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Antifibrotic action of telmisartan in experimental carbon tetrachloride-induced liver fibrosis in Wistar rats

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Abstract

Background and Aims: Liver fibrosis is the increasingly accumulation of extracellular matrix (ECM), caused by chronic liver injuries, and represents a difficult clinical challenge in the entire world. Currently, the advanced knowledge of the cellular and molecular mechanisms of liver fibrosis showed that collagen-producing cells, like activated hepatic stellate cells (HSCs), portal fibroblasts and myofibroblasts are activated by fibrogenic cytokines, such as angiotensin II, transforming growth factor-beta 1 (TGF-β1), and leptin. Because of these, we tested telmisartan, an angiotensin II (AT1) receptor blocker and a peroxisome proliferator-activated receptor-γ (PPARγ) partial agonist, for investigate its antifibrotic action, on experimental model of carbon tetrachloride-induced liver fibrosis. *Materials and Methods*: In this research, we used two groups of Wistar rats, which received intraperitoneal (i.p.) injection of carbon tetrachloride (CCl_4) 40% dissolved in olive oil, twice weekly for four consecutive weeks (initial dose of 5 mL/kg, and other doses 3 mL/kg). After one week, one group was received by gavage telmisartan (TS) dissolved in saline 0.9%, daily in dose of 8 mg/kg, for 28 days. One group of Wistar rats was used for control. The antifibrotic action of telmisartan was investigated on the pathological changes of the liver and immunohistochemical analysis for hepatic stellate (Ito) cells (HSCs) reaction using anti-alpha-smooth muscle actin (anti α-SMA) antibody and macrophages cells (Kupffer cells) reaction using anti-CD68 antibody. *Results and Conclusions*: In group treated with telmisartan, hepatic fibrogenesis process was significantly reduced, in comparison with CCl_4 group.

Keywords: hepatic fibrosis, antifibrotic action, telmisartan, myofibroblasts, hepatic stellate cells.

Introduction

Chronic liver diseases are characterized by necrotic and degenerative lesions of hepatocytes, the presence of an inflammatory reaction with varying intensity and the onset of liver fibrosis. Liver fibrosis is one of the histo-pathological elements essential in defining chronic liver disease, its intensity being correlated with the severity of liver failure. Chronic liver disease has a varying etiology: viral infections (with B and C hepatitis virus), alcohol intake, metabolic syndrome, autoimmune diseases, etc.

Worldwidely, chronic liver diseases represent a major cause of morbidity and mortality [1]. If the prevalence of chronic hepatites is difficult to appreciate all over the world, the prevalence of liver cirrhosis is estimated at about 1% of the world population [2]. Some studies showed that, worldwidely, in 2010 there were recorded about a million deaths caused by liver cirrhosis [3]. All these data show that chronic liver diseases represent a major healthcare problem.

Nowadays, liver fibrosis represents one of the treatment targets of clinical and experimental studies, as in liver diseases its progress will lead to liver cirrhosis, severe liver failure and death. At present, the research in chronic liver diseases are focused on the blocking of the liver fibrillogenesis and even reduction of liver fibrosis, some studies indicating that liver fibrosis might be a reversible process [4, 5].

Starting from the fact that previous studies showed that liver fibrosis is associated with an up-regulation of the renin–angiotensin system (RAS) [6], and the depletion of peroxisome proliferator-activated receptor gamma (PPARγ) is associated with the activation of hepatic stellate cells (HSCs) [7, 8], our aim is the study of the anti-fibrotic effect of telmisartan, an inhibitor of the angiotensin (Ang) II type-1 (AT1) receptors and an activator of PPARγ.

Materials and Methods

In this study, we used a number of 45 Wistar rats, who were divided into three groups, as follows: the control group (five animals), the carbon tetrachloride (CCl_4) group (20 animals, who received CCl_4) and the telmisartan (TS)

group (20 animals, which received CCl_4 and telmisartan). The animals obtained from the Animal Facility of the University of Medicine and Pharmacy of Craiova, Romania, were maintained in a room with a 12-hour light/dark cycle with constant temperature and humidity, and had access to food and water *ad libitum*.

The experimental protocols were approved by the Ethical Committee of the University of Medicine and Pharmacy of Craiova, and were performed according to the Directive 86/609/EEC of November 24, 1986 and according to the Ministry Order No. 143 of April 1, 2002, No. 400 of May 20, 2002 and National Sanitary Veterinary and Food Safety Authority (ANSVSA) Order No. 84 of August 30, 2005, on the protection of animal research.

Control group

Five male Wistar rats aged between 8–10 weeks, and 220 g of body weight, were kept in the above-mentioned conditions and used for the control group. In this group, we administered olive oil by i.p. injection, twice a week for four consecutive weeks, in dose of 5 mL/kg first dose, and 3 mL/kg the other doses. After four weeks, we anesthetized with sevoflurane and sacrificed the animals, and harvested blood samples and liver, for histological, immunohistochemical and biological investigations.

Carbon tetrachloride (CCl_4) group

In this group were included 20 male and female Wistar rats aged between 8–10 weeks, and 200–250 g of body weight, that received CCl_4 (40% in olive oil) by i.p. injection, twice a week for four consecutive weeks, in a dose of 5 mL/kg first dose, and 3 mL/kg the other doses. Every seven days, we anesthetized with sevoflurane and sacrificed five rats, and harvested the liver. Consequently, we watched pathological changes, another 14 days after the last dose of CCl_4.

Carbon tetrachloride (CCl_4) + telmisartan (TS) group

A total of 20 female Wistar rats aged between 15–18 weeks, and 300–350 g of body weight were used for this stage of study. The animals received CCl_4 (40% in olive oil) by i.p. injection, twice a week for four weeks, in a dose of 5 mL/kg first dose, and 3 mL/kg the other doses. From day 7 of the experiment, we daily administered telmisartan (TS), for 21 days, in dose of 8 mg/kg orally (*p.o.*) by gavage. The TS was dissolved in 0.9% saline, the solution being freshly prepared every day in a concentration of 8 mg/mL. Every seven days from the TS administration, after anesthetized with sevoflurane, there were sacrificed five rats, and harvested the liver.

We administered telmisartan seven days after administering the first doses of CCl_4, as the previous (unpublished) studies regarding the CCl_4 hepatotoxicity observed that at the end of the first week of toxic administration there appeared the first elements of liver cytolysis, granular and vacuolar degenerescence of hepatocytes and incipient fibrosis in the Kiernan space. In this way, we wanted to establish whether the telmisartan administration stopped the liver fibrillogenesis induced by CCl_4. The animals receiving CCl_4 and telmisartan were sacrificed after 14, 21 and 28 days, respectively, of CCl_4 administering.

Histopathological and immunohistochemical analysis

In order to harvested the liver, the rats were euthanized by anesthesia with sevoflurane extended until exitus. The liver was fixed in 10% formaldehyde solution for at least 48–72 hours. After paraffin processing, liver cross-sections (4 μm) were stained with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichrome method for histological evaluation of liver injury.

