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UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

FACULTAD DE ESTUDIOS SUPERIORES
CUAUTITLAN

"COLCHICINE PREVENTS D-GALACTOSAMINE-INDUCED HEPATITIS"

A R T I C U L O

QUE PARA OBTENER EL TITULO DE:

QUIMICO FARMACEUTICO BIOLOGO

P R E S E N T A :

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CUAUTITLAN IZCALLI, EDO. DE MEX. 1997

UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO

FACULTAD DE ESTUDIOS SUPERIORES CUAUTITLAN
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DEPARTAMENTO DE EXAMENES PROFESIONALES

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Con base en el art. 28 del Reglamento General de Examenes, nos permitimos comunicar a usted que revisamos El Artículo: "Colchicine prevents D-galactosamine-induced hepatitis".

que presenta el pasante: Miguel Angel Meza Martinez

con número de cuenta: 7855444-1 para obtener el TITULO de: Químico Farmacéutico Biólogo .

Considerando que dicho trabajo reúne los requisitos necesarios para ser discutido en el EXAMEN PROFESIONAL correspondiente, otorgamos nuestro VOTO APROBATORIO.

A T E N T A M E N T E .
"POR MI RAZA HABLARA EL ESPIRITU"
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Colchicine prevents D-galactosamine-induced hepatitis

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Summary

The hepatoprotective effect of colchicine in a model of liver intoxication with galactosamine (GalN), 375 mg/kg, i.p., was studied in rats. At 0.5, 1, 3, 6, 18 and 24 h after GalN intoxication the following markers of liver damage were measured: serum activity of alanine aminotransferase, alkaline phosphatase, γ-glutamyltranspeptidase, hepatic calcium and glycogen contents, liver lipoperoxidation, and liver plasma membrane activity of alkaline phosphatase, γ-glutamyltranspeptidase and high-affinity Ca^{2+}-ATPase. 24 h after GalN intoxication increases in serum levels of alanine aminotransferase, alkaline phosphatase and γ-glutamyltranspeptidase were observed along with decreases in plasma membrane activities of alkaline phosphatase, γ-glutamyltranspeptidase, and high-affinity Ca^{2+}-ATPase. A sharp increase of lipoperoxidative processes measured as malondialdehyde production was also observed. Pretreatment of rats with colchicine 10 μg/rat/day p.o. for 7 days before GalN injection prevented partially the toxic effects of GalN. When a dose of 50 μg/rat/day for 7 days was given the drug prevented almost completely the damage induced by galactosamine, with the exception of glycogen and serum alkaline phosphatase that remained different from controls. Time-course experiments showed that malondialdehyde formation increased 30 min after intoxication while all other changes became apparent from 6 h after treatment, suggesting that lipoperoxidation may be a prerequisite for galactosamine-induced damage. The protection offered by colchicine was related to its capacity to inhibit lipoperoxidation. Histochemical findings paralleled the biochemical results. The possible role of lipoperoxidation in galactosamine-induced liver damage is discussed.

Introduction

Among the numerous models of experimental hepatitis, D-galactosamine (GalN)-induced liver injury is very similar to human viral hepatitis. Typical laboratory findings include a rapid loss of liver glycogen, accumulation of calcium in the cytoplasm, release of intracellular liver-specific enzymes, bilirubin in blood plasma and a reduction in plasma proteins [1].

Examination of galactosamine-induced liver dam-

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age by means of light microscopy has revealed features closely resembling those seen in human viral hepatitis [1]. The biochemical processes involved in the initial stages of this disease model are connected with the metabolism of galactosamine and the biosynthesis of uridine derivatives [2,3]. Studies on the mechanism of induction of galactosamine hepatitis revealed that the protein and phospholipid metabolism of the Golgi apparatus of rat liver are already inhibited 6 h after the administration of D-galactosamine [4]. Reutter et al. have supported the hypothesis that an inhibition of the metabolism of membrane proteins and sphingomyelin of the plasma membrane is necessary for the induction of GalN hepatitis [5]. The specificity of the morphological and biochemical alterations typical of liver cells after galactosamine treatment is very convenient for studying the hepatoprotective effect of several drugs. Previous findings in our and other laboratories have already indicated that colchicine treatment can reverse chronic liver damage induced by CCl_4 in rats, and also prevent acute and chronic CCl_4 liver injury [6–11]. Therefore, the aim of the present work was to evaluate the capability of colchicine to prevent acute liver damage induced by galactosamine in rats.

