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Immunohistochemical analysis of the inflammatory reaction within the implantation bed of a collagen hemostatic biomaterial

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Table of contents

1. Zusammenfassung

Aktuelle Forschungen zu medizinischen Biomaterialien haben gezeigt, dass die entzündliche Zellreaktion des Körpers nach der Implantation eines Biomaterials durch die Biomaterialieneigenschaften stark beeinflusst wird. Das Ziel der Arbeit war es zu untersuchen, welche Auswirkung die Änderung einer Materialeigenschaft eines spezifischen hämostatischen Kollagenschwamms equinen Ursprungs (E-CHS), also vom Pferd stammenden Materials, auf die initiale Biomaterial-Zell-Interaktion und die Biomaterial-induzierte Entzündungsreaktion hat. Zu diesem Zweck wurde der hämostatische equine Kollagenschwamm durch Pressen (P-E-CHS) modifiziert und unter Verwendung von *ex vivo-, in vitro*- und *in vivo*-Methoden bewertet. Das E-CHS wurde durch 2-minütiges Anlegen eines konstanten Drucks (6,47 ± 0,85 N) unter Verwendung eines sterilen Edelstahlzylinders gepresst und in Segmente von 1 cm^2 geschnitten. Anschließend wurden E-CHS und P-E-CHS als zwei unabhängige Biomaterialien untersucht und mit einer Kontrollgruppe (CG) verglichen.

In *ex vivo* und *in vitro* Untersuchungen wurde ein Blutkonzentrat namens Plättchen-reiches Fibrin (PRF), das Entzündungszellen (Leukozyten) und Plättchen enthielt, verwendet. *Ex vivo* wurden mittels PRF die initiale Wechselwirkung zwischen Biomaterial und Zellen nachgeahmt und die Absorptionskoeffizienten der Biomaterialien gegenüber flüssigem PRF (iPAC) gemessen. *In vitro* wurden die Biomaterialien mit PRF beschichtet und 3 bis 6 Tage kultiviert, um die Induktion von proinflammatorischen Zytokinen (TNF-α und IL-8) zu messen. Zur Anwendung kamen dabei der sogenannte *enzym-linked immuno sorbent Assay* (ELISA) sowie diverse histologische Methoden. PRF kultiviert ohne Biomaterialien diente hierbei als Kontrolle (CG). In den *in vivo* Untersuchungen wurden die Biomaterialien im subkutanen Modell der Ratte implantiert und mit scheinoperierten Tieren (CG) verglichen, die eine physiologische Wundheilung darstellen. Nach 3, 15 und 30 Tagen wurden die explantierten Proben histochemisch sowie immunohistochemisch (IHC) auf Zellen und Strukturen mit positiver Expression von CD68 (Pan-Makrophagen), CCR7

(proinflammatorische Makrophagen, M1), CD206 (pro-Wundheilungsmakrophagen, M2) und α-Smooth Muscle Actin (α-SMA; Gefäßidentifikation) analysiert.

Nach 15-minütiger Inkubation von flüssigem PRF mit beiden Biomaterialien zeigten die *ex-vivo*-Ergebnisse, dass E-CHS von den Zellen durchdrungen wurde, während P-E-CHS zellokklusiv war. Obwohl das Biomaterial gepresst wurde, zeigte die Differenz des iPAC-Wertes keine statistischen Unterschiede. *In vitro* induzierte P-E-CHS nach 3 Tagen eine höhere Freisetzung von proinflammatorischen Zytokinen im Vergleich zu flüssigem PRF allein (CG) und E-CHS (P <0.05). *In vivo* induzierte die CG am Tag 3 eine höhere Entzündungsreaktion im Vergleich zu den Versuchsgruppen (EG) (P <0.05). Der Vergleich zwischen den Gruppen zeigte, dass P-E-CHS eine höhere Anzahl von Makrophagen (CD68+ / CC7+) im Vergleich zu E-CHS am Tag 3 induzierte (P <0.05). Es wurden nur mononukleäre CD68+ / CCR7+ Zellen (MNCs) ohne mehrkernige Riesenzellen (MNGCs) beobachtet. Nach 15 Tagen nahm die Anzahl der Makrophagen (CD68+ P <0.01 / CCR7+ P <0.001 / CD206+ P <0.05) in der CG erheblich ab. Im Gegenzug nahm die Entzündungsreaktion in den EGs zu (CD68+ / CCR7+). Der Intergruppenvergleich zeigte, dass dieses Inkrement statistisch signifikant war, wenn E-CHS und P-E-CHS am Tag 15 mit der CG verglichen wurden (P <0.01 und P <0.05). Zu diesem Zeitpunkt wurde eine verringerte Anzahl von MNGCs in den EGs beobachtet. In der CG wurden keine MNGCs beobachtet. Darüber hinaus zeigte E-CHS eine schnellere Abbaurate und wurde vollständig von Zellen und Gefäßen besiedelt, die sich in seiner inneren Region gebildet hatten. Andererseits blieb P-E-CHS für die Zellpenetration okklusiv und Gefäße wurden nur in der Peripherie gebildet. Nach 30 Tagen verlagerte sich die zelluläre Reaktion in allen Gruppen auf eine höhere Anzahl von M2-Makrophagen (CD260+) und verringerte das Vorhandensein von CD68-positiven und CCR7-positiven MNCs. Beide Biomaterialien wurden nach 30 Tagen abgebaut und nur kleine Fragmente wurden im Implantationsbett gefunden, das von MNGCs (CCR7+) umgeben war.

