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ORIGINAL ARTICLE

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E. coli endotoxin enhances cardiomyopathy in rats with chronic alcohol consumption

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Abstract The purpose of the study was to show whether it was possible to produce alcoholic cardiomyopathy by short-term alcohol ingestion combined with an infinitesimally low endotoxin injection. Wistar rats were fed an alcoholic liquid diet according to the formula of Lieber and Decarli, and challenged with an injection of *E. coli* lipopolysaccharide (LPS) endotoxin (1.0 μg/g body weight per day for ten weeks). After ten weeks alcohol diet combined with LPS challenge, light microscopical examination showed changes commonly seen in alcoholic cardiomyopathy such as hypertrophy, oedema and disarray of myofibers. By electron microscopy, degeneration of mitochondria and degeneration of myocardial fibers were observed, the latter showing disturbance of the myofibrilla arrangement and interstitial fibrosis. Rats on an alcoholic liquid diet and rats challenged with a single identical doses of LPS did not show characteristic histological findings of alcoholic cardiomyopathy. These results suggest that short-term alcohol ingestion combined with an infinitesimally low endotoxin injection experimentally produces alcoholic cardiomyopathy, and may support the idea that endotoxin plays an important role in the aetiology of alcoholic cardiomyopathy.

Key words Alcoholic cardiomyopathy · Endotoxin · Alcohol · Heart

Introduction

Sudden cardiac death due to cardiomyopathy is a relatively common form of sudden unexplained death. There are many problems in identifying alcohol induced cardiomyopathy in the field of forensic medicine, because there are no clear anatomical findings to identify without the case history. Although chronic alcoholic organ failure is multifactorial in origin, recently it has become accepted generally that alcoholic liver injury results in potentiating endotoxin toxicity (Bhagwandeen et al. 1987; Nanji et al. 1993, 1994). There is considerable evidence to suggest that alcohol plays an important role in the depression of Kupffer cell (KC) clearance function (Nolan and Camara 1982; Tanikawa and Sata 1990). This may promote a higher incidence of endotoxaemia in alcoholic liver injury with depressed KC function (Bode et al. 1987; Fukui et al. 1991; Adachi et al. 1994). Endotoxaemia is observed in human alcoholics and results in a variety of cardiovascular and metabolic responses (Hess et al. 1981). Although the presence of an alcoholic cardiomyopathy has been demonstrated in chronically alcoholic humans (Rubin 1976; Regan 1984; Urbano-Marquez et al. 1989; Fernandez-Sola et al. 1994), it has been difficult to reproduce in animals. Some morphological studies have been carried out to evaluate the effects of chronic administration of alcohol on the heart in experimental animals (Alexander et al. 1977; Rossi 1980). In both papers it was concluded that there is no myocardial degeneration in short-term (15 or 16 weeks) alcohol ingestion but various histological changes are observed after long-term (26 weeks) alcohol ingestion. Nanji et al. (1993) suggested that the period of alcohol ingestion paralleled the plasma levels of endotoxin, and that it is related to the degree of hepatic changes in a rat model. We therefore designed this experiment to determine whether it was possible to produce alcoholic cardiomyopathy after short-term (10 weeks) alcohol ingestion combined with an infinitesimally low endotoxin injection.

The aim of this study was to define whether endotoxin is related to the alcohol-induced cardiomyopathy as reflected by the endotoxaemia.

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Materials and methods

Animals

Twenty-eight male, 8-week-old Wistar rats (Seiwa, Ooita, Japan), average weight 200 g, were individually housed in wire cages and fed a liquid diet according to the formula devised by Lieber and Decarli (1982). This provided the only source of food and water. All rats were sacrificed under ether anaesthesia at the end of the 10-week experiment.

Feeding regime

Rats were divided into four groups of seven. Each rat in a group was weight-matched with a littermate in each of the other three groups. Two groups (Groups 3 and 4) were fed a diet supplemented with alcohol strictly following the schedule of Lieber and Decarli (1982). In brief, an increasing concentration of alcohol in the form of ethanol was introduced over five days to reach 36% of the total calorie intake by the 5th day. Rats in groups 1 and 2 were fed an alcohol-free diet with the alcohol substituted isocalorically by dextrin-maltose. Rats were pair-fed (Group 1 versus Group 3, and Group 2 versus Group 4), and the caloric requirement of the non-alcohol-fed rats was determined from the calorie intake of the alcohol-fed rats. Rats were weighed daily, and the average daily alcohol intake was 0.012 g/g body weight, which is equivalent to an alcohol consumption of more than 80 g/day in a 70 kg man. The blood alcohol levels in the alcohol-fed rats, determined at the time of sacrifice, ranged from 30 to 200 mg/100 ml, with an average of 92 mg/100 ml. These levels compared favourably with those reported by Lieber and Decarli.

