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BIOMEDICAL SCIENCES

Amniotic membrane modulates MMP9 and MMP12 gene and protein expression in experimental model of the hepatic fibrosis

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Abstract: Hepatic fibrosis is characterized by excessive deposition of collagen in the hepatic parenchyma, which disturbs the normal architecture and function. We have shown that human amniotic membrane (AM) can be used as a patch on the whole liver surface, resulting in an extremely significant reduction in collagen deposition. The aim of this study was to investigate the effects of AM on the matrix metalloproteinase 9 (MMP9) and matrix metalloproteinase 12 (MMP12) genes and proteins expression by real time quantitative PCR and immunohistochemistry, respectively, as well as image analysis on biliary fibrosis induced in rats by the bile duct ligation (BDL).Two weeks after the BDL, an AM fragment was applied onto the liver, and four weeks later, the liver samples were collected. MMP9 and MMP12 genes were significantly over expressed in group treated with AM. The immunoexpression of MMP9 and MMP12 was observed in all groups. However, the quantitative image analysis demonstrated an increase of the area occupied only by MMP12 in the livers of AM-treated rats with respect to BDL rats. These findings suggest that the AM exerts its beneficial effects on biliary fibrosis by increasing the MMP12, which in turn reduces the excessive collagen deposition on liver tissue.

Key words: Amniotic membrane, fibrosis, immunohistochemistry, gene expression, MMP9, MMP12.

INTRODUCTION

Liver fibrosis occurs following chronic liver injury from various insults including toxins (alcohol), infections (hepatitis), metabolic disease (nonalcoholic fatty liver disease) and cholestasis (Seki & Brenner 2015, Friedman & Pinzani 2022). Liver fibrosis is characterized by excessive deposition of collagen in the hepatic parenchyma, which disturbs the normal architecture and function. If do not treated, the progressive fibrosis could end to cirrhosis which is characterized by regenerative nodules of liver parenchyma separated by, and encapsulated in, fibrotic septa (Nathwani et al. 2020). An important complication of the cirrhosis is the liver cancer which, in Brazil, is the sixth cause of mortality in men and the eight cause of mortality in women (INCA 2021). Although, the studies have provided the understanding of cellular and molecular mechanisms of the development of this pathology, the liver transplant remains the only treatment option, which still has limitations, including scarce availability of donor livers and the increasing need for transplantation, lack of quality donor organ, hospitalization costs, failure or rejection of donated liver and long-term immunosuppression (Trotter 2017, Vishwakarma et al. 2018, Metin et al. 2020). Consequently, there is an urgent need of strategies to avoid or delay the need for liver transplantation.

A therapeutic strategy against fibrosis is the human amniotic membrane (AM). It is the innermost layer of the fetal membranes that are discarded with the placenta after delivery, so it has unlimited availability and has no ethical or legal barriers. The AM has been used as biomaterial in medicine for more than 100 years (Silini et al. 2015), and nowadays have widely applied in ophthalmology, dermatology, orthopedic, dentistry and urology (Nejad et al. 2021). These clinical applications prove that AM has beneficial effects on tissue repair and regeneration due to its properties including anti-inflammatory, antifibrotic, immunomodulation, antihemorrhagic, antipain, antibacterial, modulation of angiogenesis and promotion of epithelialization (Tehrani et al. 2017, Nejad et al. 2021).

Sant'Anna et al. (2011) demonstrated that the AM when applied as a patch to the liver surface immediately after bile duct ligation (BDL) resulting in decreased severity of biliary fibrosis with an extremely significant reduction in collagen deposition. Moreover, when applied AM in the liver with established biliary fibrosis was observed a reduction in fibrosis and demonstrated that one of the mechanisms of antifibrotic action was the reduction in the expression of fibrogenic cytokines, TGF-beta and IL-6, which decreased the activation of myofibroblasts (the main profibrogenic cells), and consequently the excessive collagen deposition (Sant'Anna et al. 2016).

Pradahn (2013) and Weiskirchen & Tacke (2016) have been shown that liver fibrosis and remodeling is a dynamic process and susceptible to regression and possible even full reversion. The reversal of fibrosis is characterized by a decrease in inflammatory and fibrogenic cytokine levels, shifting the balance from inflammation to resolution (leading to phenotypic adjustments of the immune cells, especially induction of restorative macrophages), increased collagenase activity by matrix metalloproteinases (MMPs), deactivation and disappearance of myofibroblasts and, finally, matrix degradation (Sun & Kisseleva 2015, Trautwein et al. 2015).

