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# Cross-linked hyaluronic acid slows down collagen membrane resorption in diabetic rats through reducing the number of macrophages

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**Abstract:** Accelerated degradation of collagen membranes (CMs) in diabetic rats is associated with increased infiltration of macrophages and blood vessels. Since pre-implantation immersion of CMs in cross-linked high molecular weight hyaluronic acid (CLHA) delays membrane degradation, we evaluated its effect on the number of macrophages and endothelial cells (ECs) within the CM.

Diabetes was induced with streptozotocin in 16 rats, while 16 healthy rats served as control. CM discs were labeled with biotin, soaked in CLHA or PBS and implanted under the scalp. Fourteen days later, CMs were embedded in paraffin and the number of macrophages and ECs within the CMs was determined using antibodies against CD68 and Transglutaminase II, respectively.

Diabetes increased the number of macrophages and ECs within the CMs (~2.5-fold and 4-fold, respectively). Immersion of CMs in CLHA statistically significantly reduced the number of macrophages ($p<0.0001$) in diabetic rats, but not that of ECs. In the healthy group, CLHA had no significant effect on the number of either cells. Higher residual collagen area and membrane thickness in CLHA-treated CMs in diabetic animals were significantly correlated with reduced number of macrophages but not ECs.
Immersion of CM in CLHA inhibits macrophage infiltration and reduces CM degradation in diabetic animals.

**Keywords:** Hyaluronic acid. Diabetes. Rats. Macrophages. Blood vessels. Collagen membranes.

## 1. Introduction

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The regeneration of tissues lost due to disease is the goal of periodontal treatment [1]. Collagen-based barrier membranes (collagen membranes; CMs) are routinely applied in guided tissue regeneration (GTR) and guided bone regeneration (GBR) procedures. Membranes create and maintain a space over a bony defect and act as a protective barrier against ingrowth of rapidly proliferating epithelial and connective tissue cells into the defect [2]. Resorption of the CM occurs via a biodegradation process. This process starts when cells within the surgical site release matrix metalloproteinases (MMPs) to the wound area during healing, continues with infiltration and colonization by fibroblasts and capillaries, leading to collagen scaffold remodeling, followed by its replacement with new extracellular matrix [3-5].

It has been established that CM longevity plays a crucial role in the success of regenerative procedures [6-9]. Furthermore, premature exposure of the CM to the oral cavity or its early degradation may jeopardize results and success of GTR or GBR procedures[10-12]. The degradation rate may be influenced by the composition, structure and dimension of the CM [1,3,13]. The degradation rate may also be influenced by systemic diseases, such as uncontrolled diabetes. Several studies have demonstrated faster CM resorption in rats with uncontrolled diabetes [14-16]. Some of these studies showed that the inflammatory infiltrate inside the CMs in diabetic rats was more marked, compared with normoglycemic rats [17,14] and that accelerated degradation of CM was associated with increased inflammatory components like macrophages and capillaries [17]. Reducing the inflammatory process within the CM plays an important factor in enhancing the longevity and integrity of the CM in diabetic conditions.

Hyaluronic acid (HA) is a natural glycosaminoglycan. It is an essential component of connective tissues and plays an important role during wound healing [16]. Moreover, HA may also be introduced as a hydrogel with inherent absorption properties that are desirable in the wound healing environment [18,19,16]. The properties of HA have been investigated since 1934. It was found that HA possesses biocompatible, biodegradable, bacteriostatic, antioxidant, anti-edematous and anti-inflammatory properties [20]. There are 2 main forms of HA: high molecular weight HAs (HMW) and low molecular weight HAs (LMW). It has been shown that LMW HAs (100-500 kDa), but not the HMW HA molecules (~4,000 kDa), stimulate inflammatory cells creating an inflammatory environment [21]. In medicine and dentistry, the use of HMW HAs is preferable for several reasons. HMW HAs exhibit higher viscosity, longer residence time, and higher biocompatibility when compared to LMW HAs [22]. HMW HAs also show anti-inflammatory effects by decreasing interleukin (IL)−1β, IL-6, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and prostaglandin $E_2$ ($PGE_2$) production [23-25], whereas LMW HAs have been reported to stimulate angiogenesis [26] and induce an inflammatory response [27]. On the other hand, HMW HAs predominate in healthy tissues and typically inhibit inflammation. Due to their desirable properties, HMW HAs have been used in various medical [28] and dental [29,30] therapies. In medicine, HMW HAs are routinely applied in the treatment of diabetic ulcers since they increase the rate of wound healing [30, 31]. Recently, it has been also demonstrated that HA enhances the proliferative, migratory and wound healing properties of several cell types involved in soft tissue wound healing without impairing the healing process by prolonging inflammation or causing excessive MMP expression . Interestingly, recent data [32 ] also revealed that HA has the potential to induce the growth of osteoprogenitors by maintaining their stemness, which may exert a possible effect on the balance between self-renewal and differentiation during bone healing/regeneration. Recent systematic reviews and meta-analysis in dentistry have shown favorable results using HA as an adjunct to periodontal surgical procedures [33,34].

