 doi:10.20944/preprints202003.0037.v1

EFFECT OF ORALLY ADMINISTERED RELAXIN IN EXPERIMENTAL NASH

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ABSTRACT

BACKGROUND Relaxin (RLX), a hormone-like molecule with pleiotropic effects, has been found to reduce matrix deposition and mediate collagen degradation in animal models of chronic liver injuries and might be considered as an adjuvant therapeutic agent for progressive liver diseases.

AIMS In the present study, we evaluated whether RLX affects the development of liver fibrosis in an experimental mouse model of NASH in C57BL6 male mice fed with a methionine-choline deficient diet (MCD). **Methods** Mice were treated per os as we intended to assess the enteric absorption and bioavailability of orally administered RLX (RLX purified from pig ovaries, IBSA SA, Lugano, Switzerland). Mice were fed the MCD diet for 6 weeks. After the initial 3 weeks, one group received drinking water supplemented with RLX (25 mcg/ml) whereas the other continued to receive regular water. A third group of mice fed a regular diet served as control. After 3 additional weeks mice were anesthetized and sacrificed.

Results A significant, reduced expression of the pro-inflammatory cytokines TNF-α and of relevant markers of active fibrogenesis and extracellular matrix deposition, Col1A1 and α-SMA, was observed in mice treated with RLX vs controls. RLX also induced a significant decrease of TIMP1 and an increase of both MMP 2 and 9 when evaluated by zymographic analysis in liver extracts.

Conclusions Although preliminary pharmacokinetics experiments showed barely detectable amounts of RLX in the blood of mice treated per os, these data support the absorption of orally administered RLX and confirm its potential therapeutic use in the management of liver fibrosis.

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INTRODUCTION

Relaxin (RLX) is a hormone-like molecule produced during pregnancy to induce specific changes in reproductive and extra-reproductive organs to support pregnancy and parturition [1-2]. When used as experimental therapeutic agent in a variety of applications, RLX has shown several beneficial pleiotropic effects, including vasodilation, insulin sensitization and anti-fibrotic activity [3-6]. The latter has been investigated in several organs, including skin, lung, kidney, and recently liver [7-8]. In these studies, RLX has been shown to reduce collagen deposition by inhibiting tumor necrosis factor-α (TNF-α) and tissue inhibitor matrix metalloproteinases (TIMPS), while increasing matrix metalloproteinases (MMPs) mediated collagen degradation. As endogenous inhibitor of fibrotic tissue accumulation, RLX may therefore be considered as potentially active in the treatment of progressive liver diseases [6, 9]. Preventing the onset and progression of fibrosis to end stage liver disease is indeed a major goal of treatment for chronic liver disease [10-11]. This is especially true for nonalcoholic steatohepatitis (NASH). NASH is most frequently caused by the metabolic syndrome via the induction of insulin resistance and, due to the epidemics of obesity, it is now the most prevalent chronic liver disease in Western countries [12-13]. Unfortunately, no treatment is yet available for this condition. In this respect, we evaluated whether RLX affects the development of liver fibrosis in an experimental mouse model of NASH in C57BL6 male mice fed a methionine-choline deficient diet (MCD). In addition, we also assessed the enteric absorption and bioavailability of RLX to evaluate the efficacy of the oral route of administration.

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Material and Methods

Six-week-old, male C57BL6 mice were purchased from Charles River (Charles River, Calco, Italy), housed at constant room temperature (23° C) under 12-hour light/dark cycles with ad libitum access to water in compliance with the Principles of Laboratory Animal Care (NIH publication 86-23), and fed a methionine choline deficient diet (MCD, Test Diet Baker AA) for 6 weeks.

Purified RLX, extracted from porcine ovaries, was obtained from IBSA, Lugano, Switzerland [14]. RLX amount was assessed by an in house developed ELISA with a detection limit of 0.4ng/ml and high specificity due to a double antibody anti RLX detection strategy. The reference standard used in the ELISA assay was a highly purified RLX (purity >99% by RP-HPLC assay) whose concentration was quantified by densitometry adsorption at 280nm (considering 1.93 the absorbance of a 1mg/ml solution of pure RLX measured at 280nm on a 1 cm optical path length).

The extinction coefficient was calculated on a porcine RLX solution with a measured absorbance at 280nm equal to 3.19, and quantified against a standard of bovine serum albumin solution after a RP-HPLC separation in acetonitrile gradient by measuring the eluate at 214nm. The areas of different known amounts of BSA were plotted against BSA amount to create a linear regression that was used to define the amount of the injected RLX.

The estimated concentration of the RLX solution with OD_{280nm}=3.19 resulted to be 1.65mg/ml. From this experiment it was calculated the extinction coefficient of porcine RLX ε=1.93[ml*mg^{-1}*cm^{-1}].

The RLX potency was assessed by measuring the Ec50 cAMP production by human embryonic kidney (HEK293T) cells transfected with the human RLX receptor (RXFP1) [15].

In a preliminary pharmacokinetic study, we treated mice with RLX both subcutaneously (sc), (1ml each of a 25µg/ml solution) and orally (RLX diluted in drinking water at the concentration of 2.5 µg/ml and 25 µg/ml).

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Blood samples were collected after 15, 30 and 60 minutes and sent to IBSA to determine the presence and concentration of RLX by immune assay.