Diese Ergebnisse sind von hoher klinischer Relevanz da sie zeigen, dass Änderungen der Biomaterialeigenschaften erhebliche Einflüsse auf ihre Interaktion mit dem Körper haben. Sie dienen auch als Einblick in die Möglichkeit, vielseitige Biomaterialien mit unterschiedlichen Anwendungen zu entwickeln. Zum Beispiel können E-CHs

angewendet werden, um die Blutstillung im Alveolarfortsatz zu unterstützen, und P-E-CHs können angewendet werden, indem ihre Zellokklusivität und verzögerte Abbaurate genutzt wird, um die Knochen- und Geweberegeneration gezielt zu steuern.

2. Abstract

Current research on medical biomaterials have shown that the physical and chemical characteristics of biomaterials determine the body inflammatory cellular reaction after their implantation. The aim of this study was to evaluate the individual effects of the physical characteristics over the initial biomaterial-cellular interaction and the inflammatory cellular reaction. For this purpose, an equine-derived collagen hemostatic sponge (E-CHS) was modified by pressing and evaluated using *ex vivo, in vitro* and *in vivo* methods.

The E-CHS was pressed by applying constant pressure (6.47± 0.85 N) for 2 min using a sterile stainless-steel cylinder and cut in segments of 1cm^2. Subsequently, E-CHS and the pressed equine-derived collagen hemostatic sponge (P-E-CHS) were studied as two independent biomaterials and compared to a control group (CG).

A blood concentrate containing inflammatory cells known as platelet rich fibrin (PRF) was used to mimic the initial biomaterial-cell interaction and to measure the absorption coefficient of the biomaterials to liquid PRF (iPAC). Additionally, the biomaterials were cultivated together with PRF for 3 and 6 days to measure the induction of pro-inflammatory cytokines (TNF-α and IL-8). The results were obtained through enzyme-linked immunosorbent assay (ELISA) and histological methods. PRF cultivated without biomaterials served as the CG. Additionally, the biomaterials were evaluated *in vivo* using a subcutaneous model in Wistar rats and compared to sham operated animals (CG) representing physiologic wound healing. After 3, 15 and 30 days, the explanted samples were evaluated using histochemical and immunohistochemical (IHC) staining using the following markers: CD68 (pan macrophages), CCR7 (pro-inflammatory macrophages, M1), CD206 (pro-wound healing macrophages, M2) and α-Smooth Muscle Actin (α-SMA; vessel identification).

After the mixture of liquid PRF with both biomaterials for 15 minutes, the *ex vivo* results showed that E-CHS was penetrated by cells, whereas P-E-CHS was cell-occlusive. Additionally, P-E-CHS induced a higher release of pro-inflammatory cytokines compared

to liquid PRF alone (CG) and E-CHS after 3 days (P< 0.05). Although the biomaterial was pressed, the difference of the iPAC value did not show statistical differences. *In vivo*, the CG induced at day 3 a higher inflammatory response compared to the experimental groups (EG) (*P*< 0.05). The intergroup comparison showed that P-E-CHS induced a higher presence of macrophages (CD68+/CC7+) compared to E-CHS at day 3 (*P*< 0.05). Only CD68+/CCR7+ mononuclear cells (MNCs) were observed without multinucleated giant cells (MNGCs). After 15 days, the presence of macrophages (CD68+ *P*<0.01 /CCR7+ *P*<0.001 /CD206+ *P*<0.05) reduced considerably in the CG. On the contrary, the inflammatory response increased in the EGs (CD68+/CCR7+). The intergroup comparison showed that this increment was statistically significant when comparing E-CHS and P-E-CHS to the CG at day 15 (*P*<0.01 and *P*< 0.05 respectively). At this time point, a reduced number of MNGCs were observed in the EGs. In the CG no MNGCs were observed. Furthermore, E-CHS showed a faster degradation rate and was fully invaded by cells and vessels formed in its interior region. On the other hand, P-E-CHS remained occlusive to cell penetration and vessels were formed only in the periphery. After 30 days, the cellular reaction shifted to a higher number of M2 macrophages (CD260+) in all groups and a reduced presence of CD68+ and CCR7+ MNCs. Both biomaterials degraded and only small fragments were found in the implantation bed surrounded by MNGCs (CCR7+).

These results are of high clinical relevance and show that changes in biomaterial properties have a significant impact on their interaction with the body. They also serve as insight into the possibility to develop versatile biomaterials with different applications. For example, E-CHs can be applied to support hemostasis in a bleeding alveolar socket and P-E-CHs by being cell occlusive and having a delayed degradation rate can be applied for guided bone and tissue regeneration.

3. List of abbreviations

ANOVA	Analysis of variance
CCR7	C-C chemokine receptor type 7
CD206	Cluster of difference 206
CG	Control group
CT	Connective tissue
E-CHS	Equine-derived collagen hemostatic sponge
GBR	Guided bone regeneration
GTR	Guided tissue regeneration
H&E	Hematoxylin and eosin
HIER	Heat-induced epitope retrieval
IFN-γ	Interferon gamma
IHC	Immunohistochemically
IL	Interleukin
iPAC	Liquid PRF absorption coefficient
LSCC	Low speed centrifugation concept
MNGC	Multinucleated giant cells
MNC	Mononuclear cells
N	Newton
P-E-CHS	Pressed equine-derived collagen hemostatic sponge
PRF	Platelet rich fibrin
SL	Sponge layer
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
WAC	Water absorption coefficient
α-SMA	α-Smooth Muscle Actin