Immersion of CM in HA is a currently new area of investigation. Recently, several in vitro and in vivo animal studies have demonstrated some advantages using cross-linked HMW HA [34]. In a recent animal study, it was concluded that HMW HA does not interfere with tissue integration and structural degradation of Bio-Guide® (Geistlich

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Pharma AG, Wolhusen, Switzerland) or OsseoGuard® [35], two CMs which are frequently used for periodontal and bone regeneration.

In another recent publication, Eliezer et al. showed that in rats with type-1-like diabetes the immersion of CMs in HMW cross-linked HA delayed membrane degradation compared to non-immersed CMs [34]. As a continuation of this study the current study aimed to investigate the effect of HA immersion on the inflammatory response within CMs, as evidenced by the number of macrophages and endothelial cells, following their implantation under the calvarial skin of rats with streptozotocin-induced diabetes.

## 2. Materials and Methods

Thirty-two 12-week-old male Wistar rats were used in the study. The institution Animal Care and Use Committee of Tel Aviv University, Tel Aviv, Israel, approved the study (TAU 1-16-031). Diabetes was induced in sixteen rats by a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Chemical Co. MO, USA). The remaining animals, which served as normoglycemic controls, were given a similar volume of citrate buffer. Pericardial CMs (Smartbrane, Regedent AG, Zürich, Switzerland) were cut with a disposable biopsy punch (Miltex, Lake Success, NY) to 8-mm-diameter discs. Membrane labeling with biotin has been described previously [14].

One week after diabetes induction animal surgeries were performed by the same experienced operator (CN). The animals were anesthetized by intramuscular injection of 0.1 mL/100 g 10% ketamine hydrochloride (Rhone Merieux, Lion, France) and 0.1 mL/100 g 2% xylazine hydrochloride (Vitamed, Binyamina, Israel). The dorsal part of the skin covering the scalp was shaved and aseptically prepared for surgery. Two reference lines for reproducibility of the incision site were made by drawing a line between the ears ("ear-line") and one between the eyes ("eye-line"), then a mid-sagittal line connecting the middle of the "eye-line" and that of the "ear-line" was drawn. A 15-mm incision was made toward the eye-line, beginning 5 mm away from the ear-line, on the mid-sagittal line. Following the incision, a subperiosteal pouch over the calvaria was created using a Kirkland periodontal knife (Hu-Friedy, Chicago, IL). One disc, immersed in a 20 mg/mL CLHA (Hyadent BG; Regedent AG) solution or in PBS, was implanted in each animal underneath the periosteum. Periosteum and skin were repositioned, covering the implanted membrane, and the skin was sutured with resorbable sutures (Vicryl Rapide, Ethicon).

Fourteen days following surgery, animals were euthanized with an overdose of ketamine and xylazine, followed by asphyxiation with carbon dioxide ($CO_2$). Dermal tissues were dissected together with the bone, leaving the scalp skin undisturbed covering the membrane discs. Specimens were fixed in 4% paraformaldehyde, decalcified for 10 weeks in a 10% ethylenediaminetetraacetic acid (EDTA, pH 7.3) solution, washed, dehydrated in ethanol and xylene, and embedded in paraffin. Sagittal 5-µm sections were made, and those that included the central area of the membrane were selected for comparative analysis of CM cell infiltration and degradation. Sections were stained with hematoxylin and eosin (H&E), and adjacent sections were stained with Horseradish peroxidase (HRP)-conjugated streptavidin (ZytoMed, Berlin, Germany) according to the protocol of the manufacturer, to detect biotinylated collagen. Briefly, slides were incubated with 2 drops of HRP-conjugated streptavidin solution for 30 minutes at room temperature, followed by detection with DAB substrate kit (ScyTek, Logan, UT, USA) and hematoxylin counterstain. All slides were mounted with an aqueous solution of glycerol vinyl alcohol (ZytoMed).