Because reversibility of liver fibrosis is mainly dependent on the collagenolytic activity of ECM-degrading MMPs (Pradahn 2013, Sun & Kisseleva 2015, Roeb 2018) and in an attempt to elucidate the mechanism(s) underlying the beneficial effects of AM, we investigate the effects of AM on the expression of the MMP9 and MMP12 gene and protein expression, on biliary fibrosis induced in rats by the bile duct ligation.

MATERIALS AND METHODS

Animals and experimental groups

This study was approved by the Animal Use Ethics Committee nº A03/CEUA/2016. Sixteen male Wistar rats, 8 weeks old of age, 200 to 250 grams, were randomly divided into the following groups: Group BDL - Bile Duct Ligature (6 rats), where the animals were submitted to BDL and euthanized after 6 weeks. BDL + AM Group (10 rats) where animals underwent BDL and after two weeks of ligation, a fresh human AM fragment was applied around all lobes of the liver.

Collection of human placenta and amniotic membrane processing

The study was approved by the Research Ethics Committee of the University of Vale do Paraiba, under protocol No. 3.617.247. Four human term placentas were obtained from elective cesarean sections of patients with normal gestation at the Santa Casa de São José dos Campos Hospital, with maternal consent and after testing negative for HIV-1 and 2, hepatitis B and C, and syphilis. The human AM was manually separated

from the chorion and washed extensively with physiological solution containing 100U / ml penicillin, 100 µg / ml streptomycin and amphotericin B. Then, the AM was cut into pieces of 9x9cm and stored at room temperature (Hennerbichler et al. 2007, Sant'Anna et al. 2011, 2016) in 50ml tubes containing DMEM culture medium without addition of serum and phenol-red and under sterile conditions until moment of application to animals. The AM fragments were used within 24h (Cargnoni et al. 2009, Sant'Anna et al. 2011, 2016, Mamede & Sant'Anna 2019).

Experimental model for the induction of fibrosis: bile duct ligation

The BDL experimental was the same as previously used by Sant'Anna et al. (2011, 2016). The rats were anesthetized with isoflurane 3% (Vetflurano®; Virbac, São Paulo, Brazil) by inhalation in camera. After trichotomy and disinfection, the rats were laparatomized for common bile duct exposure, which was double ligated with 4-0 silk suture and then sectioned between the ligatures. In the BDL + AM group, rats were subjected to BDL as described above. After 2 weeks of BDL, before abdominal wall closure, an AM fragment was placed to the liver with its mesenchymal side in contact with the liver lobes surface. After application to the liver, the extremities of the AM were joined with a drop of methacrylate glue to hold it in place, preventing its dispersion in the peritoneal cavity. After this procedure, the abdomen was closed as described above for the BDL group animals. After surgery, and during five days, the rats were submitted to antibiotic therapy (Enrofloxacin 2.5%, SC), and vitamin K (5mg / kg, SC) was injected weekly to decrease mortality from hemorrhagic diathesis.

Euthanasia of animals and collection of biological samples

Six weeks after BDL, the animals were euthanized with an overdose of the same isoflurane anesthetic (Vetflurano®; Virbac, São Paulo, Brazil), and liver samples were collected from the medial and right lobes from each rat (one sample from each lobe), totaling 12 samples in the BDL group and 20 samples in the BDL+AM group, that were snap-frozen in liquid nitrogen and stored frozen at -80°C for gene expression analysis. Again, other 2 samples per animal of the same lobes were immersed in 10% buffered formalin (Synth, São Paulo, Brazil) for 24h and embedded in Paraplast (SigmaAldrich, Germany) to obtain sections for immunohistochemical analysis.

Gene expression analysis

The total RNA was extracted using protocol: SV Total RNA Isolation System (Promega). The concentrations and integrity of the RNA samples were assessed using the ND-1000 spectrophotometer (NanoDrop Inc., Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Only samples with an RNA integrity number (RIN) ≥7 were considered for further analysis.

The gene expression analysis of the MMP9 and MMP12 genes and endogenous control α-actin were performed by Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) technique on the ABI Prism 7500 Sequence Detection System (Life Technologies, Foster City, CA, USA). Complementary DNAs (cDNA) were synthesized in the thermal cycler (Biocycler, MJ96G, USA) according to the ImProm-II Reverse Transcription System protocol (Promega), using 1µg of total RNA from each sample, 1µL of Oligo (dT) 15 (Promega), supplemented with nuclease free water to a final volume of 5µl and reverse

transcriptase mix that consisted of4 µL ImProm-IITM Reaction Buffer (5X) buffer, 1 µL ImProm-IITM Reverse Transcriptase enzyme, 4.8 µL MgCl2, 1 dNTP MIX (final concentration 0.5mM) and 3.7µL nuclease free water. Reverse transcription was carried out for 5 min at 25 °C, 60 min at 42 °C, followed by 15 min at 70 °C.

