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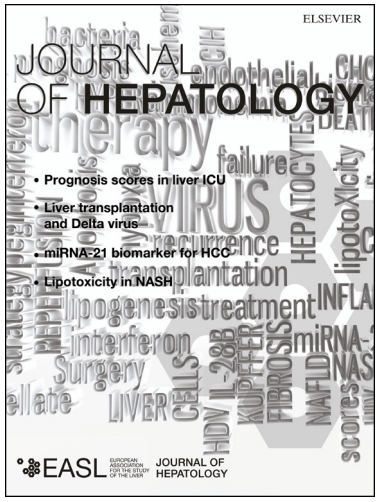

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Christopher Leung, Chandana B. Herath, Zhiyuan Jia, Michelle Goodwin, Kai Yan Mak, Matthew J. Watt, Josephine M. Forbes, Peter W. Angus

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Dietary glycotoxins exacerbate progression of experimental fatty liver disease

Authors:

Christopher Leung[1,2,3], Chandana B Herath[1], Zhiyuan Jia[1], Michelle Goodwin[1,2], Kai Yan Mak[1], Matthew J Watt[4], Josephine M Forbes[3,5], Peter W Angus[1,2].

[1]Department of Medicine, The University of Melbourne, Austin Health, Heidelberg, Victoria, Australia.

[2]Department of Gastroenterology and Hepatology, Austin Health, Austin Hospital, Heidelberg, Melbourne, Victoria, Australia.

[3]Glycation and Diabetes Group, Baker IDI Diabetes Institute, Melbourne, Victoria, Australia.

[4]Department of Physiology, Monash University, Clayton, Victoria, Australia.

[5]Glycation and Diabetes Complications Group, Mater Medical Research Institute, South Brisbane, Queensland, Australia.

Corresponding Author:

Christopher Leung, MBBS, FRACP

Department of Gastroenterology and Hepatology

Austin Health, Heidelberg 3084, Victoria, Australia

Email: chris.leung@y7mail.com

Tel + 61 (0)3 9496 5000

Fax +61 (0)3 9496 3487

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Conflicts of interest:

No conflicts of interest exist for any of the authors of this manuscript.

List of abbreviations

AGEs, advanced glycation end-products; RAGE, receptor for AGEs; ROS, reactive oxygen species; NAFLD, non-alcoholic fatty liver disease; MCD, methionine choline deficient; MCR, methionine choline replete; CML, N-(carboxymethyl)lysine; ALT, alanine transaminase; IL-6, interleukin-6; TNF-α, tumour necrosis factor α; α-SMA, α-smooth muscle actin; COL1A, collagen-1A; CTGF, connective tissue growth factor; HSCs, hepatic stellate cells; BrDU, bromodeoxyuridine; MCP-1, monocyte chemotactic protein-1; NADPH, nicotinamide adenine dinucleotide phosphate; NASH, non-alcoholic steatohepatitis; BSA, bovine serum albumin; HNE, 4-hydroxy-2-nonenal; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance.

Keywords:

advanced glycation end-products; steatohepatitis; non-alcoholic fatty liver disease; oxidative stress; hepatic stellate cell.

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Abstract

Background & Aims

Advanced glycation end-products (AGEs) levels are high in western diets and contribute to tissue injury via activation of RAGE (receptor for AGEs) and generation of reactive oxygen species (ROS). Here, we determined if high dietary AGE intake worsens progression of non-alcoholic fatty liver disease (NAFLD).

Methods

Male Sprague Dawley rats were fed a methionine choline deficient (MCD) diet for 6 weeks before 6 weeks of a high AGE MCD diet through baking. They were compared with animals on MCD diet or a methionine choline replete (MCR) diet alone for 12 weeks. Hepatic ROS, triglycerides, biochemistry, picro-sirius morphometry, hepatic mRNA expression and immunohistochemistry were determined. Primary hepatic stellate cells (HSCs) from both MCR and MCD animals were exposed to AGEs. ROS, proliferation and mRNA expression were determined.

Results

The high AGE MCD diet increased hepatic AGE content and elevated triglycerides, NADPH dependent superoxide production, HNE adducts, steatosis, steatohepatitis (CD43, IL-6, TNF-α) and fibrosis (α-SMA, CTGF, COL1A, picrosirius) compared to MCD alone. In HSCs, AGEs significantly increased ROS production, bromodeoxyuridine proliferation and MCP-1, IL-6, α-SMA and RAGE expression in HSCs from MCD but not MCR animals. These effects were abrogated by RAGE or NADPH oxidase blockade.

Conclusions:

In the MCD model of NAFLD, high dietary AGEs increases hepatic AGE content and exacerbates liver injury, inflammation, and liver fibrosis via oxidative stress and RAGE dependent profibrotic effects of AGEs on activated HSCs. This suggests that pharmacological and dietary strategies targeting the AGE/RAGE pathway could slow the progression of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world, largely due to the burgeoning rates of diabetes and obesity[1]. Although most patients with NAFLD are asymptomatic and do not develop significant liver injury, many progress to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer. However, the host and/or environmental risk factors that determine whether patients with simple hepatic steatosis go on to develop NASH and its complications remain unclear[2].

Advanced glycation end products (AGEs), also known as glycotoxins, are a complex group of compounds that are formed via the Maillard reaction, where sugar moieties become bound to proteins and lipids, causing browning and other irreversible modifications[3]. Foods that are highly processed or dry heated at high temperatures, such as broiled foods, are particularly high in AGEs[4]. AGE formation occurs at an increased rate in diabetes owing to the excess of reducing sugars available as a consequence of hyperglycaemia. The rate is also influenced by the concentration of reducing sugars in the serum, the turnover of the proteins, and the extent of the oxidative stress in the environment.