Two neighboring sagittal sections were used for immunohistochemistry. Endogenous peroxidase activity was blocked with 3 % $H_2O_2$ for 10 min. Antigen retrieval was performed with 0.1 % proteinase K in PBS for 15 min at 37°C (for CD68) or by heating under pressure of the sections in a citrate buffer, pH = 6, for a total of 12 min (for TGII). Nonspecific binding sites were blocked by incubation with Background Buster (Innovex,

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Richmond, CA, USA) for 40 min. Primary antibodies used were a mouse anti-rat CD68 monoclonal antibody (Millipore Corporation, Billerica, MA, USA) (at a 1:100 dilution) for macrophage identification and a mouse monoclonal anti-transglutaminase II (TGII) antibody (ThermoFisher Scientific, Waltham, MA, USA) (at a 1:150 dilution) for the identification of endothelial cells [36]. Both antibodies were diluted in an antibody diluent (Zytomed) and were incubated with the sections for one hour at room temperature. Bound primary antibodies were detected with a goat anti-mouse HRP-conjugated antibody (Zytomed), incubated for 30 min at room temperature, and followed by a detection with DAB substrate kit (ScyTek, Logan, UT, USA) and hematoxylin counterstain. Negative controls were performed by omitting the primary antibody. All slides were mounted with an aqueous solution of glycerol vinyl alcohol (Zytomed).

Histological evaluation was performed by the same experienced investigator (DB). Stained sections were photographed with a digital camera (AxioCam MRC, Carl Zeiss) mounted on a light microscope (AxioImager M2, Carl Zeiss) with a X20 objective.

Measurements of CM thickness and collagen content in streptavidin-stained sections (reported previously ([34]) were made by superimposing three rectangular regions of interest (ROIs) per disc, one anterior, one middle and one posterior. Each ROI had a dimension of 0.625 mm × 0.4 mm (= 0.25 $mm^2$). A grid containing 40 µm x 40 µm cells was superimposed on the ROIs (figure 1) and the number of cell-intersections in the grids that hit collagen within the ROI was registered. Two non-implanted biotin-labeled discs (one immersed in CLHA and the other one in PBS) were processed in the same manner and served as baseline. Mean residual collagen content (% of baseline) and CM thickness (mm) were determined for each disc. H&E stained sections were used to better identify the surrounding tissues (Figure 2). For macrophage and endothelial cell counts, identical ROIs were used and positive cells were counted per ROI and the mean per disc was calculated.

Data were analyzed for statistical significance using the non-parametric Kruskal-Wallis test ($p$ values $< 0.05$ were considered significant), where CM treatment with CLHA/PBS was the within-subject variable and health status (normo- vs hyper-glycemia) was the between-subject variable. The Dunn's multiple comparison test was used to test differences between groups. The Spearman's correlation procedure was used to test correlations between collagen area or thickness and the number of endothelial cells or macrophages. All statistical calculations were made using the GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA).

**3. Results**

Diabetes increased ~2.5-fold the number of CD-68 positive macrophages, which infiltrated the PBS-immersed membrane discs, compared to the healthy group (from 44.3 ± 35.1 per $mm^2$ to 109.2 ± 41.7 (figures 3 and 4). Immersion of the collagen discs placed in diabetic animals in CLHA statistically significantly reduced (~3.5-fold) the number of CD-68 positive macrophages compared to the PBS-immersed discs (from 109.2 ± 41.7 per $mm^2$ to 30.9 ± 14.7). In CM placed in control animals, immersion in CLHA had no significant effect on the number of macrophages. Therefore, CLHA immersion reduced macrophage number only in the diabetic animals.

Diabetes statistically significantly increased (by more than 4-fold) the number of TGII-positive endothelial cells within the PBS-immersed membrane discs compared to the healthy group (from 26.8± 13.4 per $mm^2$ to 115.9 ± 32.6) (figures 5 and 6). Unlike macrophages, immersion of the discs in CLHA had no effect on the growth of TGII-positive endothelial cells into the collagen discs in both the control and diabetic animals.

Since we have previously shown that immersion of CM in CLHA statistically significantly increased residual collagen content in the diabetic but not in the normoglycemic animals, we tested whether this effect correlated with the number of

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macrophages or endothelial cells in this group. Figures 7 and 8 show a significant negative correlation between membrane thickness or residual collagen and the number of macrophages in the diabetic animals. Thus, reduced membrane resorption in diabetic rats seems to be associated with a reduction in macrophage infiltration.

In contrast, figures 9 and 10 show no correlation between membrane thickness or residual collagen and the number of endothelial cells, positive for TGII, within the CM in diabetic animals. Thus, the protective effect of CLHA against CM resorption was not correlated to changes in the infiltration of blood vessels into the membrane.