RT-qPCR reactions were performed in duplicate in ABI Prism 7500 Sequence Detection System (Life Technologies, Foster City, CA, USA) using BRYT Green do Go Taq qPCR Master Mix (Promega) according to manufacturer's instructions. The primers for MMP9 (sense: 5'-GGGCGCTCTGCATTTCTT-3'and anti-sense: 5'-CCTCCGTGATTCGAGAACTTC-3'), MMP12 (sense: 5'-GGAGCCCCAGTGAAAAACC-3' and anti-sense: 5'-CACACAGTTGATGGTGGACTTC-3') and reference gene α-actin (sense: 5'-ACCCACACTGTGCCCATCT-3' and anti-sense: 5'-AGACGCAGGATGGCATGAG-3') were designed using Primer Express software (version 3.0) (PE Applied Biosystems, Foster City, CA, USA). In order to avoid amplification of contaminating genomic DNA, the primers were placed at the junction between the two exons or in a different exon. The concentration-use of the primers was 10µM. RT-qPCR conditions consisted of an initial denaturation at 95° C for 10 minutes followed by 40 cycles of three steps: denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 60° C for 1 minute; and finally, a final step at 4°C.

For all primers, amplification curves were constructed with serial dilutions of healthy liver and liver fibrosis cDNA (100, 20, 4, 0.8, and 0.16 ng/ul). The standard curves of the targets and reference gene showed similar amplification efficiencies (>90%). The RT-qPCR amplification data were analyzed using the Sequence Detection System software (version 2.1) (PE Applied Biosystems, Foster City, CA, USA) and plotted on a graph (fluorescence intensity versus number of cycles). Only the replicates with low variability (i.e., Δ cycle quantification <0.5) were considered for further analyses.

The relative expression levels of the MMP9 and MMP12 targets genes were calculated using the Delta-Delta Ct (ΔΔCt) method (Pfaffl 2001), in which, for each sample, the mean cycle threshold (Ct) value of the target gene is subtracted from the mean Ct value of the reference gene to determine the ΔCt value. The normalized expression levels (ΔCt value) of the MMP9 and MMP12 targets genes of each sample (test sample) was subtracted from the average ΔCt value of control samples and determine ΔΔCt, that was added to formula $2^{-\Delta\Delta Ct}$ and obtained the relative quantification (QR) values. QR values refers to the amount of transcript for each sample, where the decreased gene expression was considered when QR values were lower than 0.5 (QR < 0.5) and over expression when QR values were higher than 2.0 (QR > 2.0).

The RT-qPCR results were statistically analyzed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The Mann-Whitney test was used to find out the statistical significance of the expression levels (QR values) evaluated by RT-qPCR among the groups of tissues analyzed. The results were considered statistically significant with $p \leq 0.05$.

Immunohistochemistry (IHC)

The IHC was performed on 4µm-thick tissue sections placed on salinized slides (StarFrost Adhäsiv-Silan, Knittel Glass, Germany). Briefly, each section was deparaffinized with xylene and hydrated in graded ethanol. Antigen retrieval was performed using 10mM sodium citrate buffer (pH 6.0) for 5 min at 121 °C, followed by endogenous peroxidase blocking with 3% hydrogen peroxide for 15 min, followed by 3 washes in PBS containing 0.05% Tween 20 (Sigma, Saint Louis, MO, U.S.). Sections were

then incubated for 2h at room temperature with the following primary antibody: anti-MMP12 (# ab231109; rabbit polyclonal, dilution 1:1000) and anti-MMP9 [EP1254] (# ab76003; rabbit monoclonal, dilution 1:1000), both from Abcam (Cambridge, MA). The sections were again washed and incubated with the ImmPRESS Universal Reagent Anti-Mouse / Rabbit IgG Secondary Antibody Detection Kit (avidin and biotin free polymer) (Vector, Laboratories) according to the manufacturer's instructions. After 3 washes in PBS containing 0.05% Tween 20, visualization of the immunohistochemical reaction occurred by incubating the sections for 1 min and 30sec with DAB chromogen (ImmPACT DAB, brown,Vector Laboratories). After washing in running and distilled water, slides were counterstained with Harris Hematoxylin for 35s, dehydrated in alcohol, cleared in xylol and mounted with Permount. The specificity of the antigen-antibody reaction was tested by replacing the primary antibodies with the PBS + 0.05% Tween 20 buffer.

