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Antifibrotic effects of total or partial application of amniotic membrane in hepatic fibrosis

KARINA M. MAMEDE and LUCIANA B. SANT'ANNA

Laboratório de Histologia e Terapia Regenerativa, Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba, Campus Urbanova, Av. Shishima Hifumi, 2911, Urbanova, 12244-000 São José dos Campos, SP, Brazil

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Abstract: Liver fibrosis is the final common pathway of chronic liver diseases, having cirrhosis as a possible progression, which has liver transplantation as the only effective treatment. Human amniotic membrane represents a potential strategy as a therapy for liver fibrosis, due to its anti-inflammatory, anti-fibrotic and immunomodulatory properties. The aim of this study was to evaluate amniotic membrane effects as a treatment for hepatic fibrosis induced in rats by bile duct ligation (BDL), verifying alterations between two different forms of amniotic membrane application, around all the lobes of the liver and around only one lobe of the liver. Two weeks after inducing fibrosis, an amniotic membrane fragment was applied to the surface of the liver, covering it either totally or partially. Four weeks later, the animals were euthanized and liver samples were collected. Histopathological and quantitative analyses demonstrated fibrosis severity decrease and an extremely significant reduction in the deposition of collagen in the groups treated with amniotic membrane, particularly when the amniotic membrane was applied in only one liver lobe. It is concluded that the amniotic membrane acted on the repair of liver fibrosis in both modes of application, with the application of the amniotic membrane around only one hepatic lobe being more effective in reducing the severity / extent of fibrosis.

Key words: Amniotic membrane, histopathology, immunohistochemistry, liver fibrosis, myofibroblasts.

INTRODUCTION

Chronic liver diseases (CLDs) represent a significant global public health problem, due to their high prevalence and incidence, and an increasing cause of morbidity and mortality. They have been responsible for about 2 million deaths per year (Marcellin and Kutala 2018). These pathologies can be caused by different etiologies, such as viral infections, alcoholism, chemical toxicity, or metabolic and biliary disorders, presenting liver fibrosis as a frequent implication (Li and Crawford 2004).

Liver fibrosis is characterized by an excessive accumulation of extracellular matrix (ECM) in the hepatic parenchyma, which distorts the normal liver architecture, forming a scar tissue that encapsulates the injured area. With disease progression, rings of scar tissue surround regenerative nodules of the liver parenchyma, which characterizes the final stage of the disease, known as cirrhosis (Schuppan 2015, Koyama and Brenner 2017). Cirrhosis is the worst consequence of liver fibrosis, and the only curative treatment is organ transplantation, which still has several limitations, including scarce availability of liver donors, risk of immune rejection, immunosuppressive therapy

Correspondence to: Luciana Barros Sant'Anna
E-mail: lucianabsa@gmail.com
ORCid: https://orcid.org/0000-0002-5128-7414

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for the whole lifetime, the complications of long-term immunosuppression, such as infection and renal failure, and legal, ethical, economic and religious implications. In addition, patients with cirrhosis exhibit a high risk of developing portal hypertension, irreversible liver failure, and hepatocellular carcinoma (Meirelis et al. 2015, Koyama et al. 2016, Jadlowiec and Taner 2016, Azzam 2018, Rawla et al. 2018).

Recent studies indicate that liver fibrosis is reversible when the causative agent is removed. The reversal of liver fibrosis is characterized by decreased inflammatory and fibrogenic cytokine levels, increased collagenase activity and the disappearance of myofibroblasts and fibrous scars (Sun and Kisseleva 2015, Campana and Iredale 2017, Aro braz et al. 2017, Ebrahimi et al. 2018). Despite advances in understanding fibrosis progression and its possibility of regression, as well as several potential antifibrotic therapies that target fibrosis regression, there is not a standard treatment for all patients.

The amniotic membrane (AM) represents an alternative approach to manage hepatic fibrosis (Sant'Anna et al. 2011a, 2016, 2017). AM is the innermost layer of the fetal membranes, usually discarded after birth, as part of the placenta, making it readily available and a highly abundant tissue. Moreover, it is cost-effective and free from ethical concerns. Basically, the AM is characterized by an epithelial layer, a basement membrane and an avascular stroma (Niknejad et al. 2008). Amniotic membrane-derived cells present a pluripotent/multipotent potential capability of differentiating, *in vitro*, toward tissues of all three germ layers, and have low immunogenicity, allowing transplantation without acute rejection by the host (Kubo et al. 2001, Bailo et al. 2004, Parolini et al. 2009, Silini et al. 2015). Moreover, the AM cells produce bioactive factors that contribute to the benefits of AM, including anti-inflammatory, anti-fibrotic, antitumoral, pro-epithelization, and wound healing properties (Manuelpillai et al. 2011, Magatti et al. 2018).

Due to its properties, the AM has been used as biomaterial in medicine for more than 100 years (Silini et al. 2015), and has nowadays been widely applied in ophthalmology for ocular surface reconstruction (Rahman et al. 2009), repair of burn wounds (Mohammadi et al. 2013), dental (Mohan et al. 2017) and orthopedic surgery (Heckmann et al. 2016), and wound healing (Kogan et al. 2018). The AM is a useful tool in the treatment of several conditions with different purposes, which makes it necessary to study the best way of applying AM in relation to the goal. Thus, there is no standard form in AM application, and several techniques have been developed with the purpose of adapting AM application to the intended purpose (Rahman et al. 2009).

Several variables may influence the response of the damaged tissue or organ to the action of the AM, including its preservation mode (fresh, lyophilized, cryopreserved, hypothermic), and the mode of application in the tissue (patch, graft, layered, with the mesenchymal or epithelial side in contact with the tissue or organ) (Hennerbichler et al. 2007, Rahman et al. 2009, Niknejad et al. 2013). In this context, the aim of this study was to evaluate the effects of two different forms of application of the AM patch, when applied totally (around all liver lobes) or partially (around only one lobe of the liver), in hepatic fibrosis induced in rats by bile duct ligation.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL GROUPS

The study was approved by the Ethics Committee on Animal Use of the Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba (protocol n° A03/CEUA/2016). Twenty six male Wistar rats, weighing 200–250g, were randomly divided into three experimental groups: group

BDL (*n*=6) - the rats were subjected to the BDL (Bile Duct Ligation) procedure; group BDL+AM_0 (*n*=10) - the rats were subjected to the BDL and two weeks later, a fragment of fresh AM was applied around all lobes of the liver; BDL+AM_1 (*n*=10) - the rats were subjected to the BDL and two weeks later, a fragment of fresh AM was applied around only a portion of the liver lobe.