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**Figures**

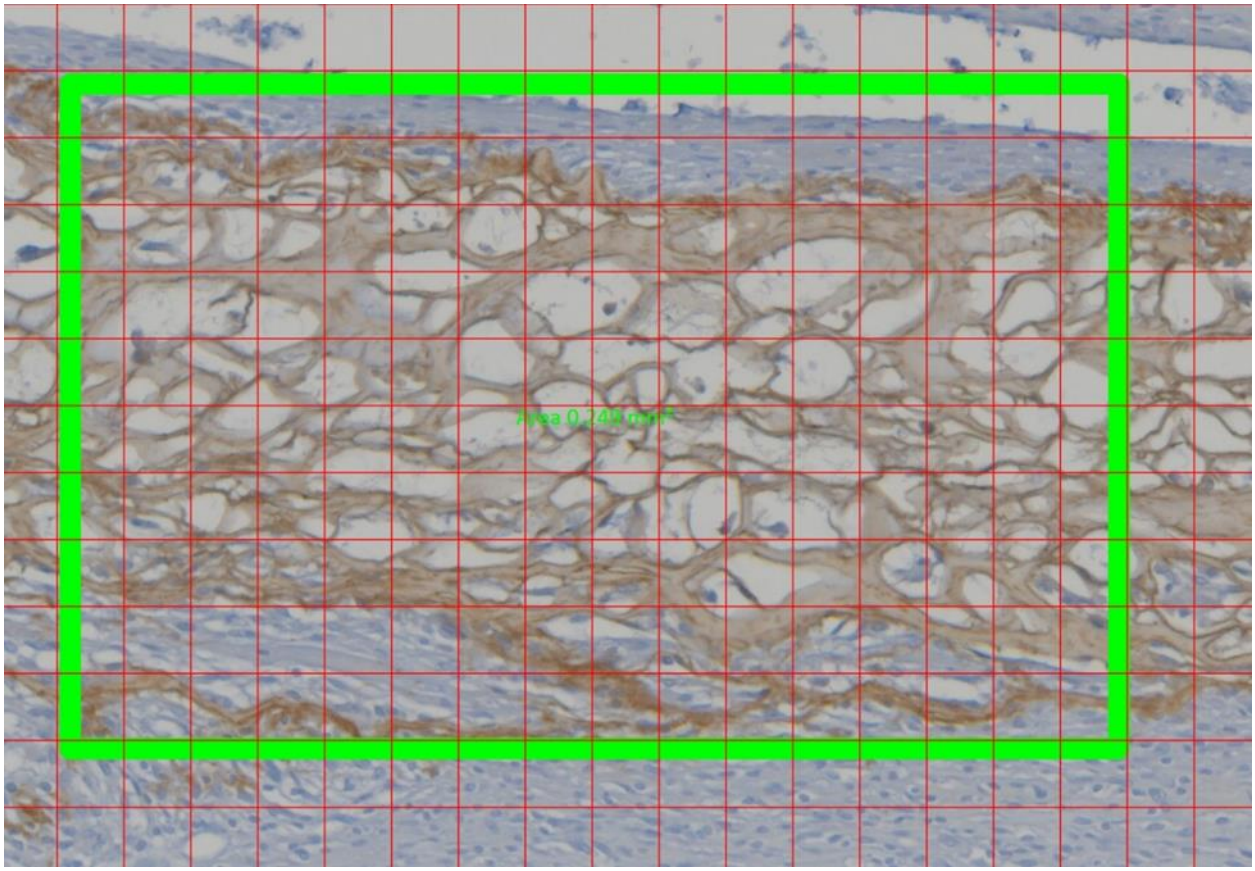

**Figure 1.** Presentation of the grid superimposed on the membrane ROI (stained in red/brown with Avidin-biotin-HRP reaction, Magnification X100)

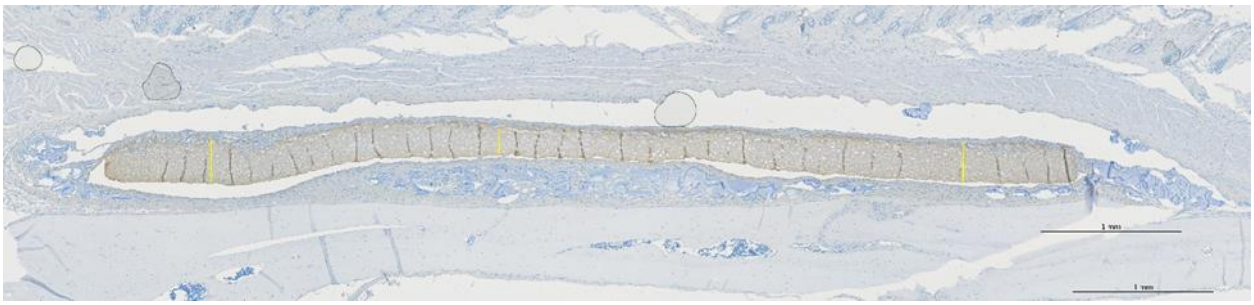

**Figure 2.** Photomicrograph of the CM stained with hematoxylin and eosin. HA appears as amorphous or round light purple-bluish material. (magnification × 20)