After the results obtained in the preliminary study, mice were divided into two groups, A and B, of 5 animals each. Both were fed the MCD diet; after 3 weeks, mice in group A received drinking water supplemented with RLX at the concentration of 25 µg /ml and dispensed in bottles refilled daily or earlier as needed, whereas group B continued to receive regular water. Based on an average 1-2 ml consumption of drinking water per mouse per day, we estimated a daily dose of 25 µg per animal.

Two additional groups of mice fed a regular, standard diet (SD) with and without RLX served as controls (5 in each group). To avoid overcrowding, mice were housed in individual cages. Body weight was measured weekly. An insulin tolerance test (ITT; 0.6 U/Kg) was performed after overnight fasting at 6 weeks; afterward animals were anesthetized and sacrificed. Blood was collected, and tissue samples dissected and flash frozen in liquid nitrogen or stored in formalin for inclusion in paraffin.

Biochemical tests

Blood glucose was measured by an Accu-Check glucometer (AVIVA, Roche Diagnostics, Mannheim, Germany). Plasma triglycerides, total cholesterol and alanine aminotransferases levels were measured with an automated analyzer (Cobas Modular, Roche Diagnostics, Mannheim, Germany) while fasting plasma insulin levels using an ELISA kit (EZRMI-13K, Millipore, Billerica, MA).

Determination of the administered dose based on the biological activity measured as EC50 by an in-vitro assay

The in-vitro test to quantify the cAMP production, after the stimulus generated by RLX on engineered HEK cell expressing the RFXP1 receptor, showed that different RLX purification

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batches had different EC50 values, when expressed in protein weight per ml (e.g.: ng/ml). In order to normalize the amount of administered RLX, we defined the concentration of 1U/ml as the amount required to obtain an effect equal to 50% of the maximal possible one (1U/ml = EC50). Each mouse was then treated with an amount of RLX equivalent to 200 U per day by oral administration. With the measured EC50, 200U are equivalent to 25µg [17]. In addition, stability of RLX in water at room temperature (20°C ± 5°C) was assessed a) by measuring the EC50 of a 25 ug/ml concentration at time 0, 44 and 164 hours and b) by analyzing the RP-HPLC RLX profile at time 0, 24, 48, 72, 144 hours. No relevant variations were observed with differences comprised between 5 and 5.3%.

Isolation of tissue RNA and qPCR

RNA was isolated from tissues by the RNeasy Plus mini kit (Qiagen). First-strand cDNA was synthesized starting from 1 mcg of total RNA using the VILO random hexamers synthesis system (Life Technologies, Carlsbad, CA). Gene expression was evaluated in triplicate by real-time quantitative PCR (qPCR), using the SYBR green chemistry (Fast SYBR Green Master Mix, Life Technologies), for Tumor Necrosis Factor-α (TNF-α), Collagen type I A1 (Col1A1), α-smooth muscle actin (α-SMA), MMP-8, MMP-9, MMP-13 and TIMP1 (Table 1). Data were normalized using the β-actin housekeeping gene and expressed as arbitrary units (AU) [17].

Matrix Metalloproteinase Extraction

Liver samples were homogenized in an ice-cold extraction buffer pH 7.6 (1:10 wt/vol) containing TRIS (50mM), NaCl (0.15 M), CaCl2 (10mM) and triton X-100 (0.25%) [18]. The homogenate was then centrifuged (30 min at 12,000 rpm) and supernatant protein concentration was measured with the colorimetric Lowry method [19]. Samples were stored at −20◦ C before use.

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Gelatinases Zymography

To detect tissue MMP-2 and MMP-9 activity, the homogenate protein content was normalized to a final concentration of 400 μg/mL in sample loading buffer (0.25M Tris-HCl, 4% sucrose w/v, 10% SDS w/v, and 0.1% bromphenol blue w/v, pH 6.8). After dilution, samples were loaded onto electrophoretic gels (SDS-PAGE) containing 1mg/mL of gelatin under no reducing conditions followed by zymography as described previously [20]. The zymograms were analyzed by densitometry (GS 900 Densitometry BIORAD, Hercules, CA, USA) and data were expressed as optical density (OD), reported to 1mg/mL protein content.

Histological analysis

Liver tissue samples were fixed in 10% PBS buffered formalin and embedded in paraffin within 48 hours of formalin fixation. Histological staining was performed using H&E and hepatic fibrosis was evaluated by Sirius Red staining as previously described [21]. Diagnosis of NASH was based on the presence of steatosis with lobular necro-inflammation and ballooning or fibrosis. Disease activity was assessed according to the NAFLD activity score and fibrosis staged according to Kleiner [22].

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U-test or the Student t-test for continuous variables and the χ^2 or Fisher exact probability test for categorical data. A probability value of $p< 0.05$ was considered statistically significant.

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RESULTS

Evaluation of oral RLX absorption

Circulating RLX levels following oral or subcutaneous administration were determined by Elisa assay in a preliminary pharmacokinetic study

In these experiments, we detected RLX in the blood of mice treated with 25µg (equivalent to 200 units of biological activity) by subcutaneous injection. The amount of RLX detected by ELISA assay ranges between 27 and 48ng/ml. Mice were bled at the time of injection and every 15 minutes for 1 hour. The maximal concentration in blood was measured at 15 and 30 minutes, indicating a short window of high detectable RLX followed by a subsequent sharp drop (fig. 1)

In the mice which received 25µg and 2.5µg (respectively equivalent to 200 and 20 U of biological activity) by oral administration, the measured level of RLX in the blood ranges from 0.6ng/ml to 7.8ng/ml which is very close to the detection limit of the method (fig. 1).