COLLECTION AND PROCESSING OF AM

The study was approved by the Research Ethics Committee of UNIVAP, under the protocol number 1. 647.871. Human term placentas were obtained from cesarean sections with maternal consent and after negative serological tests for HIV-1, Hepatitis B and C, and syphilis at the Santa Casa Hospital in the city of São José dos Campos. The AM was manually separated from the chorionic membrane and washed extensively with saline containing 100 U/mL of penicillin, 100 mg/mL of streptomycin, and amphotericin B. The AM was then cut into pieces of adequate dimension (6×9 cm and 9x9 cm) and flagged with a cut at the top right of each patch to identify the mesenchymal side of the membrane and stored at room temperature in vials containing 50 mL of DMEM culture medium without serum and phenol red. The AM fragments were used within 24 h.

RAT MODEL OF LIVER FIBROSIS: BILE DUCT LIGATION

The model of liver fibrosis induced by common bile duct ligation is a widely accepted model in rodents, which reproduces and allows the study of the pathologic features of biliary fibrosis caused by cholestasis (Kontouras et al. 1984, Li and Crawford 2004). As previously described (Sant'Anna et al. 2011a, 2016, 2017), the animals were anesthetized with 3% isoflurane (Vetflurano®; Virbac, São Paulo, Brazil) by inhalation, in-camera. Subsequently, the surgical procedure started with trichotomy and disinfection of the abdominal region with 70% alcohol and povidone, followed by a midline incision and exposure of the common bile duct, which was doubly connected with a 4-0 silk suture. The first ligature was performed in the junction of the hepatic ducts, and the second ligature was made above the entrance of the pancreatic duct (Figure 1a). The common bile duct was then sectioned between the ligatures (Figure 1b). The abdominal incision was closed in two layers with 4-0 and 3-0 suture silk. In the group BDL + MA, the rats were submitted to BDL, as described above, and after 2 weeks of surgery, an AM fragment was applied to the liver with the mesenchymal side in contact with the hepatic surface, so that the whole liver lobes were covered with an AM fragment of 9x9 cm, (group BDL+ AM_0) (Figure 1c) or only the medial lobe, with a fragment of 6x9 cm (group BDL+AM_1) (Figure 1d). After wrapping either the whole liver or only a portion of the liver, the membrane extremities were attached to each other with a drop of methacrylate glue, to keep it in position, avoiding its dispersion into the peritoneal cavity. After this procedure, the abdomen was closed, as described for the animals of the group BDL. During the five days following surgery, antibiotic therapy with 2.5% Enrofloxacin was performed and vitamin K (5mg / kg) was injected weekly to decrease mortality from hemorrhage, both administered subcutaneously.

COLLECTION OF BIOLOGICAL SAMPLES AND HISTOLOGICAL PROCESSING

Six weeks after BDL, the rats from each experimental group were euthanized with an excess of isoflurane (Vetflurano®; Virbac, São Paulo, Brazil), and liver samples were collected from the medial lobe and right lobe. The specimens were fixed in 10% buffered formalin for 48 h and embedded in Paraplast (SigmaAldrich, Germany). Four-µm-thick sections were obtained with a semi-automatic microtome, and stained with Masson's trichrome for fibrosis and collagen analysis,