4. Comprehensive summary of the study

4.1 Introduction

The use of collagen biomaterials is widely spread because of its natural abundance and biocompatibility[1]. Despite the ubiquity of collagen in its original form, it has been shown that the source of extraction (avian, porcine, bovine, equine) and the manufacturing processes modify the resultant physicochemical characteristics, such as pore morphology, topography, stiffness and surface chemistry[2,3]. Additionally, supplementary manufacturing processes like chemical cross-linking are being implemented in order to increase the stiffness of collagen biomaterials[4]. For this reason, previous studies have pursued to understand how the physicochemical characteristics of biomaterials influence the inflammatory body response after their implantation. Macrophages (mononuclear cells; MNCs) and multinucleated giant cells (MNGCs) play main roles in the inflammatory cellular response to implanted biomaterials. Macrophages are recognized as part of a physiological cellular reaction and are characterized by their functional polarization into pro-inflammatory (M1) and anti-inflammatory (M2)[5]. On the contrary, MNGCs are described to be part of a pathological cellular reaction towards implanted biomaterials[6]. In this regard, our study group has conducted a series of studies and identified different inflammatory cellular responses to biomaterials[7]. One group of biomaterials induce a mononuclear inflammatory cellular reaction after their implantation and undergo degradation. A second type of biomaterials includes biomaterials, which induce the formation of MNGCs and undergo disintegration or integration in the implantation bed[8,9]. However, the mechanism that trigger the pathway taken by the cellular response is still unknown.

The categorization of the aforementioned inflammatory cellular response is the result of observational studies in which the physical and chemical characteristics of biomaterials were studied simultaneously. This makes it difficult to discern between their individual effect over the inflammatory response. It is hypothesized that modifying the original presentation of a sponge biomaterial, i.e. by pressing, could alter the initial biomaterial-cell interaction and the inflammatory cellular reaction. Therefore, the

present study investigated the influence of modifying a non-cross-linked, equine-derived collagen hemostatic sponge (E-CHS) over the inflammatory cellular reaction *ex vivo, in vitro* and *in vivo*.

4.2 Materials and methods

Material preparation

Parasorb fleece HD® is an E-CHS used to achieve hemostasis after tooth extraction and soft tissue injuries. To evaluate the interaction of the biomaterial's physical characteristics with its environment, the native E-CHS was altered by applying constant pressure (6.47 ± 0.85 N) for 2 min using a sterile stainless-steel cylinder until the material was uniformly flattened. Both the E-CHS in its original form and the pressed form (P-E-CHS) were cut into segments of 1 cm^2 and assessed *ex vivo, in vitro* and *in vivo*. A final correlation between the *ex vivo* and *in vivo* results was performed.

***Ex vivo* and *in vitro* evaluation**

A blood concentrate containing a high number of leukocytes and platelets known as platelet rich fibrin (PRF) was obtained after centrifugation and used as an instrument to mimic and evaluate the initial biomaterial-cell interaction (Table 1)[10,11]. Ethical approval for the PRF preparation and usage in research was obtained from the ethical committee of the Goethe University of Frankfurt (IRB No. 265/17) and blood was collected from three healthy donors after signing an informed consent.

Table 1: Centrifugation protocol to obtain liquid PRF known as the "low- speed centrifugation concept" (LSCC).

Angel of centrifugation	Radius-max	RCF	Time	Tube
Fixed angle 41.3°	11 cm	44 *g*	8 min	10-ml plastic tubes

The biomaterials were placed in cell culture plates in segments of 1 cm^2 and covered with 1 ml of liquid PRF. The following parameters were evaluated:

a) the fibrins' and cell's penetration within the biomaterial by histological observations.

b) the functionality of the biomaterials to transport liquid PRF. This measurement was named liquid PRF absorption coefficient (iPAC). After mixing the biomaterials with liquid PRF for 15 minutes, 2 segments were removed from the culture plates and the iPAC was calculated by subtracting the biomaterial's weight at dry state from the biomaterial's weight combined with liquid PRF divided by the biomaterial's weight at dry state (gravimetric method)[12].

c) the biomaterial's influence over the inflammatory cells within the liquid PRF. The release of the inflammatory cytokines Interleukin-eight (IL-8) and tumor necrosis factor-alpha (TNF- α) was measured after 3 and 6 days using a quantitative sandwich DuoSet ELISA kit (R&D Systems, Minneapolis, USA). Additionally, PRF was cultured without biomaterials as a control group (CG).

***In vivo* evaluation**

The animal experiments were designed and conducted according to the ARRIVE guidelines[13]. Approval for animal experiments according to applicable law was obtained from the responsible governmental authorities (RP Darmstadt) of the State of Hessen (No. FK/1023). Thirty-six Wistar rats (Rattus norvegicus) were purchased from Charles River (Sulzfeld, Germany) aged 6 to 8 weeks, weighting approx. 200 g. The animals were divided into 2 experimental groups (EG) and 1 CG, each with 12 animals; group 1 was implanted with E-CHS; group 2 was implanted with P-E-CHS; group 3 was sham operated (CG). Four animals per group were euthanized at 3, 15 and 30 days post-operatively and the explanted area was preserved in 4% buffered formalin (Roti-histofix, Carl-Roth, Germany) for 24 h[14].

Histochemical and immunohistochemical staining

The samples were divided in 5-10 mm segments, processed using an automatic tissue processor (Leica TP1020), embedded in paraffin blocks and cut using a microtome (Leica RM2255, Wetzlar, Germany) in 3-4 µm slides. One slide from each block was stained with hematoxylin and eosin (H&E) and the block representing the best cross-section of the biomaterial was selected for further analyses. Seven consecutive slices were cut, transferred on slides, and stained as follow: 1st H&E; 2nd Azan; 3rd Masson Goldner. The remaining 4 slides were immunohistochemically (IHC) stained as follow: 4th CD68 (pan

marker for monocyte/macrophages); 5th α-Smooth muscle actin (α-SMA; vessel identification); 6th CD206 (M2 macrophages) and 7th CCR7 (M1 macrophages). The stained slides were qualitative evaluated using a transmitted light microscope (Nikon Eclipse 80i, Tokyo, Japan)[15].