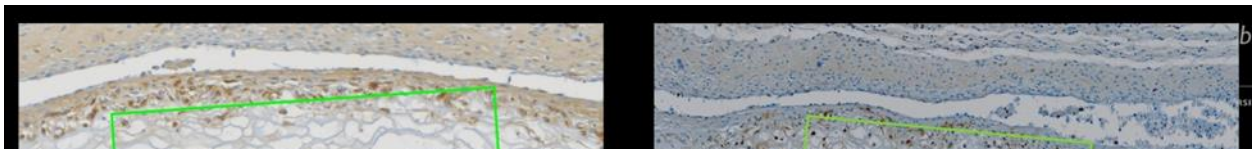

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**Figure 3.** Photomicrographs of CD-68 positive cells within the CM placed in a normoglycemic (c-/c+) or a diabetic (d-/d+) rat, showing fewer macrophages in CLHA-immersed CM in the diabetic animal (d+ vs d-). C- = control group, no CLHA. C+=control group, with CLHA. d-=diabetic group, no CLHA. d+=diabetic group with CLHA. Green rectangles represent the ROI. Magnification = ×40

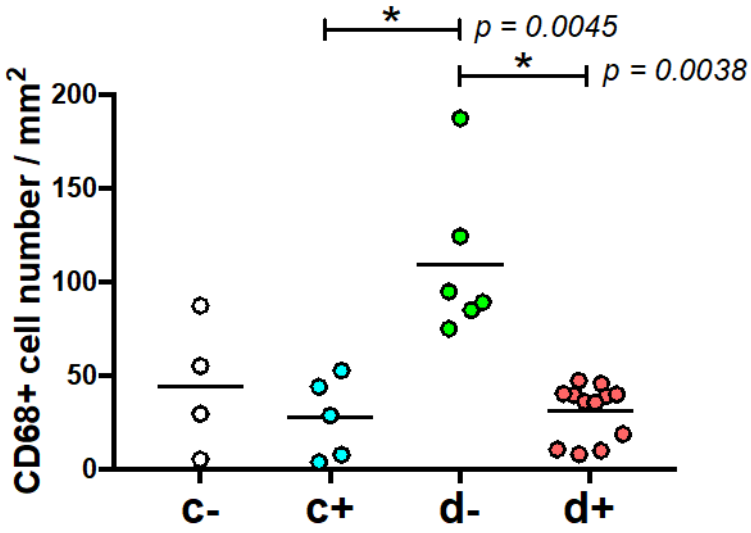


**Figure 4.** Number of mononuclear CD-68 positive cells within the CM discs immersed in PBS (c-/ d-) or CLHA (c+/ d+) in normoglycemic (c) and diabetic (d) rats. Horizontal lines represent group means. * denotes significant difference between groups.

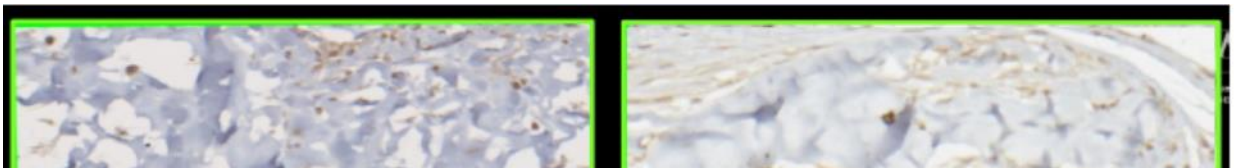

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**Figure 5.** Photomicrographs of TGII-positive endothelial cells within the membrane placed in a normoglycemic (c-/ c+) or a diabetic (d-/ d+) rat. C- = control group, no CLHA. C+=control group, with CLHA. d-=diabetic group, no CLHA. d+=diabetic group with CLHA. Green rectangles represent the ROI. Magnification = ×60.

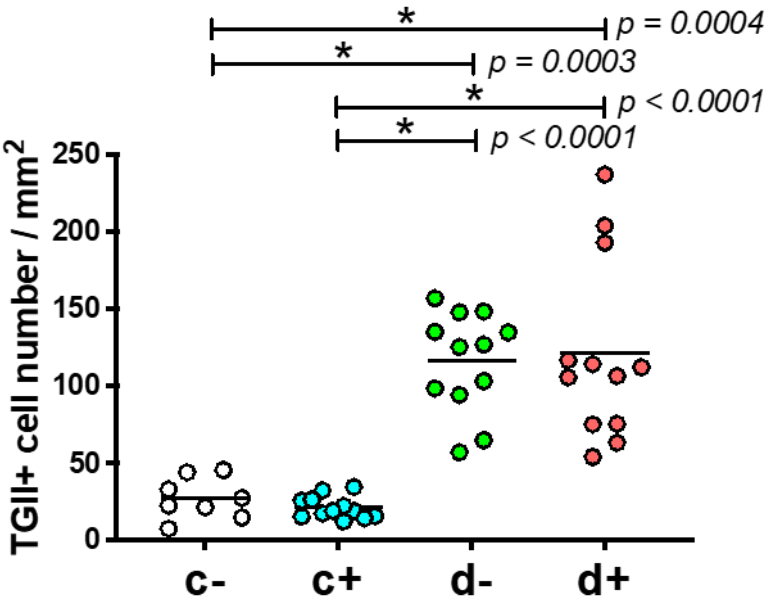


**Figure 6.** Number of TGII-positive endothelial cells within the membrane discs immersed in PBS (c- / d-) or CLHA (c+ / d+) in normoglycemic (c) and diabetic (d) rats. Horizontal lines represent group means. * denotes significant difference between groups.

