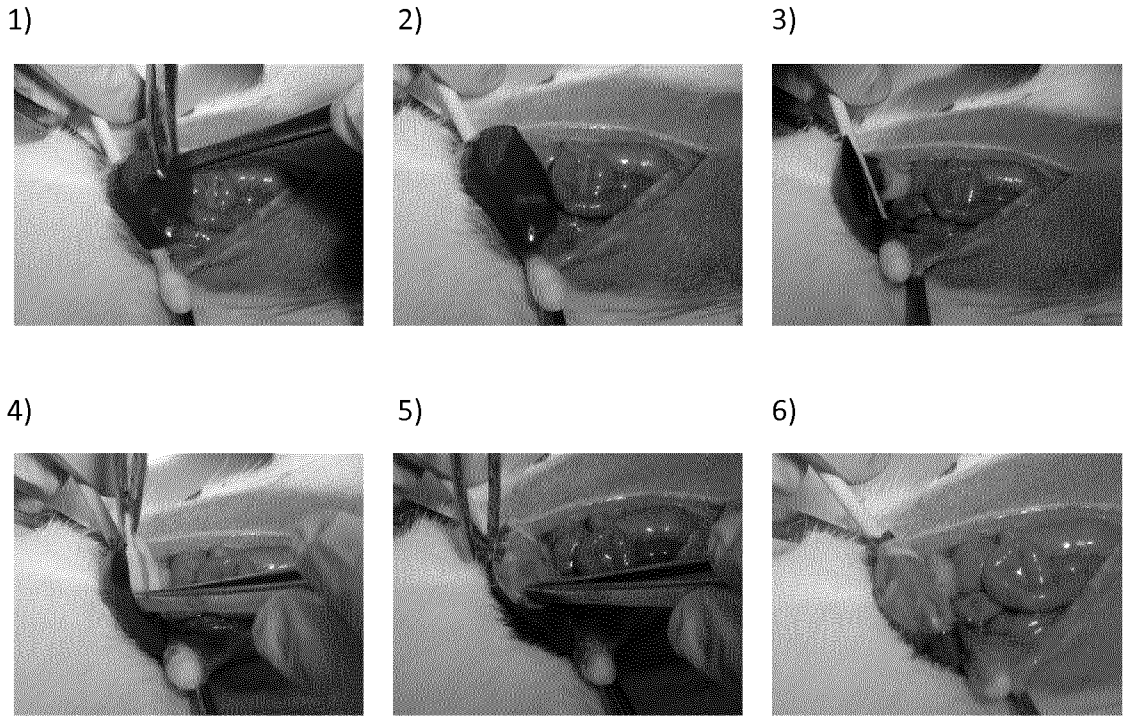

1)
2)
3)
4)
5)
6)