These data suggest that RLX has a very short half-life but can still be detected after enteric administration although at borderline concentrations.

Impact of chronic RLX administration on MCD fed mice

Mice were fed the MCD diet for 3 weeks and then supplemented with or without RLX per os for 3 additional weeks, to investigate a possible effect in preventing the onset of fibrosis or its progression after an initial induction of liver damage. Serum samples collected at the end of the study were analyzed for the presence of circulating RLX but no detectable amount could be measured.

We then analyzed serum samples collected from RLX and placebo treated mice and did not observe any significant difference for the levels of glucose, triglycerides, insulin and ALT (alanine transaminase) (data not shown). While no differences were found in the amount of steatosis

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between mice fed the MCD diet in the presence or absence of RLX (Table 2), the latter induced vasodilation and a slight increase in endothelial basal membrane as shown in sections stained by Sirius Red (Fig. 2).

Impact of chronic RLX administration on fibrogenesis in MCD fed mice

Gene expression analysis showed a reduced expression of the main pro-inflammatory cytokines (TNF-α) and of some of the most important markers of active fibrogenesis and extracellular matrix deposition (Col1A1 and α-SMA) in mice treated with RLX vs. controls (Fig. 3 a, b and c). Also the expression of the ECM remodeling metalloproteinases appeared to be reduced with a significant decrease in the case of MMP13 in mice fed the MCD diet plus RLX compared to those receiving the MCD diet alone. In this group the MCD diet alone caused a significant increase compared to mice fed the SD with and without RLX. (Fig. 3 d). On the other hand both MMP 2 and 9 showed a increased activity when evaluated by zymographic analysis in liver extracts (Fig. 4-5). As expected, also TIMP1 showed significantly lower levels in the presence of RLX (Fig. 3e).

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DISCUSSION

The anti-fibrotic activity of RLX, previously showed in several organs, makes its potential effect in liver diseases worth exploring [3, 6, 8]. Treatment of fibrosis and its progression to cirrhosis is still an unmet need in the management of chronic liver diseases [23-25]. In this regard, we have investigated the efficacy of RLX in preventing the onset and progression of fibrosis in a mouse model of NASH, currently the most prevalent chronic liver disease [12-13]. In addition, we have analyzed the enteric absorption and bioavailability of RLX administered per os, to demonstrate the efficacy of the oral route of administration so far only indirectly proven in preliminary studies in humans receiving gastro-protected encapsulated formulations [26].

Our data confirm the ability of RLX in reducing the expression of the main mediators of ECM deposition, including Col1A1 and α-SMA as well as TIMP1 [27]. These results are consistent with those previously reported in experiments where RLX was shown to inhibit HSC activation in vitro [28]. While RLX also decreased the levels of the pro-inflammatory cytokine, TNF-α, a general reduced expression of the investigated MMPs was observed particularly in the case of MMP 13, a finding previously associated with a reduced expression of inflammatory markers, including TNF-α and TGF-β1, and liver inflammation [29]. Indeed, the marked elevation of MMP13 observed in the MCD fed mice seems to confirm its role in the initial phase of liver inflammation favored by an alteration of the normal ECM and liver cells homeostasis. In this respect RLX may exert a positive regulatory effect in the lack of relevant collagen deposition as observed in MCD fed mice. On the other hand, zymographic analyses showed a significant higher activity of both MMP 2 and MMP9 in the liver of mice treated with RLX. MMP 2 and 9 are the key gelatinase mediators already known to be increased in ECM remodeling in the liver [30-31]. As expected RLX, induced sinusoidal vasodilatation, an effect known to be part of its pregnancy related activities, mainly induce by NO release [32]. NO elevation as well as the increase of fatty acids oxidation related genes have been also reported in a recent study in MCD fed mice treated with recombinant RLX [33].

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Taken together, these results may suggest that RLX was indeed adsorbed when provided in drinking water with no enteric coating. The lack of detectable circulating amounts of RLX in sera of mice that were sacrificed at the end of the study, may be due to its very short half-life that we observed in the preliminary pharmacokinetics experiments.

This represents the first evidence in support of an oral formulation of RLX that could facilitate its use in the experimental treatment of hepatic fibrosis and its progression in chronic liver diseases.

Acknowledgements

We thank Dr. L. Valenti, P. Dongiovanni and Dr. M. Meroni for contributing to the design, implementation of the study and data collection. This study was supported by an unrestricted research grant from IBSA, Institute Biochemique, Lugano, Switzerland.

Ethical approval: “All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.” The study was approved by the Ethical Committee Milano Area 2, ID 485, on May 15, 2018.

 doi:10.20944/preprints202003.0037.v1

REFERENCES

[1] Bathgate RA, Samuel CS, Burazin TC, et al. Relaxin: new peptides, receptors and novel actions. Trends Endocrinol Metab, **2003**, 14, 207-13.

[2] Bani G, Maurizi M, Bigazzi M, Bani Sacchi T Effects of relaxin on the endometrial stroma. Studies in mice. Biology of Reproduction, **1995**, 53, 253–262.

[3] Bani D. Relaxin: a pleiotropic hormone. General Pharmacology, **1997**, 28, 13–22.

[4] Samuel CS. Relaxin: Antifibrotic Properties and Effects in Models of Disease. CM&R, **2005**, 3, 241-249.

[5] Samuel CS, Summers R J, Hewitson T D. Anti-fibrotic Actions of Serelaxin-New roles for an Old Player. Trends Pharmacol Sci, **2016**, 37(6), 485-497.