For the immunohistochemical study of the biological material included in paraffin, there were performed cross-sections of 4 μm thickness that were harvested on histological blades covered by poly-L-lysine (Sigma). The histological cups were transferred in an incubator, at 45ºC and kept over night (for 18 hours), for increasing the adherence of the biological material on the histological blade surface. Then, there was applied the classical immunohistochemical protocol for highlighting the proposed antigens. The immunohistochemical marking was performed by using 3.3'-diaminobenzidine (DAB) (Dako), followed by the contrasting of the nuclei with Mayer's Hematoxylin. For the immunohistochemical study, there were used the anti-alpha-smooth muscle actin (α-SMA) antibodies (clone 1A4, 1:100 dilution, Dako) for highlighting the Ito cells transformed in myoblasts and anti-CD68 (clone KP1, 1:200 dilution, Dako) for highlighting the Kupffer cells and macrophages.

The cross-section processing was performed within the Research Center for Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova.

Statistical analysis

Statistical analysis followed the presence of Kupffer cells and of the myofibroblasts in all animal groups (control, CCl_4 group, and the CCl_4 + telmisartan group). For each animal, using a Nikon 55i microscope equipped with a 5 Mp CCD (charge coupled device) camera and the Image ProPlus AMS software, we captured 10 images with a 20× objective from the areas with the highest cellular densities. The cells were manually tagged and counted, all values averaged per animal and then for each pathology, and the resulting data have been compared utilizing an ANOVA (analysis of variance) testing. The value of $p<0.05$ was considered significant.

Results

The study of histological samples coming from the control group (that received only olive oil i.p.) showed that the lobular architecture of the liver parenchyma was not affected (Figure 1a). In the Kiernan space, the GS trichrome staining allowed us to observe the presence of a small quantity of fibrillary collagen, perfectly normal, with no inflammatory reaction (Figure 1b). The hepatocyte cordons had normal shapes and sizes, and the sinusoidal capillaries and the center lobular vein did not present any histopathological alterations. Still, the study of the histological samples with strong microscopic lens allowed us to remark that some hepatocytes presented an incipient process of granulovacuolar degenerescence. Using the anti-α-SMA antibody, allowed us to remark the fact that

the reaction was positive only in the pericytes of the vessel walls in the Kiernan space (Figure 1c), while the cells of the liver parenchyma presented a negative reaction. Using the anti-CD68 antibody, allowed us the selective highlighting of the Kupffer cells from the liver parenchyma structure. As observed on our samples (Figure 1d), the Kupffer cells had a normal distribution, slightly heterogeneous in the liver lobe structure. They presented normal shapes and sizes, which showed a normal metabolic, phagocytosis and immunological activity.

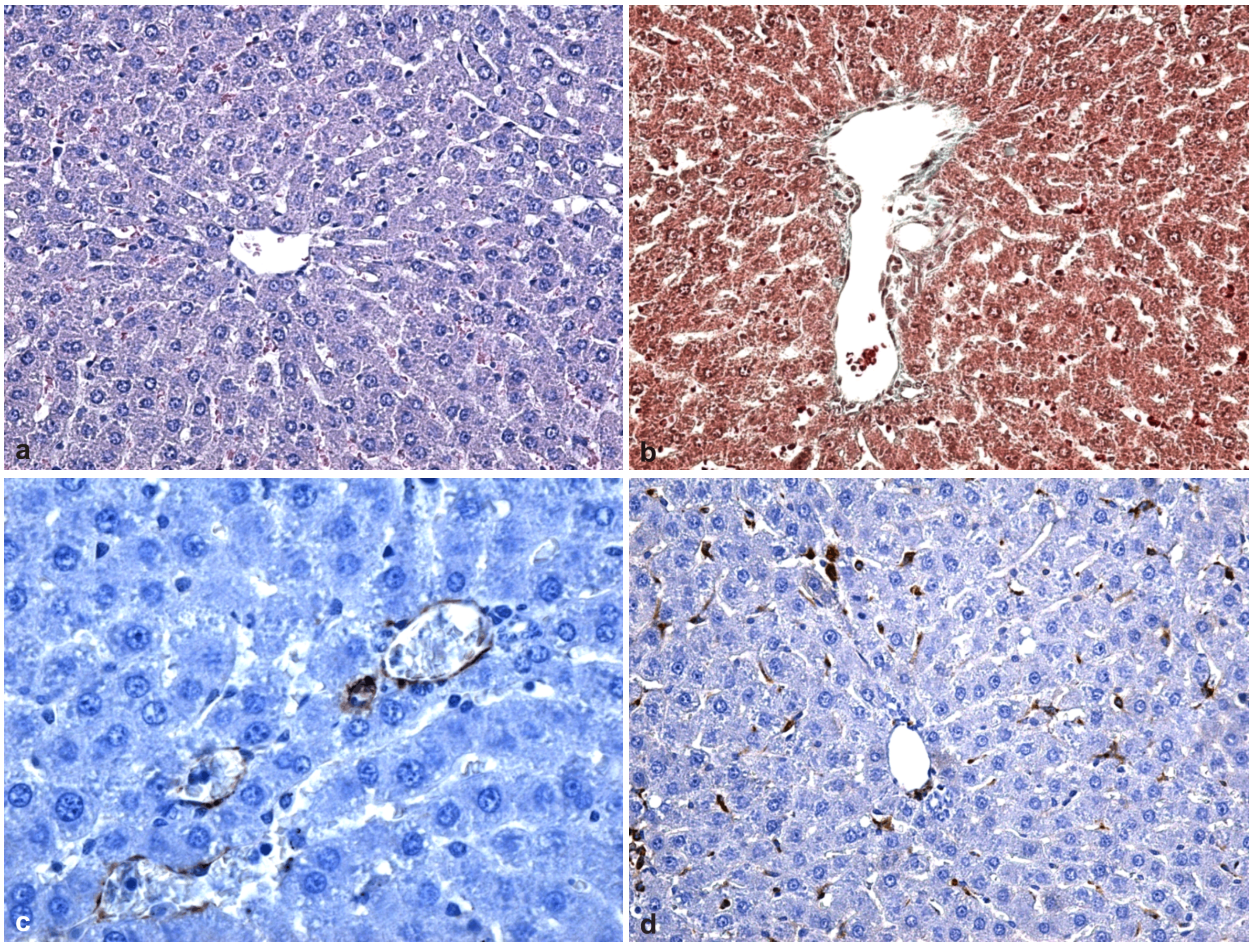

Figure 1 – ***Microscopic images of the liver parenchyma in the control group: (a) Normal aspect of the lobular architecture, without any histopathological changes (HE staining, ×200); (b) Kiernan space in the control group – there highlights a fine perivascular and pericanalicular fascia of collagen fibers (GS trichrome staining, ×200); (c) The cells in the blood vessel walls present in the Kiernan space (pericytes) positive to α-SMA (Anti-α-SMA antibody immunomarking, ×400); (d) Normal aspect of the distribution of Kupffer cells in the liver parenchyma (Anti-CD68 antibody immunomarking, ×200).***

In the animal group treated with CCl_4 for seven days, the histopathological examination showed the presence of small sizes of hepatocytolysis, and also the presence of extended lesions of granulovacuolar degenerescence mainly disseminated around the center lobular vein (3rd lobular) (Figure 2a). In the Kiernan spaces, there was observed a moderate increase of the fibrillary collagen quantity (Figure 2b) and the presence of a high number of cells with elongated nucleus, probably fibroblasts or myofibroblasts. The immunohistochemical examinations identified in the liver parenchyma the presence of cells with a positive reaction to α-SMA (myofibroblasts) arranged relatively heterogeneously, mainly in the walls of the sinusoidal capillaries (Figure 2c). The use of the anti-CD68 antibody allowed us to remark a moderate increase of the Kupffer cells, especially in the areas where the hepatocyte lesions were more intense (Figure 2d).