Quantitative histomorphometric evaluation

A so called ‘‘total scan’’ representing a complete image of a slide, was constructed from a total of 175 ± 25 images per slide captured using a Nikon Eclipse 80i histological microscope. In the EGs and CG four slides per time point were used to quantify the cellular inflammatory reaction and the vascularization pattern. The degradation rate of E-CHs and P-E-CHS was measured at each time point by recording fifteen cross-sectional measurements from each slide. The category of the cellular inflammatory reaction was measured using the IHC-stained slides (CD68; CCR7; CD206). From each slide the number of positive MNCs (M1 or M2) were counted and the total number of cells were divided by the total area of the implantation bed. Vascular structures were identified by the formation of a lumen, cell deposition and positive immunostaining. The results were expressed as density vascularization and percentage vascularization.

Statistics

Sample size (n=4) was calculated according to previous studies[16,17]. The results of the *ex vivo* and *in vivo* experiments were presented as the mean ± standard deviation (SD) and analyzed using two-way analyses of variance (ANOVA) with a Tukey multiple comparison test of all pairs (α = 0.005; 95% CI of diff.) Intragroup and intergroup differences re considered statistically significant if the *P* values were < 0.05 •, •/* and highly significant if the *P* values were < 0.01 ••, ••/**, < 0.001 •••, •••/***, and <0.0001 ••••, ••••/****.

4.3 Results

Ex vivo and *in vitro* evaluation

Histologically the pores' geometry of E-CHS was of irregular shape and of bigger size when compared to P-E-CHS. The fibrin and cells from PRF entered E-CHS but stayed on the surface of the P-E-CHS. Furthermore, E-CHS and P-E-CHS exhibited an iPAC of 8-fold and 5.6-fold of their original form without statistical differences (Figure 1).

The release of TNF- α and IL-8 were barely measurable in the CG. Both EGs showed a statistically significantly higher release of the proteins at day 3 and 6 compared to the CG (P<0.05). The PRF+P-E-CHS group showed a statistically significantly higher release of TNF- α and IL-8 when compared to E-CHS after 3 days (P<0.05) (Table S1-S2).

In vivo evaluation

Qualitative histological analyses

The CG induced the highest presence of CD68+/CCR7+ cells at day 3 compared to the EGs. Thereafter, the inflammatory cells progressively reduced until day 30. At this timepoint, the CG showed a higher number of CD206+ cells (M2) than CCR7+ cells (M1). MNGCs were not observed in the CG.

At day 3, both biomaterials maintained the space of implantation without a change of their structure. Mainly CD68+/CCR7+ mononuclear cells penetrated the outer third of E-CHS but remained in the surface of P-E-CHS. At day 15, E-CHS was fully invaded by CD68+/CCR7+ mononuclear cells and vascular structures were identified in the biomaterial's center. On the contrary, these cells remained in the surface of the P-E-CHS with new vascular structures in the periphery. Few MNGCs were observed in the EGs starting at day 15 and finally at day 30 surrounding the remaining fragments of the biomaterials (Figure 2 and 4).