[6] Williams EJ, Benyon RC, Trim N, et al. Relaxin inhibits effective collagen deposition by cultured hepatic stellate cells and decreases rat liver fibrosis in vivo. Gut, **2001**, 49, 577-583.

[7] Bennett RG, Heimann D G, Tuma D J. Relaxin Reduces Fibrosis in Models of Progressive and Established Hepatic Fibrosis. Ann N Y Acad Sci, **2009**, 1160, 348–349.

[8] Fallowfield JA, Hayden AL, Snowdown VK, et al. Relaxin Modulates Human and Rat Hepatic Myofibroblast Function and Ameliorates Portal Hypertension In Vivo, Hepatology **2014**, 54, 1492-1504.

[9] Bani D, Yue SK., Bigazzi M. Clinical Profile of Relaxin, a Possible New Drug for Human Use, Current Drug Safety **2009**, 4, 238-249.

[10] Pinzani M. Pathophysiology of Liver Fibrosis. Dig Dis **2015**, 33(4), 492-497.

[11] Trautwein C., Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: Concept to treatment, J Hepatol, **2015**, 62, S15-24.

[12] Polyzos SA, Mantzoros CS. Nonalcoholic fatty future disease, Metabolism, **2016**, 65(8), 1007-1016.

[13] Zounossi ZM, Koenig AB, Abdelatif D et al. Global epidemiology of nonalcoholic fatty liver disease: meta-analytic assessment of prevalence, incidence, and outcomes, Hepatology **2016**, 64, 73-84.

[14] Frankshun AL, Ho TY, Reimer DC, Chen J et al. Characterization and biological activity of relaxin in porcine milk, Reproduction, **2011**, 141(3), 373-80.

[15] Sudo S, Bathgate RA, and Hsueh AJ. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2, J Biol Chem, **2003**, 278, 7855–7862.

[16] Ren X, Wang C, Wu L, et al. Bioactivity assay of porcine relaxin based on cAMP accumulation in THP-1 cells quantified by LC-MS/MS, J Pharm Biomed Anal, **2015**, 111, 320-323.

[17] Dongiovanni P, Meroni M, Baselli GA et al. Insulin resistance promotes Lysyl Oxidase Like 2 induction and fibrosis accumulation in non-alcoholic fatty liver disease, Clin Sci (Lond), **2017**, 131(12), 1301-1315.

[18] Kossakowska AE, Edwards DR, Lee SS et al. Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis, American Journal of Pathology, **1998**, 153, 1895–1902.

[19] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent, The Journal of Biological Chemistry, **1951**, 193, 265–275

[20] Tozzi R, Palladini G, Fallarini S et al. Matrixmetalloprotease activity is enhanced in the compensated but not in the decompensated phase of pressure overload hypertrophy. American Journal of Hypertension, **2007**, 20, 663–669.

[21] Dongiovanni P., Meroni M., Baselli G. L. et al. Insulin resistance promotes Lysyl Oxidase Like 2 induction and fibrosis accumulation in nonalcoholic fatty liver disease, Clin Sci, **2017**, 18 (7), 1301-1315.

[22] Kleiner DE, Brunt EM., Van Natta M, et al. Design and Validation of a Histological Scoring System for Nonalcoholic Fatty Liver Disease. Hepatology, **2005**, 41, 1313-1321.

[23] Bataller R, Brenner DA. Liver fibrosis. J Clin Invest, **2005**, 115(2), 209–218.

[24] Gandhi CR, Hepatic stellate cell activation and pro-fibrogenic signals, J Hepatol, **2017**, 67(5), 1104–1105.

[25] Iredale JP, Pellicoro A, Fallowfield JA. Liver Fibrosis: Understanding the Dynamics of Bidirectional Wound Repair to Inform the Design of Markers and Therapies, Dig Dis, **2017**, 35(4), 310-313.

[26] Milia P. Caserio M, Bani D, et al. Efficacy of Relaxin on functional recovery of post stroke patients. Ital J Anat Embryol, **2013**, 118, 92-97.

[27] Benyon RC, Iredale JP, Goddard S, et al. Expression of Tissue Inhibitor of Metalloproteinases 1 and 2 Is Increased in Fibrotic Human Liver. Gastroenterology , **199**), 110, 821–831.

[28] Bennett RG, Kharbanda KK, Tuma DJ. Inhibition of markers of hepatic stellate cell activation by the hormone relaxin, Biochemical Pharmacology **2003**, 66(5), 867-874.

[29] Uchinami H, Seki E, Brenner DA, D'Armiento J. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. Hepatology, **2006**, 44(2), 420-9.

[30] Kurzepa J, Mądro A, Czechowska G, et al. Role of MMP-2 and MMP-9 and their natural inhibitors in liver fibrosis, chronic pancreatitis and non-specific inflammatory bowel diseases. Hepatobiliary Pancreat Dis Int, **2014**, 13(6), 570-9.

[31]. Chow BS, Chew EG, Zhao C, et al. Relaxin signals through a RXFP1-pERK-nNOS-NO-cGMP-dependent pathway to up-regulate matrix metalloproteinases: the additional involvement of iNOS. PLoS One, **2012**, 7(8), e42714.

[32] Conrad KP. Unveiling the vasodilatory actions and mechanisms of Relaxin. Hypertension, **2010**, 56 (1), 2-9.