The evaluation of the liver histopathological changes in the animals receiving CCl_4 for two weeks allowed us to remark that the hepatocyte lesions increased in intensity. The liver cytolysis foci increased in size (Figure 3a), and the granulovacuolar lesions affected much more intensely and more extended the hepatocytes in the 3rd area of the liver lobe (Figure 3b). In some animals, there was observed a high quantity of collagen fibers and a high number of fibroblast and myofibroblast cells in the Kiernan space (Figure 3c), with the outline of porto-portal bridging fibrosis. The immunostaining with the anti-α-SMA and anti-CD68 antibodies showed a moderate increase of myofibroblast cells in the liver parenchyma and the Kiernan space and also an increase of the macrophage cells number (Kupffer cells). Macrophages were found in a large number in the areas with hepatocyte lesions; also, there was observed an increase in the size of Kupffer cells, which makes us believe that the intensity of the phagocytosis process increased.

In the group treated with CCl_4 for 14 days and with telmisartan for seven days, the hepatocyte lesions were similar to the ones in the group treated with CCl_4. The most intense hepatocyte lesions were recorded in the peri-portal area of the liver lobe. The process of collagen fibrillogenesis developed in the porto-billiary spaces (the Kiernan spaces) with a tendency of forming porto-portal and porto-central fibrous bridges. The reaction of myo-fibroblasts and Kupffer cells was similar to the one present in the animals treated only with CCl_4 for 14 days (Figure 4).

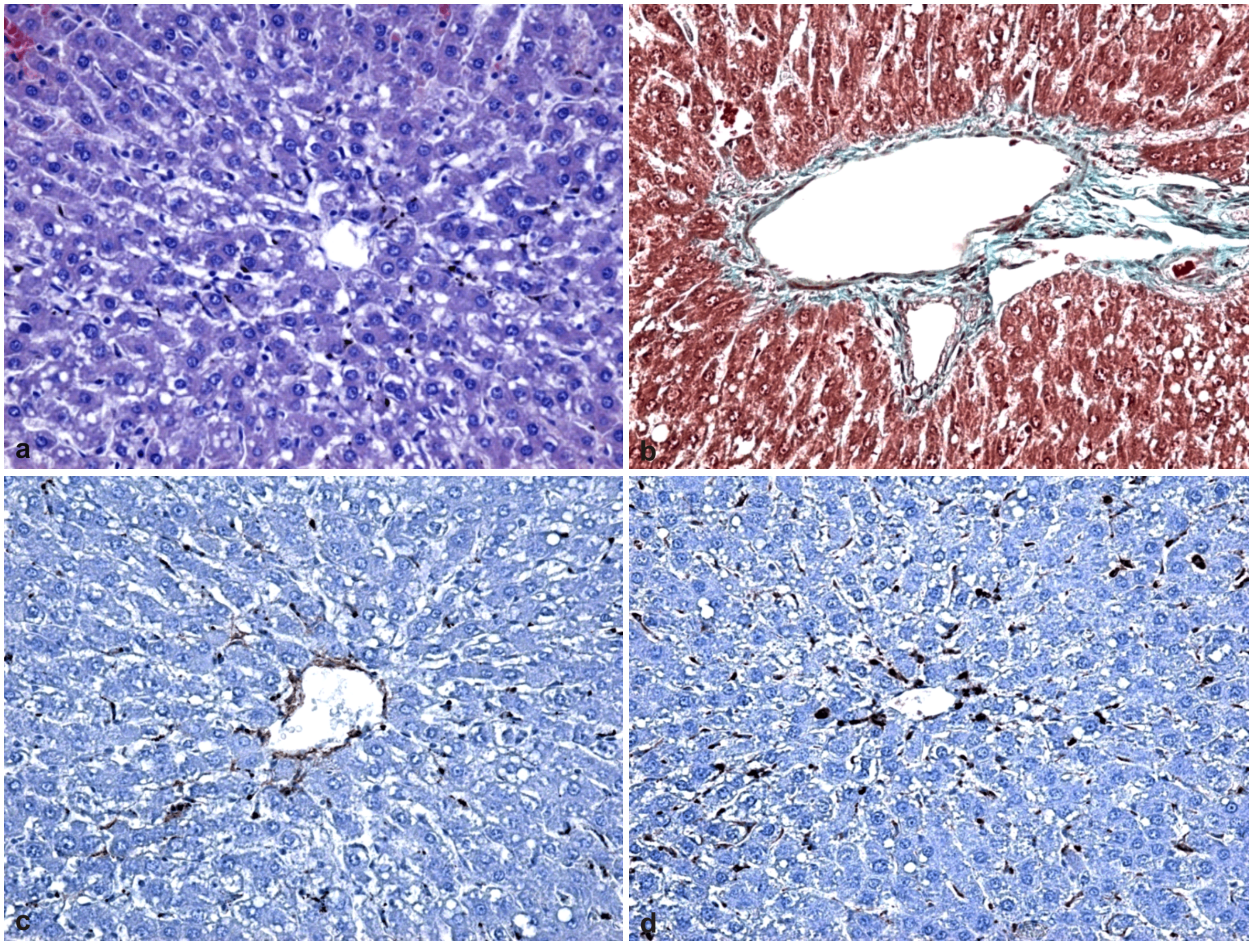

Figure 2 – ***Histopathological aspects of the liver parenchyma after seven days in the group treated with CCl_4: (a) Lesions of granulo-vacuolar degenerescence mainly found around the central lobular vein (HE staining, ×200); (b) Image of the Kiernan space, in which there is observed a moderate increase of the fibrillary collagen quantity (GS trichrome staining, ×200); (c) α-SMA positive cells present along the sinusoid capillaries, possibly Ito cells transformed into myofibroblasts (Anti-α-SMA antibody immunomarking, ×200); (d) Kupffer cells mainly present in the areas with more intense lesions of hepatocytosis or granulo-vacuolar degenerescence (Anti-CD68 antibody immunomarking, ×200).***