Quantitative image analysis

The quantitative evaluation of the area occupied by the immunostainings with MMP9 and MMP 12 antibodies was performed using the image analysis program CellProfiler cell image analysis software (Lamprecht et al. 2007; Sant'Anna et al. 2011). The images of all stainings were visualized in the Leica DM2500 Trinocular Microscope and captured by a digital camera (LEICA DFC 425) and digitized at 1024x768 pixels, 24 bits / pixel resolution with a global magnification of 200x. The digital images were processed by CellProfiler, to identify, isolate, and measure areas occupied by MMPs immunostained cells over the total liver parenchyma area. The mean percentage taken from 10 non overlapping random fields per section was used to generate a single value for each specimen of each experimental group. Data were subjected to Shapiro-Wilks normality test, and then to the parametric two-tailed Student's *t*-test. The p value <0.05 was considered statistically significant (*). Data analysis and graph construction were performed using GraphPad Prism software version 6.0.

RESULTS

Gene expression

In this study, RT-qPCR analysis performed for the MMP9 and MMP12 genes on two liver samples per animal (one sample from the medial lobe and one from the right lobe), totaling 12 samples in the BDL group (n=6) and 19 samples in the BDL+AM group (n=10), showed significant increase in the expression of the MMP9 (p = 0.0037) and MMP12 (p = 0.0037) genes in the BDL+AM group when compared to the BDL group (Figure 1a,b).

The MMP9 gene analysis showed in the BDL group, four samples with increased expression (QR = 5.63 to 9.54), three samples with decreased expression (QR = 0.17 to 0.24) and five samples with normal expression (QR = 0.66 to 1.96) when compared to the QR normal values. In the BDL+AM group, 14 samples showed increased expression (QR = 2.95 to 845.97), no sample showed decreased expression and five samples showed normal expression (QR = 0.85 to 1.74) when compared to the QR normal values.

The MMP12 gene analysis showed in the BDL group with fibrosis, 12 samples with over expression (QR = 5.63 to 2295.74), no sample with decreased or normal expression when compared to the QR normal values. In samples with membrane-treated fibrosis, 19 samples showed increased expression (QR = 36.44 to 998 023.93) and no sample showed decreased or normal expression when compared to the QR normal values.

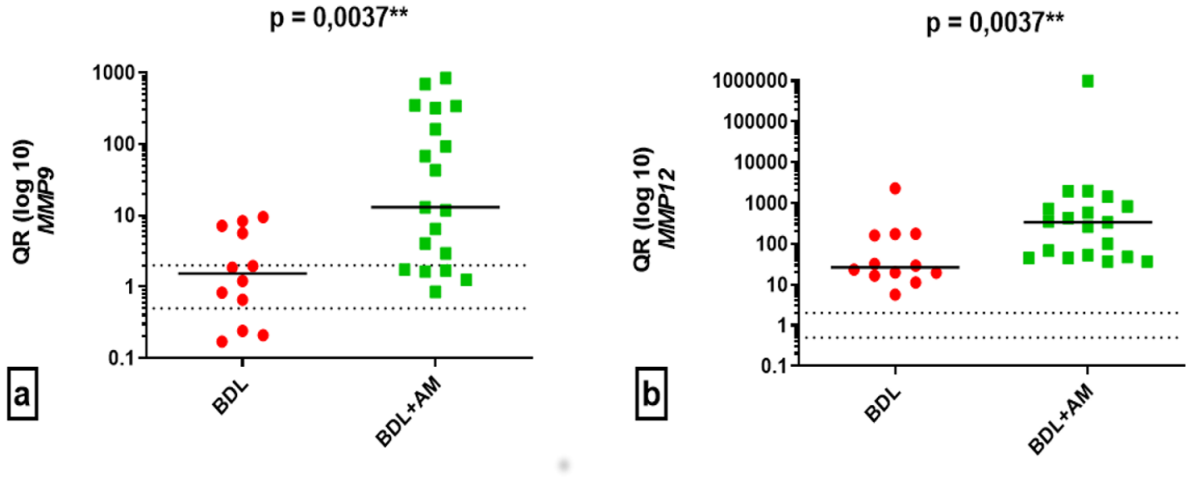

Figure 1. Comparison between medians of gene expression levels by the RT-qPCR technique of MMP9 (a) and MMP12 (b) transcripts between the BDL group and the BDL+AM group. BDL: Bile Duct Ligature, BDL+AM: BDL with application of the amniotic membrane. **p<0.01 versus BDL group.