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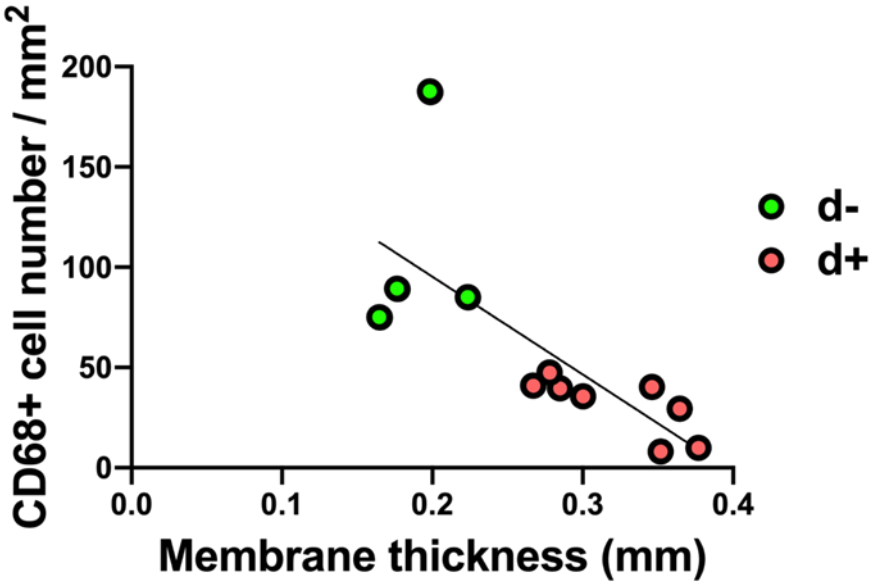


**Figure 7.** Scatter diagram showing a significant negative correlation (r = -0.9021, *p* = 0.0002) and linear regression line between the number of macrophages and membrane thickness in the diabetic group. d+= CLHA-immersed CM; d-= PBS-immersed CM.

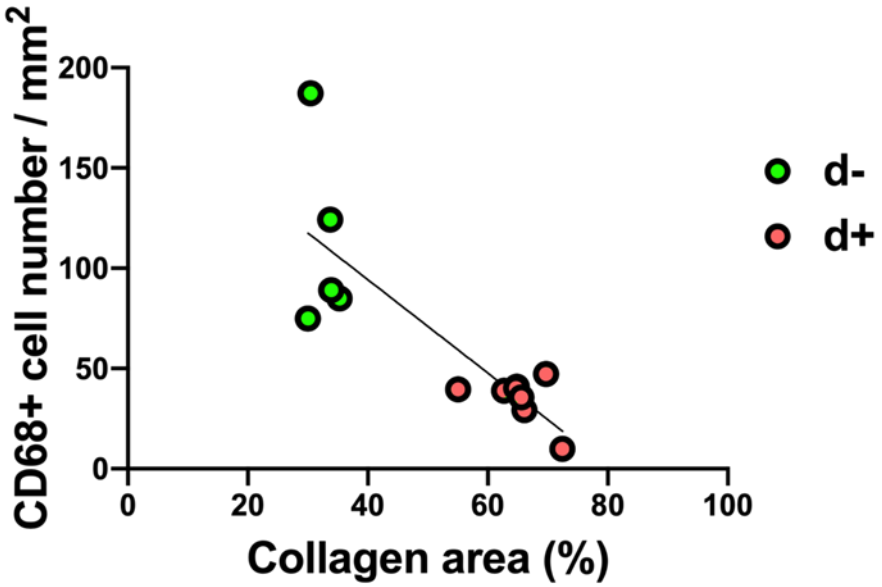


**Figure 8.** Scatter diagram showing a significant negative correlation (r = -0.7802, *p*= 0.0025) and linear regression line between the number of macrophages and collagen area in the diabetic group. d+= CLHA-immersed CM; d-= PBS-immersed CM.