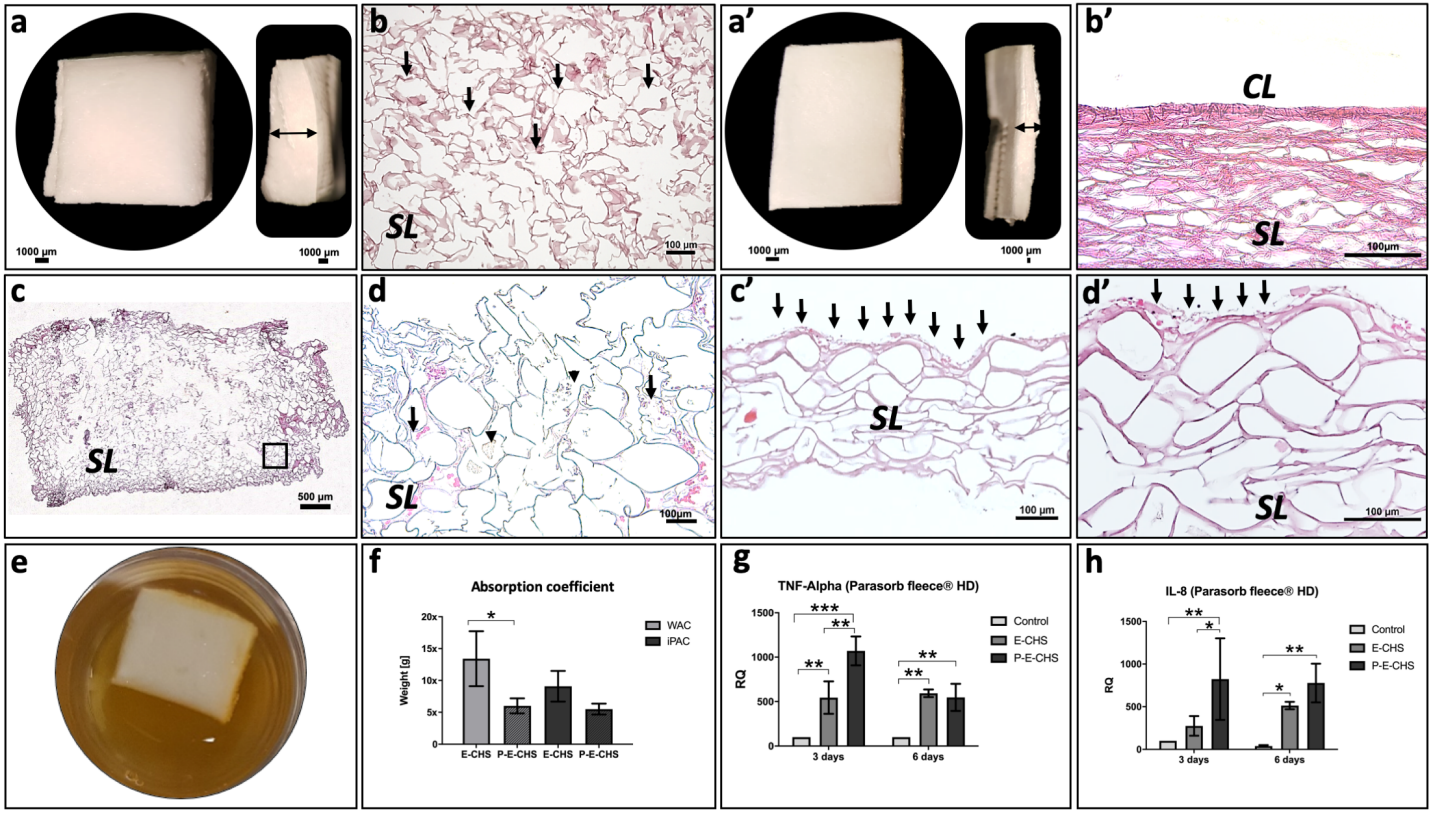

Figure 1: Histological evaluation of the collagen hemostatic sponge in its original form (E-CHS; a-b) and pressed (P-E-CHS; a'-b'). Interaction of the fibrin and cellular content in liquid PRF with both biomaterials (E-CHS; c-d) (P-E-CHS; c'-d'). Representative image of the *in vitro* evaluation (e). Liquid PRF absorption coefficient (iPAC) and water absorption coefficient (WAC) as control measurement (f). Quantification of the release of the pro-inflammatory cytokines TNF-alpha and IL-8 from the inflammatory cells within the liquid PRF (g-h). PRF was cultured without biomaterials as a control group (CG)[18].

Quantitative histological analyses

Biomaterial thickness

At day 3, there was no difference between the two experimental groups. However, after 15 days, the structure of E-CHS disintegrated by 0.7-fold, which was statistically significant when compared to P-E-CHS (P<0.0001). At day 30, only small fragments of both biomaterials were found in the implantation area (Figure 2) (Table S3).

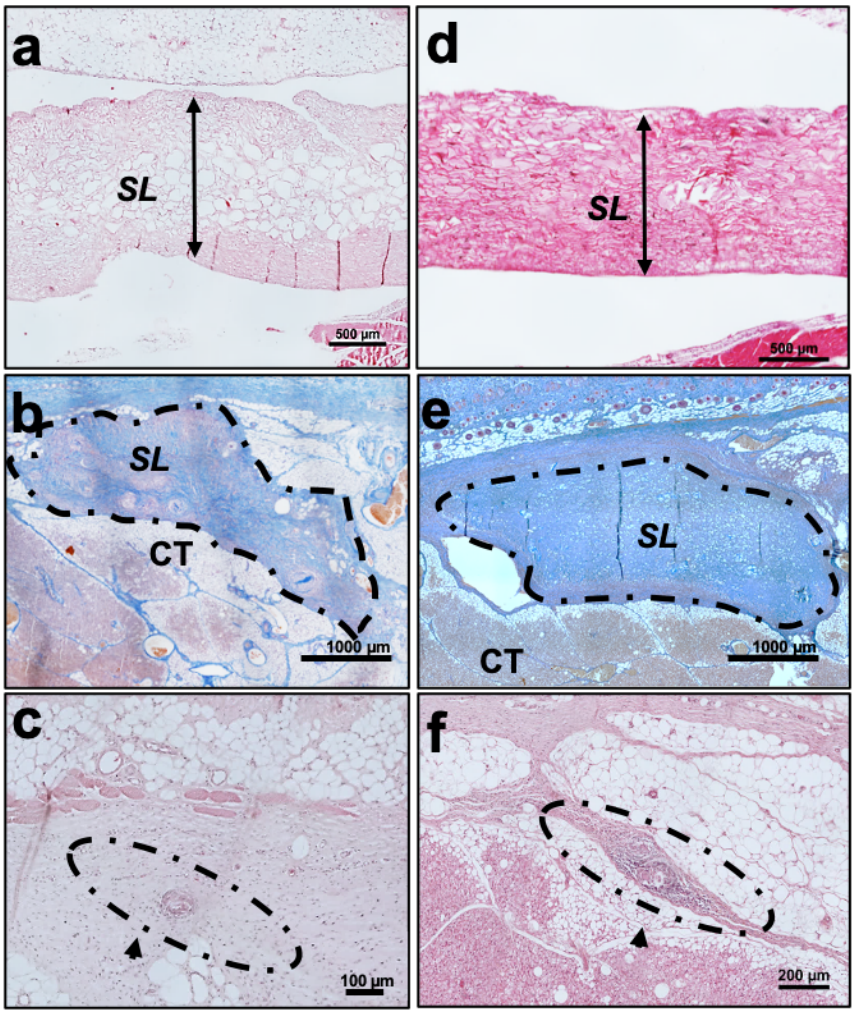

Figure 2. Histological images of the explanted areas after subcutaneous implantation in Wistar rats. The sequence of images show the inflammatory cellular reaction and the biomaterial's degradation up to day 30[18].