Table 1 The dietary regime and LPS injection

Group	1	2	3	4
Diet	Alcohol free	Alcohol free	Alcohol	Alcohol
Injection	Diluent	LPS	Diluent	LPS
Body weight+	392.9 ± 6.0	385.7 ± 3.7	362.1 ± 9.6**	352.9 ± 7.9**

+: Body weights of animals, at the end of the 10-week experiment
**: Differences is significant from group 1 ($P < 0.01$,) Data are expressed as the mean ± SD for 7 rats

Endotoxin treatment

Rats in groups 2 and 4 were injected subcutaneously with lipopolysaccharide (LPS: Escherichia coli 026: B6; Difco, Detroit, Mich.) at a dosage of 1.0 μg/g body weight per day fot ten weeks. Rats in Groups 1 and 3 were injected with sterile water only. The dietary regimen and LPS challenge schedule are summarized in Table 1.

Light microscopy

For light microscopy (LM), animals were sacrificed by bleeding and the heart was isolated, cut into 2-mm-thick slices, fixed in 10% buffered formalin, and prepared for LM. Sections were stained with haematoxylin and eosin.

Electron microscopy

For transmission electron microscopy (TEM), animals were perfused with a solution of sterile saline (37° C) and then with a Karnovsky solution at 4° C from the inferior vena cava. The heart was isolated and tissues were taken from two areas of the left ventricle, including the ventricular septum and anterior wall. These tissues were than cut into 2-mm-thick slices and post-fixed in 2% osmium tetroxide in 0.1 M PBS at 4° C for 1 hr. Specimens were dehydrated in an ascending ethyl alcohol series and embedded in Quetol 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM 1200 EX electron microscope.

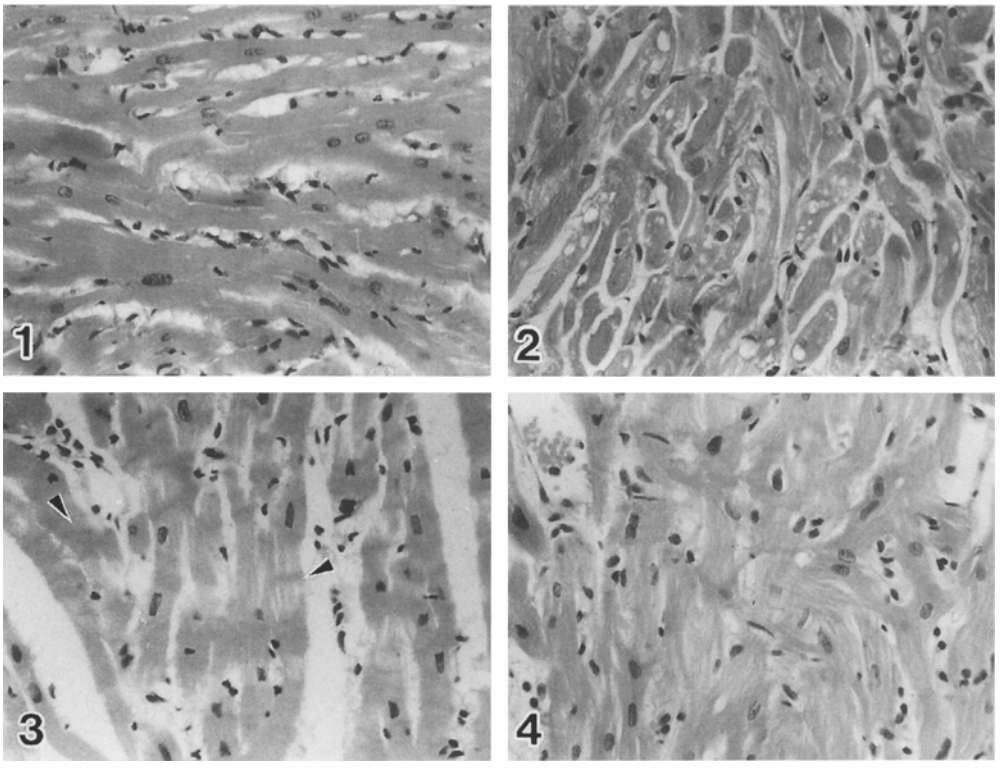


Fig. 1 Light micrograph of the heart of an alcohol diet rat combined with LPS-challenge for 10 weeks, showing hypertrophic myofibers with eosinophilic changes. H&E; magnification × 350