These compounds act on many receptors, including macrophage scavenger receptor type I and II, oligosaccharyl transferase-48 (AGE-R1), 80k-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3) and the receptor for advanced glycation end products (RAGE). RAGE, a member of the immunoglobulin superfamily of cell-surface molecules, is the best characterised of these receptors[5]. It is expressed in a number of cell types, including endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells, macrophages including

Kupffer cells, and hepatic stellate cells (HSCs). There is now strong evidence that accumulation of AGEs results in changes to extracellular matrix structure and function, and they have been implicated in the pathogenesis of diabetic renal, neurological, retinal and vascular complications[6]. Cellular responses to AGEs are driven by its engagement with RAGE, which is thought to be the main way in which AGEs impart their pathogenic effects. RAGE activation increases inflammation and the generation of ROS[7].

There are a number of lines of evidence to suggest that in NAFLD, AGEs may be a factor that contributes to the progression from simple steatosis to NASH and liver fibrosis[8]. A number of studies have shown that RAGE plays a role in acute liver injury and that blockade of RAGE can ameliorate toxic, ischaemic and cholestatic liver damage[9-11]. In chronic liver injury, hepatic expression of RAGE is significantly increased[12] and in NAFLD, AGE levels correlate with the severity of fibrosis, leading to speculation that they play a primary role in disease pathogenesis[13]. Furthermore, diabetes, which increases AGE formation and RAGE expression, worsens the progression of fibrosis in a number of human liver diseases, including NAFLD and hepatitis C[14].

In our study, we explored if excess dietary AGE consumption could exacerbate the development of NASH in experimental NAFLD in both normal and fatty livers. The MCD diet model was chosen as it does not produce the potentially confounding effect of peripheral insulin resistance which might increase endogenous AGE production. We also performed complementary in vitro studies in HSCs to determine mechanistic pathways to AGE mediated effects seen in our liver disease model.

Methods

Experimental design

Experiments were approved by the Austin Health Animal Ethics Committee and performed according to the National Health and Medical Research Council (NHMRC) of Australia Guidelines for animal experimentation. To explore the role of dietary AGEs in the normal liver, rats were randomised into two groups (n=10/group) and followed for 12 weeks.

1) A control group; fed a MCR diet (MP Biomedicals, Solon, Ohio, United States);

2) A baked diet in normal animals; fed a MCR diet for the first 6 weeks but in the last 6 weeks this was baked (MCR baked) to increase AGE content as previously described by our group[15, 16].

To explore the role of AGEs in NAFLD, steatosis was induced in 10 week old Sprague Dawley rats by depleting methionine and choline from their diet as previously described[17]. This model induces accumulation of VLDL in the liver leading to classical histopathological features of NASH without peripheral insulin resistance[18].

Rats were randomised into three groups (n=10/group) and followed for 12 weeks.

1) A group fed the MCR diet

2) A steatotic group; fed a methionine and choline deplete diet (MCD) (MP Biomedicals);

3) A steatotic group with increased oral AGEs (MCD baked) where animals were fed an MCD diet for 12 weeks and in the last 6 weeks had this diet baked to increased dietary AGE content.

AGE production and content

A high AGE diet was produced by baking the diet at 160^{o}C for 1 hour. AGEs for *in vitro* studies were produced as previously described (supplement1)[19]. CML is the

predominant AGE in food and the extent of advanced glycation was measured by ELISA [16] and this showed that baking the MCD diet increased CML levels more than 4 fold from 31 to 137nmol/100mg lysine, as in previous studies[20]. Liver CML content was also assessed by ELISA as previously reported[21] (Supplementary 1).

Assessment of biochemistry, superoxide production, hepatic triglyceride content and histology.

Serum biochemistry, superoxide, lipids and histology were quantified as reported[11, 22]. (Supplementary 1). To ensure that there was no peripheral insulin resistance or diabetes that could confound the effects of exogenous dietary AGEs via formation of endogenous AGEs, the HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) was measured across all groups.

Quantification of gene expression and protein localisation

Gene expression and protein localisation was performed using quantitative real-time PCR and immunohistochemistry, respectively, and as previously reported[11]. The details of fluorescent labeled oligonucleotide probes and their target specific primers is given in Table 1 in Supplementary 1. Immunohistochemical staining methods are also expanded in Supplementary 1.

In vitro experiments

HSCs were isolated and cultured from livers in both MCR and MCD animals as reported[23]. Cells were seeded in 6 well plates with BSA at 100μg/mL (vehicle) or AGE-BSA at 100μg/mL (intervention) from 2 to 8 days post-isolation. Two additional

groups were treated with AGE-BSA along with NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (5μM, Sigma-Aldrich); and AGE-BSA along with goat anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA). (Supplementary 1)

Measurement of reactive oxygen species (ROS) and cell proliferation.

Measurement of intracellular ROS was performed as reported[24]. Proliferative response was assessed using a BrdU assay (Roche Applied Science, Indiana, USA) as per manufacturer's instructions.

Statistical analysis

Data (mean ± SEM) were analyzed by (ANOVA) and Student's two-tail, unpaired t-test and log transformation where appropriate (Prism 5, GraphPad, San Diego, USA). $P < 0.05$ was considered significant.

Results

High dietary AGEs in the normal liver (MCR animals) did not result in steatohepatitis or fibrosis.

Both MCR and MCR baked groups had similar food intake and no difference in weight gain, liver weight or liver to body weight ratio. Baking the MCR diet did not worsen liver biochemistry, inflammation or fibrosis (Supplementary 2). We then explored the role of increased dietary AGEs in NAFLD using the MCD model.

High dietary AGEs induce hepatic accumulation of CML and worsen liver steatosis and inflammation in steatohepatitis without affecting insulin resistance.