Fig. 4

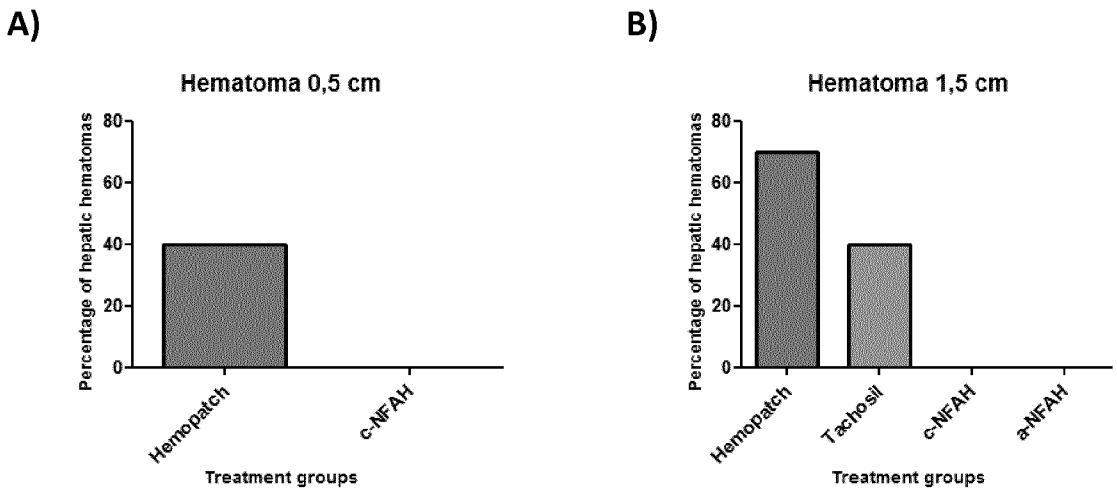

A)
Hematoma 0,5 cm
Percentage of hepatic hematomas
0
20
40
60
80
Hemopatch
c-NFAH
Treatment groups
B)
Hematoma 1,5 cm
Percentage of hepatic hematomas
0
20
40
60
80
Hemopatch
Tachosil
c-NFAH
a-NFAH
Treatment groups

Fig. 5

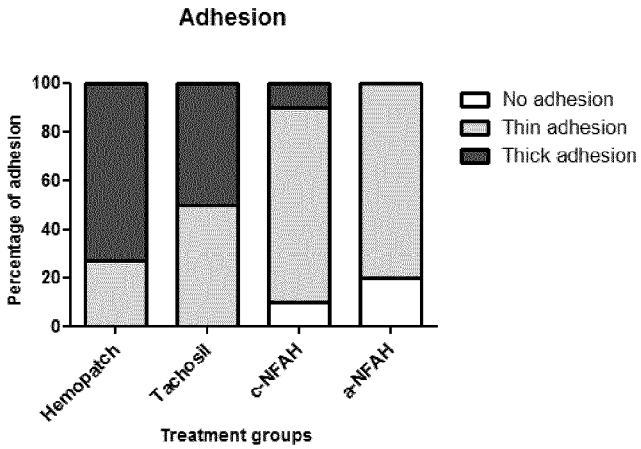

Adhesion
Percentage of adhesion
0
20
40
60
80
100
Hemopatch
Tachosil
c-NFAH
a-NFAH
Treatment groups
No adhesion
Thin adhesion
Thick adhesion