Fig. 2 Light micrograph of the heart of an alcohol diet rat combined with LPS-challenge for 10 weeks, showing oedematous myofibers, which included vacuole, inflammatory infiltrates and interstitial oedema. H&E; magnification × 350

Fig. 3 Light micrograph of the heart of an alcohol diet rat combined with LPS-challenged for 10 weeks, showing hypertrophic myofibers containing partly contraction band myolysis (arrowheads). H&E; magnification × 350

Fig. 4 Light micrograph of the heart of an alcohol diet rat combined with LPS-challenge for 10 weeks, showing a disarray of hypertrophic myofibers. H&E; magnification × 350

Table 2 Histological findings of light microscopy

Findings	Group			
	Non-alcohol non-LPS	Non-alcohol LPS	Alcohol-fed non-LPS	Alcohol-fed LPS
Eosinophilic change	± (2)	+ ~ ++ (5)	± (5)	++ ~ +++ (7)
Hypertrophy		+ ~ ++ (3)	± (3)	++ ~ +++ (5)
Atrophy				+ ~ ++ (3)
Disarray				+ (3)
Contraction band				+ ~ ++ (3)
Myolysis		+ (4)		++ ~ +++ (5)
Vacuolation		+ (3)		+ ~ ++ (4)
Cell infiltration		+ (3)		+ ~ +++ (5)
Interstitial oedema		+ (3)		+ ~ ++ (4)
Interstitial fibrosis				+ (3)

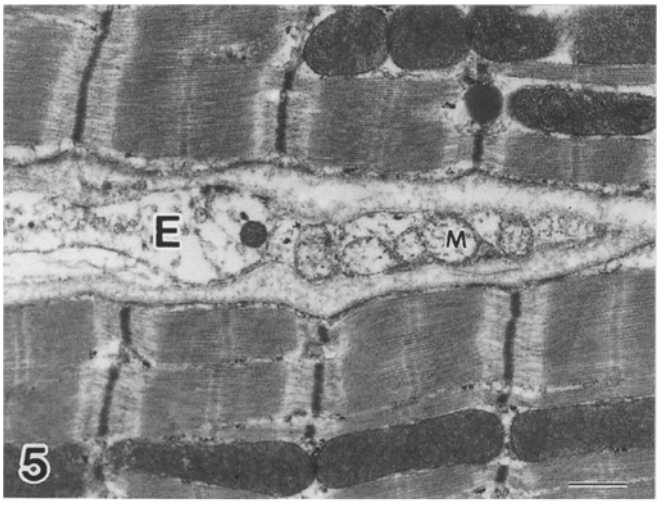
slight: ±, mild: +, moderate: ++, severe: +++ (frequency)

Results

No animals died during the 10-week experiment. The body weights of animals at the end of the 10-week period of the experiment were approximately 11% lower in the alcohol group than in the non-alcohol group (Table 1). A non-paired t-test was used to determine the significance (significance was accepted if $P < 0.01$).

The histological LM findings in alcohol-fed-non-LPS rats were almost identical to those of the normal control (non-alcohol-fed-non-LPS) rats. Slight eosinophilic changes and hypertrophy in myofibers were only seen in alcohol-fed-non-LPS rats, and mild or moderate hypertrophy of myofibers accompanied with eosinophilic changes were seen in non-alcohol-fed-LPS rats. In alcohol-fed-LPS rats, the changes described were more severe and frequently observed in the left ventricle (Fig. 1). In the LPS groups, the cardiac musculature showed partly myolysis, including vacuole formation, inflammatory infiltrates and interstitial oedema (Fig. 2). In alcohol-fed-LPS rats, these changes were more severe and frequently observed, and added to these changes, contraction band (Fig. 3), atrophy, interstitial fibrosis and occasionally disarray of myofibers (Fig. 4) were also seen. The histological LM findings of this experiment are shown in Table 2.