Materials and Methods

Male Wistar rats (180–220 g) were used for all experiments. D-Galactosamine hydrochloride and all other reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

The experimental animals were divided into 5 groups of 6 animals each as follows: group 1 received GalN (375 mg/kg) dissolved in NaCl 0.9% given intraperitoneally; group 2 was given the same dose of GalN after a 7 day colchicine treatment (10 μg/animal/day dissolved in water); animals of group 3 received GalN after a 7 day treatment of colchicine (50 μg/animal/day dissolved in water); groups 4 and 5 received the same doses of colchicine as groups 2 and 3 respectively but received NaCl instead of GalN. Since water was the vehicle for colchicine we felt that there was no need to form an additional control group. In all groups colchicine was given by gavage. Galactosamine was administered 1 h after the last dose of colchicine and all animals were sacrificed 24 h after GalN intoxication.

An additional group of 40 rats was administered GalN (375 mg/kg) and subgroups of 5 animals each were sacrificed at 0, 0.5, 1, 3, 6, 15, 18 and 24 h after intoxication to follow the time-courses of lipoperoxidation, alanine aminotransferase (ALT) serum activity, glycogen content and hepatic calcium accumulation. Blood was collected by heart puncture under diethyl-ether anesthesia and serum was obtained by centrifugation. The livers were quickly removed and were used to prepare homogenates and plasma membranes by the method described by Neville [12]. Small liver sections fixed in formalin (10%) were used for hematoxylin-eosin staining.

The serum activity of ALT was determined according to Reitman and Frankel [13]. Alkaline phosphatase activity was measured according to the method of Berger and Rudolph [14] and γ-glutamyl transpeptidase (GGTP) according to Glossmann and Neville [15] in both serum and plasma membranes. The Ca^{2+}-dependent ATPase was determined in isolated liver plasma membranes according to the method described by Lotersztajn et al. [16]. Protein contents were determined according to Bradford [17]. Glycogen liver content was determined using the anthrone reagent method [18]. Lipoperoxidation was estimated in liver homogenates by measuring the malondialdehyde (MDA) formation by the thiobarbituric acid method [19]. The hepatic calcium content was determined in tissue samples prepared for analysis by nitric acid digestion [20]. Total calcium was determined by flame spectrophotometry and the results were expressed as μg of Ca^{2+} per g of liver, dry weight [21].

For statistical analysis, data were analyzed by one-way analysis of variance. Differences were considered significant when $P < 0.01$.

Results

Fig. 1 (panel A) shows the representative histolog-

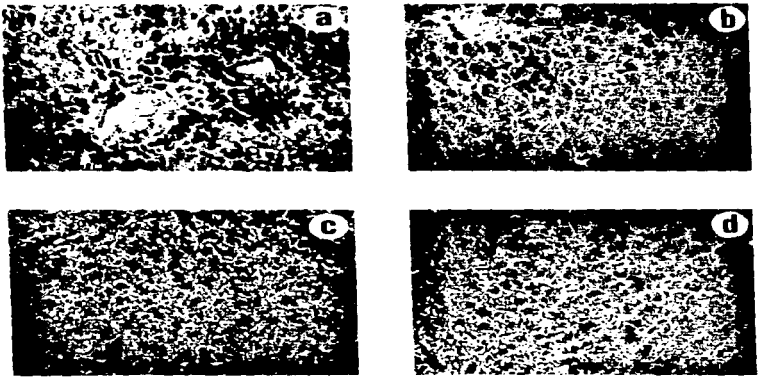

Fig. 1. Panel A: liver section from a rat treated with GalN (375 mg/kg, i.p.), showing intense, predominantly mononuclear cell infiltration of the periportal region. Panel B: liver section from a normal rat. Panel C: liver section from a rat pretreated with colchicine (10 μg/rat day, for 7 days, p.o.); lower mononuclear cell infiltration is observed. Panel D: liver section from a rat pretreated with colchicine (50 μg/rat day, for 7 days, p.o.); the liver parenchyma is normal. (H & E, ×80).

ical findings of the liver 24 h after an acute dose of GalN and panel B represents normal liver. Panel C shows a liver section from an animal pretreated with colchicine for 7 days at the dose of 10 μg/animal and panel D shows a representative liver section from those rats pretreated with 50 μg of colchicine before the administration of GalN.