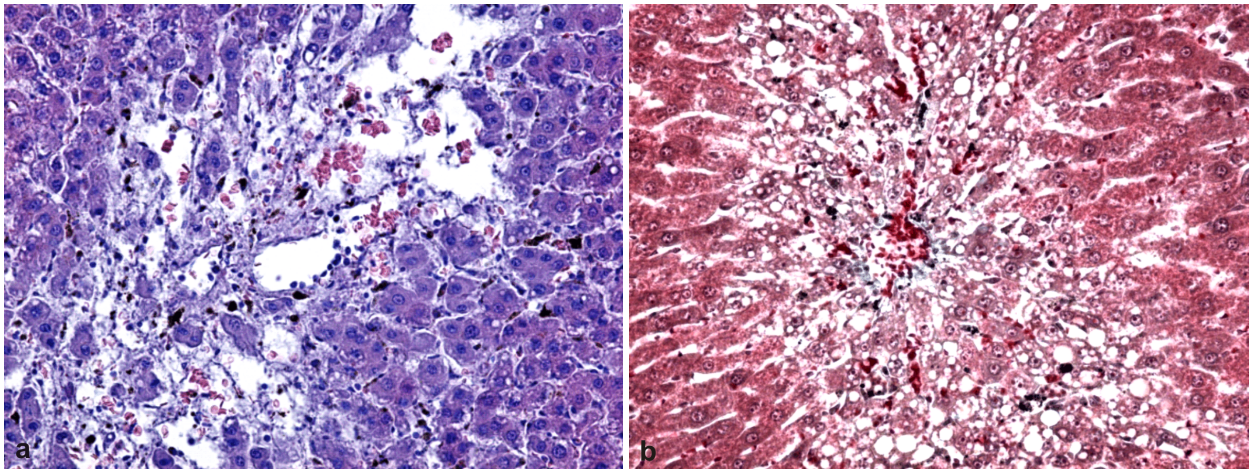

Figure 3 – ***Histopathological changes of the liver parenchyma after 14 days, in the group treated with CCl_4: (a) Extended area of hepatocytolysis (HE staining, ×400); (b) Image of liver parenchyma highlighting the intensity of hepatocytolysis lesions and granulo-vacuolar degenerescence disseminated around the central lobular vein (GS trichrome staining, ×200).***

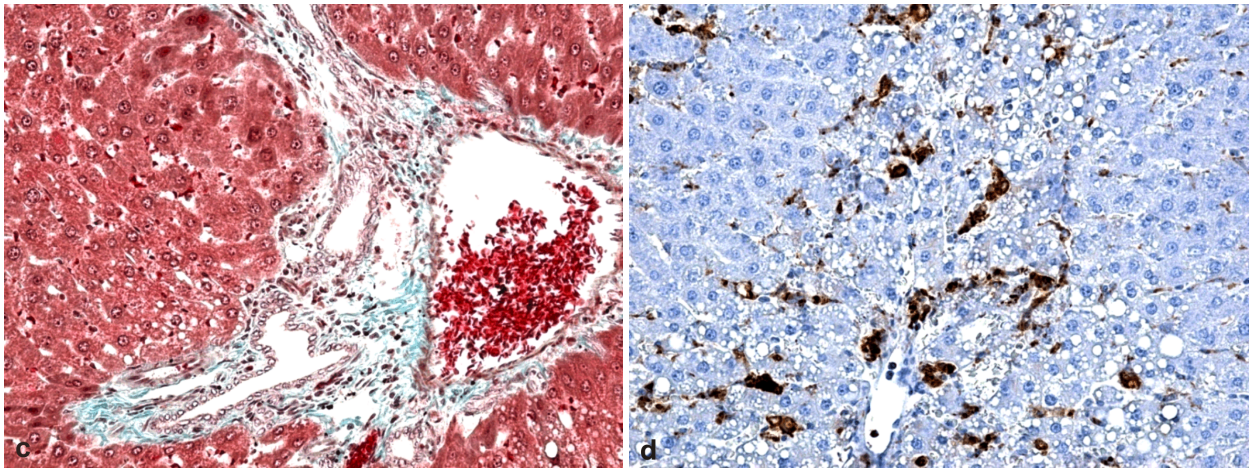

Figure 3 (continued) – ***Histopathological changes of the liver parenchyma after 14 days, in the group treated with CCl_4: (c) Moderate fibrosis in a Kiernan space with a tendency in performing porto-portal fibrous bridges (GS trichrome staining, ×200); (d) Intense reaction of Kupffer cells in the areas with lesions of hepatocytolysis and granulo-vacuolar degenerescence (Anti-CD68 antibody immunomarking, ×200).***

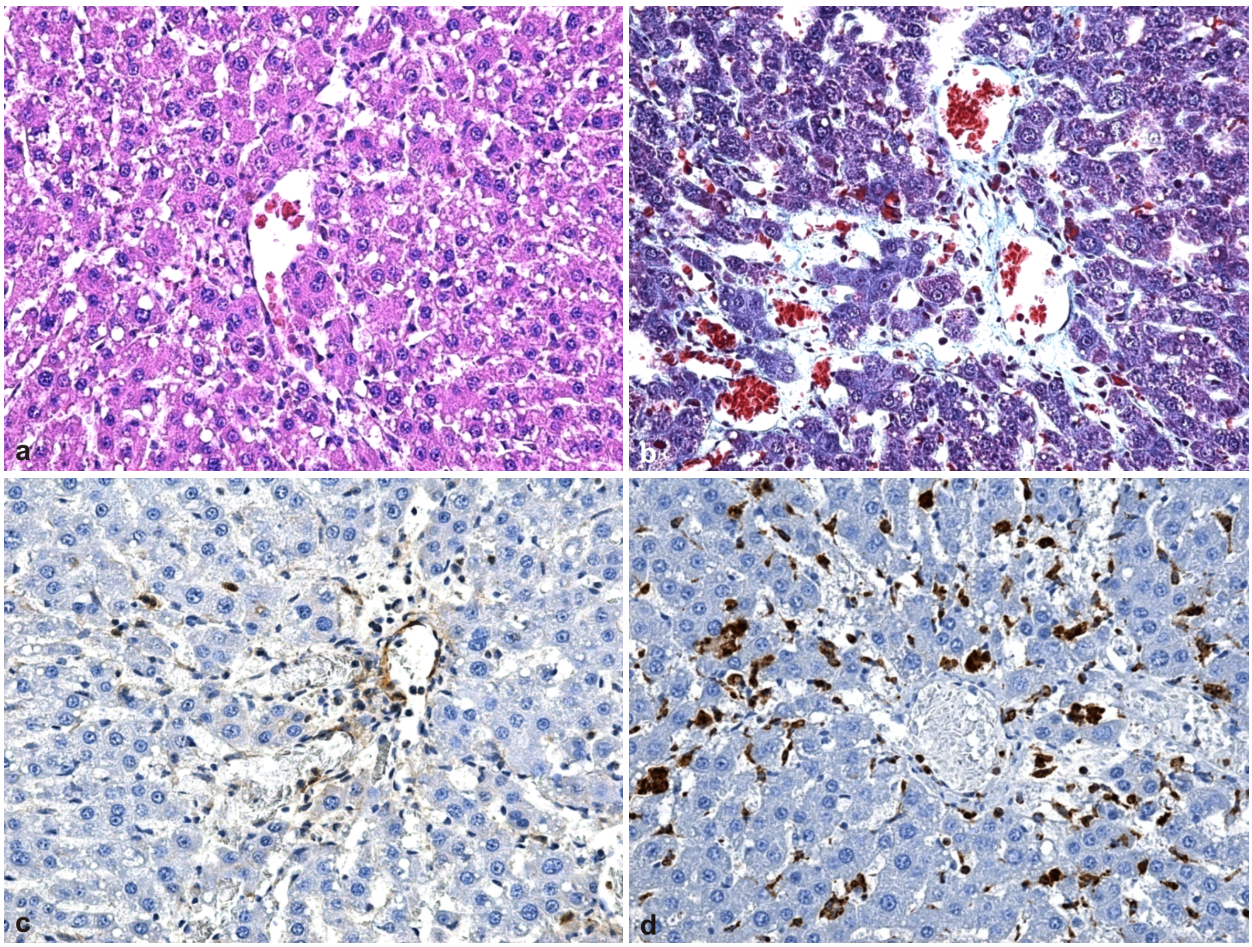

Figure 4 – ***Histopathological changes of the liver parenchyma after 14 days in the group treated with CCl_4 and telmisartan: (a) Area of liver parenchyma around the central lobular vein with intense lesions of granulo-vacuolar degenerescence (HE staining, ×200); (b) Kiernan space with moderate fibrosis, with conjunctive fine porto-central septa and with hepatocytolysis lesions in the periportal area (GS trichrome staining, ×200); (c) Moderate reaction of the myofibroblast cells around the sinusoid capillaries and around the central lobular vein (Anti-α-SMA antibody immunomarking, ×200); (d) Intense reaction of Kupffer cells in the areas with hepatocyte lesions around the central lobular vein (Anti-CD68 antibody immunomarking, ×200).***

