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Original Article

Preclinical analysis of nonsteroidal anti-inflammatory drug usefulness for the simultaneous prevention of steatohepatitis, atherosclerosis and hyperlipidemia

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Abstract: Nonalcoholic steatohepatitis (NASH) is currently one of the primary liver diseases. Recent studies have shown a clinical relation between NASH and atherosclerosis. There is much interest in these two diseases because they are both associated with great morbidity and mortality. Inflammation and the overexpression of COX-2 participate in the pathophysiology of the two diseases, and therefore simultaneous treatment is feasible. The role of the four NSAIDs, meclofenamate, mefenamate, flufenamate, and aspirin, was analyzed in a mouse model of NASH, as well as preclinical atherosclerosis induced by a high-fat diet (HFD). Six mouse groups were formed. Five of the groups were fed a high-fat diet for 6 months and one group was fed a standard diet, acting as the normality reference. Of the five groups fed a high-fat diet, four received a NSAID, each of them identified by the specific drug administered. One group received no treatment. Serum markers (cholesterol, triglycerides, ALT, and AST) and histologic changes in the aorta and liver were analyzed for the study. Aspirin significantly reduced the hepaticsteatosis. All the drugs significantly reduced the hepatic inflammatory infiltrate. In relation to atherosclerosis, there were significant reductions in all the study variables with the use of aspirin and flufenamate. The four medications were able to stop steatosis from progressing into steatohepatitis by reducing inflammation. However, aspirin was the most beneficial, simultaneously reducing steatosis, atherosclerosis, and serum cholesterol levels.

Keywords: Nonalcoholic fatty liver disease, atherosclerosis, meclofenamate, mefenamate, flufenamate, aspirin

Introduction

Nonalcoholic fatty liver disease (NAFLD) is emerging as a pathology of great importance, given that it is the most common liver disease in the Western world. It is closely related to metabolic syndrome. Histologically, nonalcoholic fatty liver disease covers a spectrum of diseases from simple steatosis (accumulation of fat in the hepatocytes) to nonalcoholic steatohepatitis (NASH). The latter is characterized by hepatocyte injury, inflammation, and varying grades of liver fibrosis [1] and can progress to cirrhosis in approximately 20% of the patients [1]. An association between NAFLD, metabolic syndrome, and cardiovascular disease (CVD) has recently been suggested. Indeed, different clinical studies have demonstrated that NAFLD patients present with increased preclinical atherosclerosis compared with non-steatosic individuals; these analyses are supported by follow-up studies revealing that atherosclerotic CVD is the second most common cause of death in NAFLD patients [2].

Atherosclerosis, whether combined with NAFLD or not, is an important public health problem that is characterized by vascular site-specific chronic inflammation initiated in response to retained and modified lipids within the arterial wall. Preclinical atherosclerosis, clinically detected by the thickening of the intima-media layers of the carotid artery or the aorta, is a generalized atherosclerosis indicator, as well as one of coronary artery disease, and it is also related to metabolic control [3].

Despite the known fact that elevated cyclooxygenase-2 (COX-2) is involved in the development of NAFLD, as well as of atherosclerosis, the role of COX-2 inhibitors has not been evaluated with regard to the simultaneous treatment of these two pathologies. Celecoxib, nitroaspirin, cilostazol, and atorvastatin are drugs that have been shown to protect against the development of NASH in models of animals fed a high-fat diet [4].

Independently, aspirin has been shown to inhibit platelet aggregation, preventing the formation of a thrombus and infarction following a tear in atherosclerotic plaque [5]. Nevertheless, there has not been much study on the use of NSAIDs for long-term prevention of the formation of atherosclerosis.

At present, no medication has been approved for the simultaneous prevention of NAFLD and atherosclerosis development. Thus, the search for and validation of treatments for these diseases are a necessity [6].

The present study analyzed the biochemical profile and the effect on the liver and aorta of these four NSAIDs: meclofenamic acid, mefenamic acid, flufenamic acid, and acetylsalicylic acid in a model of BALB/c mice fed a high-fat atherogenic diet.