The initial body weights of the three groups were similar. Both the MCD and MCD baked groups lost weight as expected compared with MCR rats, but there was no difference in weight loss, liver weight or liver to body weight ratio between the 2 MCD groups. The daily weight of chow consumed each day was the same in all the groups. MCD did not increase insulin resistance or cause diabetes. Indeed, there was a small but significant decrease in HOMA-IR in the MCD groups likely reflecting the lower weight gain in these animals compared to those fed an MC replete diet. Most importantly, there was no increase in peripheral insulin resistance or hyperglycaemia in the MCD AGE diet group compared to MCD alone that might lead to increased *endogenous* AGE production (Supplementary 2).

The effect of baking increased dietary CML content by more than 4-fold compared to the non-baked MCD diet. Importantly, animals exposed to this high AGE diet had a major increase in liver CML content which was 2.2 times ($p<0.0001$) greater than that in MCD and MCR controls (Fig. 1A). This indicated that the increased dietary CML in the baked diet group was absorbed and accumulated in the liver.

Both MCD groups had increased ALT compared with MCR ($p < 0.05$). However the high AGE diet further elevated plasma ALT and bilirubin compared with MCD alone ($p < 0.05$). (Fig. 1B). This was associated with increased portal inflammation as detected by CD43 staining of leukocytes. Similarly, triglyceride content as assessed by thin layer chromatography was increased in the MCD model and further increased with the addition of dietary AGEs. (Fig. 1C)

IL-6 and TNF-α are predominant proinflammatory cytokines that are upregulated in steatohepatitis[25]. IL-6 is also an nuclear factor kappa B (NF-κB) dependent cytokine that contributes to hepatocyte proliferation and survival[26]. Both these cytokines have been found to be higher in patients with NASH than those with simple steatosis. Consistent with the results of liver biochemistry and histology, expression of both IL-6 and TNF-α were significantly elevated by a high AGE diet compared to MCD without baking ($p<0.05$) (Fig. 1D). Both MCP-1 and RAGE were significantly ($p<0.05$) upregulated in the two MCD groups compared with MCR controls.

High dietary AGEs exacerbate oxidative stress in the MCD model.

The production and accumulation of superoxide is a key mediator of liver injury and cellular dysfunction associated with the progression of fatty liver disease. Moreover, oxidative stress plays a central role in downstream pathways activated by AGEs[7]. NADPH appears to be the major cytosolic source of ROS in diabetes[27] and there has been evidence in murine HSCs that this pathway is activated with AGEs[24]. HNE, an end-product of peroxidation of membrane N-6-polyunsaturated fatty acids, is a particularly good marker of lipid oxidation during liver injury and is related to the intensity of necroinflammation[28]. Assessment of oxidative stress in the liver was therefore performed by measuring cytosolic NADPH-dependent superoxide production and HNE adducts.

Cytosolic superoxide production was found to be significantly ($p<0.01$) increased by increased AGE oral intake in the MCD baked group compared to both the MCR and MCD groups (Fig. 2A). Supporting this was the finding of significantly elevated HNE content in

both the MCD groups with the highest levels detected in the high AGE group ($p<0.05$, Fig. 2B and C).

High dietary AGEs increased markers of liver fibrosis in steatohepatitis.

As expected, hepatic fibrosis as assessed by picrosirius red staining was significantly ($p<0.05$) increased in both MCD groups compared with MCR control. Importantly, the proportional area stained was further increased ($p<0.05$) by high oral intake of AGEs in rats in the MCD baked group compared to MCD alone (Fig. 3A and B).

In keeping with the results of picrosirius red staining, COL1A gene expression was significantly increased in both MCD groups compared with MCR controls and the expression levels were higher ($p<0.05$) in the rats with high oral intake of AGEs compared with MCD alone (Fig. 3C). CTGF has been strongly implicated in the progression of NASH, and was significantly upregulated in both MCD groups and in keeping with other findings, the levels were highest in the livers of rats with high oral intake of AGEs (Fig. 3D).

Activation of myofibroblasts, as assessed by α-SMA immunohistochemical staining, was also significantly increased in the MCD model and further increased ($p<0.05$) with high oral intake of AGEs (Fig. 3E). Based on these findings, we proceeded with in vitro studies of the effects of AGEs on primary hepatic stellate cells isolated from both control animals and those on the MCD diet for 12 weeks.

Disease-activated primary hepatic stellate cells induce RAGE and NADPH oxidase-dependent increases in ROS generation, cell proliferation and expression of proinflammatory and profibrotic genes

HSCs were isolated from both non-diseased (MCR) and diseased (MCD) animals to help clarify the controversy in the literature concerning whether AGEs have profibrotic effects on HSCs and whether AGEs act primarily on activated HSCs.[24] Primary HSCs were used to avoid the problems with phenotype in cell lines. Given the effects of AGEs on ROS generation in other disease states, the effect of AGEs on stellate cell ROS production was studied. This showed that the addition of physiological doses of AGE-BSA to preactivated hepatic stellate cells from animals with MCD induced steatohepatitis markedly increased ROS generation but had no effect on stellate cells from healthy livers (Fig. 4A). This effect was completely abrogated by RAGE blockade or by inhibition of NADPH oxidase (Fig. 4A). HSC cell proliferation, as measured by BrdU incorporation, was also increased by AGEs but only in disease preactivated hepatic stellate cells (Fig. 4B) and the effect was inhibited by RAGE blockade or DPI.

Similarly, MCP-1, IL-6 and α-SMA gene expression were significantly ($p<0.05$) increased by AGEs in preactivated HSCs. MCP-1 and α-SMA but not IL-6 gene expression were attenuated by blocking RAGE or NADPH oxidase. Importantly, RAGE gene expression was also increased by AGEs in preactivated HSCs but not in control HSCs (Fig. 4C-F). This provided one possible explanation for the increased sensitivity of disease activated cells to AGEs.