By electron microscopy, a slight degeneration of mitochondria which also showed an increase in number, swelling and cristolysis, was observed in the alcohol-fed-non-LPS rats. Mild or moderate degeneration of mitochondria was seen in non-alcohol-fed-LPS rats. In alcohol-fed-LPS rats, these changes were more severe and frequently observed, and giant mitochondria were occasionally observed. In the LPS groups, the endothelial cells occasionally showed cytoplasmic oedema (Fig. 5), and myocardial fibers were frequently edematous and contained widely separated myofibrils in association with swelling of the mitochondria (Fig. 6). In alcohol-fed-LPS rats, these changes were more severe and frequently observed, along with occasional observations of disturbance of the myofibrillar arrangement (Fig. 7), disorder of the intercalated discs (Fig. 8), dilatation of the tubules (both transverse and sarcoplasmic reticulum) and pigment granules in the perinuclear area of the myocardial fibers (Fig. 9) and interstitial fibrosis.



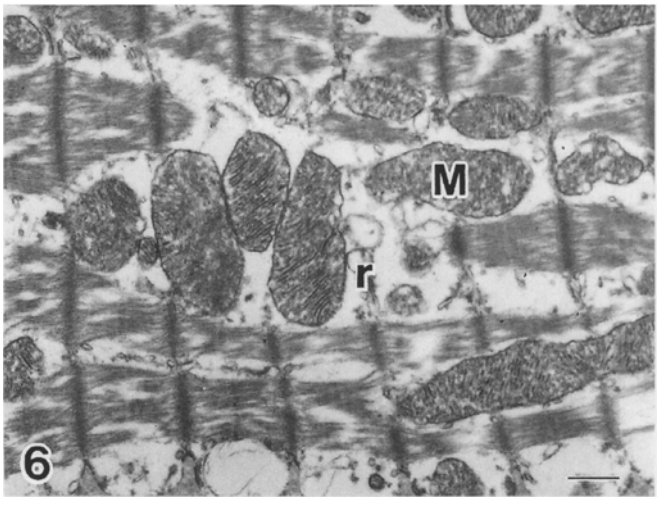


Fig. 5 Electron micrograph showing endothelial cells (E) of the myocardial capillary in the alcohol diet rat combined with LPS-challenge for 10 weeks. The endothelial cells appear to be oedematous having swollen mitochondria (M). Bar = 500 nm

Fig. 6 Electron micrograph showing myocardial fibers in the alcohol diet rat combined with LPS-challenge for 10 weeks. The myofibers are widely separated and the myofilaments have disappeared. The sarcoplasm shows oedematous changes accompanied with swollen mitochondria (M) and dilated sarcoplasmic reticulum (r). Bar = 500 nm

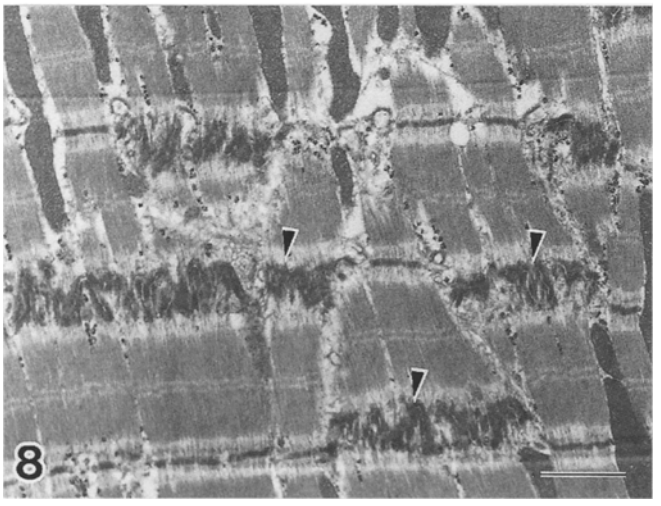


Fig. 7 Electron micrograph showing myocardial fibers in the alcohol diet rat combined with LPS-challenge for 10 weeks. The arrangement of myofibrils are irregularly disturbed. Bar = 200 nm

Fig. 8 Electron micrograph showing myocardial fibers in the alcohol diet rat combined with LPS challenge for 10 weeks. Distortion of the zig-zag intercalated disc (arrowheads) can be observed. Bar = 1 μm