As previously reported by Keppler et al. [1], we observed that GalN intoxication provokes foci of hepatocellular necrosis and pronounced accumulation of inflammatory cells. The GalN-induced liver damage observed in rats pretreated with colchicine (10 μg/rat) was lower than that observed in rats receiving GalN alone. The liver damage was histologically absent in those animals pretreated with the highest dose of colchicine used (50 μg/rat).

The treatment with colchicine alone, 10 μg/rat for 7 days or 50 μg/rat for 7 days, did not change any of the indicators of liver damage measured either in serum or liver samples. The absence of triglyceride accumulation in livers excludes the possibility of the disruption of hepatocyte microtubules by colchicine in these studies.

Fig. 2 (panel A) shows the time-course of lipoperoxidation measured as MDA formation in liver homogenates from GalN-intoxicated rats. As shown, lipoperoxidative processes increased as early as 30 min after GalN intoxication and remained elevated throughout the observation period. However, from 0 to 3 h after GalN no changes were observed in ALT serum activity nor liver glycogen and calcium contents (panel 2B–D). At 6 h after GalN administra-

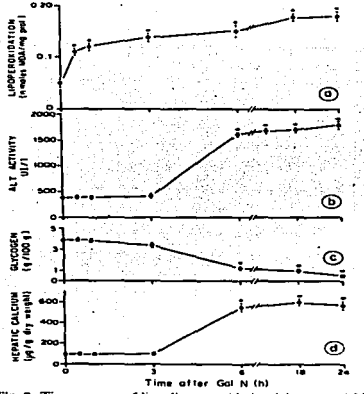

Fig. 2. Time-courses of liver lipoperoxidation (a), serum ALT activity (b), glycogen content (c) and hepatic calcium (d) determined in samples from rats treated with GalN. Each value represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.001$ as compared to control group.

tion significant increases in serum ALT activity and hepatic calcium content were observed along with a sharp decrease in glycogen content. Because the greatest alterations induced by GalN were observed at 24 h we selected that time to investigate the effect of colchicine.

Fig. 3 shows the effect of colchicine pretreatment on lipoperoxidation 24 h after galactosamine intoxication. As shown, 10 μg/day for 7 days partially prevented that increase while 50 μg/day for 7 days completely abolished it. The effect of 50 μg/day of colchicine was also examined at 0.5, 2 and 6 h after GalN administration and no increases in lipoperoxidation were observed in any pretreated rat.

Twenty-four hours after GalN administration, sharp

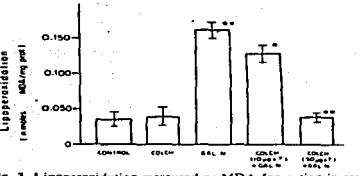

Fig. 3. Lipoperoxidation measured as MDA formation in rat liver homogenates from rats treated with: GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each bar represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; $^{**}P < 0.001$ as compared to GalN-treated group.

and significant ($P < 0.01$) changes in the serum enzymes activities were observed as depicted in Figs. 4, 5 and 6. Along with the serum findings, GalN produced statistically significant decreases in alkaline phosphatase, GGPT, and Ca^{2+}-ATPase activities in the hepatocyte plasma membranes (see Figs. 5, 6 and 7, respectively). In all cases, the yield of membrane preparations was about 1 mg protein/g of wet liver. When animals were pretreated with colchicine (10 μg/rat for 7 days) a partial but significant prevention

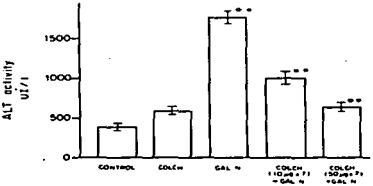

Fig. 4. ALT activity determined in serum of rats treated with GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each value represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; $^{*}P < 0.05$ and $^{**}P < 0.001$ when compared to GalN group.