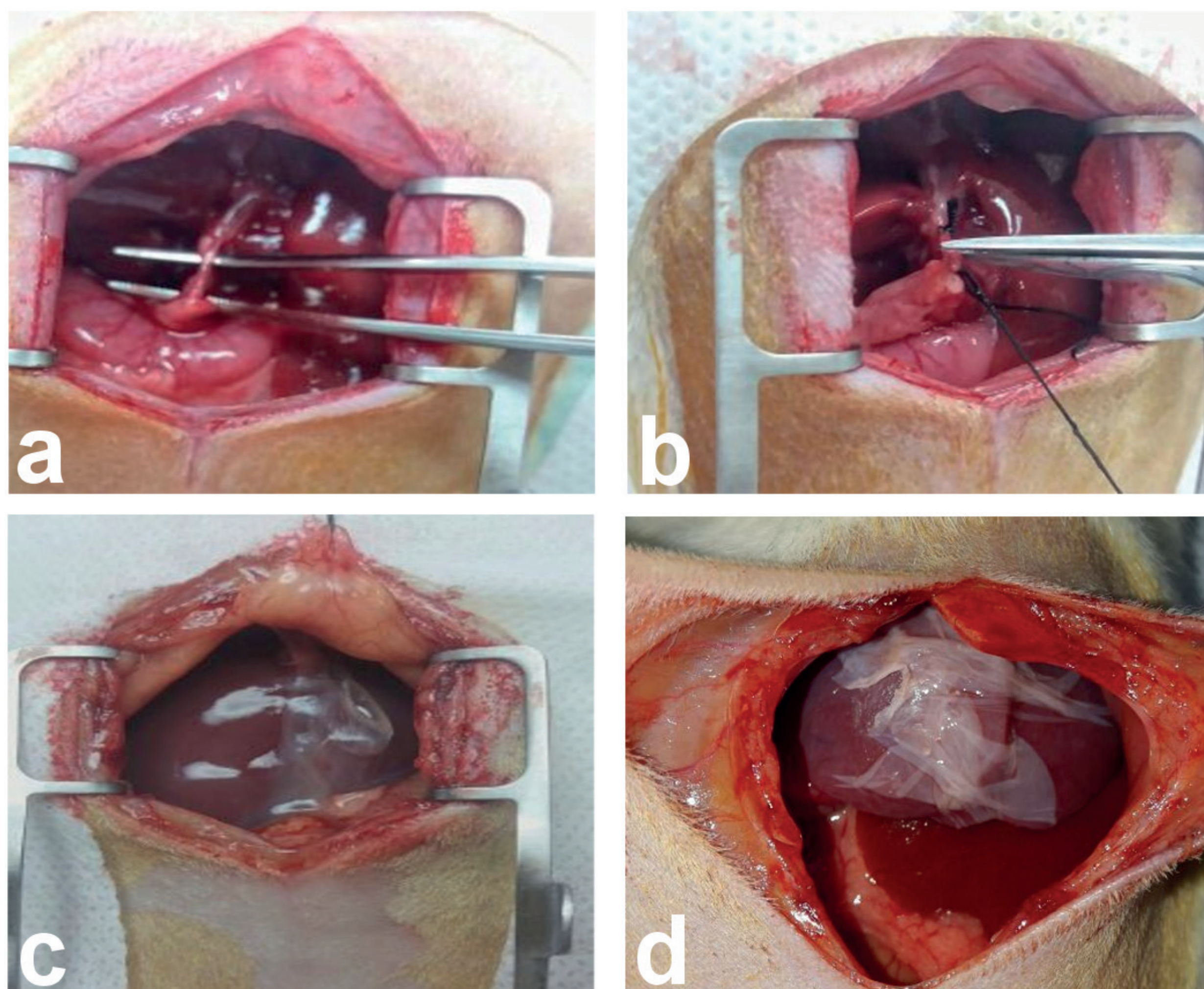

Figure 1 - Bile duct ligation: Exposure of the bile duct (**a**); Bile duct ligation (**b**); Application of AM around all lobes of the liver – Group BDL+AM_0 (**c**); Application of AM around the medial lobe – Group BDL+AM_1 (**d**).

according to the manufacturer's instructions or placed on polysinine-L coated slides for immunohistochemical assessment.

QUALITATIVE ANALYSIS OF THE FIBROSIS DEGREE

The degree of liver fibrosis was qualitatively assessed using a Nikon Eclipse E200 optical microscope at 100x magnification by the Knodell scoring system, which classified the histopathological alterations, as follows (Brunt 2000): score 0: no fibrosis; score 1: fibrous portal expansion; score 3: bridging fibrosis (portal-portal or portal-central); score 4: cirrhosis. The average score taken from 10 non-overlapping random fields per section was used to generate a single score for each specimen, in each experimental group (Sant'anna et al. 2011a).

IMMUNOHISTOCHEMISTRY

In order to evaluate activated myofibroblasts and macrophages, α-SMA and CD68 antibodies were used, respectively. Briefly, each section was deparaffinized and hydrated in graded ethanol. Then, sections for α-SMA were immersed into the preheated retrieval solution (10 mM citrate buffer, pH 6.0) for 20 min at 98°C. The sections

for CD68 immunostaining were immersed into the preheated retrieval solution (10 mM citrate buffer, pH 6.0) for 20 min at 96°C, followed by a further digestion of 10 min in trypsin solution (Gibco®, Life Technologies, São Paulo, Brazil) at 37°C. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min followed by three washes in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), and the unspecific labeling was reduced by a 20-min incubation with normal horse serum (ImmPRESS Reagent Kit; Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated overnight in a humidity chamber at 4°C with primary mouse monoclonal antibodies against anti-human α-SMA (clone 1A4; dilution 1:300; Dako Corporation, Glostrup, Denmark) and against anti-rat CD68 (clone ED1; dilution 1:600; AbD serotec, Oxford, UK). The sections were washed again and incubated with a secondary antibody using the ImmPRESS Universal Reagent Anti-Mouse/Rabbit Ig Polymer Detection Kit (Vector Laboratories) for 30 min. For the visualization of the reactions, the slides were incubated with ImmPACT DAB (Vector Laboratories) chromogen substrate for the α-SMA antibody and with ImmPACT VIP (Vector Laboratories) for CD68. Finally, the sections were counterstained with Harris hematoxylin, being ready for microscopic evaluation.

QUANTITATIVE IMAGE ANALYSIS

The quantitative evaluation of the area occupied by collagen deposition in the sections stained with Masson's trichome and the immunostainings with α-SMA and CD68 antibodies was performed using the image analysis program CellProfiler (Sant'anna et al. 2011b). The images of all stainings were captured by Leica DM2500 Trinocular Microscope, and digitalized at 1024×768-pixel, 24 bit/pixel resolution with a global magnification of 100x. The digital images were processed by CellProfiler, to identify, isolate, and measure areas occupied by immunostained cells or by collagen deposition over the total area of liver parenchyma. The mean percentage of 10 histological fields, randomly chosen per sample, was used to generate a single value for each animal in each experimental group.

STATISTICAL ANALYSIS

The results obtained were statistically evaluated in the program GraphPad Prism 6, which also generated the graphical presentation of the results. Data were submitted to the Shapiro-Wilk normality test and Kruskal-Wallis non-parametric analysis of variance followed by Dunn's multiple comparison test. The confidence level was 95% ($p < 0.05$).

RESULTS

QUALITATIVE ANALYSIS

The histopathological changes are represented in the photomicrograph of the three experimental groups (Figure 2). In the BDL group, liver architecture is seriously compromised with bile duct hyperplasia, a large amount of interstitial collagen in the parenchyma, and the formation of hepatic nodules that are characteristic of the stage of cirrhosis (Figure 2a). The group BDL+AM_0 exhibited characteristics usually found in fibrosis score 3 with the presence of fibrotic septa ("bridging fibrosis") connecting portal spaces and directing to the central vein; however, it is observed that the tissue is more conserved when compared to the group BDL, with little interstitial collagen in the hepatic parenchyma (Figure 2b). Group BDL+AM_1 showed fibrosis confined along the portal tracts with a consequent greater preservation of the tissue structure (Figure 2c). The semiquantitative Knodell scoring of these experimental results is reported in Figure 3. Fibrosis score in group BDL+AM_0 was lower than in group BDL, but without a significant difference. In contrast, group BDL+AM_1 presented