Fig. 6

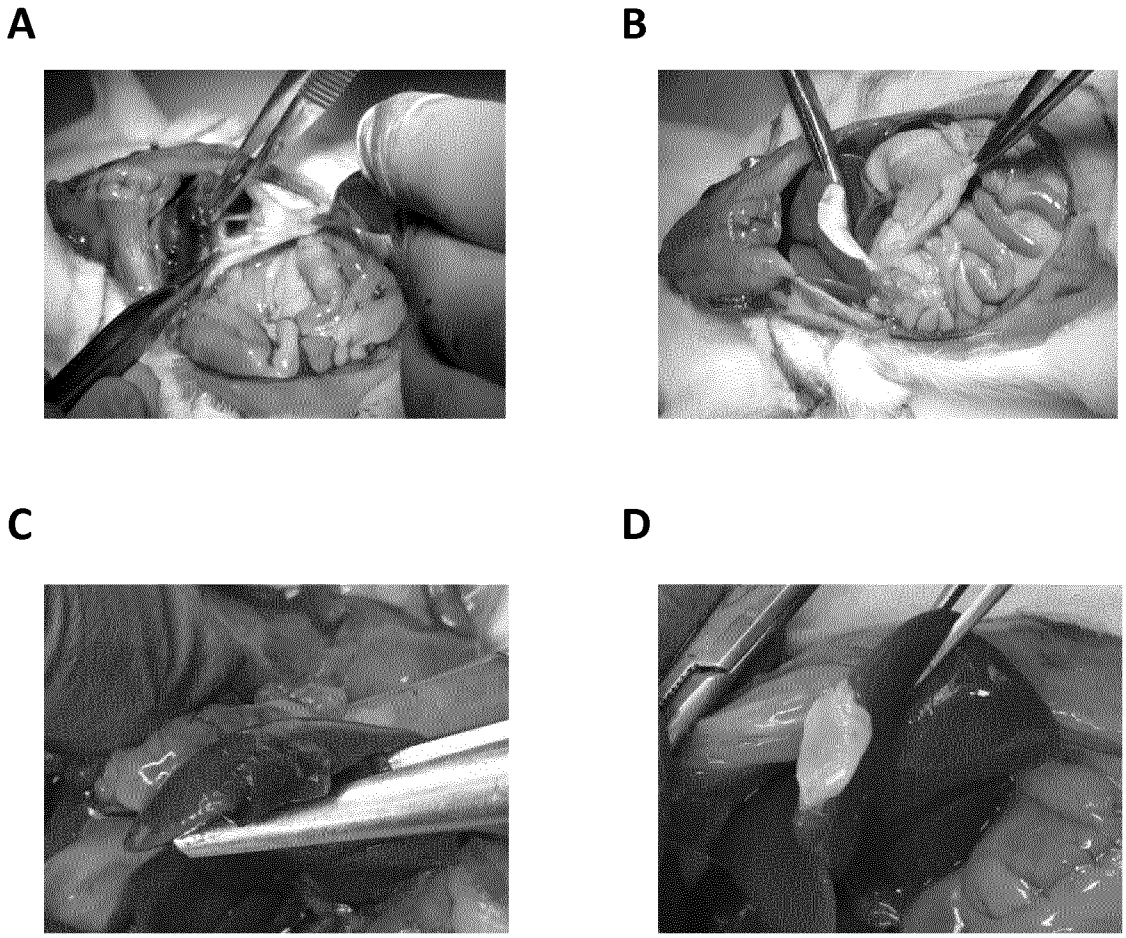


Fig. 7

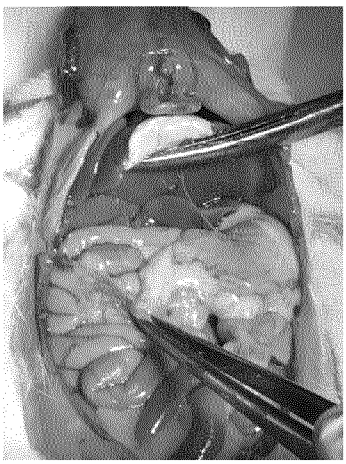
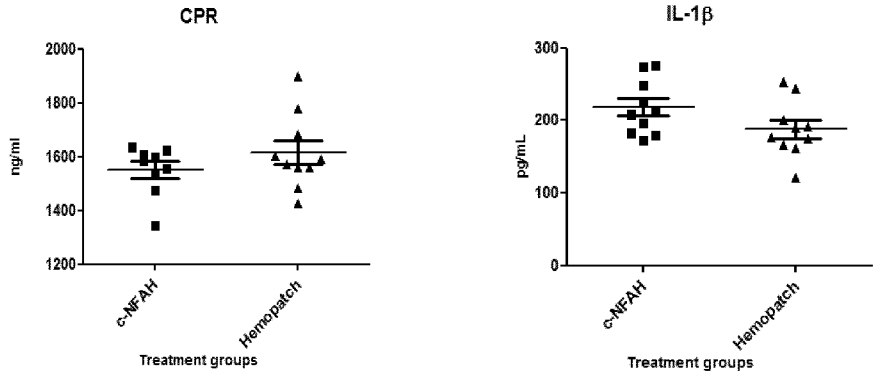