IHC and quantitative image analysis

The results of IHC demonstrated the distribution pattern of MMPs in the liver tissue of the two experimental groups (Figures 2a,b and 3a,b). MMP12 immunoexpression was observed in several cell types, including hepatocytes, cells located in the capillaries and central vein lumen, Kupffer cells and/or macrophages near the sinusoid capillaries with different shapes (oval, circular, elongated) and sizes, which were located mainly in the Pericentral and Intermediate zones of hepatic parenchyma. In the BDL group a mild immunostaining for MMP12 was observed in some cells near the sinusoids and in some hepatocytes. In this group the liver architecture was compromised by various biliary structures surrounding small nodules of hepatic parenchyma but without immunostaining for MMP12 (Figure 2a). In contrast, in the BDL+AM group the architecture of the liver parenchyma was preserved, the hepatocytes are arranged in branching plates separated by sinusoids, and in both cells a moderate immunostaining for MMP12 was observed (Figure 2b). Regarding MMP9, the immunoexpression was observed in cells with oval or round morphology located in the portal and periportal regions around and near biliary structures. The hepatocytes and biliary structures were not labeled for MMP9 (Figure 3a,b).

Quantitative data for the area occupied by MMP12 and MMP9 obtained by the image analysis are shown in Figure 4a, b. The results of the quantitative image analysis of MMPs immunoexpression demonstrated a significant increase in the percentage of area occupied by MMP12 in the group treated with AM (BDL + AM) when compared to the group BDL without treatment. The area occupied by MMP9 expression in the BDL + AM group was similar to the AM group. The AM treated BDL group presented the highest mean of area occupied by MMP12 when compared to the untreated group BDL, respectively (8.75 ± 3.65% x 4.00 ± 1.41%, p = 0.0092). In contrast, the area occupied by expression of MMP9 was similar in the two groups (BDL+AM: 0.91 ± 0.29% x BDL: 1.06 ± 0.66%, p = 0.567)

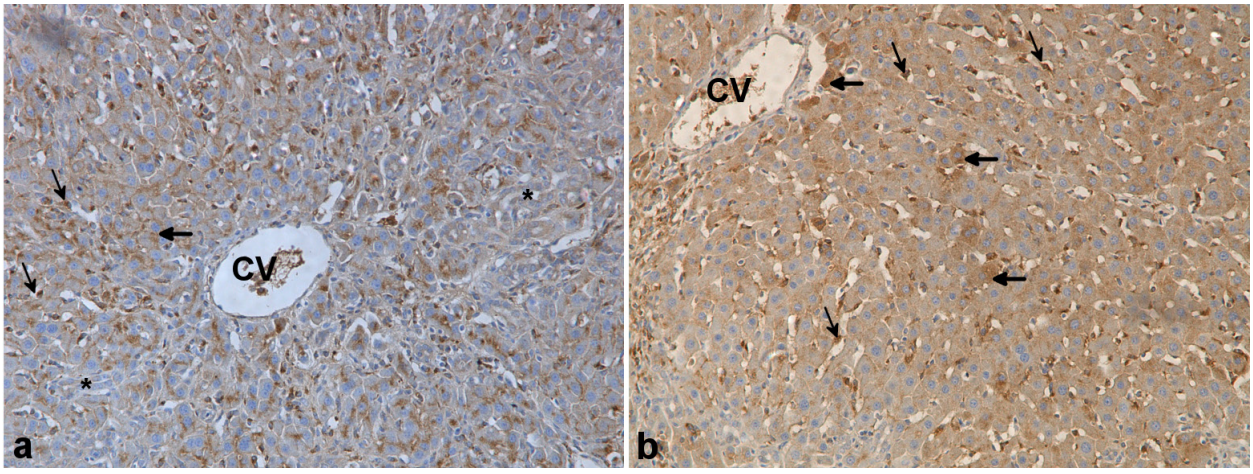

Figure 2. Immunohistochemical of MMP12 staining in the experimental groups. (a) BDL group: mild staining in some cells (↽) with irregular morphology near the sinusoids and in in some hepatocytes (↽); and no staining in biliary structures (*). (b) BDL + AM group: moderate staining in cells near the sinusoids and in hepatocytes. BDL: Bile Duct Ligature, BDL+AM: BDL with application of the amniotic membrane, CV: central vein. Original magnification x200.