Discussion

The present study demonstrates for the first time that a diet high in AGEs exacerbates liver injury, inflammation and fibrosis in experimental fatty liver disease. These findings are consistent with previous studies suggesting possible pro-inflammatory and pro-fibrotic effects of AGEs in a number of organs[6]. Importantly, a high AGE containing diet had no effect on liver biochemistry or histology in animals without hepatic steatosis, suggesting that they may act as a cofactor that increases injury in diseased tissues but have little pathogenic effect in the normal liver. Moreover, the finding that RAGE was significantly upregulated in this model and that RAGE antibody completely blocked the effects of AGEs in cultured stellate cells suggest a possible role for the AGE/RAGE axis in the diseased liver.

Our choice of model deserves further discussion. Although deficiency of methionine and choline does not normally happen in human NASH, the MCD model causes accumulation of lipids in the liver leading to classical histopathological features of steatosis, mixed inflammatory lobular infiltrate and fibrosis as seen in human NASH. It has therefore been viewed as a very useful model for examining the intrahepatic mechanisms involved in steatotic liver injury[18]. We chose the MCD model because, in contrast to obesity related NAFLD models, it does not cause insulin resistance[29]. We specifically wished to avoid hyperglycaemia, since this would be expected to increase endogenous AGE production through protein glycation and cause accumulation of AGEs in body tissues, confounding the effects of dietary AGE exposure [30]. The HOMA-IR scores in our animals confirmed that insulin resistance was not increased in any of the treatment groups.

There has been major interest in the pathogenic effects of AGEs in a range of diseases, including most importantly diabetes, but also atherosclerosis and chronic renal disease. In diabetes, AGEs have been shown to exert their harmful effects through several receptors, the best studied of which is RAGE. Activation of RAGE results in generation of ROS from activated NADPH oxidase[31]. This culminates in the activation of NF-κB, a redox sensitive transcription factor, which in turn translocates into the nucleus, leading to the transcription of growth factors, inflammatory cytokines, chemokines and adhesion molecules. The promoter region of the RAGE gene contains an NF-κB binding site and therefore, one of the important consequences of NF-κB activation and translocation is upregulation of RAGE itself. This sets up a positive feedback loop and ensures maintenance of RAGE signaling. On the other hand, the generation of ROS via NADPH oxidase[7] triggered by RAGE activation causes increased AGE formation and contributes to a vicious cycle of AGE formation, generation of oxidative stress and further RAGE activation.

The two-hit hypothesis of NASH pathogenesis suggests that a second injury or cofactor is required for progression from simple benign steatosis to harmful steatohepatitis which can subsequently lead to fibrosis, cirrhosis and hepatocellular carcinoma. There has been considerable interest in factors which could serve as this "second hit". The generation of oxidative stress is a central mechanism in NASH pathogenesis and our study suggests that, as in other diseases[32], AGEs-driven increases in oxidative stress and tissue inflammation could drive disease progression. This is reflected in our finding that AGEs significantly increased NADPH dependent superoxide production and HNE content *in vivo* and is complemented by our *in vitro* work suggesting that in disease activated primary murine HSCs, ROS production and proinflammatory cytokine production were greatly increased in the presence of AGEs. Our finding of significantly increased IL-6 and TNF-α expression in

MCD livers that was further increased by AGEs both *in vivo* and *in vitro* is in keeping with human studies showing that clinically significant NASH is associated with increased plasma levels of TNF-α and IL-6.[33] In stellate cells, most of the effects of AGEs were inhibited by both RAGE blockade and NADPH oxidase inhibition. This suggests that RAGE dependent non-phagocytic NADPH oxidase activation was responsible for the effects of AGEs and this occurs much more readily in preactivated HSCs. Interestingly, IL-6 increases were not abrogated, perhaps due to the activation of autocrine loops involved in the perpetuation of stellate cell activation.[34]

It has been documented that RAGE is expressed on HSCs and upregulated during cell activation[35]. However studies which have examined the effects of AGEs on cultured stellate cells have produced conflicting results.[36] To our knowledge, this is the first study comparing the effects of AGEs in cells taken from normal and steatohepatitic livers. Our findings may clarify previous controversies in the literature as to whether AGEs have an effect on HSCs [12, 35] based on the suggestion that cellular activation by disease, possibly involving upregulation of RAGE and its pathways, was needed for these compounds to produce meaningful changes in HSC responses. Consistent with the two-hit hypothesis, these findings also help explain the lack of effect of AGEs in the healthy livers of animals fed the MCR diet.

The formation of AGEs in foods involves the condensation of an amino group with the carbonyl group of a reducing carbohydrate (glucose, fructose, maltose, lactose or ribulose) to form intermediate Amadori products. Oxidation of Amadori products leads to a more stable compound, CML, used in studies as an indicator of the AGE levels in foods[37]. Although the estimated bioavailability of orally ingested AGEs is low, there is increasing interest in the

possible harmful effects of long-term high AGE containing diets[4] and thus, our study has implications for this concern. A common method of AGE formation in our diet is via caramelisation (which occurs by heating sugars without involvement of amines) and via lipid peroxidation. High fat foods such as animal products, rich in phospholipids, can thus contain advanced lipoxidation end products contributing to the AGE pool. Due to these inter-relationships between the Maillard pathway and lipid oxidation, AGE formation can be considered a process initiated by both fats and carbohydrates[38]. The temperature of cooking is important, with foods cooked with dry heat at high temperatures (such as broiling) being particularly high in AGEs[37]. Given this, baked, roasted, fried and broiled foods are potent promoters of advanced glycation. High levels of AGEs are thus found in many common foods such as baked breads and biscuits/cookies, toasted breakfast cereals, grilled steak, brewed beer, and roasted coffee beans.