[33] Lee K. C., Hsieh Y. C., Chan C. C. et al. Human relaxin-2 attenuates hepatic steatosis and fibrosis in mice with non-alcoholic fatty liver disease. Laboratory Investigation**, 2019, 99,** 1203–1216.

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Gene	FW primer	Rv primer
Col1A1	5'- GAGCGGAGAGTACTGATCG-3'	5'- TACTCGAACGGGAATCCATC -3'
α-Sma	5'- GGAAAAGATCTGGCACCACT – 3'	5' – GAGTCCAGCACAATACCAGTTG -3'
MMP8	5'- TGCAGAGAAGCTTAAAGAGA -3'	5' - AGTCTCTGCTAAGCTGAAGAA -3'
MMP9	5'- GGATCCAAAACTACTCTGAAGA -3'	5'- ACCAAACTGGATGACAATGT – 3'
MMP13	5'- TTGGGACTAAAGAACATGGTGA -3'	5'- CGCCTGGACCATAAAGAAAC -3'
TIMP1	5'-TCAAGATGACTAAGATGCTAAAA-3'	5'-ACCCCACAGCCAGCACTAT-3'
TNF- α	5'- CCCCAAAGGGATGAGAAGTT -3'	5'- GTGGGTGAGGAGCACGTAGT -3'
β-actin	5' – GCTACAGCTTCACCACCACA -3'	5'- AAGGAAGGCTGGAAAAGAGC -3'

Table 1. PCR amplification primers specific for Col1A1, α-SMA, MMP8, MMP9, MMP13,TIMP1, TNF-α and β-actin.

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Mice	Steatosis	Ballooning	Lobular inflammation	Fibrosis
MCD+RLX	3	2	2	1
STD+RLX	0	0	0	0
MCD	3	2	2	1
STD	0	0	0	0

Table 2. NAS (NAFLD Activity Score) mean score of mice (5 in each group) fed the MCD or SD diet with or without RLX supplementation, according to Kleiner [22].

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Figures

Fig. 1. Pharmacokinetics of Relaxin (RLX) as assessed at time 0, 15, 30 and 60 minutes after subcutaneous (sc) and oral (os) administration. Results are expressed as RLX ng/ml.

Fig. 2. Blood vessels vasodilation observed in the liver parenchyma of mice fed the standard and the MCD diet (SD) with Relaxin (RLX) compared to controls. Sirius red staining, 40 x.

Fig. 3a. Reduced expression of TNF-α in mice fed the methionine choline deficient diet (MCD) diet plus Relaxin (RLX) compared to MCD alone (* P <0.05 MCD+RLX vs MCD). Results are expressed as fold changes (arbitrary units, AU) mean ± SD of 5 mice in each group.

Fig. 3b. Reduced expression of Col1A1 in mice fed the methionine choline deficient diet (MCD) plus Relaxin (RLX) compared to MCD alone. (* P <0.05 MCD+RLX vs MCD). Results are expressed as fold changes (arbitrary units, AU) mean ± SD of 5 mice in each group.

Fig. 3c. Reduced expression of α-SMA in mice fed the methionine choline deficient diet (MCD) plus Relaxin (RLX) compared to MCD alone. (^{o} P <0.05 MCD+RLX vs MCD). Results are expressed as fold changes (arbitrary units, AU) mean ± SD of 5 mice in each group.

Fig. 3d. Decreased expression of MMP13 in mice fed the methionine choline deficient diet (MCD) plus Relaxin (RLX) compared to MCD alone (**P <0.05 MCD+RLX vs MCD). The latter caused a significant increase in MMP13 expression compared to SD (*P <0.05 MCD alone vs SD). Results are expressed as fold changes (arbitrary units, AU) mean ± SD of 5 mice in each group.

Fig. 3e. Decreased expression of TIMP1 in mice fed the methionine choline deficient diet (MCD) plus Relaxin (RLX) compared to MCD alone. (* P <0.05 MCD+RLX vs MCD). Results are expressed as fold changes (arbitrary units, AU) mean ± SD of 5 mice in each group.

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Fig. 4. Increased activity of MMP9 (a) and MMP2 (b) in mice fed the methionine choline deficient diet (MCD) plus Relaxin (RLX) compared to MCD alone. (*P <0.05 MCD+RLX vs MCD). Results are expressed as optical density (OD), reported to 1mg/mL protein content, mean ± SD of 5 mice in each group.

Fig. 5. Gelatin zymograms of MMP9 and MMP2 in liver extracts from mice fed the methionine choline deficient diet (MCD) with (lanes 1-5) or without (lanes 6-10) Relaxin (RLX) administration.