Inflammatory pattern

In the CG the highest number of macrophages (CD68+; CCR7+; CD206+) was observed after 3 days with statistically significant difference compared to the EGs (P<0.05). The number of macrophages reduced progressively from day 3 until day 30. However, at day

30, the number of M2 macrophages was higher than M1 macrophages. No MNGCs were observed during the evaluation period in the CG. The experimental groups showed different inflammatory patterns. At day 3, the number of macrophages (CD68+) in the P-E-CHS was higher in comparison to the E-CHS (P<0.05). After 15 days, the numbers of macrophages (CD68+; CCR7+) increased in both experimental groups and were statistically significantly higher than the CG (P<0.05). At day 30, a significant reduction of CD68+ and CCR7+ macrophages was observed in both experimental groups. Remarkably, there was a shift of the polarization pattern of all groups with a higher number of M2 macrophages (CD206+) (Figure 3) (Table S4).

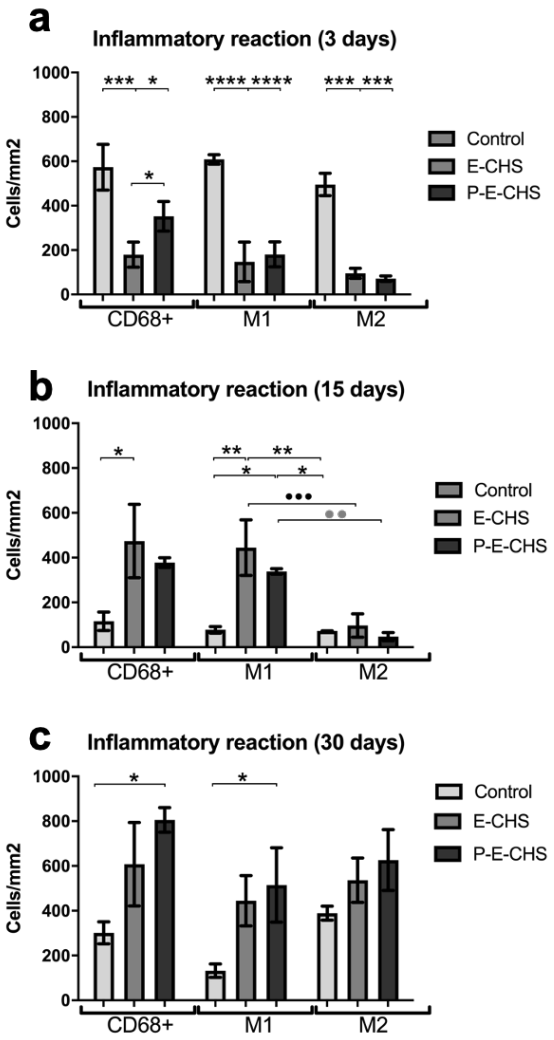

Figure 3: Histomorphometric evaluation of the cellular inflammatory reaction. The number of MNCs (C68+), M1 macrophages (CCR7+) and M2 macrophages (CD206+) were quantified[18].

Vascularization

The results at day 3 from all groups did not show statistical differences. At day 15 the intragroup comparison showed that the vessel density and percentage vascularization

in the experimental groups significantly increased compared to day 3 (P<0.05). The intergroup comparison showed higher vessel density in the E-CHS group when compared to the CG (P<0.05) and a higher percentage vascularization of both experimental groups in comparison to the CG (P<0.05). At day 30 only a reduced number of vessels were observed in the implantation bed of all groups (Figure 4) (Table S5).

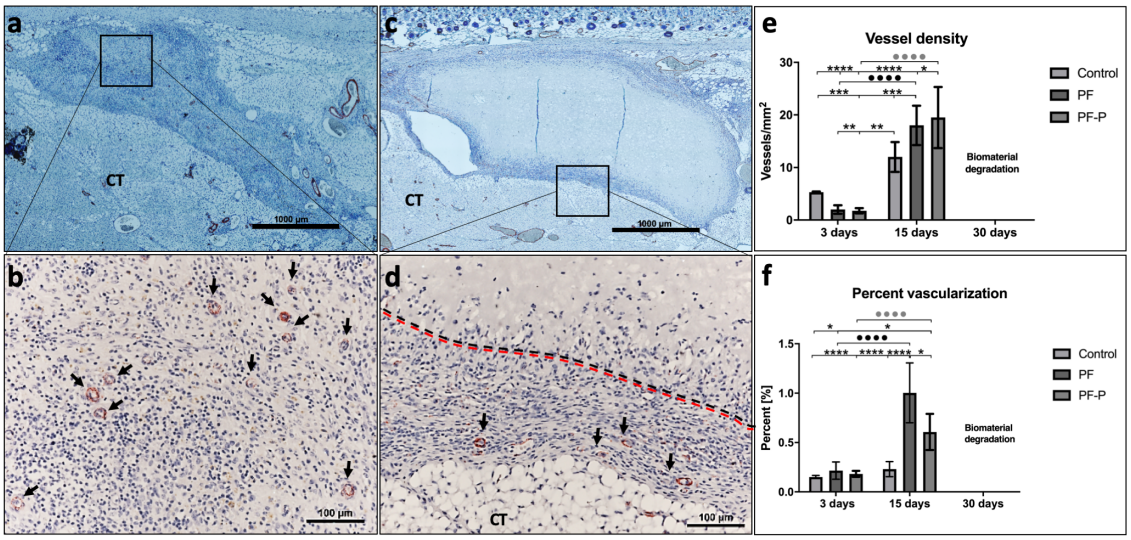

Figure 4. Vascularization of E-CHS (a-b) and P-E-CHS (c-d). Arrows signal vessel formation. The dash lines delineate the biomaterial's area (black) and the periphery (red). Histomorphometric analyses of vessel formation was reported in density (e) and percent of vascularization (f)[18].