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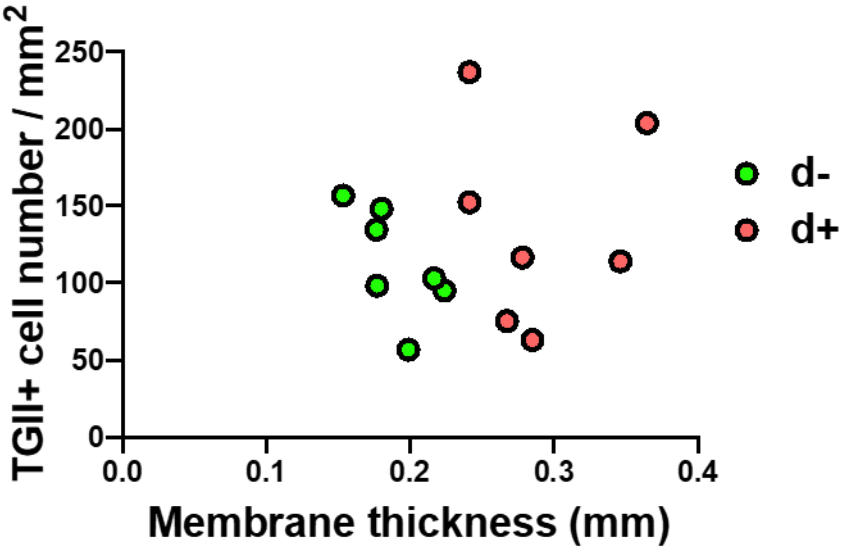


**Figure 9.** Scatter diagram showing no correlation (r = -0.0264, $p$ = 0.9307) between the number of endothelial cells and membrane thickness in the diabetic group. d+= CLHA-immersed CM; d-= PBS-immersed CM.

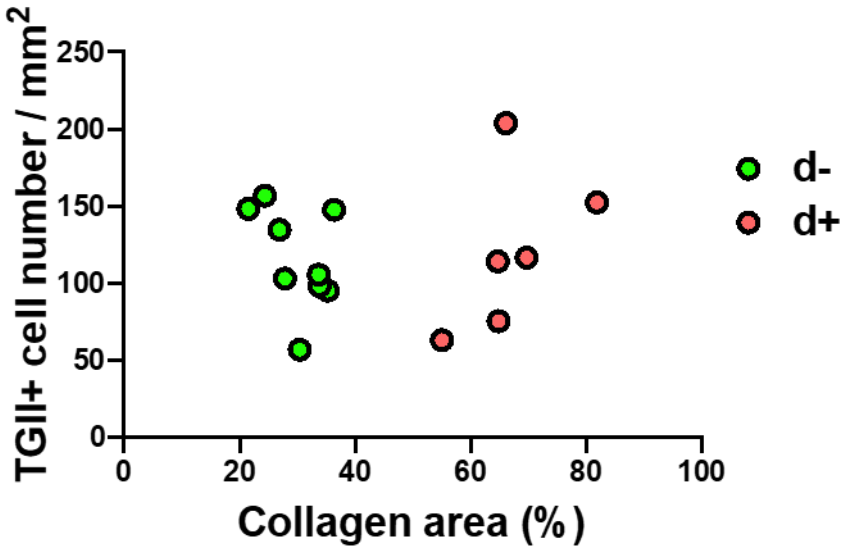


**Figure 10.** Scatter diagram showing no correlation (r = 0.0250, $p$ = 0.9336) between the number of endothelial cells and collagen area in the diabetic group. d+= CLHA-immersed CM; d-= PBS-immersed CM.

## 4. Discussion

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Several studies, including our recent publication, demonstrated that CM degradation is markedly enhanced in uncontrolled diabetes compared to a healthy situation [14,34,17]. Those studies showed distinctly more inflammatory cells penetrating the CMs that were implanted in diabetic rats compared to health rats. This observation, together with a finding of a 4-fold increase in the number of macrophages inside the CM in diabetic rats suggested an association between faster CM degradation in diabetic rats and the presence of inflammation in and around the CM [14,34,17]. The current study, showing that diabetes increased 2.5-fold the number of macrophages inside the CM discs is in agreement with these studies. In support of a hyperglycemia-induced local inflammation, a recent study using the same model reported that the expression and abundance of several inflammatory molecules (IL-6, TNF$\alpha$, MMP-9, MIF, MIP-1$\alpha$, and MIP-2$\alpha$) in the tissues around and within CMs implanted in diabetic rats was markedly elevated, compared with those in normoglycemic rats [37].