A comparative evaluation of the liver changes after 21 days of CCl_4 administration and CCl_4 administration for 21 days + telmisartan administration for 14 days allowed us to observe the presence of significant changes of the liver lesions. Thus, if the hepatocytolysis and granulo-vacuolar lesions persisted and even intensified in the group of animals treated with CCl_4 (Figure 5a), in the group of animals treated with CCl_4 and telmisartan, the

hepatocyte lesions diminished their intensity (Figure 5b). Also, in the group treated only with CCl_4 there was observed an intensification of the collagenous fibrillogenesis in the portobilliary and periportal spaces (Figure 5c), while in the group treated with CCl_4 and telmisartan there was observed a diminishing of the fibrillogenesis process (Figure 5d). The immunohistochemical examinations highlighted a relatively equal number of myofibroblasts and Kupffer cells in the liver parenchyma in both animal groups. Still, in the animals treated only with CCl_4 there was observed the presence of larger, more intensely reactive macrophages (Kupffer cells) present especially in the areas with granulo-vacuolar lesions (Figure 5, e–h).

After 28 days of treatment with CCl_4 and CCl_4 + 21 days of treatment with telmisartan, the histopathological changes in the two groups of animals were more intense. Thus, in the group treated only with CCl_4 the liver lesions had a higher intensity in comparison to the anterior groups, thus proving the fact that the severity of liver lesions is correlated with the quantity of administered CCl_4. Thus, there was observed the presence of large areas of hepatocytosis, with the onset of thick fibrous septa, with numerous fibroblasts and myofibroblasts (Figure 6a), while in the group treated with CCl_4 and telmisartan, the hepatocyte lesions were less intense, granulo-vacuolar degenerescence persisting in some hepatocytes (Figure 6b). In the Kiernan space, in the group treated only with CCl_4, there was observed a massive process of collagen fibrillogenesis, with the formation of thick porto-portal and porto-central bridges (Figure 6c), with a tendency of forming cirrhotic nodules, while in the group treated with CCl_4 and telmisartan, the process of collagen fibrosis was much more reduced in comparison to the previous groups (Figure 6d).

The immunohistochemical study using the anti-α-SMA antibody showed that in the animals treated only with CCl_4 the number of myofibroblasts significantly increased in comparison to the previous groups, and their arrangement was mainly in the porto-portal and porto-central septa, thus contributing to the genesis of cirrhotic nodules (Figure 6e). On the contrary, in the animals treated with CCl_4 and telmisartan, the number of myofibroblasts significantly dropped in comparison to the previous group, being observed only across the liver sinusoid capillaries (Figure 6f). The Kupffer cells in the group treated only with CCl_4 appeared in large number, of high sizes, with intense reaction, mainly arranged in the areas with hepatocytolysis lesions or granulo-vacuolar degenerescence, around the central lobular vein and also in the Kiernan space (Figure 6g). In the group treated with CCl_4 and telmisartan, the number of Kupffer cells was lower, most cells being highlighted in the wall of the sinusoid capillaries. Still, also in this group, some Kupffer cells presented larger sizes and a more intense reaction, denoting that the phagocytosis activity persisted in this group, as well.

We need to show that the histopathological lesions induced by CCl_4 and CCl_4 + telmisartan, were not identical in all the animals of the same group, sometimes presenting considerable variations from one animal to another, which makes us believe that every animal, just like in humans, had a particular reactivity related to various internal factors.

The statistical evaluation of the reaction of Kupffer cells and myofibroblasts showed that in the group treated only with CCl_4 their number significantly increased alongside with the quantity increase of CCl_4 administered, while in the group treated with CCl_4 and telmisartan, their number decreased in relation to the increase of administration period of telmisartan (Tables 1 and 2; Figure 7, a and b).

Table 1 – ***Reaction of Kupffer cells for experimental groups***

Time	Average of Kupffer cells/field		
	Control group	*CCl_4 group*	*CCl_4 + TS group*
7 days	57	79	–
14 days	58	94	83
21 days	60	98	70
28 days	59	122	51

Table 2 – ***Reaction of myofibroblasts cells for experimental groups***

Time	Average of myofibroblast cells/field		
	Control group	*CCl_4 group*	*CCl_4 + TS group*
7 days	0	24	–
14 days	0	35	27
21 days	0	49	33
28 days	0	151	22

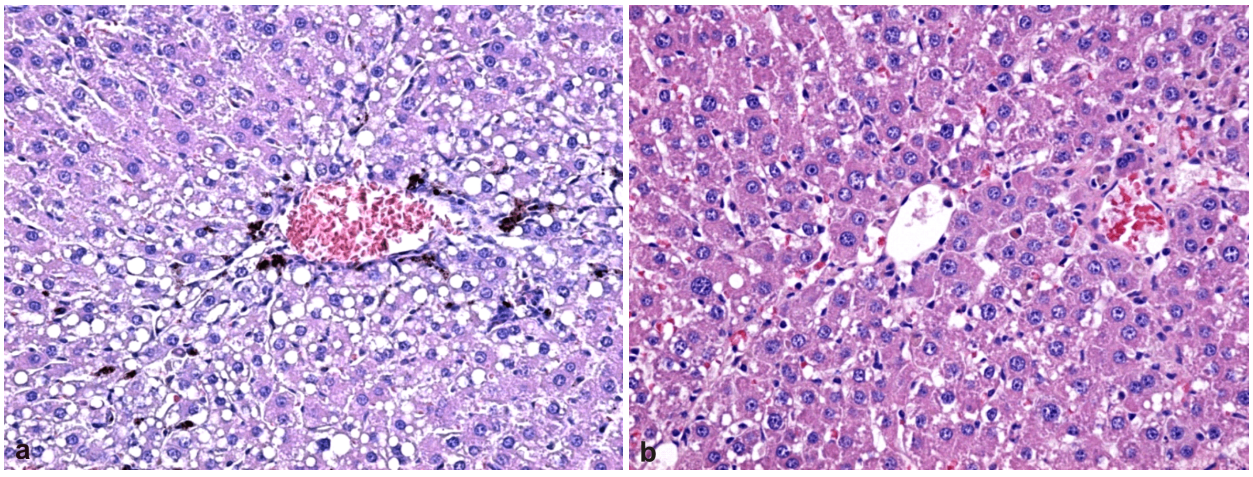

Figure 5 – ***Histopathological changes of the liver parenchyma after 21 days: (a) Liver parenchyma with intense lesions of granulo-vacuolar degenerescence and reduced lesions of hepatocytolysis, localized around the central lobular vein in the animals treated with CCl_4 (HE staining, ×200); (b) Liver parenchyma with moderate lesions of granulo-vacuolar degenerescence in the animals of the group treated with CCl_4 + telmisartan (HE staining, ×200).***