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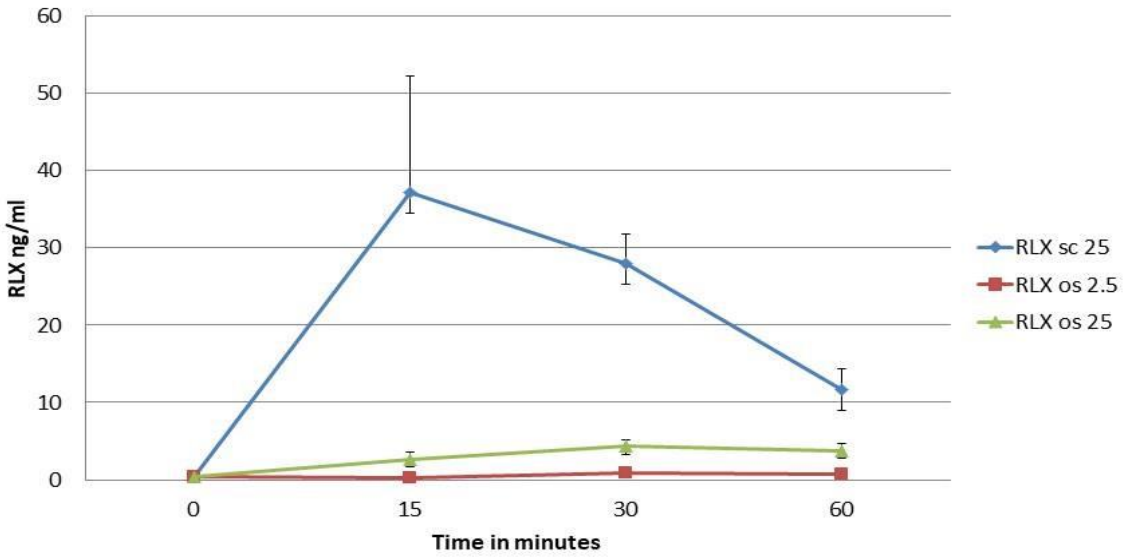

Fig. 1

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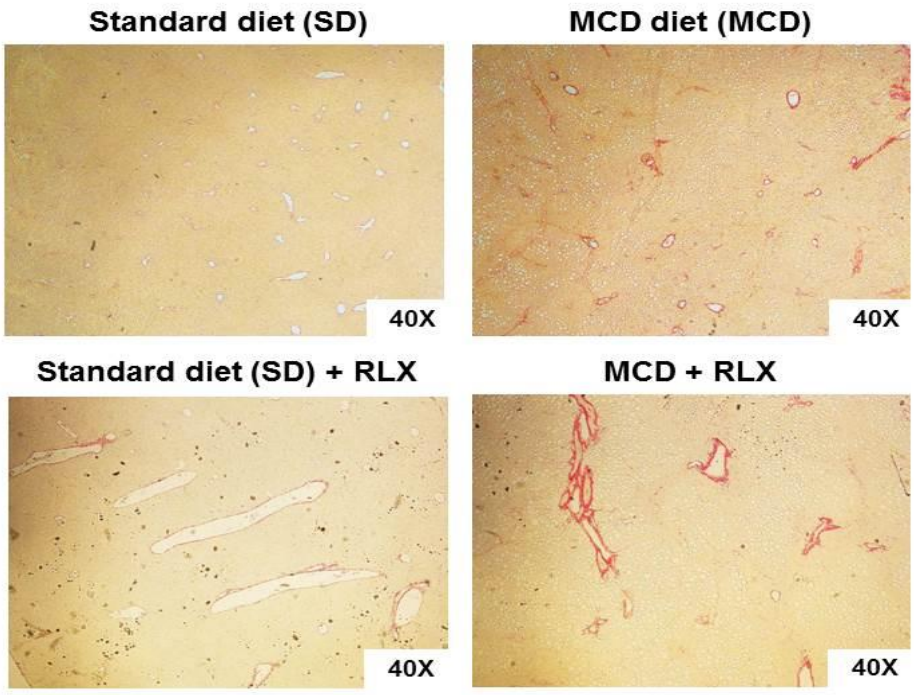

Fig. 2

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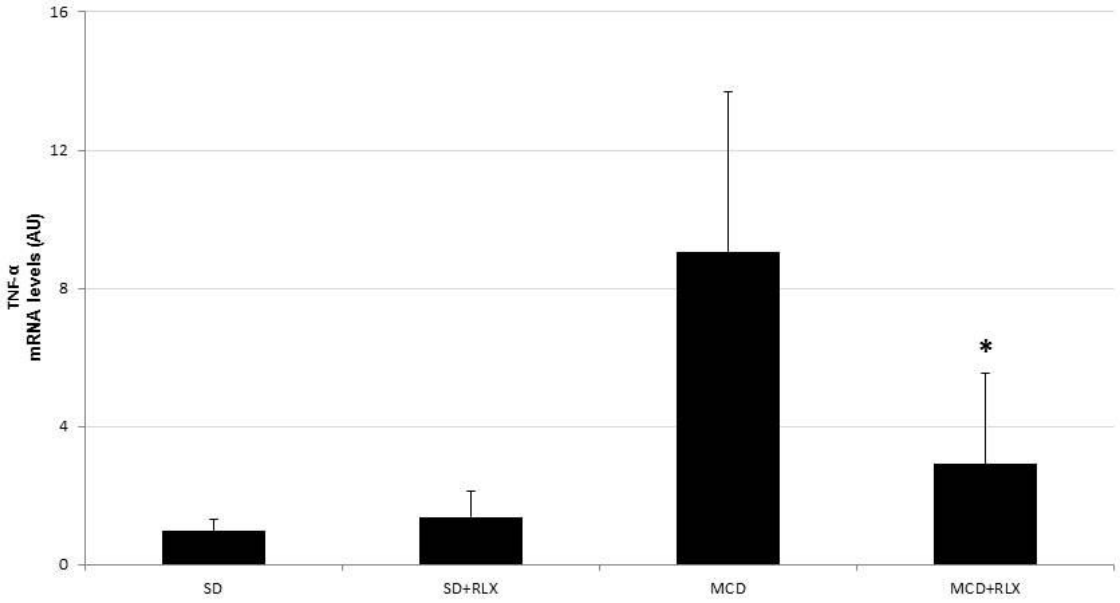