Fig. 8

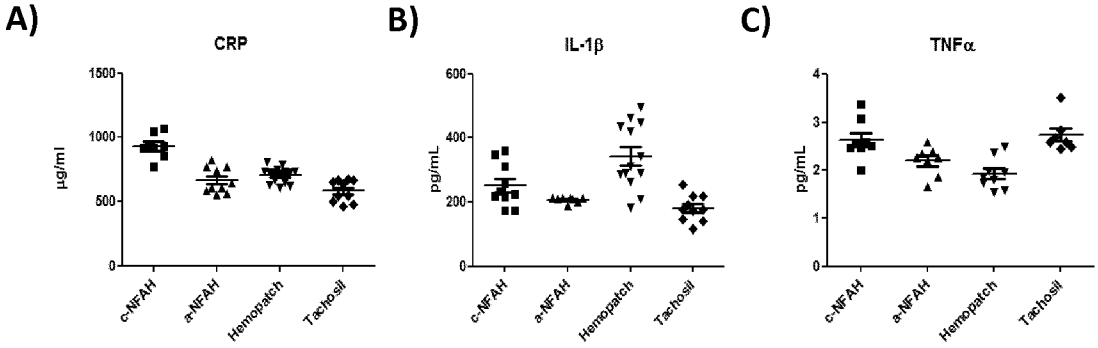
Inflammatory factors in 0.5 cm hepatic resection



	CRP	IL-1B
Normality	Non parametric data	Non parametric data
Statistic Test	Mann-Whitney	Mann-Whitney
P Value	p = 0,549	p = 0,0892
	Are medians signif. different? (P < 0.05) No	Are medians signif. different? (P < 0.05) No

Fig. 9

Inflammatory factors in 1.5 cm hepatic resection



	CRP	IL-1B	TNFa
Normality	Parametric data	Non parametric data	Non parametric data
Statistic Test	Newman-Keuls	Kruskal-Wallis-Dunn's Multiple Comparation	Kruskal-Wallis-Dunn's Multiple Comparation
P Value	p < 0.0001	p = 0.0002	p < 0.0001

	Significance		Significance		Significance
Tachosil vs c-NFAH	***	c-NFAH vs a-NFAH	ns	c-NFAH vs a-NFAH	ns
Tachosil vs Hemopatch	**	c-NFAH vs Hemopatch	Ns	c-NFAH vs Hemopatch	**
Tachosil vs a-NFAH	*	c-NFAH vs Tachosil	Ns	c-NFAH vs Tachosil	ns
a-NFAH vs c-NFAH	***	a-NFAH vs Hemopatch	*	a-NFAH vs Hemopatch	ns
a-NFAH vs Hemopatch	ns	a-NFAH vs Tachosil	Ns	a-NFAH vs Tachosil	ns
Hemopatch vs c-NFAH	***	Hemopatch vs Tachosil	***	Hemopatch vs Tachosil	*