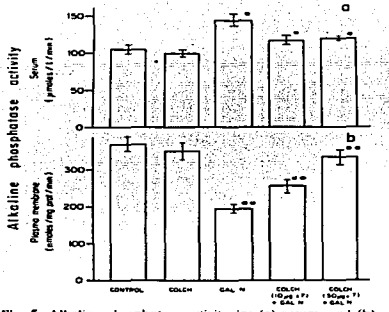

Fig. 5. Alkaline phosphatase activity in: (a) serum, and (b) plasma membranes of rats treated with GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each bar represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; $^{*}P < 0.05$ and $^{**}P < 0.001$ when compared to GalN group.

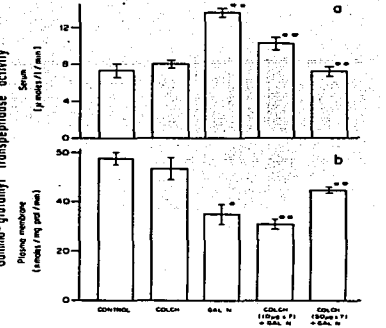

Fig. 6. GGTP activity in: (a) serum, and (b) plasma membranes of rats treated with GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each value represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; $^{*}P < 0.05$ and $^{**}P < 0.001$ when compared to GalN group.

of the serum and plasma membrane changes induced by GalN administration was observed. The pretreatment with colchicine 50 μg/day for 7 days completely prevented most of the changes induced by GalN intoxication; however, serum alkaline phosphatase activity still remained above control values and glycogen content remained below that of controls (Fig. 8).

Since diarrhea could have been a side effect of colchicine [22] we deliberately avoided such a risk by spreading colchicine administration over a period of 7 days before giving GalN. On the other hand, the slow accumulation of colchicine in the body [23] provided enough colchicine in the liver for the expected effects to be observed.

Discussion

In the liver cell cytoplasm of rats injected with

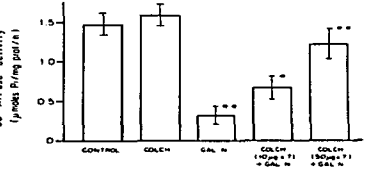

Fig. 7. High-affinity Ca^{2+}-ATPase activity in plasma membranes of hepatocytes isolated from rats treated with GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each bar represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; and $^{**}P < 0.001$ as compared to GalN group.

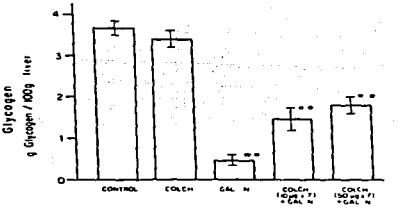

Fig. 8. Liver glycogen content determined in rats treated with: GalN, colchicine (10 μg × 7) + GalN, colchicine (50 μg × 7) + GalN, and controls. Each value represents the mean of 6 experiments performed in duplicate ± S.E.M. $^{*}P < 0.01$ and $^{**}P < 0.001$ as compared to control groups; and $^{*}P < 0.05$ as compared to GalN group.

GalN one can see periportal infiltration with lymphocytes and plasma cells. The morphological changes reflect disturbances of liver cell metabolism, which lead to characteristic changes in the serum enzyme activities. The increased values of ALT, alkaline phosphatase, and GGTP may be interpreted as a result of the liver cell destruction or changes in the membrane permeability. These enzymes are characteristic of liver damage and are not detected in normal serum, therefore their release into the serum confirmed the GalN-induced liver damage. The increases in the serum levels of alkaline phosphatase and GGTP are paralleled with decreases in their respective activities in the plasma membrane. Moreover, Ca^{2+}-ATPase plasma membrane activity also decreased after GalN injury.