Correlation of the inflammatory cellular reaction

The results of the *ex vivo* and *in vivo* evaluation showed similar patterns of inflammatory reaction. At day 3, the P-E-CHS induced a higher release of TNF- α and IL-8 *ex vivo* when compared to E-CHS. This result resembles the inflammatory cellular reaction *in vivo*, as P-E-CHS induced a higher number of macrophages (CD68+) in comparison to the E-CHS (P<0.05).

4.4 Discussion

Current observational studies describe the initial biomaterial-cell interaction and the inflammatory cellular reaction to the physicochemical characteristics of biomaterials[19,20]. However, with these studies, it is difficult to discern between the individual effects of the physical and chemical characteristics of biomaterials. The

present study focused on the effect of the physical characteristics over the inflammatory cellular reaction. The rationale behind the selection of a collagen sponge to conduct the present study lies in the possibility to change the biomaterial's original physical characteristics without further chemical modifications. The biomaterial was evaluated *in vivo* using a subcutaneous animal model and *ex vivo* by means of a blood concentrate called liquid PRF. Liquid PRF was used as an *ex vivo* and *in vitro* model because of its high number of inflammatory cells[10].

It was observed histologically, that after pressing the collagen biomaterial, the sizes of its pores were reduced and it resembled the characteristics of a membrane[21]. Additionally, pressing E-CHS reduced only partially its absorption coefficient. Once both biomaterials came into contact with liquid PRF *ex vivo*, the size of their pores increased. Interestingly, cells enter E-CHS while P-E-CHS was cell-occlusive, as the cells remained on the surface. The same biomaterial-cell interaction was observed *ex vivo* and *in vivo*. Furthermore, the *ex vivo* and *in vitro* results showed that the combination of liquid PRF and P-E-CHS induced a higher release of the pro-inflammatory cytokines TNF- α and IL-8 when compared to PRF+E-CHS. The higher release can be explained by the stimulation of the immune cells caused by the biomaterial's physical characteristics, being that the release of TNF- α and IL-8 was not modified in the CG. The literature describes these cytokines as potent inducers of neutrophil migration, which together with our findings, provide a rationale behind the observed higher inflammatory cellular reaction induced by P-E-CHS *in vivo* at early time points[22]. Previous studies have shown that during the first contact between blood and biomaterials, the absorbed proteins regulate the functionality of the immune cells[23,24]. The results of this study support the assumption that the biomaterial-cell interaction is highly determined by their initial contact. Furthermore, this work is the first to show that when combining a xenogeneic collagen biomaterial and liquid PRF, the physical characteristics of the biomaterial modifies the interaction and the biological response of the inflammatory cells within liquid PRF. This is of relevance in a clinical setting, since liquid PRF is an autologous blood derivate implemented to enhance wound healing and its combination with xenogeneic biomaterials could be altering its regenerative capacity[25]. Therefore, further studies are required in this regard.

The *in vivo* investigation showed that E-CHS entered an earlier degradation process compared to P-E-CHS. However, both E-CHS and P-E-CHS induced a chronic inflammatory cellular reaction and an increase in the vascular formation that extended until day 15. The reaction was led by macrophages with a small number of MNGCs and the biomaterials underwent degradation after 30 days. Moreover, macrophages polarization was dominated by M1 macrophages (pro-inflammatory) during the first two time points of the study. After 30 days, the implantation bed seemed to have entered a reparative stage, as the polarization shifted to a higher presence of M2 (pro-wound healing) macrophages. These postulations derive from the observations of the EGs compared to those of natural wound healing in the CG. The CG also induced an acute inflammatory reaction after 3 days, but seemed to have entered a reparative stage already at 15 days due to the reduction of inflammatory cells (CD68+/CCR7+). Similar to the EGs, a shift to a higher presence of M2 macrophages took place. Nevertheless, this shift was only observed at day 30. It can be inferred from the results, that M2 macrophages are playing a regulatory role that avoids exacerbation of inflammation. However, it remains unknown if a higher presence of M2 macrophages compared to M1 macrophages at an earlier stage induce a faster healing process. As observed, at 15 days the CG showed signs of healing, but the M2 macrophages remained a lower number that M1 macrophages until day 30. Recent studies aimed to induce a higher polarization of M2 macrophages at early stages of wound healing with the intention to accelerate the regeneration process[26]. However, further studies need to be conducted to confirm whether this translate into clinically observations of accelerated wound healing.

Both biomaterials induced the formation of a small number of MNGCs, which were observed at 15 and 30 days of implantation. The IHC revealed that these cells expressed the pro-inflammatory marker CCR7. Unlike the macrophages, the MNGCs did not polarized to M2 (pro-wound healing) and remained pro-inflammatory, confirming their involvement in chronic inflammatory processes[14]. This result goes not in line with recent studies stating an observed reversible plasticity in the phenotype of MNGCs at least in the time period of 30 days [27]. This contradiction could be due to different material characteristics of the analyzed materials in both studies.

In the present study, the authors sought to simulate the manipulation of a collagen biomaterial in the clinical practice and perform a histological analysis focused on the inflammatory cellular reaction. Nevertheless, the absence of the mechanical characterization and surface chemistry may be considered a limitation. Taken together, the study adds to the growing body of evidence that the initial biomaterial-cell interaction and the inflammatory cellular reaction are modulated by the physical characteristics of a biomaterial. It is clinically relevant that pressing a collagen sponge before its use reduces its absorption coefficient, delay the early degradation rate and avoid cell penetration. In this sense, within the limitations of this study, a pressed collagen sponge could be applied for GBR/GTR.