Baking the MCD diet increased CML content approximately 4 fold, which compares well with our previous studies[20] This is in keeping with the effects of processing and cooking of the foods in a typical Western diet which has been shown to increase AGE content by at least 3-4 fold. As an example, simply toasting bread increases it's AGE content 3.3 times whilst pan-frying beef for human consumption (in olive oil) increases its CML content by more than 10 fold[39]. Interestingly, acidifying such foods prior to cooking by marinating them in acetic acid (vinegar) or lemon juice, reduces their CML content substantially without compromising significantly on palatability[40]. In developed countries, industrialized methods of food processing have dramatically changed diets. Meals now contain many highly processed foods, often with excess fat and sugar.[41] In fact, many foods which are considered highly palatable are high in AGEs, making them a large component of the consumed Western diet.

Even in foods usually considered healthy, heating, processing or long-term storage results in AGE modifications from Amadori products resulting in a relatively high AGE content in coffee, wine, beer or honey. Long-term storage or chemical processes such as emulsification may also produce AGEs in foods such as heated low fat milk[4]. Thus there are a number of modifications in both food preparation and selection that could help mitigate against the possible compounding effects of a diet high in AGEs in patients with fatty liver disease. Of note, it has been shown that patients with NASH have higher levels of circulating AGEs than those with simple steatosis[36]. However whether this reflects an increased dietary exposure to AGEs or greater endogenous AGE production in patients who have both fatty liver and glucose intolerance is unclear. Studies examining the relationship between dietary exposure to AGEs and the histological severity of liver injury in non-diabetic patients will help clarify this issue.

In conclusion, we show for the first time that high dietary AGE exposure worsens liver pathology in rats fed a MCD diet, and implicate the AGE/RAGE axis in fatty liver disease progression. If confirmed in human studies, then our findings have broad implications for the way we process foods and the dietary advice given to patients with NAFLD. They also suggest possible therapies targeting the AGE/RAGE pathway in the treatment and prevention of NASH.

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Figures Legends

Figure 1. High dietary AGEs induce hepatic accumulation of CML and worsen liver injury and inflammation in steatohepatitis. Increased dietary AGEs lead to significant CML accumulation in the liver (A). ALT and bilirubin were significantly elevated in animals on the MCD diet and were further increased with high dietary AGEs (B). Triglyceride content was increased in the MCD model and further increased with increased dietary AGEs. CD43, a marker of inflammatory cell infiltrate, was significantly increased in portal areas in the MCD model and was further with increased dietary AGEs (C). Proinflammatory genes IL-6, TNF-α and MCP-1 as well as RAGE gene expression were upregulated in the MCD model of fatty liver disease; and there was further gene upregulation in IL-6 and TNF-α with increased dietary AGEs (D). ** P<0.0001 versus MCR and MCD. * P<0.05 versus MCD. # P<0.05 versus MCR.

Figure 2. Increased dietary AGEs increased hepatic oxidative stress via increased cytosolic (NADPH dependent) superoxide production (A). This is also reflected in increased HNE adduct quantification in the liver (B) with representative photomicrographs showing increased positive staining for HNE adducts around lobular inflammatory cells and ballooned hepatocytes (C). * P<0.01 versus MCR and MCD. # P<0.05 versus MCD. ** P<.05 versus MCR.

Figure 3. The MCD model of fatty liver disease had increased liver collagen and fibrosis. Picrosirius staining in the liver was significantly increased in rats fed on MCD diet compared with that in rats fed on control MCR diet, which was further increased by high dietary intake of AGEs (A, B).Hepatic gene expression of COL1A (C) and CTGF (D), was significantly increased in the MCD model and further increased with increased dietary AGEs. α-SMA, a marker of hepatic stellate cell activation, was also significantly increased in the MCD model and further with increased dietary AGEs on immunohistochemistry (E). * P<0.05 versus MCR. # P<0.05 versus MCD.

Figure 4. AGEs significantly increased ROS generation and cell proliferation in preactivated HSCs but not in control HSCs (A). This effect was abrogated by inhibition of RAGE with RAGE antibody (RAGE Ab) and NADPH oxidase with DPI (A). AGEs increased HSC proliferation, as measured by BrdU incorporation, only in disease preactivated HSCs and this effect was abrogated by inhibition with both RAGE and NADPH oxidase blockade (B). The addition of AGEs significantly increased MCP-1 (C), IL-6 (D), α-SMA (E) and RAGE (F) gene expression in preactivated HSCs but not in control HSCs. This effect on MCP-1 and α-SMA was also abrogated by inhibition of RAGE and NADPH oxidase. ** P<0.05 versus BSA, AGEs with RAGE antibody and AGEs with DPI. * P<0.05 versus BSA. # P<0.001 versus MCD HSCs with AGEs. ~P<0.001 versus MCR HSCs with AGEs.