We also showed in this paper that calcium is accumulated in the liver of rats after acute administration of GalN, as reported before [24]. Normally, there exists a calcium concentration gradient across the hepatocyte plasma membrane. Although several mechanisms have been described that contribute to the compartmentation of calcium in the liver cell and maintenance of the gradient across the membrane [25], it has been considered that the ultimate regulation of the total content of calcium is dependent upon the plasma membrane calcium ATPase [26]. The high-affinity Ca^{2+}-ATPase that we measured in our study has been qualified as the plasma membrane calcium pump responsible for the final regulation of the total calcium content of the hepatocyte [26]. Therefore, the decrease in the Ca^{2+}-ATPase activity may play an important role in determining the coagulative necrosis induced by GalN. The changes in plasma membrane enzyme activities induced by GalN observed herein are in agreement with the hypothesis proposed by Reutter et al. [5,27], indicating that a defect in plasma membrane composition may be necessary to induce GalN hepatitis because membrane compositon modulates the activity of associated enzymes which are sensitive to changes in lipid microenvironment.

It is important to note that the early increase in lipoperoxidation after GalN intoxication had not been reported before. Our findings clearly established that the lipid peroxidative decomposition of the membranes in GalN toxicity should also be considered. Our results demonstrated that the increase in lipoperoxidation preceded the increases in serum ALT activity, hepatic calcium accumulation and the decrease in glycogen. Lipoperoxidation, in turn, could also explain the changes in lipid-embedded enzymes of the hepatocyte plasma membrane.

Furthermore, with the help of several tests which are considered to give an overall assessment of the principal disorders found in liver pathology we have shown that colchicine had a protective effect in GalN-induced hepatitis. Indeed, pretreatment with colchicine inhibited the increase in blood enzymes. This effect was linked to the dose of colchicine administered. The protective effect of colchicine herein observed cannot be explained by its action on microtubules because it has been shown that higher doses of colchicine are needed to produce an antimicrotubular effect [28]. Moreover, pilot experiments with vinblastine (2.5 and 5.0 mg/kg) demonstrated that GalN toxicity was not prevented by disruption of microtubules.

Additionally, it is well known that the action of colchicine on microtubules is tightly coupled to a reduction in triglyceride secretion [28]. This effect was not

observed in colchicine-treated rats even when the high dose was used (50 μg/rat). It is interesting to note that the protective effect of colchicine is apparent in two types of intoxication: CCl_4 [6–11], and GalN (present results), which are identical with regard to their final effect, necrosis, but very different in their mechanisms of action.

Decker et al. attributed the hepatic toxicity of GalN to its metabolism in the liver, which caused a decrease in the level of several uracil nucleotides, resulting in an inhibition of RNA synthesis and disturbance of the biosynthesis of glycoproteins, leading to deterioration of the cell membranes [29]. Although in the present work we did not estimate the uracil nucleotides in the liver, we cannot exclude the possibility that colchicine may be able to prevent the lowering of the uracil nucleotides following the administration of GalN.

The capability of colchicine to inhibit lipoperoxidative processes may play a key role in the good protection observed. Several protective mechanisms not specific for GalN are reported in the literature, i.e. stimulation of hepatic regeneration [30], activation of the reticuloendothelial system [31,32], inhibition of protein biosynthesis [33,34], etc.

Our findings support the hypothesis that the protection offered by colchicine is mainly due to inhibition of lipoperoxidation and its consequences on hepatocyte plasma membranes. We recently found that colchicine can prevent CCl_4-induced lipoperoxidative processes, perhaps acting as a free radical scavenger [10] or acting as a membrane stabilizing agent [7]. Some other drugs have been demonstrated to have protective effects against GalN-induced hepatitis, among others silymarin, cyanidanol and vitamin E [35–37]. It is worth noting that the protective effects of colchicine have some properties in common with flavonoids and vitamin E, such as the capture of free radicals [38], stabilization of membranes [39], prevention of necrosis [40], and inhibition of lipid peroxidation [37].

Further work is necessary to clarify these various possibilities for the mechanism of the prevention of GalN-induced hepatitis by colchicine.

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