Fig. 10

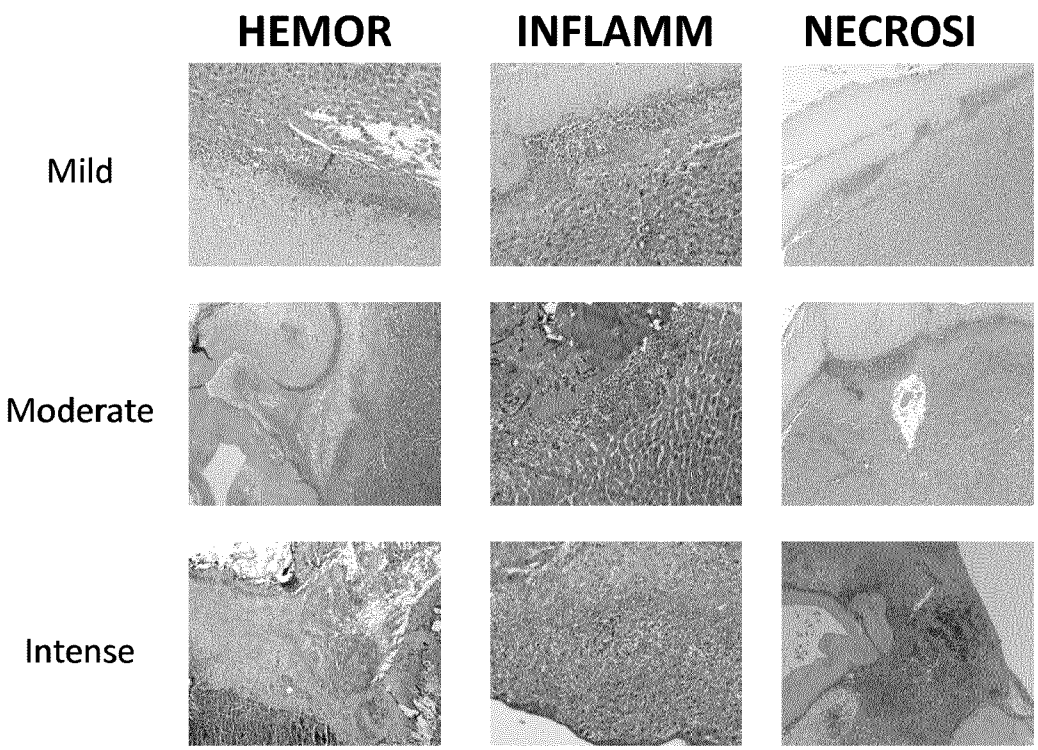


Fig. 11

Histological analysis of 0,5 cm hepatic resection

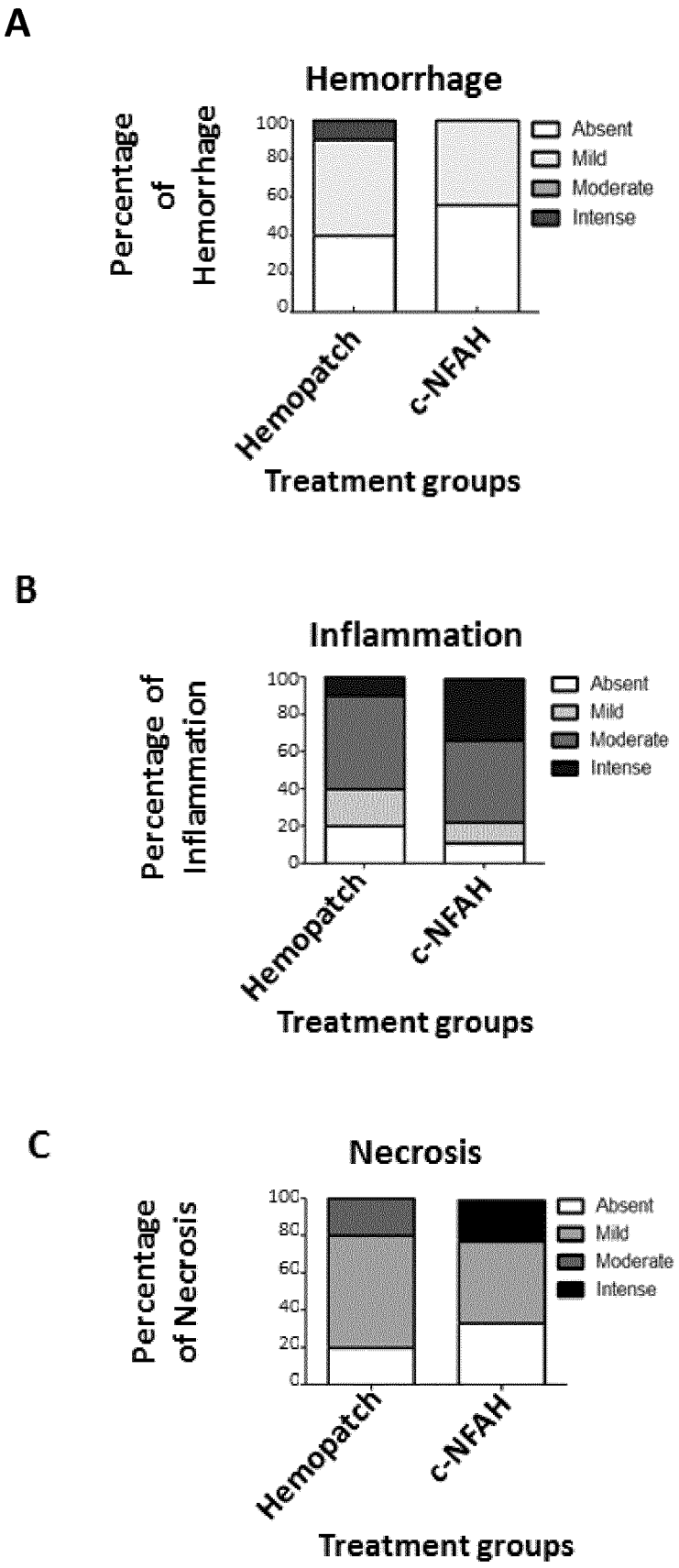