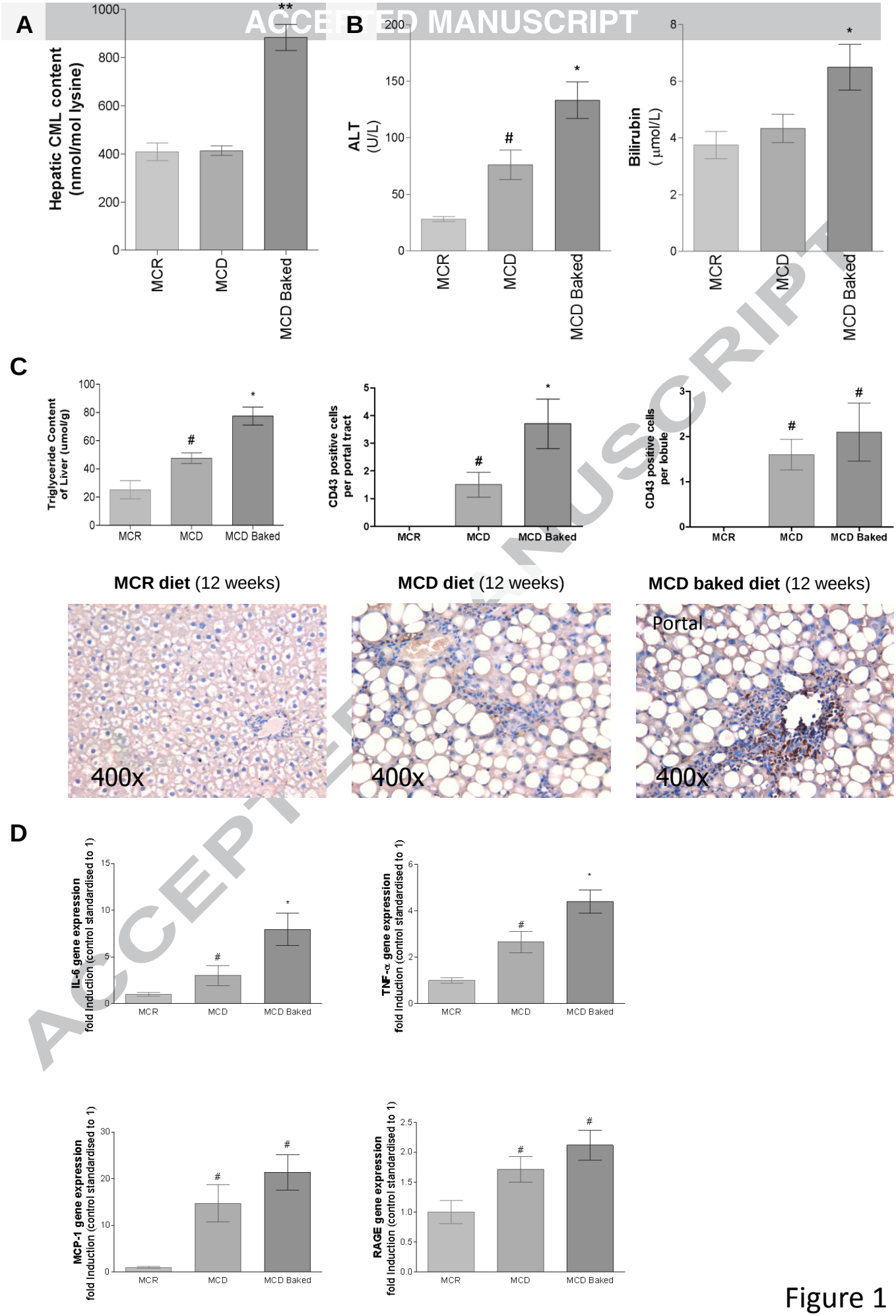
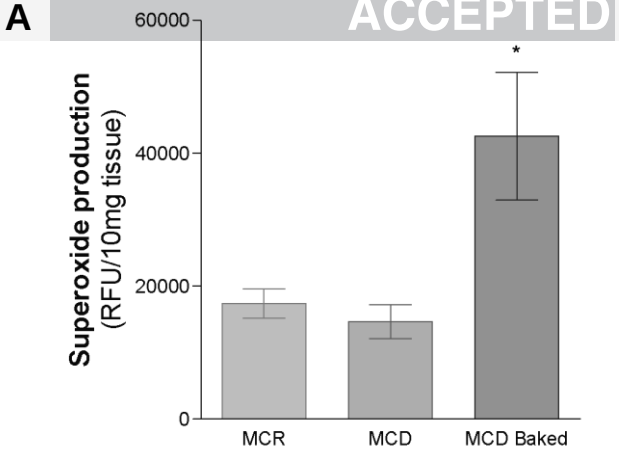
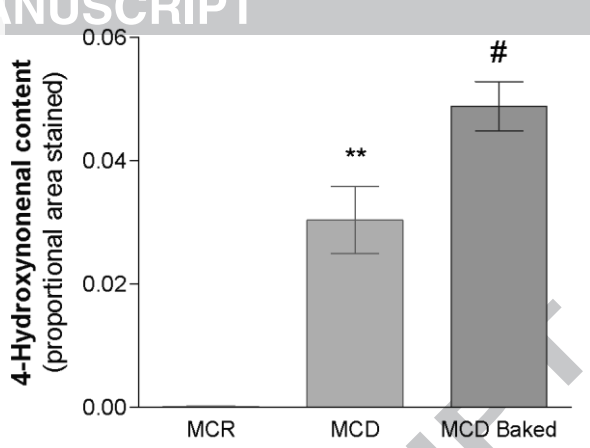