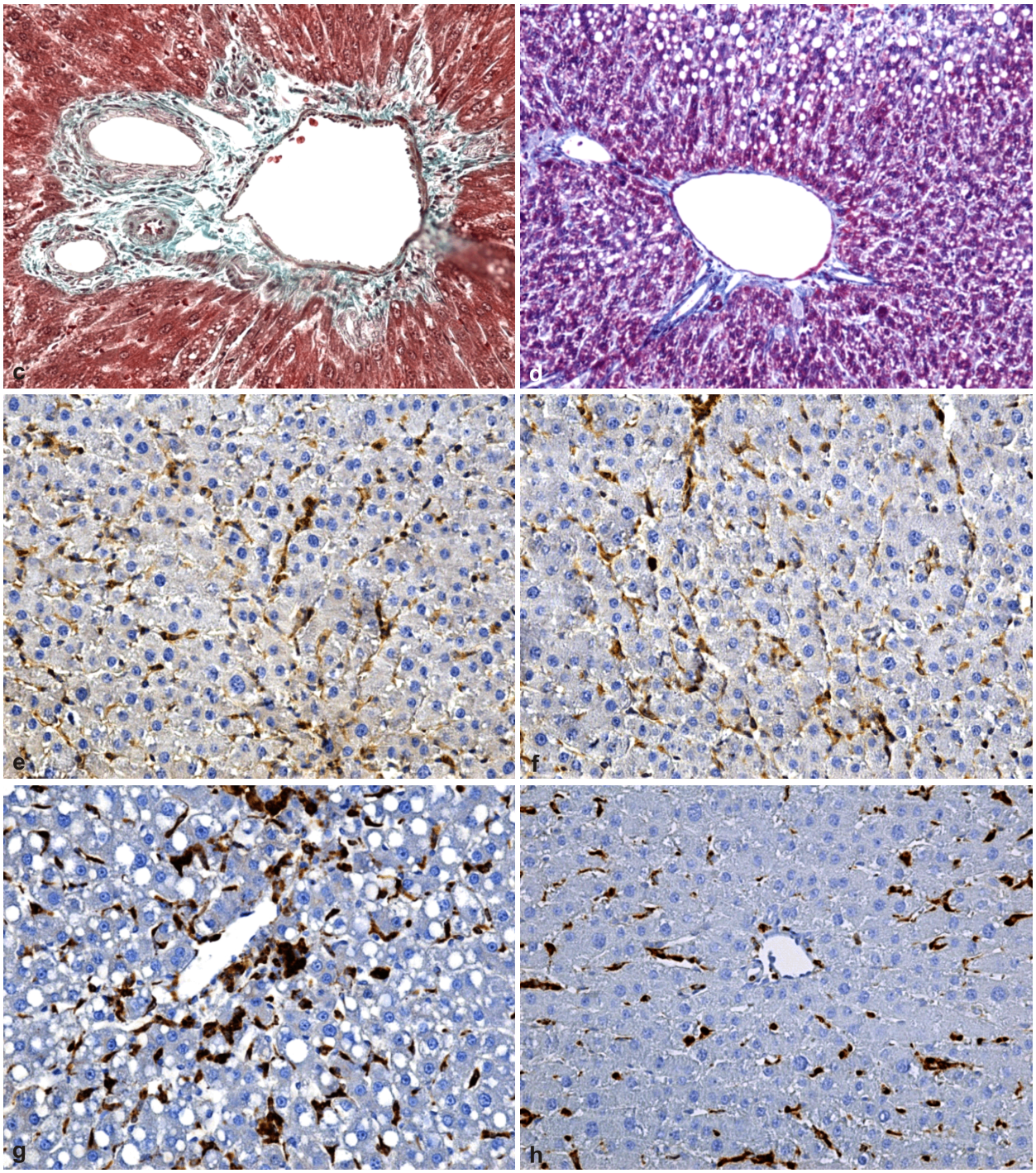

Figure 5 (continued) – ***Histopathological changes of the liver parenchyma after 21 days: (c) Image of Kiernan space with intense portal and periportal fibrosis in the animals treated with CCl_4 (GS trichrome staining, ×200); (d) Kirnan space with reduced fibrosis in the animals treated with CCl_4 and telmisartan (GS trichrome staining, ×100); (e) Intense reaction of myofibroblasts in the liver parenchyma in the animals treated with CCl_4 (Anti-α-SMA antibody immuno-marking, ×200); (f) Moderate reaction of myofibroblasts in the animals treated with CCl_4 and telmisartan (Anti-α-SMA antibody immunomarking, ×200); (g) Liver parenchyma with intense reaction of Kupffer cells in the animals treated with CCl_4 (Anti-CD68 antibody immunomarking, ×200); (h) Moderate reaction of Kupffer cells in the animals treated with CCl_4 + telmisartan) (Anti-CD68 antibody immunomarking, ×200).***

