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THE EFFECT OF LONG-TERM ETHANOL FEEDING ON *BRUCELLA MELITENSIS* INFECTION OF RATS

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Abstract — The adverse effects of ethanol on *Brucella melitensis* have not been studied previously. In this study, a new model of *B. melitensis* infection was used in the setting of chronic ethanol administration in rats. It was found that the chronically ethanol-receiving rats exposed to *B. melitensis* infection had significantly greater numbers of *B. melitensis* in their spleen and liver than the rats in the control group.

INTRODUCTION

Alcoholic patients display an increased susceptibility to bacterial infections. Once such infections develop, they are usually more severe, and some are associated with a higher mortality than that found in the non-alcoholic population (reviewed in Sternbach, 1990). Ethanol exposure adversely affects the infections caused by bacteria such as *Listeria monocytogenes* (Saad *et al*., 1993), *Streptococcus pneumonia* (Davis *et al*., 1991; Lister *et al*., 1993), *Legionella pneumophilia* (Yamamoto *et al*., 1993), and *Mycobacterium avium* (Bermudez and Young, 1991). One of the main reasons for such adverse effects is that ethanol predominantly impairs the ability of mononuclear phagocytes to control the growth of the intracellular organisms (Jerrells and Sibely, 1995). Moreover, the infection with intracellular pathogens is prevalent because immunological abnormalities after both chronic and acute alcohol consumption appear to be consistent with a decreased Th1-type immune response based on reduced antigen-specific T cell proliferation and increased antibody and autoantibody levels (reviewed in Szabo, 1999).

On the other hand, *Brucella* species and the disease spectrum are partially explained by the ability of the organism to evade host defence mechanisms by virtue of its intracellular existence. The spectrum of disease depends on many factors, including the immune status of the host, the presence of other underlying diseases or conditions and the species of infecting organism (Koneman *et al*., 1997). Brucellosis is a disease of domestic and wild animals (zoonosis) and is transmittable to humans; the disease exists worldwide, especially in the Mediterranean basin, the Arabian Peninsula, the Indian subcontinent, parts of Mexico and Central and South America (Young, 1999). This infection is considered to be a problem, because *Brucella abortus* vaccines do not protect effectively against *B. melitensis* infection. Thus, bovine *B. melitensis* infection emerges as an increasingly serious public health problem in some countries (Corbel, 1997).

Since the clinical picture in human brucellosis can be misleading and ethanol abuse is a serious health problem, much attention should be paid to these two intersecting groups. Therefore, the aim of the present study was to investigate whether chronic ethanol consumption affects the course of *B. melitensis* infection in a rat model.

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MATERIALS AND METHODS

Chronic ethanol administration procedures

Wistar rats weighing 210–290 g were obtained from a local supplier (Kocaeli, Turkey). The rats were housed individually. The rats were then matched by weight and paired so that one rat of the pair received the ethanol diet (n = 17) while the second rat was pair-fed (n = 12) isocaloric liquid diet containing sucrose as caloric substitute of ethanol. The nutritionally adequate liquid diet used in this study was formulated as previously described (Uzbay and Kayaalp, 1995). The maximum contribution of ethanol to total energy intake was 42.2% in the diet of ethanol-receiving rats. The liquid diets were prepared daily and given to rats every morning (10:00) throughout the study. For *B. melitensis* infection studies, rats were maintained on this dietary regimen for 16 days before challenge and 16 days after challenge. Five rats from the ethanol-receiving group were used to determine blood ethanol levels.

Determination of blood-ethanol levels

Blood ethanol levels were determined by headspace gas chromatography (Kumar and Gow, 1994). Blood samples were taken by intracardiac puncture from the rats under very light diethyl-ether anaesthesia.

Bacteria and culture conditions

A standard smooth strain of *B. melitensis* (16M) used in this study was provided by Pendik Institute of Veterinarians (Turkey). Bacteria were cultured on *Brucella* agar (Difco, Detroit, MN, USA) at 37°C to the logarithmic phase, which was assumed to have occurred after 48-h incubation (stationary phase entered after a 4-day incubation) and stored at 4°C until use. Identification of *B. melitensis* isolated from experimental animals was based on typical colonial morphology, growth pattern and gram stain appearance.

Experimentally induced B. melitensis infection

Brucella melitensis strain 16M cells were suspended in saline (cells in the logarithmic phase were used) and were adjusted

(based on live counts) to yield 2×10^4 to 4×10^4 colony-forming units (CFU). Inoculation was performed by injecting one dose of 0.5 ml saline containing 2×10^5 to 4×10^5 intraperitoneally. Sixteen days after challenge to *B. melitensis*, rats were weighed and assessed for the weight of the spleen, liver and the number of *B. melitensis* isolated from those organs. Spleens and livers were aseptically removed, weighed and a small piece of each organ was homogenized in 1.0 ml of sterile saline. Aliquots of 0.1 ml of the homogenates were diluted 10-fold in saline and plated on to *Brucella* agar plates to obtain a viable count. Plates were incubated at 37°C for 72–96 h and a colony count was performed. Each procedure was repeated in triplicate. The mean count was calculated and expressed as $\log_{10}$. If no bacterial growth was apparent after 4 days of incubation, the plates were incubated for 3 additional days before being considered sterile (modified from Shasa *et al*., 1994).