Fig. 12

Histological analysis of 1,5 cm hepatic resection

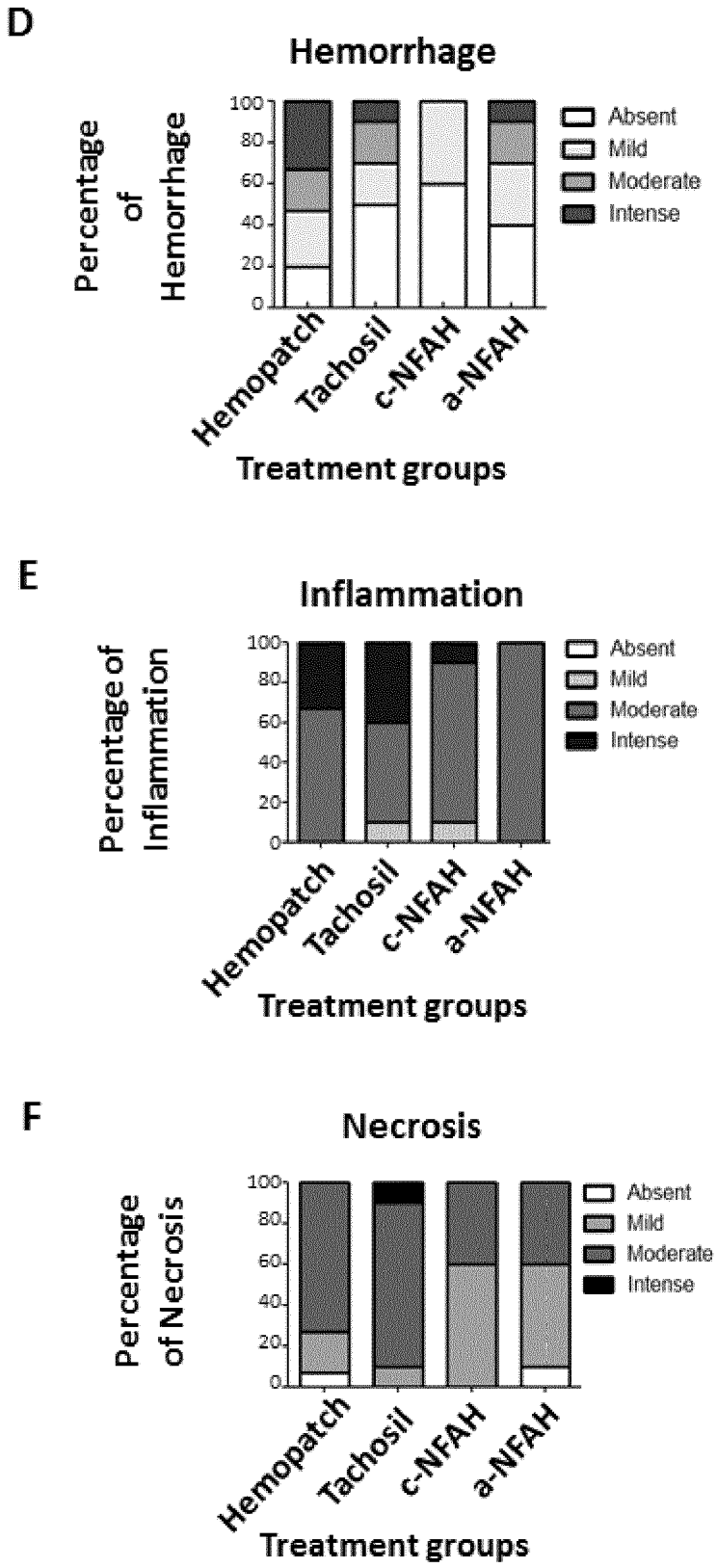


Fig. 12 (Cont.)