Figure 1





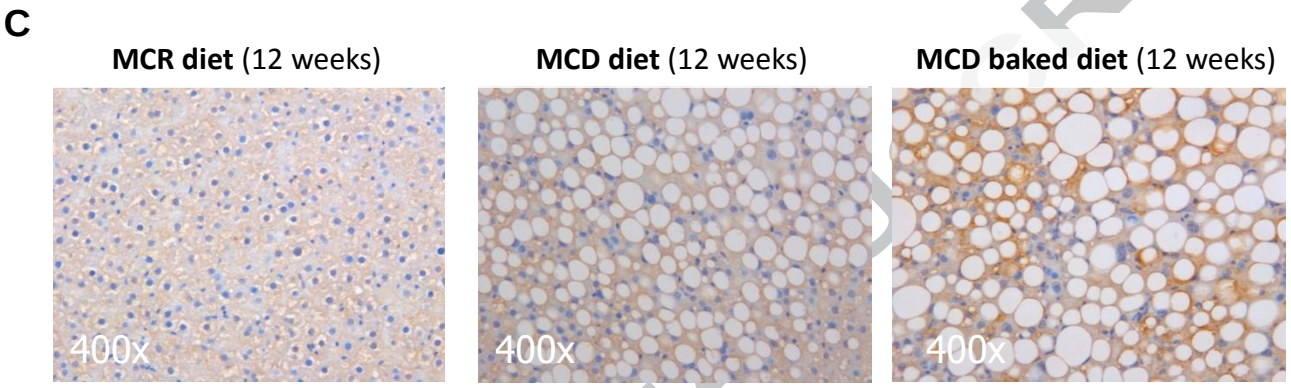


Figure 2

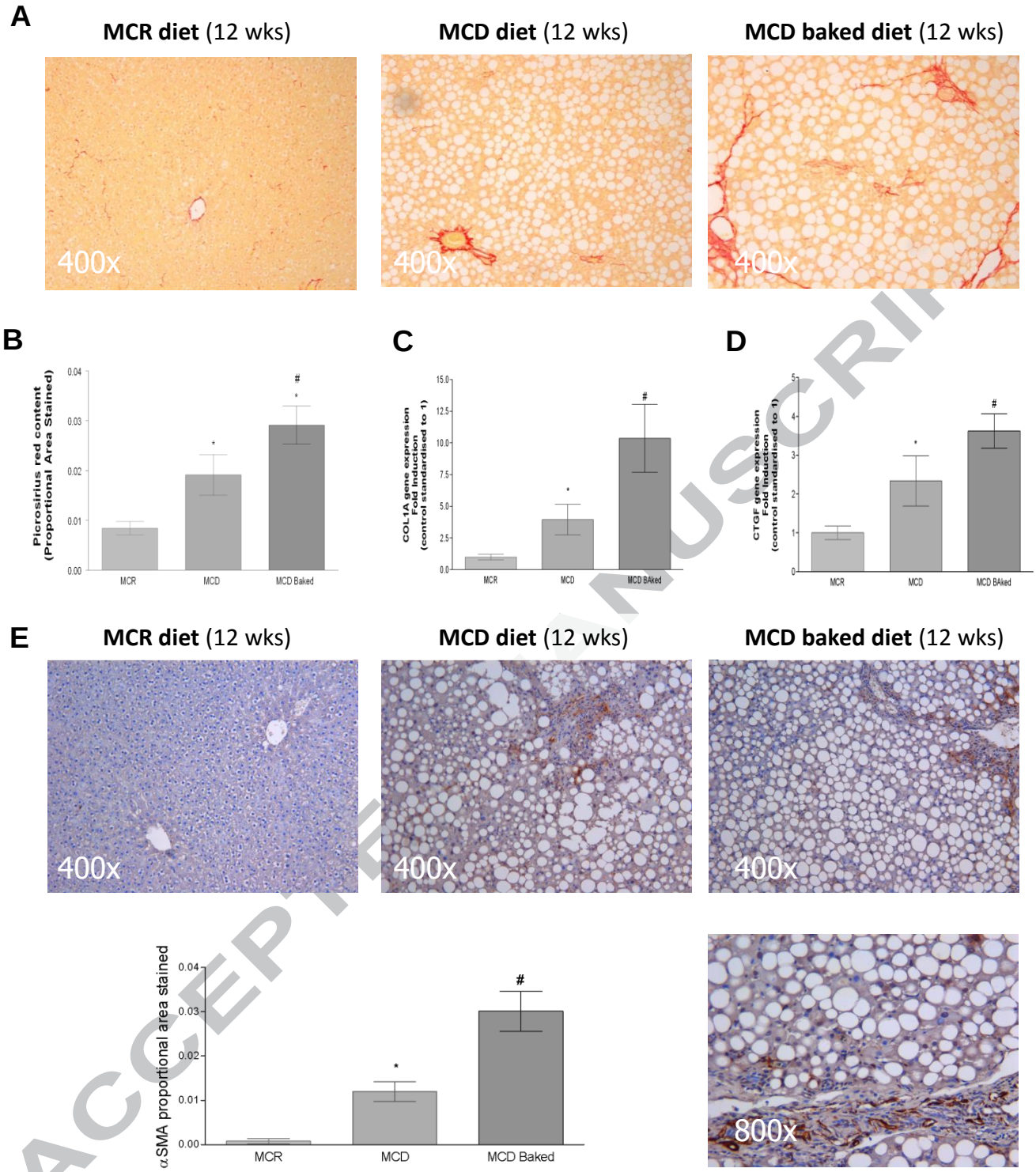


Figure 3