Fig. 3a

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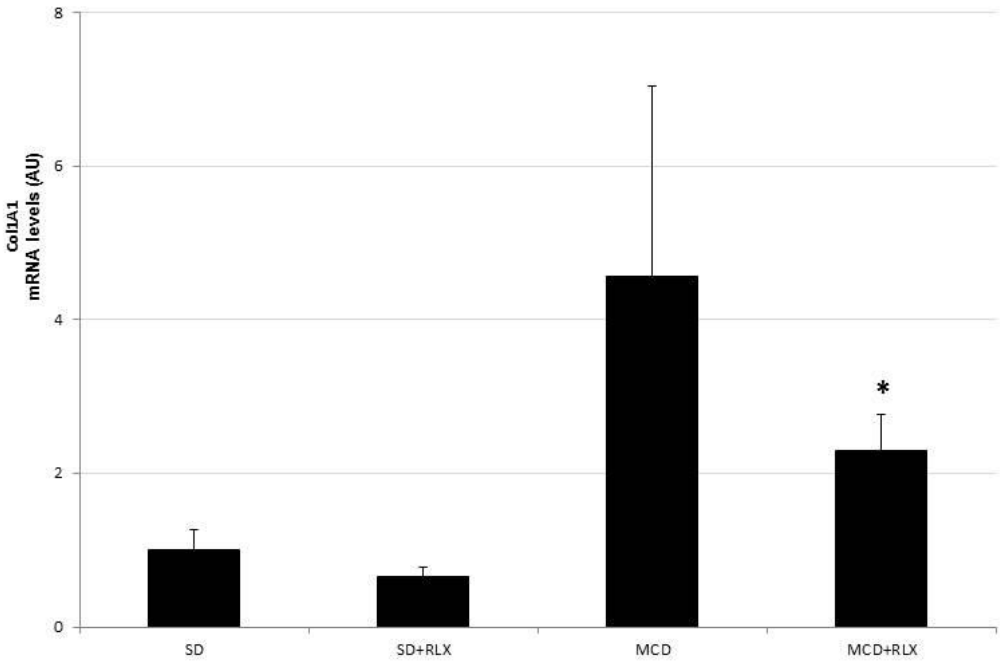

Fig. 3b

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 March 2020 doi:10.20944/preprints202003.0037.v1

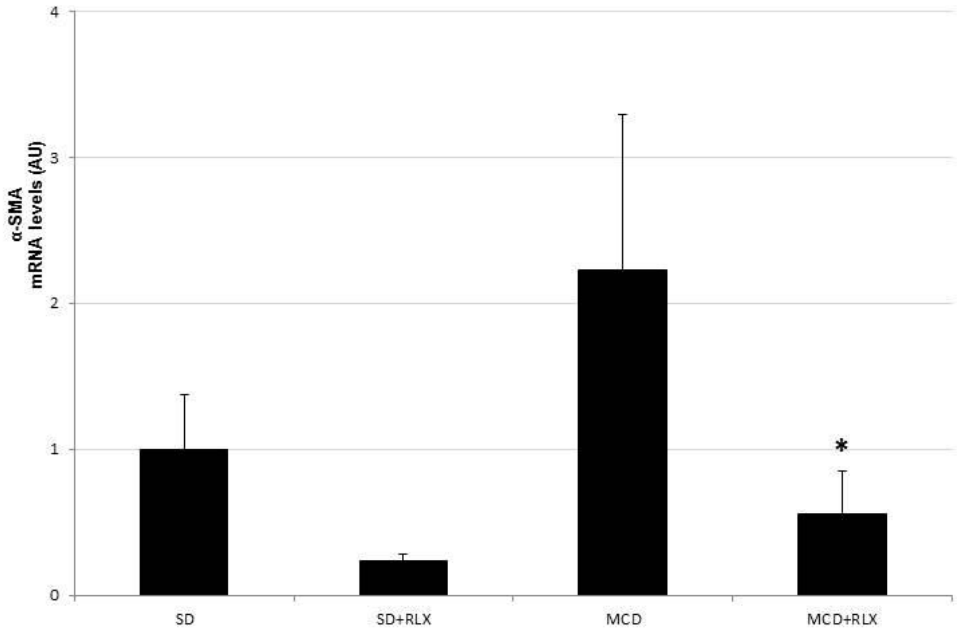

Fig. 3c

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 March 2020 doi:10.20944/preprints202003.0037.v1