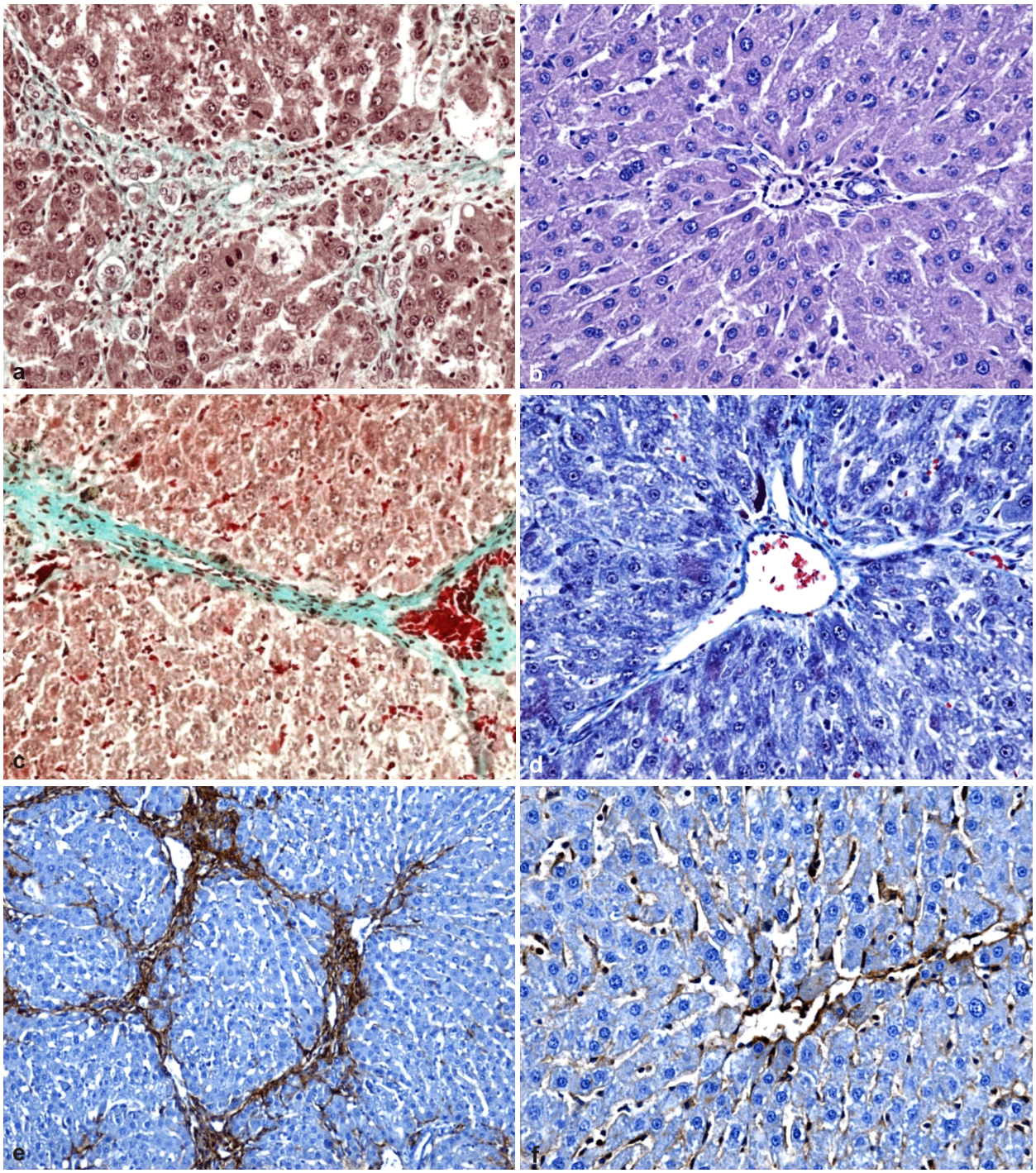

Figure 6 – ***Histopathological changes of the liver parenchyma after 28 days: (a) Image of liver parenchyma with intense lesions of hepatocytolysis and granulo-vacuolar degenerescence associated with intense collagen fibrosis and increase of the number of fibroblasts and myofibroblasts in the Kiernan space in the animals treated with CCl_4 (GS trichrome staining, ×200); (b) Liver parenchyma with minimum lesions of granulo-vacuolar degenerescence in the animals treated with CCl_4 and telmisartan (HE staining, ×200); (c) Image of porto-portal fibrous bridge in the animals treated with CCl_4 (GS trichrome staining, ×200); (d) Minimum lesions of collagen fibrosis in the portal space in the animals treated with CCl_4 and telmisartan (GS trichrome staining, ×200); (e) Intense reaction of myofibroblasts with formation of cirrhotic nodules in the animals treated with CCl_4 (Anti-α-SMA antibody immunomarking, ×100).***

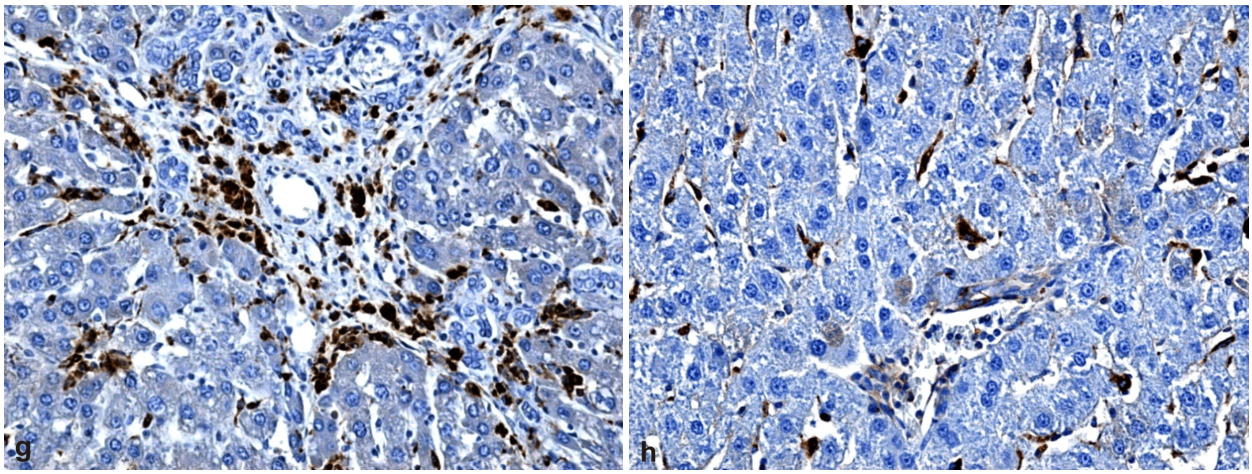

Figure 6 (continued) – ***Histopathological changes of the liver parenchyma after 28 days: (g) Intense reaction of macrophages in the Kiernan space and Kupffer cells in the animals treated with CCl_4 (Anti-CD68 antibody immunomarking, ×200); (h) Minimum reaction of Kupffer cells in the animals treated with CCl_4 and telmisartan (Anti-CD68 antibody immunomarking, ×200).***

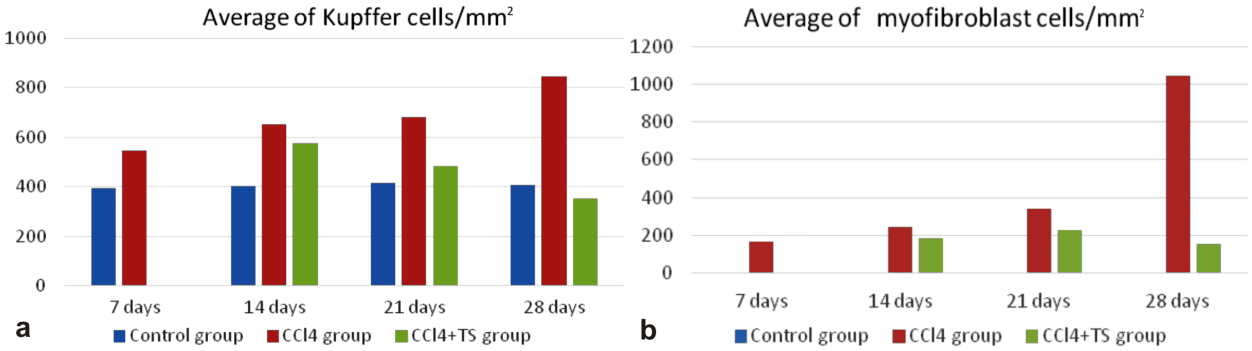

Figure 7 – ***Statistical evaluation of the reaction of Kupffer cells (a) and myofibroblasts (b) for experimental groups. TS: Telmisartan.***

Discussion

The liver plays a central part in preserving the general homeostasis of the body, also playing a part in the general metabolism, in the storage, synthesis and redistribution of nutrients. It has a great ability to regenerate, so that the liver may undergo a full recovery in a few weeks, even after losing 70% of its structure (partial hepatectomias) [9, 10]. Still, in chronic liver lesions, caused by hepatotropic viruses (mainly, B and C hepatitis viruses), toxic substances/drugs intake (especially alcohol chronic intake), in some autoimmune conditions or metabolic syndromes, the liver regeneration fails and this thing leads to an excessive accumulation of extracellular matrix (ECM), especially fibrillary collagen, thus leading to liver fibrosis. The ECM accumulation changes the liver architecture by forming fibrous scars, as well as its subsequent development of hepatocyte nodules, thus leading to the onset of liver cirrhosis [11].