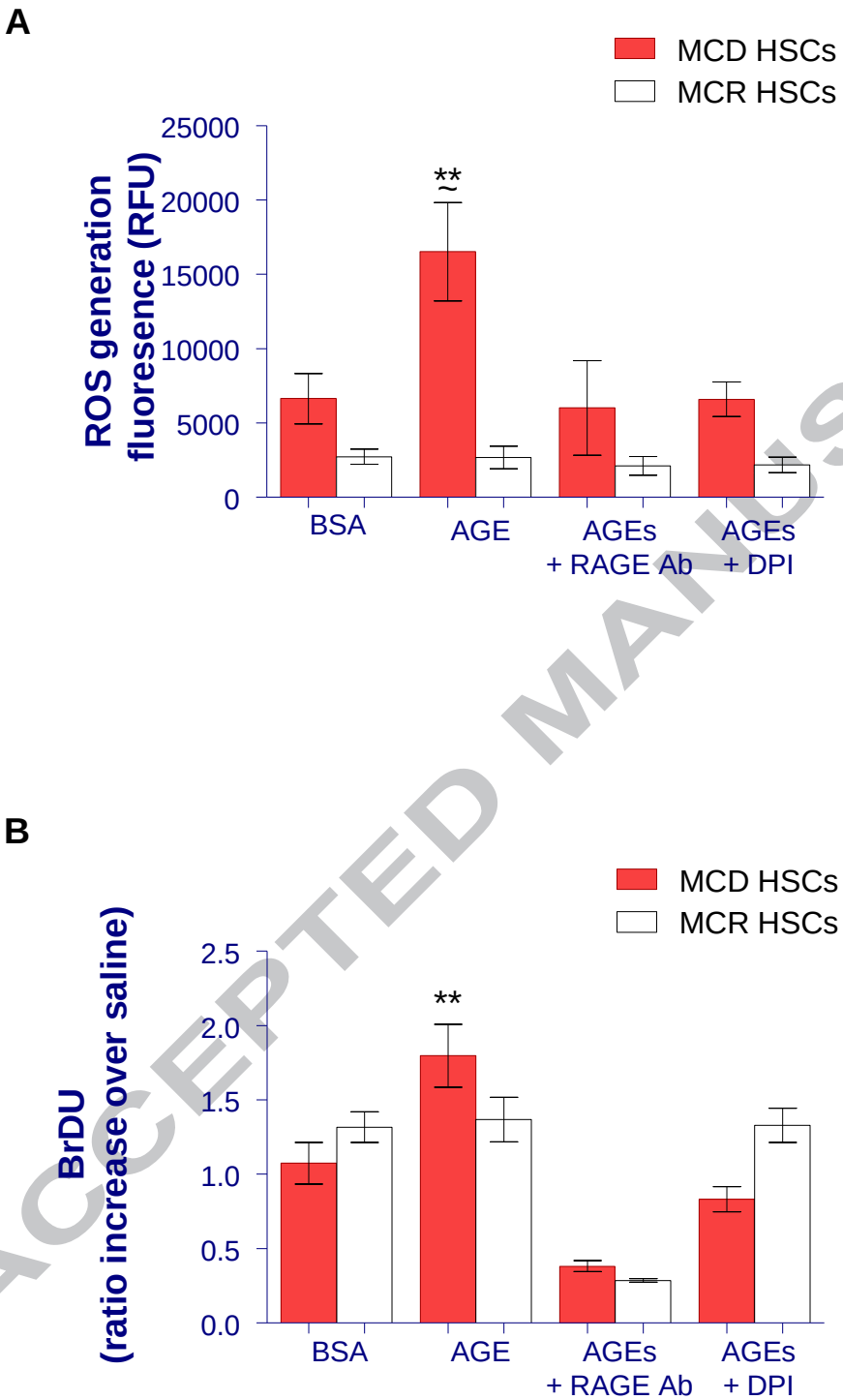


Figure 4

Figure 4

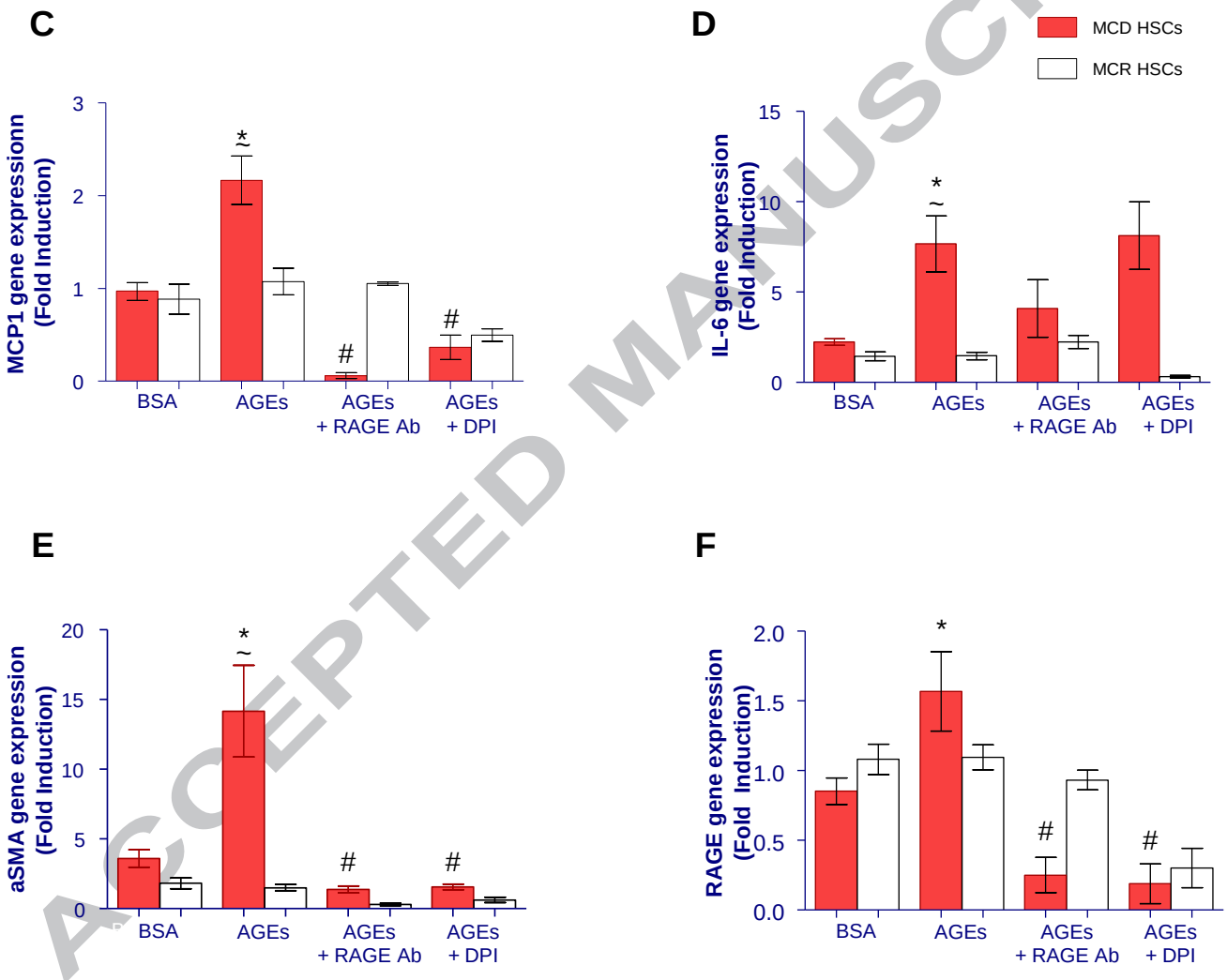


Figure 4