Material and methods

Reagents and animal treatments

Six groups of 6 to 8-week-old male BALB/c mice (Harlan, Mexico), weighing 25-30 g, were included in the experiment. Five groups of mice were fed a high-fat diet (Atherogenic Rodent Diet, TD.02028, Harlan®, USA) and the sixth group was fed a standard diet (the SD group) (2018S Tekl and Global 18% Protein Rodent Diet, Harlan®, USA), and therefore was the normality reference. This model has been shown to be capable of the simultaneous production of preclinical steatohepatitis and atherosclerosis within a period of 6 months [3]. From the 5 groups fed the high-fat diet, four were each given a NSAID; each of these four groups was identified by the specific drug administered, leaving one group without treatment (the HFD group), which was the point of reference for the pathologic alterations caused by diet. The NSAIDs used were acetylsalicylic acid (Bayer aspirin) and three fenamates: flufenamic acid (Sigma, St. Louis, MO, USA), mefenamic acid (Sigma, St. Louis, MO, USA), and meclofenamic acid (Sigma, St. Louis, MO, USA).

The mice were kept in cages, with a maximum of 5 mice per group. Light and temperature were controlled; the animals had access to food and water *ad libitum*. The drug to be administered was dissolved in drinking water, calculating a daily doseof 10 mg/kg/day. The amount of water the mice had consumed daily and their weight were determined every 15 days for the purpose of recalculating the drug concentrations in the drinking water and maintaining the established doses. Fresh water containing the drug was provided every 48 hours. The mice were given the diet and drug for a period of 6 months, after which they were killed by decapitation.

Blood samples were collected for the biochemical analyses and the liver and the thoracic and abdominal aorta were extracted, weighed, and processed for histopathologic analyses. The trial complied with the national and international legal and ethical requirements applicable to pre-clinical research. The animals were manipulated according to institutional guidelines and the Mexican Official Norm regulating laboratory animal use (NOM-062-ZOO-1999) and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.The ethics committee of the Colima State Cancer Institute of Colima, Mexico, approved the study.

Histopathologic analysis

The tissues were fixed in solutions of 10% formaldehyde. Three cross-sectional liver slices were included, two from the right lobe (the central region and external third) and a slice from the left lobe. The aorta was dissected from the

Table 1. Biochemical and morphometric parameters

	Standard Diet	High-Fat Diet	Meclofenamate	Mefenamate	Flufenamate	Aspirin	P
Cholesterol (mg/dL)	138±21	156±51	165 ±84	139±33	169± 91	78±53	0.006[a]
Triglycerides (mg/dL)	224±76	182±49	231±135	176±55	198±110	144±62	0.16[a]
ALT (IU)	144±21	190±58	162±78	153± 87	134±55	93±49	0.04[a]
AST (IU)	1001±166	1049±354	821±420	652±259	597±307	505±293	0.001[a]
Thoracic Aorta thickness (μm)	59.9 (53-69)	70.5 (62-79)	66.5 (55-79)	61.6 (55-75)	59.8 (53-70)	58.9 (51-67)	0.001[b]
Abdominal Aorta thickness (μm)	61.2 (54-70)	72.2 (62-87)	66.7 (54-79)	66.9 (61-74)	61.9 (52-74)	58.8 (48-69)	0.001[b]
Thoracic Aorta Stary lesion	1.68±0.4	1.72±0.9	1.40±0.5	1.10± 0.3	1.00±0	1.27±0.4	0.038[b]
Abdominal Aorta Stary lesion	1.62±0.5	1.69±0.6	1.40±0.5	1.22±0.4	1.00±0	1.18±0.4	0.000[b]
Liver weight (g)	1.45±0.15	1.79±0.19	1.67±0.15	1.97±0.30	1.73±0.22	1.53±0.22	0.000[a]
Liver steatosis	5.1±7	55.7±10	45.9±19	47.7±13	51.4±19	37.9±17	0.000[a]
Microvesicular	4.7±7	20.4±6	15.6±9	25.6±11	19.5±8	12.2±7	0.000[a]
Macrovesicular	0.4±1	35.3±6	30.3±12	22.1±4	31.9±12	25.7±10	0.000[a]
Inflammation	5.6±10	35.3±17	10.7±9	6.3±4	4.2±4	5.9±7	0.000[a]

[a]ANOVA; [b]Kruskal-Wallis[b]; The values are expressed as the means ± SD, except thoracic aorta thickness and abdominal aorta thickness which are expressed as median values (25-75 percentile); Stary lesion scores result from assigning a numeric value of 1, 2, and 3 to the lesion types I, II, and III, respectively, so the mean values could be calculated. Liver steatosis (total, micro or macrovesicular) and inflammation are shown as the percentage of hepatic tissue occupied by these alterations.