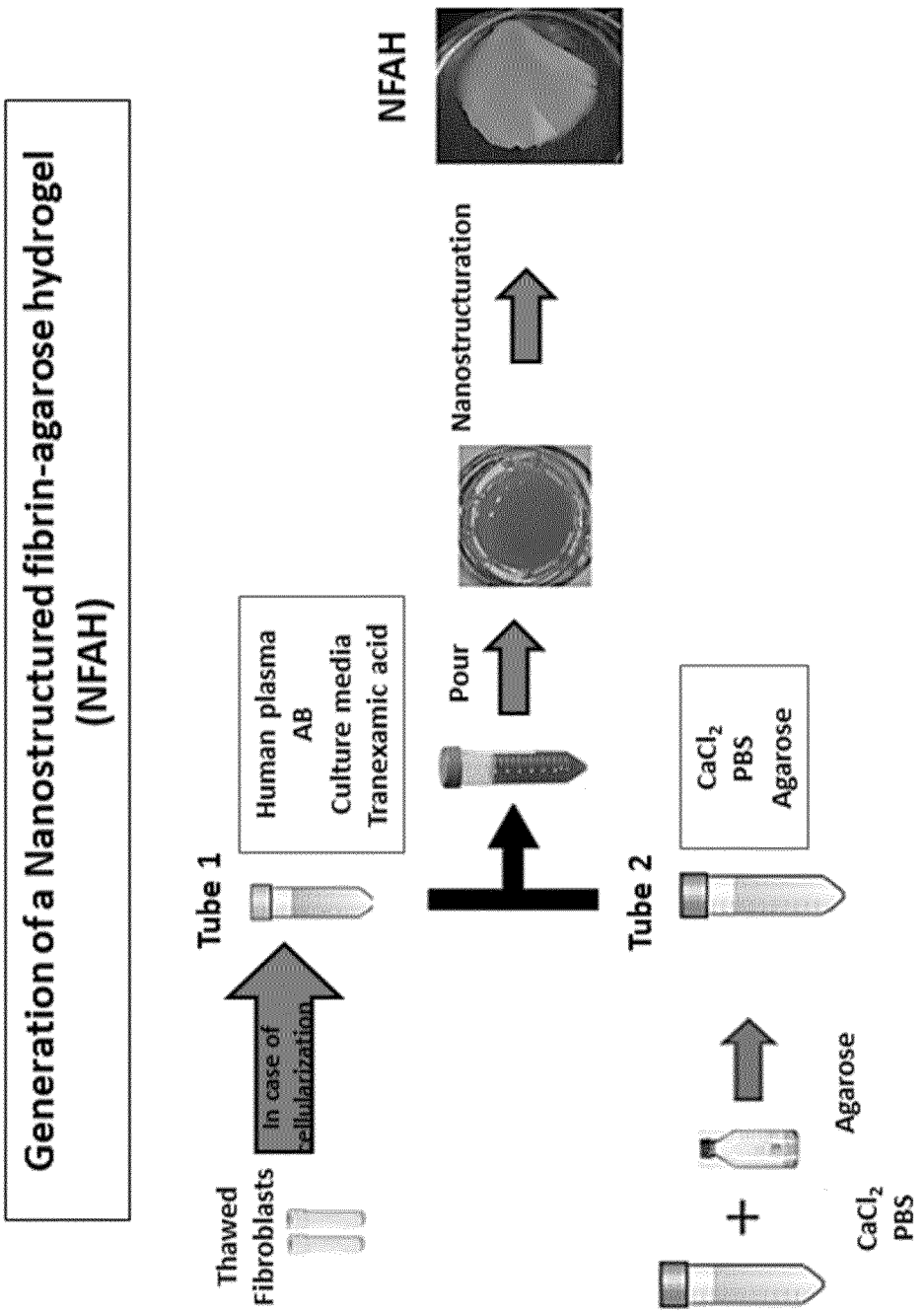

Generation of a Nanostructured fibrin-agarose hydrogel (NFAH)
Thawed Fibroblasts
In case of cellularization
Tube 1
Human plasma AB
Culture media
Tranexamic acid
Pour
Tube 2
$CaCl_2$ PBS Agarose
Agarose
$CaCl_2$ PBS
Nanostructuration
NFAH

Fig.13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058822

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L26/00 A61L31/04 A61L31/14 A61L24/00 A61L24/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/123346 A1 (FALUS GEORGE D [US]) 6 October 2011 (2011-10-06) page 2, paragraph Second - page 5, last paragraph claims; examples	1-8
A	I.GALANAKIS ET AL.: "A REVIEW OF CURRENT HEMOSTATIC AGENTS AND TISSUE SEALANTS USED IN LAPAROSCOPIC PARTIAL NEPHRECTOMY", REVIEWS IN UROLOGY, vol. 13, no. 3, 2011, - 2011, pages 131-138, XP002793237, the whole document	1-8
A	EP 2 471 902 A2 (SERVICIO ANDALUZ DE SALUD [ES]; UNIV GRANADA [ES]) 4 July 2012 (2012-07-04) the whole document	1-8

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 2019	09/08/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Espinosa y Carretero

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058822