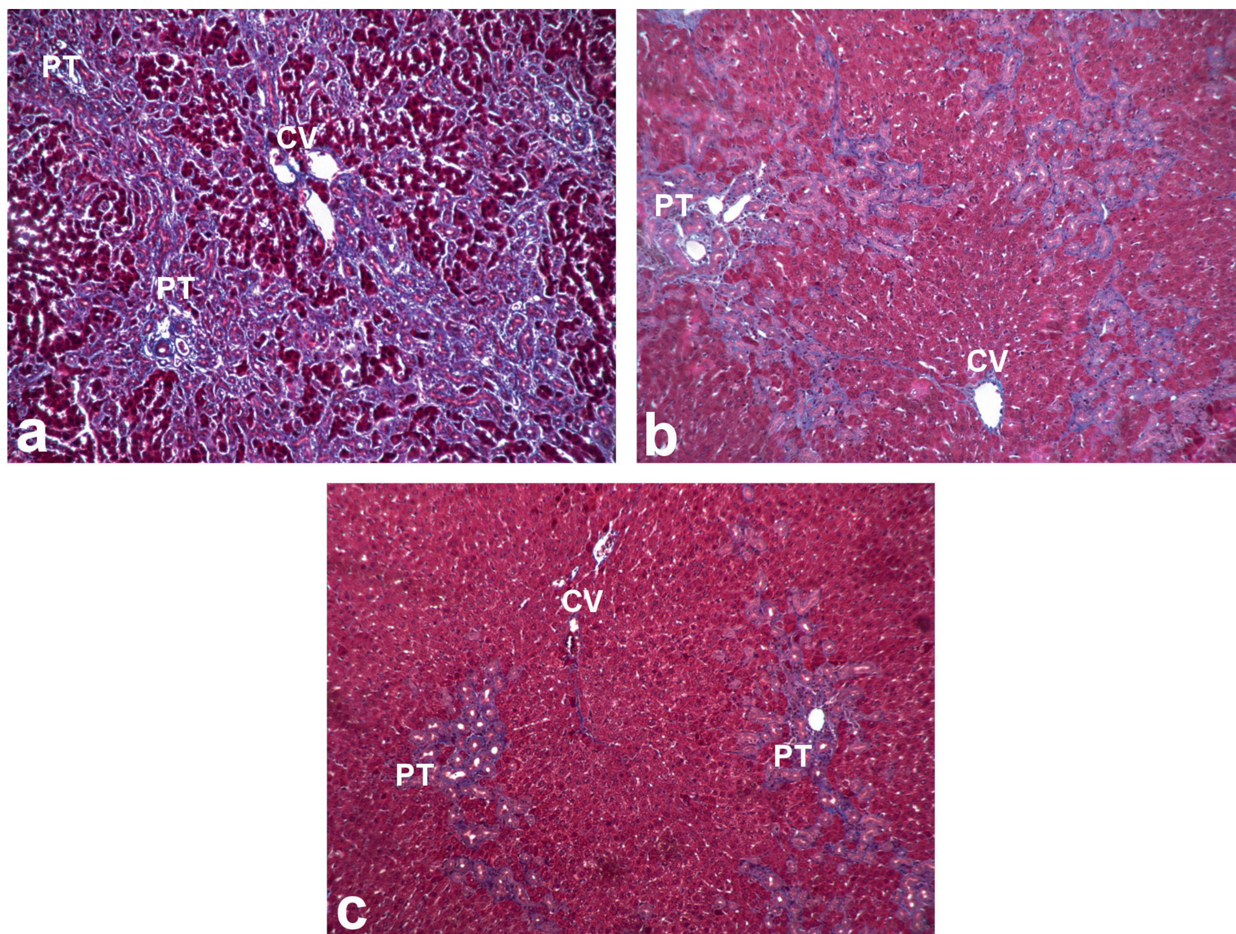

Figure 2 - Semiquantitative evaluation of fibrosis in the experimental groups after 6 weeks of BDL. **a**) Group BDL; **b**) Group BDL+AM_0; **c**) Group BDL+AM_1. Portal tract (PT); Central Vein (CV). Masson's trichome staining with a magnification of 100 x.

the lowest score, and a significantly lower score in comparison to group BDL+AM_0, and an extremely significant difference in relation to group BDL.

QUANTITATIVE ANALYSIS - COLLAGEN DEPOSITION

Quantitative data for the area occupied by collagen deposition obtained by image analysis are shown in Figure 4. Group BDL presented the highest median of area occupied by collagen (28.73%), almost twice the value of collagen deposition in group BDL+AM_0 (12.94%). In group BDL+AM_1, the percentage of area occupied by collagen was lower (4.30%) in comparison to the other two groups,

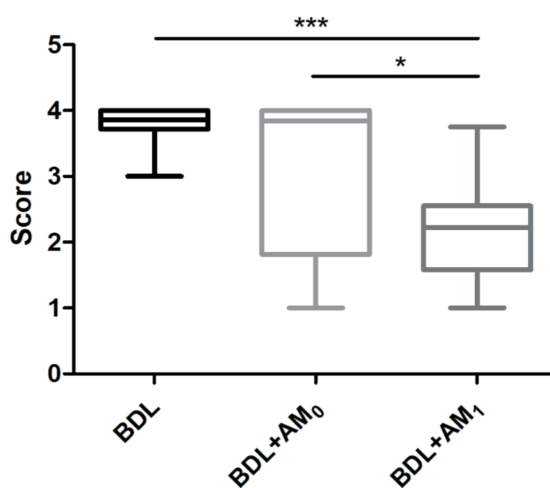

Figure 3 - Analysis of fibrosis degree by the Knodell classification system. Median and interquartile range of fibrosis score in the experimental groups. * $p < 0.05$ *** $p < 0.001$.

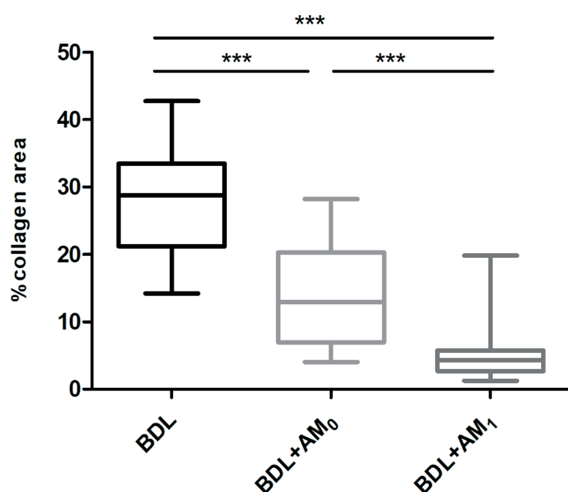

Figure 4 - Quantitative evaluation of collagen deposition. Median and interquartile range of collagen deposition in the experimental groups, after 6 weeks of BDL. *** $p < 0.001$.

with extremely significant ($p < 0.001$) differences among the groups.