In the current study we found a significant negative correlation between the number of macrophages and the amount of residual collagen in the implanted CMs. Macrophages are known to participate in the host defense by their production of inflammatory cytokines, nitric oxide, and toxic oxygen metabolites contributing to collagen degradation [38]. These cells also have the capacity to secrete several members of the matrix metalloproteinase (MMP) family including collagenase-1 (MMP-1) [39], stromelysin (MMP-3) [40], gelatinase B (MMP-9) [41], and macrophage elastase (MMP-12) [42]. While the activity of these proteinases likely contributes to both the host defense function of macrophages and to normal tissue remodeling and repair [38], various reports have shown that the levels of tissue MMPs in diabetes are elevated and those of the tissue inhibitors of matrix metalloproteinases (TIMPs) are lower [43,44]. Excessive MMP-driven proteolysis has been associated with several pathological conditions including arthritis, cancer growth and metastasis, chronic obstructive pulmonary disease [45-47], and diabetes [43,44]. It is well known that CM degradation depends on collagenolytic activity of the host cells, specifically on MMPs [48,49]. Moreover, macrophage accumulation is attributed to increased non-enzymatic glycation of proteins (formation of advanced glycation end products (AGEs)) and oxidative stress (OxS) [50]. AGEs may affect many cell types by binding the receptor for AGEs (RAGE) and inducing the local formation of molecules that attract and retain macrophages such as monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) [50]. These activated macrophages, in turn, secrete a variety of pro-inflammatory mediators such as interluekin-1 (IL-1) and TNF$\alpha$ and contribute to diabetic tissue injury by producing reactive oxygen species (ROS) and MMPs [50].

The first part of the current experiment [34] showed the delaying effect of HMW CLHA on the degradation of the CM. CMs that were immersed in CLHA showed slower degradation in diabetic rats than those immersed in PBS. In contrast, such an effect of CLHA was not observed in the healthy rats. The fact that HMW CLHA had a significant effect only in the animals with uncontrolled diabetes is in agreement with other studies that showed the effect of HMW HA only in inflamed conditions (like uncontrolled diabetes [31]. Another proof that HMW HA is beneficial in chronic inflammatory situations was found in a systematic review and meta-analysis that showed that HA significantly improved healing of chronically inflamed wounds, including most difficult-to-treat diabetic foot ulcers [31].

One of the explanations for better outcomes with HMW CLHA in a chronic inflammatory state could be its effect on macrophages. Chronic inflammation is often associated with persistently activated macrophages, leading to most detrimental effects [51]. Therefore, macrophage reduction may help to reduce collagen degradation. This study examined the influence of CLHA on the number of macrophages present in the CM implanted in uncontrolled diabetic rats. Our results clearly showed the effect of HMW CLHA on the degradation of the CM in uncontrolled diabetic conditions through the reduction of macrophages. The reason for this is likely linked to a phenotypic switch

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macrophages undergo when they are in contact with HA. Macrophage phenotypes depend on the molecular weight of HA. At LMW of HA, macrophages have pro-inflammatory response and at HMW of HA, macrophages have anti-inflammatory and pro-resolving responses [52]. HMW HA can suppress inflammation induced by lipopolysaccharides (LPS) [53] and by pro-inflammatory cytokines like TNF-$\alpha$.

The current study also aimed at evaluating the effects of CLHA on the endothelial cells invading the CM. It is well known that aberrant angiogenesis in different tissues can play a role in the pathogenesis of many complications of diabetes (e.g., retinopathy, nephropathy) [54]. The enhancing effect of diabetes on blood vessel invasion into CM was found in several of our studies, where the number of endothelial cells was significantly higher in the diabetic group compared to the healthy group [14,17]. A 2- to 3-fold increase in the number of TGII positive endothelial cells was found within the CM discs that were implanted in diabetic animals. The data of the current study (a 2.5-fold increase) support these observations. The current study also found that adding CLHA to the CM had no effect on the number of endothelial cells inside the CM in the diabetic group and hence, no correlation was found between the number of endothelial cells and the residual collagen content and thickness of the CM.

## 5. Conclusions

Accelerated degradation of implanted CMs in rats with uncontrolled diabetes is strongly associated with increased infiltration of macrophages. Pre-implantation immersion of the CMs in cross-linked HMW HA slowed down their degradation most probably by reducing the number of infiltrating macrophages. Therefore, immersion of CM, to be applied in diabetic conditions, in CLHA can lead to a longer maintenance of CM thickness and collagen density, better preserving the barrier functions of CM.

**Author Contributions:**

**Conceptualization**, M.E.; **methodology**, M.E., O.M., R.M. and C.N; **software,** D.B. and M.K..; **validation**, R.M., A.S. and C.N..; **formal analysis**, D.B., M.E. and M.K..; **investigation**, R.M., M.E. and D.B..; **resources**, A.S..; **data curation**, M.K. and M.E.; **writing—original draft** preparation, M.E..; **writing—review and editing**, M.E., O.M., A.S., D.B. and M.W..; **visualization**, M.E..; **supervision**, A.S., O.M. and M.W..; **project administration**, M.E..; **funding acquisition**, A.S. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The institution Animal Care and Use Committee of Tel Aviv University, Tel Aviv, Israel, approved the study (TAU 1-16-031**).**

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**Conflicts of Interest:** "The authors declare no conflict of interest."

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