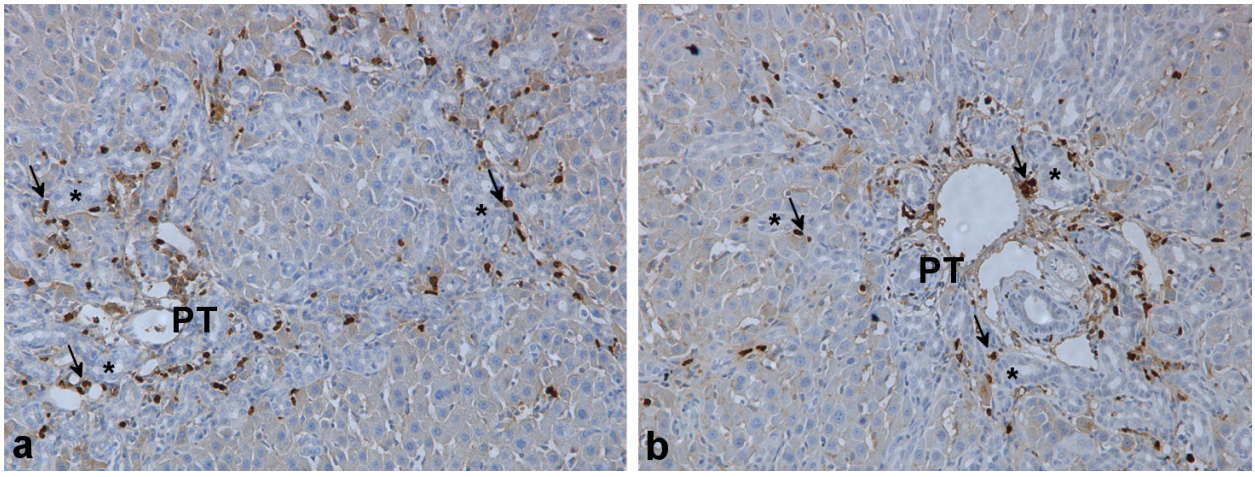

Figure 3. Immunohistochemical of MMP9 staining in the experimental groups. (a) BDL group; (b) BDL +AM groups: staining in oval and round cells (↽) located in the portal and periportal regions around and near of biliary structures without immunostaining (*). BDL: Bile Duct Ligature, BDL+AM: BDL with application of the amniotic membrane, PT: portal tract. Original magnification x200.

DISCUSSION

To understand the mechanism of action of AM in the reduction of hepatic fibrosis, the present study evaluated the expression of two genes, belonging to a family of 28 endopeptidases known as MMPs, which are zinc metalloproteinases with multiple domains specialized in degradation of the different components of the ECM, including fibrillary and non-fibrillary collagens and elastin. These proteins control the formation and degradation of ECM, which is an essential event in the biological processes involved in maintaining homeostasis and tissue regeneration, such as healing and fibrosis (Weiskirchen & Tacke 2016, Galliera et al. 2015).

In the present work the expression of the MMP9 and MMP12 genes demonstrated a significant increase in the group treated with AM compared to the BDL group. The MMP9