For a long time, liver fibrosis was considered a passive and irreversible process, caused by the destruction of liver parenchyma and its replacement with a conjunctive tissue rich in collagen fibers [12, 13]. Clinical and experimental studies in the last decades proved that liver fibrosis is reversible in early stages, before damaging the normal architecture of the liver and deteriorating the liver function [14, 15].

Starting from the observation that the renin–angiotensin system plays a major part in liver fibrinogenesis [16–18], we proposed to investigate the effects of telmisartan, an antagonist of angiotensin II receptors, in the process of blocking the EMC synthesis in the liver. Like other authors [19], we consider that, at present, there is no clinically efficient available treatment for treating liver fibrosis, but there are a high number of preclinical studies performed experimentally on animal models for evaluating the therapeutic efficiency of certain drugs with an antifibrotic action.

For reaching liver fibrosis, we used CCl_4 as a toxic substance, an experimental model largely used for evaluating the therapeutic effect of various hepatoprotective drugs [20]. There was shown that CCl_4, administered for a long time in the experimental animal, causes liver lesions (hepatocyte necrosis, granulo-vacuolar necrosis, steatosis and liver fibrosis), because through its metabolization there are released toxic liver compounds (free radicals triclormethyl and oxygen reactive species) [21, 22].

In our study, a repeated administration of CCl_4 for four weeks (twice a week) to the experimental animals (common Wistar rats) led to a progressive deterioration of liver architecture, with a subsequent liver fibrosis bridge, similar to a precirrhotic stage.

We monitored the liver changes weekly, qualitatively

evaluating the hepatocyte lesions and onset of liver fibrosis, and qualitatively and quantitatively the changes of Kupffer cells and myofibroblasts. The intensity of liver lesions increased along with the increase of the total administered quantity of CCl_4. If after the first week of CCl_4 administration the liver lesions had a low intensity, after four weeks, the hepatocyte lesions and fibrosis had a maximum intensity, which proved that the effects of CCl_4 administration were cumulative ones.

The toxic effects of CCl_4 were highlighted even from the first week of the experiment, when in the hepatocytes of the 3rd area in the liver lobe (around the central lobular vein), there were identified lesions of granulo-vacuolar degenerescence, and in the Kiernan spaces, there was observed an early process of collagen fibrosis. We consider that the damaging of the hepatocytes in the 3rd area of the liver lobe may be due to the fact that this area is slightly hypoxic in comparison to other areas of the liver lobe. Other studies support the idea that in this area the hepatocytes contain cytochrome P450, which interferes in CCl_4 metabolization [23, 24].

The onset of minor liver lesions caused a sudden reaction of Kupffer cells and the emergence of positive α-SMA cells, even after a week since the treatment started. In the following groups, the reaction of Kupffer cells and myofibroblasts was more and more intense.

Various studies showed that myofibroblasts are the main cells involved in the process of liver collagen fibrillogenesis [25–29]. The normal liver does not contain myofibroblasts. Still, in the Disse spaces there are hepatic stellate cells (HSCs) also called Ito cells that are storage cells for lipids and vitamin A. These cells, in case of chronic injuries, change their phenotype becoming myofibroblasts capable of proliferation, contraction, mobilization and fibrogenesis [1, 30–32]. Also, other types of liver cells, besides HSCs, may have a fibrogenic potential, including the fibroblasts in the portal spaces [33, 34]. In the last decades, there was proven that hematopoietic stem cells CD34+CD38- of the hematogenous bone marrow in cell cultures, stimulated by various growth factors, may generate HSCs and myofibroblasts. In other words, it is possible that certain stem cells of the hematogenous bone marrow should infiltrate the human liver during the process of tissue remodeling [34, 35].

In our study, at the beginning of the experiment, when the quantity of administered CCl_4 was low, the myofibroblasts appeared diffuse in the liver parenchyma; at the end of the experiment (after 28 days), when the quantity of CCl_4 received by the experimental animals was high, the myofibroblasts appeared in very high quantity, at the periphery of liver regeneration nodules, in the areas of maximum fibrillary collagen. These morphological aspects make us consider that myofibroblasts are the cells producing the highest quantity of collagen where the liver chronic injuries appear.

Regarding the reaction of the Kupffer cells, we observed that their number progressively increased together with the increase of the total quantity of administered CCl_4. Moreover, we observed that Kupffer cells appeared in a higher number in the areas where the hepatocytes presented severe lesions of hepatocyte necrosis or granulo-vacuolar degenerescence. Also, Kupffer cells had larger sizes, and the intensity of the reaction to the anti-CD68 antibody was more intense. These morphological aspects showed an exacerbation of the phagocytosis activity of Kupffer cells and an increase of the lysosome quantity, as the anti-CD68 antibody mainly marks the lysosomes of the macrophage system cells. An important quantity of macrophages appeared in the porto-billiary spaces, showing their involvement in the EMC remodeling in this area.

The studies performed up to now show that the Kupffer cells are resident macrophages in the liver, representing about 20–25% of the non-parenchymal cells of the liver [36, 37]. They play an essential part in liver defense by eliminating foreign (toxic and infectious) substances from the portal blood, by the synthesis and secretion of numerous interleukins and cytokines, through which they participate to the stimulation of the immune system or influence other cells in the liver (including hepatocytes). Numerous studies showed that Kupffer cells are involved in the pathogenesis of various liver conditions, including in the process of liver fibrogenesis [38–41]. Some studies showed that the macrophage cells present in the acute or chronic liver diseases are not exclusively Kupffer cells, but also macrophages originnating in the hematogenous bone marrow or spleen, that participate in the immune reactions in which the liver is involved [42, 43].

In the animal group, where there was administered CCl_4 and telmisartan, the liver lesions had a different evolution. If after 14 days since the beginning of CCl_4 administration and after seven days since the start of telmisartan treatment, the liver lesions present in the animals of this group were similar to the lesions present after 14 days in the animals receiving only CCl_4, in the sacrificed animals after 21 and 28 days, respectively, the liver lesions (liver necrosis, granulo-vacuolar dystrophy) significantly decreased in intensity, leading to a normal aspect of the liver. The number of myofibroblasts and Kupffer cells significantly decreased in the animals treated with CCl_4 + telmisartan; also, the process of collagen fibrosis was significantly reduced. These histopathological data show the hepatoprotective and antifibrotic effects of telmisartan.

In the last years, several studies approached the part played by telmisartan in chronic liver pathology. Thus, Jim *et al.* (2007), Nakagami *et al.* (2010), Atawia *et al.* (2016) showed that telmisartan inhibits the process of liver fibrogenesis, reduces the liver infiltration with Kupffer cells and inflammatory cells and favors liver regeneration [44–46].

We consider that liver fibrosis is a dynamic process, a complex response of the organism, mediated by many cellular and molecular factors (some still unknown) that strive to maintain liver homeostasis. We believe that telmisartan may become a basic component in the treatment of some chronic liver diseases.

Conclusions

Our study showed that telmisartan has a hepatoprotective and antifibrotic effect in chronic hepatitis caused by the CCl_4 administration. It reduces the number of myofibroblasts and Kupffer cells and allows the hepatocyte regeneration process.

Conflict of interests

The authors declare that they have no conflict of interests.

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