sinuses of Valsalva up to the iliac bifurcation. Cross-sectional slices (1-2 mm thick) were cut at six different portions of the aorta: three at the level of the thoracic aorta (ascending aorta, aortic arch, and descending aorta) and three portions of the abdominal aorta (the proximal, middle, and distal third). They were dehydrated in ethanol, embedded in paraffin wax, sectioned (5 μm thick), and stained with hematoxylin and eosin. The evaluations of the aortic and liver slices were carried out through images taken with an Axiocam MRC-5 model digital camera (Zeiss®, Germany) attached to an AxioPlan 2 M model bright field optical microscope (Zeiss®, Germany) with a motorized stage and A-plan X5 and X20 objective (total magnification X50 for the aorta and X200 for the livers). Images of the entire sample surfaces were scanned using MosaiX and Autofocus modules. All the shots were taken under the same conditions of light and exposure. The analyses were done in a blinded manner by one pathologist.

Steatosis was considered in relation to the percentage of liver tissue with fat accumulation. The percentage of liver tissue with inflammation was evaluated by functional histologic zones, according to the oxygen supply (zone 1 encircles the portal tracts where the oxygenated blood from the hepatic arteries enters; zone 3 is located around the central veins, where oxygenation is poor; zone 2 is located between zones 1 and 3). The Masson trichrome stain was used for evaluating fibrosis.

Atherosclerosis of the thoracic and abdominal aorta was evaluated separately. Only the slice qualitatively showing the lesion with the highest grade of disease according to the Stary classification (grades I-VI) [7] was selected and a blinded analysis was carried out byonepathologist. The same slice was quantitatively evaluated by measuring the intima-media thickness (from the interior edge of the endothelium to the exterior edge of the middle layer) [8].

This was done at eight equidistant sites per section, selected through systematic uniform random sampling, regardless of the presence or absence of atherosclerotic lesions at the measuring site.

Biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, and triglyceride (TG) were determined using an automatic biochemical analyzer (Cobas c111, Roche ®, Mexico).

Statistical analysis

Means, medians and percentiles were used for the descriptive statistics. For the inferential statistics, the normal data distribution was first determined through the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA, with Dunnett's post hoc test) was used to compare the quantitative data and normal distribution (biochemical parameters). The data with non-normal distribution or on an ordinal scale were

Table 2. Results of the post hoc analysis (*p*-values) upon comparing diverse parameters of the high-fat diet with the rest of the groups

Parameters	High-Fat Diet vs				
	Standard Diet	Meclofenamate	Mefenamate	Flufenamate	Aspirin
Cholesterol (mg/dL)[a]	0.527	0.904	0.560	0.931	0.007*
Triglycerides (mg/dL)[a]	0.987	0.989	0.744	0.912	0.363
ALT (IU)[a]	0.153	0.423	0.309	0.108	0.004*
AST (IU)[a]	0.679	0.197	0.025*	0.008*	0.002*
Thoracic AortaThickness (μm)[b]	0.006*	0.352	0.137	0.004*	0.008*
Abdominal Aorta Thickness (μm)[b]	0.003*	0.264	0.239	0.019*	0.002*
Thoracic Aorta Stary Lesion[b]	0.828	0.476	0.082	0.014*	0.233
Abdominal Aorta Stary lesion[b]	0.839	0.262	0.040*	0.001*	0.022*
Liver weight (g)[a]	0.000*	0.269	1.000	0.572	0.006*
Liver steatosis[a]	0.000*	0.202	0.302	0.557	0.009*
Microvesicular[a]	0.000*	0.280	0.997	0.746	0.037*
Macrovesicular[a]	0.000*	0.278	0.002*	0.452	0.019*
Inflammation[a]	0.000*	0.000*	0.000*	0.000*	0.000*

*Statistically significant. The values are expressed as *p* values. [a]Dunnett's post hoc test; [b]Mann-Whitney U post hoc test.

evaluated using the *Kruskal-Wallis and Mann-Whitney U post hoc* tests. Statistical tests were performed with SPSS version 20 software. A 95% confidence interval (CI) was used in all the tests, and *p* values of <0.05 were considered statistically significant.

Results

No significant differences in mouse body weight were observed between the different groups during the study. **Table 1** shows the biochemical and morphometric parameters analyzed. Significant differences were found in all the variables, with the exception of triglycerides, when the intergroup analysis (ANOVA orKruskal-Wallis tests) was carried out. The post hoc analysis is shown in **Table 2**.

The post hoc analyses produced the following results: with respect to the biochemical parameters, there was an increase in AST in the HFD group compared with the SD group. The mice that were given aspirin showed significantly lower levels of serum cholesterol, AST, and ALT than the HFD and SD groups (P<0.05). The groups that received flufenamate or mefenamate also had significantly reduced levels of AST when compared with the HFD and SD groups.