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FERNANDO CAMPOS ET AL: "characterization of a novel tissue-like cross-linked fibrin-agarose hydrogel for tissue engineering applications", BIOMEDICAL MATERIALS, INSTITUTE OF PHYSICS PUBLISHING, BRISTOL, GB, vol. 11, no. 5, 29 September 2016 (2016-09-29), page 55004, XP020309218, ISSN: 1748-605X, DOI: 10.1088/1748-6041/11/5/055004 [retrieved on 2016-09-29] abstract -----	1-8
X,P	R. CAMPOS CUERVA ET AL.: "NANOSTRUCTURED FIBRIN AGAROSE HYDROGEL AS A NOVEL HAEMOSTATIC AGENT.", JOURNAL OF TISSUE ENGENEERING AND REGENERATIVE MEDICINE, vol. 13, 22 February 2019 (2019-02-22), - 22 February 2019 (2019-02-22), pages 664-673, XP002793238, DOI: 10.1002/term.2831 the whole document -----	1-8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/058822

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011123346 A1	06-10-2011	AU 2011232907 B2	17-09-2015
		CA 2829787 A1	06-10-2011
		CN 103037783 A	10-04-2013
		EP 2552326 A1	06-02-2013
		KR 20130009826 A	23-01-2013
		US 2011066182 A1	17-03-2011
		WO 2011123346 A1	06-10-2011
EP 2471902 A2	04-07-2012	DK 2471902 T3	06-08-2018
		EP 2471902 A2	04-07-2012
		ES 2677945 T3	07-08-2018
		JP 6010460 B2	19-10-2016
		JP 2013502915 A	31-01-2013
		JP 2016165280 A	15-09-2016
		TR 201809713 T4	23-07-2018
		US 2012269776 A1	25-10-2012
		WO 2011023843 A2	03-03-2011