QUANTITATIVE ANALYSIS - ACTIVATED MYOFIBROBLASTS

Considering that activated myofibroblasts are the main cells responsible for the synthesis and secretion of collagen in liver fibrosis, the potential of AM in reducing the activation of these cells was evaluated by monitoring the presence of α-SMA positive cells in the 3 experimental groups. After 6 weeks of fibrosis induction, α-SMA expression in group BDL was intense around the biliary ducts going deeply into the hepatic lobule and into perisinusoidal spaces (Figure 5a). In the groups BDL+AM_0 and BDL+AM_1, α-SMA immunoexpression was reduced around the biliary structures, with a rare presence in the perisinusoidal spaces (Figures 5b and c). Quantitative data for the area occupied by α-SMA demonstrated a lower presence of activated myofibroblasts in groups BDL+AM_0 and BDL+AM_1 in relation to the group BDL, respectively (8.00%; 6.79% vs. 13.29%; $p<0.001$) (Figure 6).

QUANTITATIVE ANALYSIS - CD68 POSITIVE MACROPHAGES

Considering the role of macrophages in the resolution of fibrosis, we eventually investigated the immunoexpression of hepatic macrophages. Figure 7 shows the immunolocalization of CD68 positive macrophages in the periportal region of the hepatic tissue in all experimental groups. It was observed that macrophages (Kupffer cells) are located on the walls of sinusoid capillaries, with a diverse and irregular morphology. In the groups BDL+MA_0 and BDL+MA_1, Kupffer cells have a large and diffuse distribution in the hepatic parenchyma, and the area occupied by CD68 positive macrophages was similar between the treated groups (3.81% vs. 3.85% $p>0.05$, respectively). In contrast, between BDL and AM treated groups, the value of CD68 positive macrophages was extremely significant ($p<0.001$) (Figure 8).

After obtained the results of the therapeutic effects of AM on biliary fibrosis, we had also verified what the AM treatment did in the normal livers. The results demonstrated that the hepatic parenchyma presented the histological characteristics of normal liver. In addition, the AM patch gradually reduced in thickness over 4-week observation period (Figure S1- Supplementary Material).

DISCUSSION

This study demonstrated that the human AM applied as a patch in the surface of the liver was able to reduce the progression of biliary fibrosis in the two forms investigated, either with AM wrapping the whole liver with a fragment of 9x9 cm in size, or when AM application was restricted to a portion of the liver, covering only the medial lobe with a fragment of 6X9 cm in size, which reduced fibrosis more significantly. In both treated groups, the qualitative and quantitative analyzes for all fibrosis parameters were performed in medial (covered) and right lobes (uncovered), and the results showed