In relation to the atherosclerosis analysis, all the groups, compared with the HFD group, had less thoracic and abdominal aortic thickness, but statistical significance was reached only in the SD, flufenamate, and aspirin groups (P=0.000). Regarding the atherosclerotic lesions evaluated with the Stary classification that includes type I to VI lesions, only types I to III (less advanced lesions) were present. Compared with the HFD group, there was a reduction in the grade of the thoracic and abdominal aortic lesions with all the anti-inflammatory drugs and these reductions were statistically significant in the mefenamate (P=0.040 in the abdominal aorta), flufenamate (P=0.014 in the thoracic aorta and P=0.001 in the abdominal aorta), and aspirin (P=0.022 in the abdominal aorta) groups.

In regard to the liver, only the SD and aspirin groups had significant reductions in hepatic weight (P=0.000 and 0.006) and steatosis percentage (P=0.000 and 0.01), compared with the HFD group. The microvesicular or macrovesicular fat accumulation pattern was significantly reduced in the SD and aspirin groups. However, it should be mentioned that mefenamate significantly reduced macrovesicular fat accumulation, but not the total steatosis, whereas unlike the rest of the groups, microvesicularsteatosis was the predominant pattern. Finally, it is important to point out that all the drugs significantly reduced the hepatic inflammation (P=0.000), compared with the HFD group, being similar to that of the SD group.

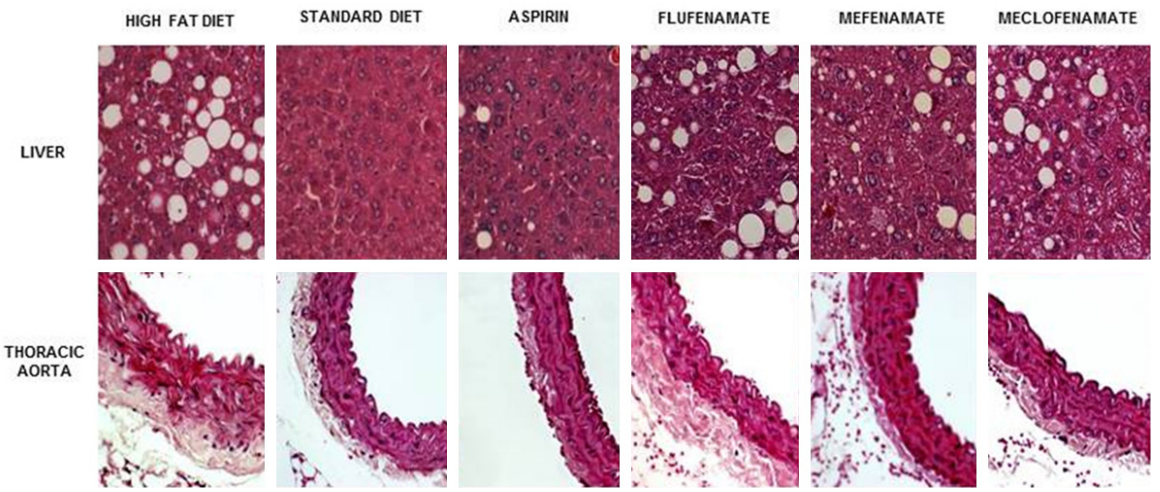

Figure 1. Representative images of the liver and thoracic aorta that show reduction in steatosis and atherosclerosis among the groups treated with the anti-inflammatory drugs, compared with the high-fat diet group. Liver and thoracic aorta tissues in the standard diet show no pathologic changes. Images magnified by ×200.

Discussion

All the anti-inflammatory drugs tested were shown to reduce the hepatic inflammatory infiltrate and were able to stop the progression of steatosis to steatohepatitis and they simultaneously reduced the formation of atherosclerosis, as shown in **Figure 1**. Nevertheless, only aspirin was able to significantly reduce hepatic-steatosis and serum levels of cholesterol, ALT, and AST; flufenamate and mefenamate only reduced the AST enzyme. Thus it was apparent that aspirin was the analyzed drug that provided greater simultaneous benefits with respect to hepatic and arterial alterations caused by a high-fat diet.