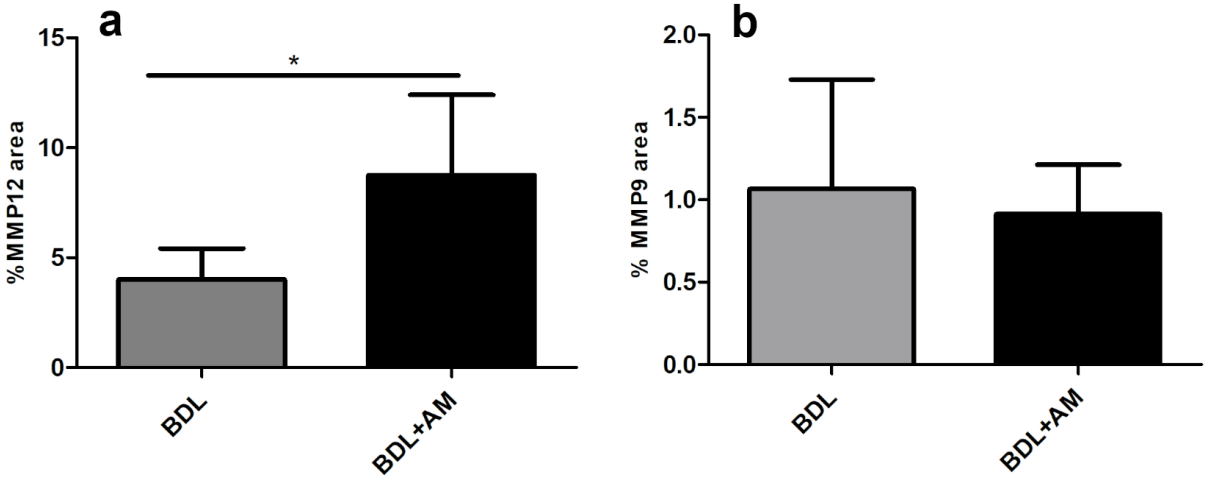

Figure 4. Quantitative evaluation of MMPs. Mean ± standard deviation (SD) of area occupied by MMP12 (a) and MMP9 (b) are represented as bars for BDL (gray) and BDL+AM (black). *p<0.05 versus BDL group.

gene is responsible for encoding the matrix 9 metalloproteinase enzyme, which plays important roles in wound healing, arthritis, angiogenesis and tumor metastasis (Nagase & Frederick Woessner 1999, Duarte et al. 2015, Chen et al. 2017). In the liver, during the first week of recovery from fibrosis the expression of MMP-9 has been demonstrated on mRNA and protein levels in rodent models, involved in the proteolytic degradation of cleavage products from fibrillary collagens, but no activity against collagen type I. MMP-9 may also contribute to recovery from fibrosis by accelerating HSC apoptosis (Roderfeld 2018). The MMP12 gene was described as macrophage metalloelastase, responsible for encoding the characteristic enzyme of macrophages, which is mainly involved in the degradation of elastin that is the major component or characteristic feature of advanced fibrosis (Pellicoro et al. 2012).

Manuelpillai et al. (2012) used the cell therapy with amniotic epithelial cells (hAEC) in rats with tetrachloride-induced fibrosis (CCL4), detecting an increase in MMP9 mRNA expression levels, but a reduction in MMP12 gene mRNA. Madala et al. (2010) examined the role of MMP12 in S. mansoni egg-induced inflammation and fibrosis in the liver and revealed an important counter-regulatory link between MMP12 and other ECM degrading metalloproteinases. According to this study, the reduction of the MMP12 gene could be positive, which is considered an inhibitor of MMP2, MMP9, and MMP13, enzymes that promote the degradation of the ECM in this model of fibrosis. Thus, the authors showed that the MMP12 gene can act in the progression of fibrosis, but it is worth mentioning that the fibrotic model used by these authors was different from our study. Madala et al. (2010) evaluated fibrosis caused by *Schistosoma mansoni*, and did not use any treatment, while Manuelpillai et al. (2012) used a fibrosis model inducing CCL4 and used the treatment with hAEC. Sugiura et al. (2018), using the fibrosis induction method by alfa-naftil-isocianato (ANIT) observed decreased expression of the MMP9 gene in the groups treated by transplantation of amnion-derived human mesenchymal stem cells (hAMSCs) and through conditioned medium obtained from cultures of hAMSCs. The discrepancy of the results obtained among studies may be due to the different methods employed, both in the treatment and in the induction of liver fibrosis, which may behave differently depending on

its origin and the degree of severity (mild to moderate) (Lee et al. 2015, Campana & Iredale 2017).

Hepatic macrophages account for the largest non-parenchymal cell population in the liver and play a crucial role in both fibrogenesis and fibrosis resolution (Li et al. 2016). Although an oversimplification, studies have suggested that polarized macrophages are often referred to as M1 or classically activated for pro-inflammatory macrophages, and as macrophage type 2 (M2) or alternatively activated for macrophage associated with remodeling and repair (Weiskirchen & Tacke 2016, Li et al. 2016). Manuelpillai et al. (2012), after transplanted hAEC in mice with advanced hepatic fibrosis, also related increased mRNA expression of the YM-1, CD206 and IL-10 genes associated to the M2 alternatively activated phenotype associated with hepatic wound healing. Our previous study (Teixeira et al. 2020), using the same animal model to induce fibrosis was also demonstrated increase in CD206 gene expression after intact AM application, suggesting that AM may stimulate CD206 gene expression, which is related to macrophage M2 and fibrosis repair. The results obtained for these authors is in line with the over expression of the MMP9 and MMP12 genes observed in our study, considering that these MMPs are mostly expressed by macrophages with the M2/restorative phenotype during fibrosis resolution phase (Weiskirchen & Tacke 2016). The restorative function of MMP-9 and MMP-12 was also confirmed in another study analyzing fibrosis resolution (Pellicoro et al. 2012).