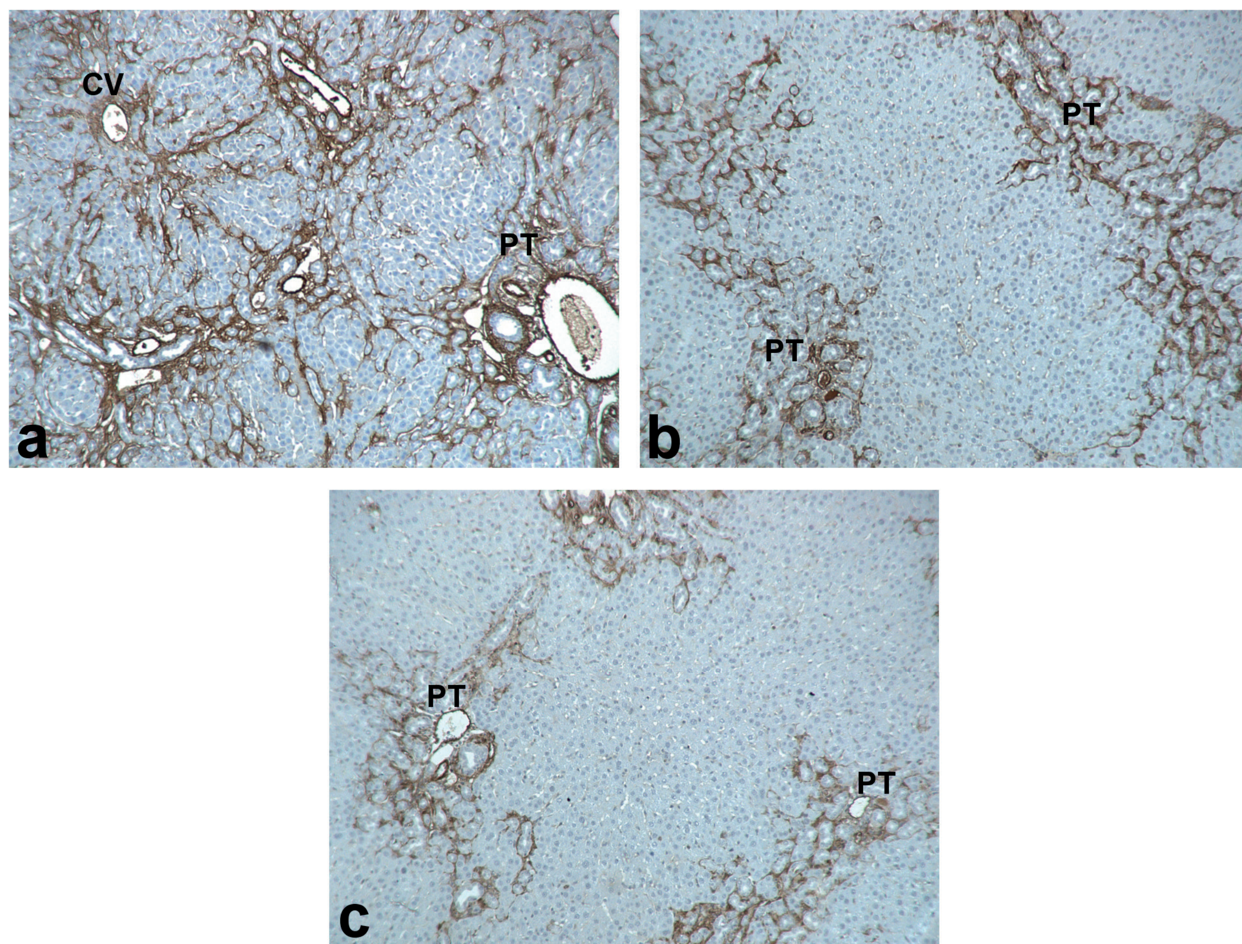

Figure 5 - Representative microphotographs of α-SMA immunoexpression in the experimental groups after 6 weeks of BDL. **a**) Group BDL; **b**) Group BDL+AM_0; **c**) Group BDL+ AM_1. Portal tract (PT); Central Vein (CV). Magnification of 100x.

no significant diferences between these two areas (“unpublished results”).

The degree of fibrosis was first evaluated by the qualitative analysis using the Knodell score system, which was applied to histopathological alterations in the hepatic tissue stained with Masson’s trichrome, indicated for the visualization of the interaction between collagen fibers and the liver tissue. This qualitative or semiquantitative evaluation allows a classification of fibrosis degree severity. This analysis is essential for the direct and rapid diagnosis of fibrosis, and is considered the gold standard for the initial diagnosis in clinical and experimental studies (Standish et al. 2006, Wang and Hou 2015).

In the groups treated with AM, fibrosis score was lower in relation to group BDL, and the liver parenchyma was more conserved. The results of this semiquantitative analysis demonstrated an extremely significant reduction when AM was applied around a portion of the liver (group BDL+AM_1), compared to group BDL, where fibrosis presented characteristics of its more advanced state, cirrhosis. Even though the group BDL+AM_0 presented a similar median of group BDL, it is fundamental to note the tendency in reducing the values of fibrosis score, which was about 47% in relation to the median. Although this analysis did not demonstrate a significant difference between groups BDL and BDL+AM_0, a

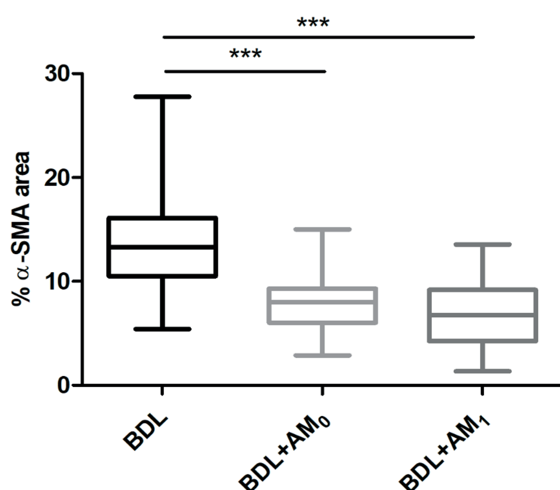

Figure 6 - Quantitative evaluation of α-SMA area. Median and interquartile range of α-SMA area in the experimental groups after 6 weeks of BDL. ***$p<0.001$.

quantitative analysis, using the Cellprofiler imaging analysis program, showed an extremely significant reduction in the area occupied by collagen deposition in both groups treated with AM. In the group BDL+AM_0, the reduction was 50% of levels observed in untreated BDL rats. In addition, when AM wrapped partially the liver, the reduction was around 80% compared to group BDL.

The reduction in the amount of collagen deposition is an important result obtained in our study, since digital quantitative image analysis is more objective, precise and specific, compared to the analysis by the Knodell score system, which can be influenced by the size of the biopsy, number of samples, and by the intraobserver and interobserver variability. According to Standish et al. (2006), the changes described in the scoring categories are largely architectural, with little reference to the actual amount of collagen in the liver sample. The quantitative image analysis measures fibrosis (amount of collagen) as a continuous numerical variable, and has been indicated for the diagnosis of advanced fibrosis, prognosis prediction, therapeutic planning and monitoring, and especially for the detection of the effects of the antifibrotic therapy (Standish et al. 2006, Wang and Hou 2015, Yegin et al. 2016).

Sant'Anna et al. (2011a) demonstrated that fresh AM cannot prevent the onset of liver fibrosis when applied at the same time as the fibrotic stimulus, but reduced around 50% of all parameters characteristic of biliary fibrosis. In 2016 the same authors, studying the action of AM in established fibrosis, verified a reduction similar to the previous study in collagen deposition, relating it to the decrease of the pro-fibrotic factors TGF-ß1 and IL-6. Recently, Garrido et al. (2018) tested the potential of cryopreserved AM in the treatment of liver fibrosis induced by BDL in young rats, suturing cryopreserved AM to the liver surface at the same time as BDL was performed, and found a decrease in the area occupied by collagen and in the expression of pro-fibrotic genes TGF-ß1 and Alepin, both quantified by real-time PCR.

Activated myofibroblasts are considered the main cells responsible for the synthesis and deposition of collagen in liver with fibrosis (Hinz et al. 2007, Brenner et al. 2012). A chronic obstruction of the bile duct causes a massive activation of myofibroblasts in the portal and periductular region (Pinzani and Luong 2018) In our study, it was possible to observe, in the AM-treated groups, a reduced immunoexpression of α-SMA around the bile ducts, portal spaces, and hepatic parenchyma. The quantitative analysis showed an extremely significant difference in the level of α-SMA between group BDL and the AM-treated groups, with a reduction of about 50% in the area occupied by myofibroblasts. These results corroborate with Ricci et al. (2013), who demonstrated the anti-fibrotic potential of fresh and cryopreserved AM in rats with liver fibrosis induced by BDL, as well as Manuelpillai et al. (2010), who applied human amniotic epithelial cells (hAEC) isolated from the membrane in immunodepressed rats, with liver fibrosis induced by carbon tetrachloride, and also with Cargnoni et al. (2018), who demonstrated the