Inflammation has been determined as an important process within the pathogenesis of atherosclerosis and NASH. Because increased COX enzymes have been found in atheromas, it has been suggested that inhibiting these enzymes using anti-inflammatory drugs could have an effect on the development of atherosclerosis. However, of the drugs analyzed in our study, even though aspirin, meclofenamate, and flufenamate are potent nonselective inhibitors of COX-1 and COX-2 [9], they may have different effects on the development of atherosclerosis [10]. This could mean that just the inhibition of the COX enzymes may not be enough to have a great anti-atherosclerotic effect and that perhaps the interaction with other molecules could also contribute to its effect.

It has recently been reported that aspirin can reduce age-induced atherosclerosis in mutated ApoE gene mice [11, 12]. This is in agreement with the findings of our study with nonmutated mice fed a high-fat diet. Flufenamate has also been reported to be able to reduce atherosclerosis in rabbits; these are the few studies carried out with fenamates on this topic [13]. The abovementioned corroborates the anti-atherosclerotic effect of the NSAIDs and also makes it clear that not all are equally efficient for preventing this pathology. With respect to the effect on hepatic tissue, our study showed that four anti-inflammatory drugs could prevent the progression of steatosis to steatohepatitis.

All the drugs significantly reduced hepatic inflammation, and it can be assumed that this is due to their COX-2 inhibiting potential. A previous study stated that the reduced steatosis and hepatic inflammation produced by celecoxibmay also be due to COX-2 inhibition [6]. However, our study showed that not all the anti-inflammatory drugs had the same effect onsteatosis reduction, and that aspirin was the only one that was significantly beneficial in that respect. It is important to point out that meclofenamate is a very potent COX-2 inhibitor [14], in this sense, better than other fenamates, and nevertheless it was the drug that produced the

least effect againststeatosis and atherosclerosis. It can thus be hypothesized that COX-2 inhibition, alone, reduces hepatic inflammation, but it is not sufficient for achieving significant changes in fat accumulation.

Celecoxib [6], nitro-aspirin [15], andother antiplatelet drugs [4] have been previously reported to be able to reduce steatosis and hepatic inflammation in experimental models. These data are comparable with those of our study. However, in relation to aspirin, the results have been controversial [4, 15].

It was previously demonstrated that different antiplatelet agents (including aspirin) reducedsteatosis in certain animal models, cilostazol being the most efficient. Given that cilostazoldoes not inhibit COX-2, it could be said that the effect against steatosis produced by aspirin might be caused by a mechanism that aspirin and cilostazol have in common, such as the suppression of mitogen-activated protein kinase activation induced by oxidative stress [16]. It is worth mentioning that cilostazolsignificantly reduces steatosis, but not inflammation, whereas there is no doubt that aspirin has the additional advantage of reducing the inflammatory infiltrate.

It is striking that aspirin impressively reduces (50%) total cholesterol levels. A previous reporthas pointed out that low doses of aspirin (1.4 mg/kg/day) do not affect total cholesterol levels, even though there are reports that they do increase beneficial LDH levels [17].

However, doses of aspirin similar to those employed in our study (8-10 mg/kg/day) have been shown to be able to reduce total cholesterol levels in studies carried out on rats [18] and rabbits [19] fed a high-fat diet. Interestingly, a published clinical trial conducted on 9 patients that were given a high dose of aspirin (6.2 g/day) for two weeks showed a significant reduction (15%) in total cholesterol, as well as in other parameters, such as triglycerides and glucose. It has been proposed that this effect can be produced by IkB Kinase (IKK) inhibition [20].

This result of aspirin's effect on serum lipids has not been reported often and its clinical usefulness has not been evaluated. Therefore, dose response studies on aspirin, or some other derivative with fewer adverse effects, would be very interesting. Celecoxib [6], nitro-aspirin [15], andaspirin at high doses [20] have been shown to have the capacity to reduce triglyceride levels. Even though aspirin reduced triglyceride levels by an average of 21% in our study, this value was not statistically significant, but it concurs with that reported for the abovementioned drugs.

Another aspect of the present study to take into consideration is that aspirin and the fenamates aided in preventing steatohepatitis and atherosclerosis when they were consumed in conjunction with a high-fat diet. Further experiments are necessary for determining whether it could revert preclinical steatohepatitis or atherosclerosis.

In conclusion, the chronic administration of different anti-inflammatory drugs was seen to be useful for preventing steatohepatitis and atherosclerosis in animals fed a high-fat diet. Aspirin was the drug that had the additional effects of reducing hepatic steatosis and serum cholesterol levels. Further studies are needed in order to evaluate the clinical usefulness of these drugs or their derivatives.

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Disclosure of conflict of interest

None.

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