4.5 Conclusion

Changing the physical characteristics of a non-cross-linked equine-derived collagen hemostatic sponge by pressing changed the size and deposition of its pores. The pressed collagen sponge avoided cell penetration *ex vivo* and induced a higher release of inflammatory cytokines from platelet-rich fibrin *in vitro*. The present study showed *in vivo* that the hemostatic collagen sponge without modifications induced a chronic inflammatory reaction led by M1 macrophages and a low number of MNGCs. Modifying the original characteristics altered the initial biomaterial-cell interaction and increased the initial inflammatory cellular reaction. The results bring to attention the need for pre-clinical studies prior to biomaterial modification in the clinical practice. Finally, the study provided insights into the possibility of developing biomaterials with shape-shifting capabilities and multiple applications.

5. Publication outline and publication

Herrera-Vizcaíno C, Al-Maawi S, Sader R, Kirkpatrick C, Choukroun J, Ghanaati S. Modification of collagen-based sponges can induce an up shift of the early inflammatory response and a chronic inflammatory reaction led by M1 macrophages: An *in vivo* study. [published online ahead of print, 2020 Feb 17]. *Clin Oral Investig*. 2020

6. Contribution to the publication

The candidate is a full member of the FORM-lab (Frankfurt Orofacial Regenerative Medicine) since 2016 and contributed to this publication and other studies related to biomaterials and platelet rich fibrin (PRF).

The candidate was accompanied throughout the study by his supervisor in the conception, design and execution of the study. He completed a FELESA B course successfully, gained sufficient training and get qualified for animal handling. He performed the animal surgeries and prepared the explanted biomaterials for histological evaluation (tissue processing, embedding and cutting). The candidate learned and carried out the histological and immunohistochemical staining. Data collection and statistical analyses were done by the candidate. He introduced the iPAC measurement as a tool to measure the functionality of biomaterials to transport liquid PRF.

The candidate prepared the images and wrote all parts of the manuscript under the guidance of his supervisor.

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8. Supplemental data

Supplemental Table S1: Mean results from the water absorption coefficient (WAC) and the liquid PRF absorption coefficient (iPAC).

Groups	WAC	iPAC
E-CHS	12,60 ± 4,32	7,88 ± 2,40
P-E-CHS	5,87 ± 1,186	5,93 ± 0,86

Supplemental Table S2: Release of the inflammatory cytokines tumor necrosis factor-alpha (TNF- α) and interleukin-eight (IL-8) from liquid PRF. Results from the experimental groups are relative quantified (RQ) to the results from the control group at day 3.

	TNF-alpha			**IL8**	
Groups	**Day 3**	**Day 6**	**Groups**	**Day 3**	**Day 6**
Control	100	100	**Control**	100	100
E-CHS	611,13 ± 182,70	593,04 ± 43,02	**E-CHS**	275,51 ± 114,42	518,90 ± 43,80
P-E-CHS	995,60 ± 162,49	598,79 ± 152,67	**P-E-CHS**	823,00 ± 478,53	667,03 ± 226,56

Supplemental Table S3: Mean measurements of the biomaterial's thickness previous to its implantation (*ex vivo*) and at different timepoints of implantation (*in vivo*).

Time	E-CHS	P-E-CHS
Ex vivo	5000 µm	500 µm
In vivo - 3 days	1536,32 ± 176,82 µm	1227,18 ± 456,43 µm
In vivo - 15 days	881,23 ± 347,45 µm	1522,98 ± 500,86 µm
In vivo - 30 days	0 µm	0 µm

Supplemental Table S4: Mean results from the histomorphometric analyses of the inflammatory cellular reaction.

Groups	Control	E-CHS	P-E-CHS
CD68+ (3 days)	573,48 ± 72,61	179,58 ± 56,29	389,45 ± 80,07
M1 (3 days)	608,46 ± 15,12	178,54 ± 62,88	180,78 ± 39,70
M2 (3 days)	495,79 ± 35,41	95,42 ± 16,16	70,98 ± 12,54
CD68+ (15 days)	116,01 ± 29,32	474,02 ± 163,86	377,55 ± 21,07
M1 (15 days)	77,65 ± 10,30	444,51 ± 123,96	338,06 ± 8,62
M2 (15 days)	72,66 ± 0,42	96,87 ± 52,10	46,72 ± 18,28
CD68+ (30 days)	301,48 ± 34,76	607,53 ± 186,41	805,72 ± 38,89
M1 (30 days)	132,28 ± 21,14	444,54 ± 112,79	514,92 ± 166,46
M2 (30 days)	388,97 ± 22,23	536,22 ± 99,14	626,06 ± 135,81

Supplemental Table S5. Mean results of vascularization during the course of the study from day 3 to day 15.

Percent vascularization

Groups	Day 3	Day 15
Control	0,15 ± 0,02	0,23 ± 0,07
E-CHS	0,19 ± 0,08	0,94 ± 0,30
P-E-CHS	0,18 ± 0,03	0,54 ± 0,18
Groups	**Day 3**	**Day 15**
Control	5,30 ± 0,14	12,01 ± 2,83
E-CHS	2,00 ± 0,81	18,00 ± 3,74
P-E-CHS	1,75 ± 0,50	19,5 ± 5,80

Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Immunohistochemical analysis of the inflammatory reaction within the implantation bed of a collagen hemostatic biomaterial

in der Klinik für Mund-, Kiefer- und Plastische Gesichtschirurgie unter Betreuung und Anleitung von Prof. Dr. Dr. Shahram Ghanaati mit Unterstützung durch Prof. Dr. Dr. Robert Sader ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht*. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden (oder werden) in folgendem Publikationsorgan veröffentlicht:
Herrera-Vizcaíno C, Al-Maawi S, Sader R, Kirkpatrick C, Choukroun1 J, Ghanaati S. Modification of collagen-based sponges can induce an up shift of the early inflammatory response and a chronic inflammatory reaction led by M1 macrophages: An in vivo study. *Clin Oral Investig*. 2020.

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(Ort, Datum) (Unterschrift)