In order to confirm the role of the MMP9 and MMP12 genes in the action of the amniotic membrane in hepatic fibrosis we analyzed the expression and distribution of MMP9 and MMP12 proteins by immunohistochemistry and quantitative image analysis. The results demonstrated a significant increase of the MMP12 protein expression in the liver tissue of the group treated with AM in relation to the group without treatment and similar expression to MMP9 protein in two groups. MMP9 is a gelatinase that has activity on collagen types IV, V, VI VII, X and XI, and some activity on elastin, but does not have a clear collagenase activity against type I interstitial collagen, although its expression increases at the beginning of the fibrosis process, thus suggesting an indirect participation in the degradation of the matrix (Campana & Iredale 2017).

In the liver, several cells express MMPs, including hepatocytes, cholangiocytes, stellate cells, Kupffer cells, neutrophils, and macrophages from blood monocytes. It is known that the increase or decrease in the expression of MMPs in different cell types also varies according to the type of fibrosis (Roeb 2018). However, hepatic macrophages are the cells that play a key role in the resolution of fibrosis through the secretion of matrix metalloproteinases in the late stages of the disease (Li et al. 2016). In our study the results of protein expression by IHC demonstrated the pattern of distribution of MMPs immunostaining in the structure of liver tissue. Considering that inflammatory cells and macrophages express MMP9 (Campana & Iredale 2017, Tag et al. 2015), and that the immunolocalization of MMP9 was observed in the cells of the connective tissue of the portal space and periportal area adjacent to some biliary structures, it can be suggested that these cells are inflammatory cells and/or macrophages. These results were partially like Carvalho et al. (2013) because these authors used the same BDL model and demonstrated MMP9 protein expression in macrophages near portal spaces and fibrous septa. However, different of our result they observed high expression of MMP9 protein in the liver tissue suggesting, that bone marrow mononuclear cells (BMMNCs) may stimulate MMP production, contributing

to ECM degradation and hepatic regeneration. This distinct result could be attributed to use of different therapies, such as transplantation of intact AM (in the present study) versus BMMNCs (Carvalho et al. 2013). Although the period of the transplantation was the same (e.g. two weeks after of BDL injury) in these studies, the time of treatment was different, four weeks versus one week, respectively.

The contradictory results observed in the gene and protein expression of the MMP9 can be explained because the protein expression does not always correspond to the expression of the gene that encodes it (Liu et al. 2016) because often not all the expressed mRNA is encoded in its corresponding protein, that is, the concentration of transcripts (RNAs) that contribute to the level of expression of a protein can vary, in addition to the existence of multiple important processes that can also influence the translation of that protein (McManus et al. 2015). Another hypothesis that explains the different results observed is the occurrence of alternative splicing in the MMP9 gene (Lee & Rio 2015) that originates two different transcripts of the gene. We observed that the primer used in the expression analysis of this gene is specific only for transcript 1, and our hypothesis is that the isoform of the MMP9 protein may have been translated from transcript 2 of the gene, and thus the increase in expression observed for the transcript 1 was not reflected in an increased expression of the MMP9 protein isoform which may have been encoded by transcript 2 which was not analyzed.

MMP12 protein immunolocalization was confirmed in cells like macrophages. In our previous study the presence of macrophages was confirmed on the wall of sinusoids (Mamede & Sant'Anna 2019). These results corroborate the studies by Pellicoro et al. (2012) and Iredale et al. (2013) that demonstrated that macrophages are cells that produce MMP12, also called macrophage metalloelastase, in addition to demonstrating that in liver fibrosis the degradation of the extracellular matrix is mediated by MMP12. Moreover, the increased expression of MMP12 protein in the group treated with AM validated it over gene expression observed in this group.

This study is pioneer in testing the hypothesis that intact AM applied around the liver exerted its antifibrotic activity on biliary fibrosis by increasing MMPs. Thus, we consider the increase in MMP12 an important result, as it strongly suggests that one of the mechanisms of antifibrotic action of AM in biliary fibrosis might be due to the action of MMP12, which has a degradation function of the main component of ECM, elastin and fibrillary collagen.

In conclusion, this study demonstrated that AM application has increased the expression of the MMP9 and MMP12 genes and of the MMP12 protein in hepatic tissue during the resolution of biliary fibrosis Therefore, we suggest that one of the mechanisms by which transplanted AM can reduce scarring in biliary fibrosis is through increased expression of the MMP12 in the liver tissue, which in turn reduces the excessive collagen deposition on liver tissue.

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