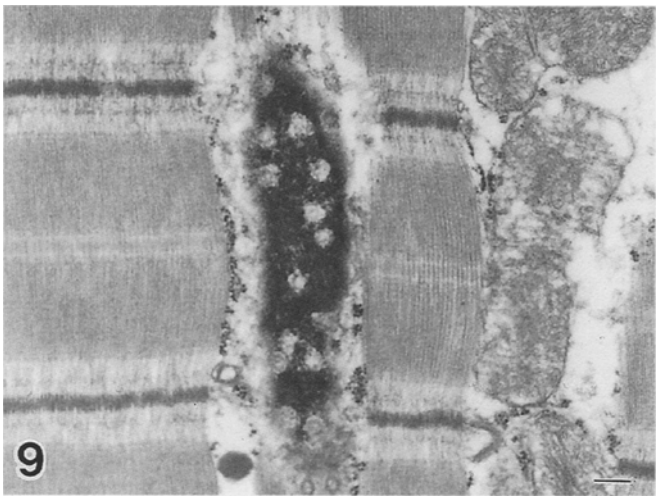


Fig. 9 Electron micrograph showing myocardial fibers in the alcohol diet rat combined with LPS-challenge for 10 weeks. Pigment granules are seen in the perinuclear area of myofibers. Bar = 200 nm

Discussion

Bhagwandeen et al. (1987) studied alcoholic liver disease and reported that alcohol-fed-non-LPS rats developed typical fatty livers of varying severity, but alcohol itself failed to induce hepatocyte necrosis in the rat model during short-term (ten weeks) alcohol ingestion. Our observations are similar to their report, but we observed that alcohol itself also failed to induce cardiac myolysis. In contrast, in the LPS groups (alcohol-fed-LPS rats and non-alcohol-fed-LPS rats) various cardiac histological changes were observed. In comparing the histological findings of alcohol-fed-LPS rats with that of non-alcohol-fed-LPS rats, the pattern of cardiac histological changes was similar, but these changes were more severe and frequent in alcohol-fed-LPS rats than in non-alcohol-fed-LPS rats. By the LM histological findings, the cardiac musculature showed myolysis, including hypertrophic and edematous myofibers. Disarray of myofibers, which characterized hypertrophic cardiomyopathy, was especially observed in alcohol-fed-LPS rats (Maron et al. 1975). By electron microscopy, degeneration of mitochondria, which showed an increase in the number and size, swelling and cristolysis was observed in all experimental groups except the control. In the LPS groups, degeneration of myocaridal fibers, with cytoplasmic oedema and widely separated myofibrils, was seen. The most impressive changes observed in the alcohol-fed-LPS rats included disturbance of the myofibrillar arrangement, disorder of the intercalated discs, pigment granules and interstitial fibrosis. The abovementioned features in alcoholic-LPS rats have been observed in human alcoholic cardiomyopathy (Hibbs et al. 1964; Alexander 1967; Kawamura and James 1971; Maron et al. 1975). In contrast, several cardiac histologocial changes were observed in alcohol-fed-non-LPS rats and non-alcohol-fed-LPS rats, but characteristic histological findings of alcoholic cardiomyopathy were not seen.

Some pathologists have shown that alcohol itself fails to induce the histological findings of cardiomyopathy in experimental animals after short-term alcohol ingestion (Alexander et al. 1977; Rossi 1980) and McDonough and Henry (1991) also suggested that it is difficult to reproduce the dysfunction of alcoholic cardiomyopathy in these short-term rat models. Nanji et al. (1993) suggested that the period of alcohol ingestion must parallel the plasma levels of endotoxin. Thus cardiomyopathy could not be experimentally induced in alcoholic liquid diet rats, and single identical doses of LPS-challenged rats. Because the resistance of the rat to endotoxin is stronger than that of humans (Caridis et al. 1972; McCuskey et al. 1984), it has been difficult to reproduce alcoholic cardiomyopathy in rats with a short-term alcohol ingestion. The facts suggest that the heart of a rat can undergo cellular changes when

exposed to chronic alcohol consumption. These changes are not obvious when the heart is exposed to a low concentration of endotoxin, they are, however, manifested when a higher concentration of endotoxin is imposed. This also suggests the importance of endotoxin in the pathogenesis of experimental alcoholic heart disease.

In our rat model of short-term alcohol ingestion combined with LPS injection, the presence of cardiac lesions similar to those seen in chronically alcoholic humans, supports the idea that endotoxin plays an important role in the aetiology of alcoholic cardiomyopathy.

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