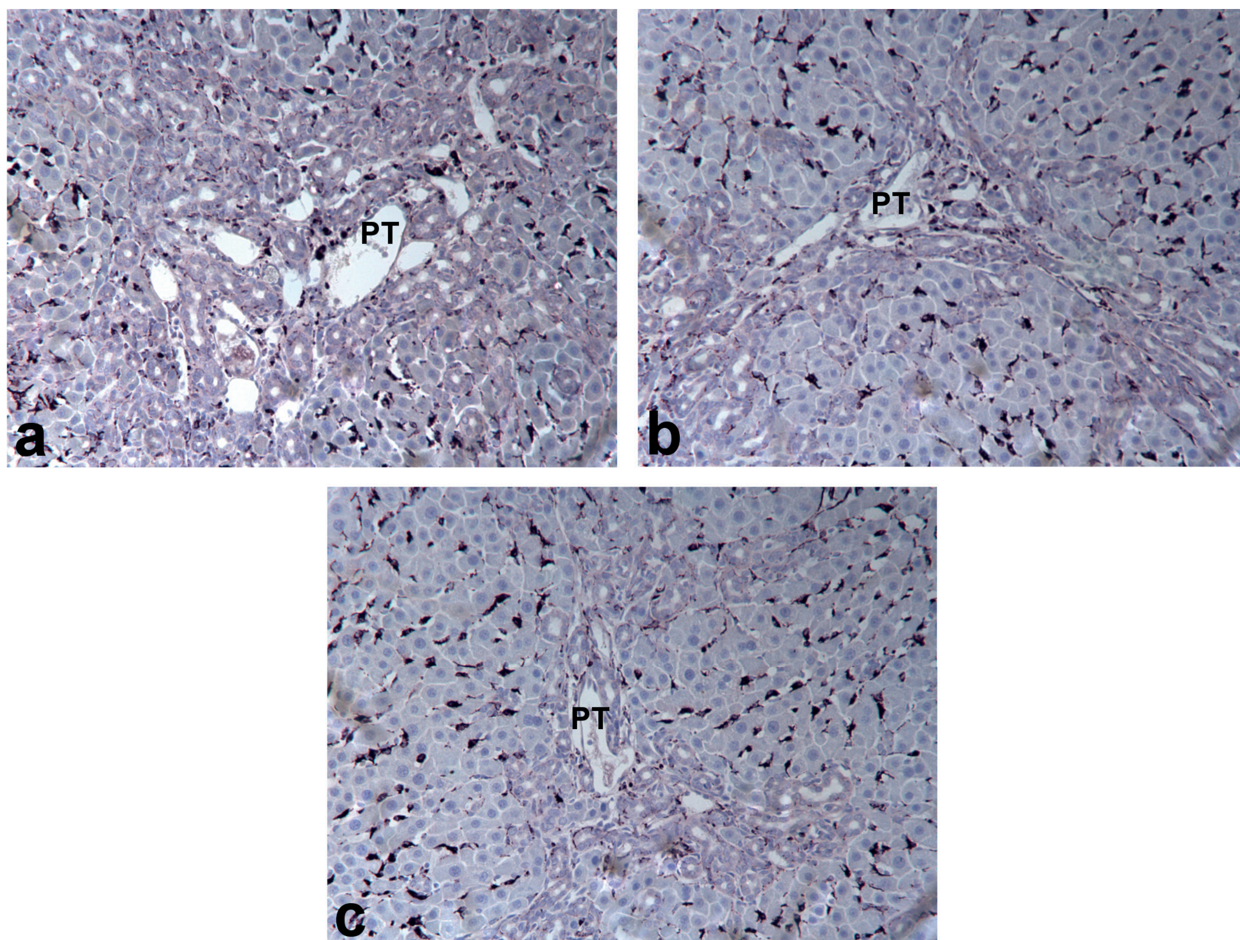

Figure 7 - Representative microphotographs of CD68 positive macrophages in the periportal area 6 weeks after BDL. **a**) Group BDL; **b**) Group BDL+AM_0; **c**) Group BDL+ AM_1. Portal tract (PT). Magnification of 100x.

antifibrotic effect of hAEC on fibrosis induced in rats also by BDL. Finally, Fu et al. (2018) observed *in vitro* that hepatic stellate cells from rats, when exposed to a conditioned medium prepared from AM mesenchymal cells, had reduced levels of α-SMA and collagen I, and increased metalloproteinases 2, 9 and 13.

Thus, all of these studies, regardless of the method used to induce fibrosis or the mode chosen for the application of AM (Manuelpillai et al. 2010, Ricci et al. 2013, Cargnoni et al. 2018, Fu et al. 2018), showed a decrease in the activated myofibroblasts, together with a reduction in the area of collagen deposition in the hepatic tissue of treated rats. This would appear to indicate that the decrease in myofibroblast activation may have contributed to the attenuation of collagen deposition in the hepatic tissue, reducing the fibrosis caused by BDL. Interestingly, when AM was applied around only a portion of the liver (group BDL+ AM_1), the reduction in collagen deposition was higher compared to the group with membrane around all lobes (BDL+ AM_0). This result suggests another possible mechanism that contributes to the reduction of collagen, which is not the direct reduction of myofibroblasts, once the immunoexpression of SMA was similar in the groups treated with AM. This hypothesis is supported by studies about hepatic fibrosis and its reversibility (Bataller and Brenner 2005, Kisseleva et al. 2012, Sun and Kisseleva 2015), which have demonstrated that the mechanism of liver fibrosis

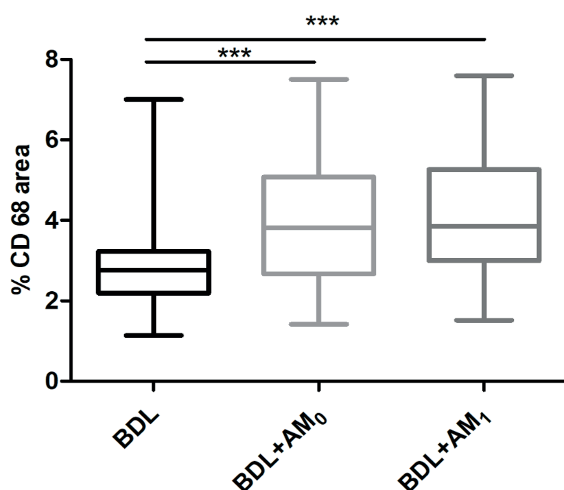

Figure 8 - Quantitative evaluation of CD68 in the periportal area. Median and interquartile range of CD68 positive macrophages in the experimental groups after 6 weeks of BDL. ***$p<0.001$.