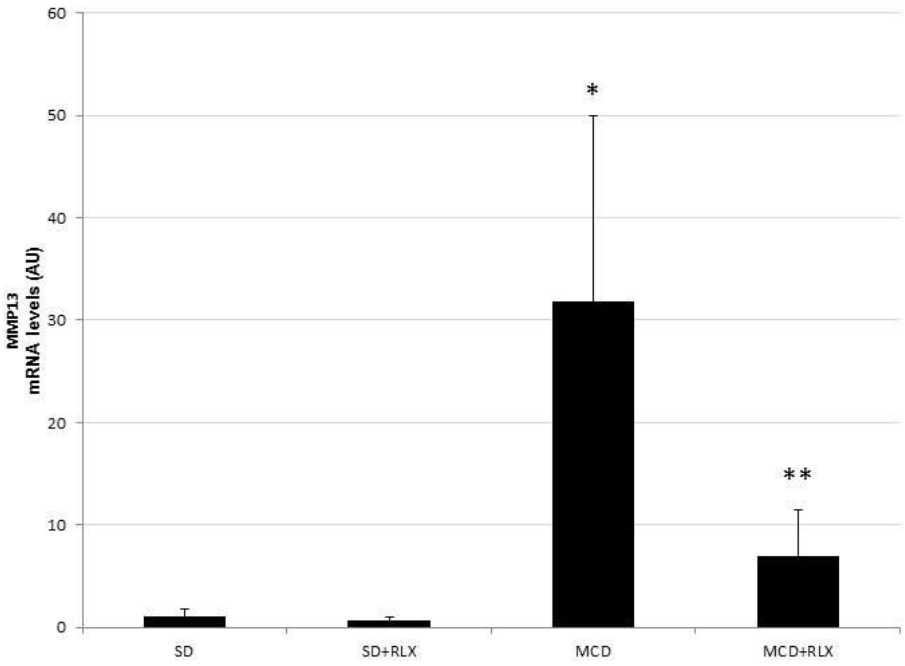

Fig. 3 d

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 March 2020 doi:10.20944/preprints202003.0037.v1

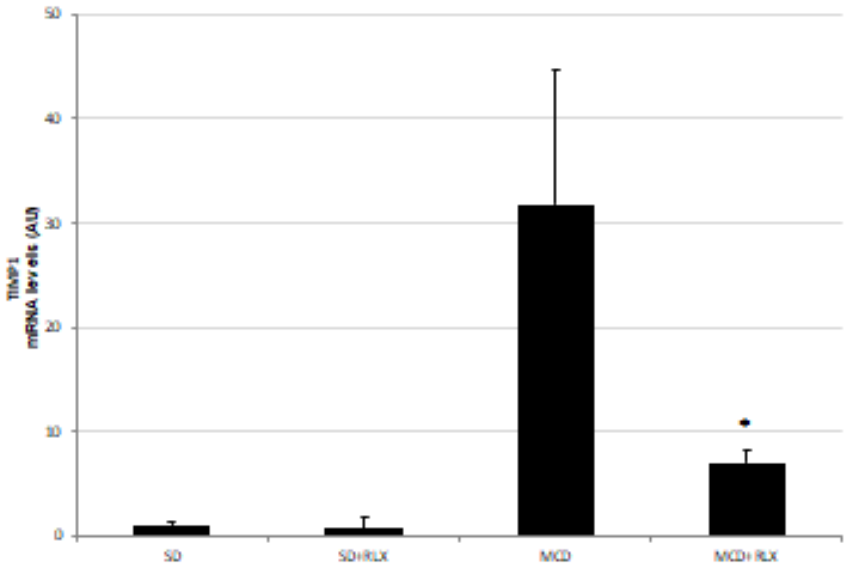

Fig. 3 e

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 March 2020 doi:10.20944/preprints202003.0037.v1

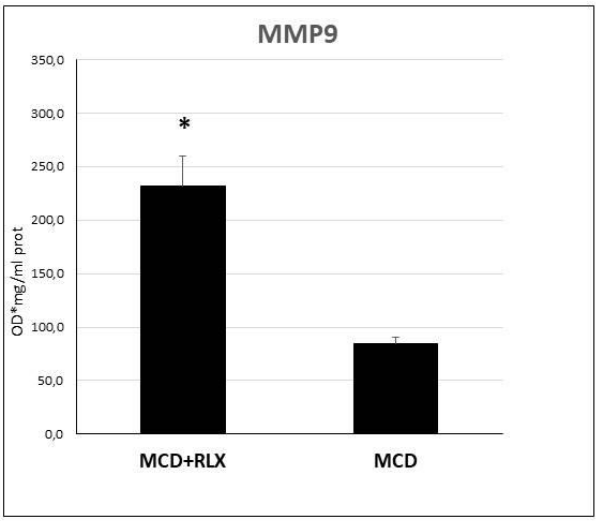

Fig. 4 a

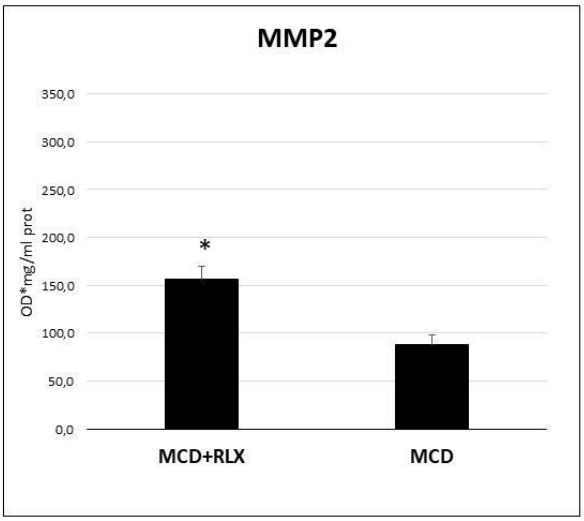

Fig. 4 b

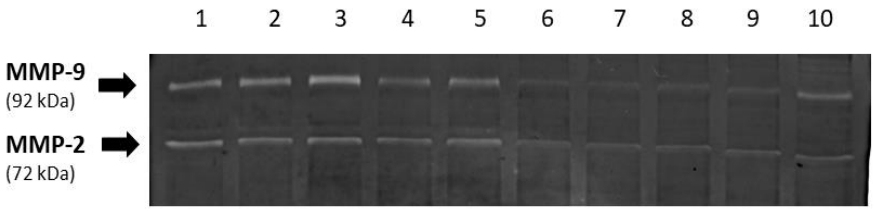

Fig. 5