regression is characterized first by the decreased inflammatory and fibrogenic cytokine levels, increased collagenase activity, that degrade ECM, and then the disappearance of myofibroblasts. Consistent with these studies, Sant'Anna et al. (2016) showed that the livers treated with AM had a 62% reduction in the main profibrotic cytokine, TGF-β1, when compared to the untreated group BDL. Indeed, Manuelpillai et al. (2010) observed the decreased expression of TGF-β1 coupled with an induction of collagen-degrading metalloproteinases, and a reduction of their tissue inhibitors of metalloproteinases, thus, reducing the progression of fibrosis to advanced stages.

Kupffer cells play an important role in the pathogenesis of hepatic lesions. These macrophages respond to the signs present in the tissue environment in a diversified manner, presenting a remarkable plasticity, being able to differentiate into different functional subtypes in the same tissue, exerting actions on the progression and cure of fibrosis (Ju and Tacke 2016, Wynn and Barron 2010). In the present study, macrophage immunostaining with CD68 antibody was performed, resulting in an extremely significant difference in the number of macrophages present in the periportal region in the treated groups, MA_0 and MA_1, compared to the group BDL. We hypothesize that the superior number of macrophages in the periportal region, together with the lower amount of collagen in AM-treated groups, could suggest the presence of restorative macrophages capable of expressing metalloproteinases and anti-inflammatory cytokines that repair liver fibrosis. This theory can be supported by studies which demonstrated that macrophages act to reduce fibrosis by multiple mechanisms, including phagocytic activity in bile ducts, and apoptotic myofibroblasts, but especially by their secretion of metalloproteinases, that degrade the excess of ECM and favor the resolution of fibrosis (Wynn and Barron 2010, Li et al. 2016). In addition, AM release immunomodulatory factors that stimulate the presence of pro-regenerative macrophage phenotype subpopulation in the resolution of the fibrotic process (Silini et al. 2016). CD68 antibody is proposed as a general marker of macrophages, not exclusive to Kupffer cells (Ju and Tacke 2016). Therefore, future studies are necessary to identify different populations of macrophages present in the hepatic tissue and their possible relationship with fibrosis regression in the AM-treated groups, correlating it with the immunoexpression of the metalloproteinases.

To the best of our knowledge, this is the first study to assess two different modes of application of AM patches in the fibrotic liver, and considering different sizes of AM fragments. Although these two modes of application reduced fibrosis, the results showed that covering only one liver lobe was more efficient than covering all lobes of the liver. This finding can be explained by the lower handling of AM patch for its partial application around one lobe, improving preservation of the smaller fragment. We support this hypothesis based on studies that showed the relationship among the processing and the handling of AM in its components, including

cytokines and growth factors, which is important to the beneficial proprierties of AM (Hopinkinson et al. 2006, Gicquel et al. 2009, Pereira et al. 2016).

The fact that AM exerted beneficial effects in reducing fibrosis on the whole liver, even when in contact with only a portion of the liver (~ 40%), may suggest that a large fragment of AM wrapping the whole liver is not necessary to its antifibrotic action. This is a very important finding of our study because it reinforces the most accepted mechanism by which AM patch reduces liver fibrosis, which is associated with the release of soluble factors secreted by the AM cells, that exert paracrine action on the hepatic tissue, reducing the expression of pro-inflammatory and pro-fibrotic cytokines, such as TGF-β, PDGF, IL-6, and increasing the anti-inflammatory cytokines, such as IL-10 and the metalloproteinases responsible for degrading the extracellular matrix (Manuelpillai et al. 2011, Silini et al. 2015).

Soluble factors released by the AM cells and present in their stroma play an important role in the treatment of inflammatory and fibrotic diseases, with their medicinal capacity being dependent on time, dosage and location of the cytokine / growth factor produced by the amnion, together with the influence of the receptor tissue microenvironment (Silini et al. 2013). Considering that, AM acts mainly as a matrix and as a source of bioactive factors that diffuse through the tissue (Manuelpillai et al. 2011, Silini et al. 2013), we apply the membrane with the mesenchymal side facing the hepatic tissue, corroborating the previous studies of our laboratory which reported better results when this protocol was followed (Sant'Anna et al. 2011a 2016, Nicodemo et al. 2017, Campelo et al. 2018).

Considering that, patients with cirrhosis have to wait for liver transplantation, and that AM reduces fibrosis by decreasing its progression to liver cirrhosis, it is possible to suggest that the application of the membrane is a therapeutic strategy against fibrosis, which can delay and / or reduce the need of liver transplantation in some cases. This suggestion is related to the new concept in the management of patients with cirrhosis, that should be the prevention and early intervention to stabilize disease progression and to avoid or delay clinical decompensation and the need for liver transplantation. The challenge in the XXI century is to prevent the need for liver transplantation in as many patients with cirrhosis as possible (Tsochatzis et al. 2014).

In conclusion, this study demonstrated that human AM, when applied to the liver, covering it either totally or partially, acted in the repair of liver fibrosis. However, the AM patch applied around only a portion of the liver was more efficient in reducing the severity / degree of fibrosis, in addition to decreasing the content of collagen deposition in the hepatic tissue, an important occurrence to avoid the progression of biliary fibrosis to the final stage of the disease, liver cirrhosis.

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AUTHOR CONTRIBUTIONS

The author Sant'Anna LB. conceived and designed the presented idea of this work, collected amniotic membrane at hospital, performed the chirurgic procedures of fibrosis induction and AM application, performed the quantitative image and the statistical analyzes, contributed to the

interpretation of the results, and provided critical revision of the article. The author Mamede KM. takes care of animals before, during and after all chirurgic procedures following the good practice of housing and handling, collected and processed liver samples of all experimental groups for histology and immunohistochemistry, discussed the results, and wrote the paper.

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SUPPLEMENTARY MATERIAL

Figure S1.