The experiments reported in this study were carried out in accordance with the declaration of Helsinki. Ethical approval was granted by Kocaeli University Ethics Committee (Kocaeli, Turkey).

Statistical analysis

The ratio of spleen and liver weight to rat weight and the mean count of bacteria isolated from the ethanol-receiving and pair-fed groups were calculated. The mean count of bacteria and the spleen/body weight and liver/body weight isolated from ethanol-receiving rats were compared to that of pair-fed rats by using Student's *t*-test for independent groups. $P > 0.01$ was considered to be insignificant. The relationship of daily ethanol consumption and the number of *B. melitensis* in spleen and liver were analysed by using Pearson correlation test with a level of significance of $P = 0.05$.

RESULTS

The weights of the rats were recorded on a daily basis and daily ethanol intake was also measured and expressed as g per kg body weight per day.

Daily ethanol consumption of the ethanol-receiving rats was on average 14.0 ± 0.4 (mean ± SEM) (range 12.5–14.9) g/kg. The blood-ethanol level was 203 ± 16 mg/dl (mean ± SEM) on day 16 of the ethanol exposure. Ethanol consumption decreased body weight in the ethanol-fed group compared to pair-fed rats. Thus, the body weight gains (in g) minus initial experimental body weights were 32.0 ± 11.7 and 52.5 ± 16.1 (mean ± SEM) for alcohol-fed and pair-fed rats, respectively.

The number of *B. melitensis* isolated ($\log_{10}$ CFU) from spleen (Table 1) and liver (Table 2) in ethanol-receiving rats was significantly ($P < 0.01$) greater than in control rats. However, although there was a moderate correlation between level of ethanol consumption and the number of *B. melitensis* in spleen ($r = –0.062$), it was not significant ($P > 0.05$) (Fig. 1). There were no physical signs of infection observed in rats after they were challenged with *B. melitensis*. In order to show any possible enlargement of spleen and liver, the organs/body weights ratio were calculated. There were no significant differences found in the spleen ($P = 0.204$) and liver ($P = 0.977$) body wt ratios between the ethanol and control groups.

DISCUSSION

In this study, a new model of *B. melitensis* infection was used in the setting of chronic ethanol administration in rats. It was found that the chronically ethanol-receiving rats exposed to *B. melitensis* infection had a significantly greater number of *B. melitensis* in their spleen and liver than the rats in the control group. The reason for this might be related to decreased Th1-type immune response due to ethanol consumption. Moreover, the organisms can survive in phagocytic cells and multiply to high concentrations. Besides, chronic alcoholics are thought of as 'immuno-compromised hosts', and infection with intracellular pathogens is prevalent (reviewed in Szabo, 1999). Thus, the differences between the number of the *B. melitensis* in rats' spleens and livers in both groups can be attributed to the adverse effects of the ethanol

Table 1. The number of *B. melitensis* isolated from spleen

	Ethanol-treated rats					Pair-fed controls				
	Spleen weight (g)		CFU/spleen weight*			Spleen weight (g)		CFU/spleen weight*		
Pair no:	Total	Pieces**	Mean	SD	Median	Total	Pieces**	Mean	SD	Median
1	0.9275	0.2149	7015	3	7015	0.7632	0.1598	664	14	663
2	0.8966	0.2133	6066	1	6067	0.7390	0.3064	507	12	500
3	0.9640	0.1782	5024	3	5025	0.6627	0.2110	821	16	813
4	0.7530	0.1665	4747	4	4749	0.7639	0.2166	697	19	695
5	0.7989	0.2289	6183	5	6181	0.5637	0.2190	556	1	556
6	0.8178	0.2254	3136	3	3137	0.5657	0.2151	615	4	614
7	1.3248	0.1795	4977	2	4977	0.5845	0.1616	326	4	326
8	0.5845	0.2232	5744	9	5739	0.4382	0.1626	460	2	461
9	0.3950	0.2054	5583	11	5587	0.4324	0.1304	563	1	563
10	0.6405	0.1569	7329	10	7329	0.4237	0.1330	902	13	899
11	0.5042	0.1708	7611	10	7610	0.4989	0.1115	896	1	895
12	0.3906	0.2431	4977	15	4982	0.8720	0.3255	307	4	305
Mean for group + SEM			5699 ± 363					609 ± 57		

CFU, colony forming units.
*Ethanol group values significantly different from pair-fed groups values at $P < 0.01$.
**Pieces that were homogenized in 1.0 ml of sterile saline to obtain a viable count.

Table 2. The number of *B. melitensis* isolated from liver

	Ethanol					Pair-fed				
	Liver weight (g)		CFU/liver weight*			Liver weight (g)		CFU/liver weight*		
Pair no:	Total	Pieces**	Mean	SD	Median	Total	Pieces**	Mean	SD	Median
1	9.1781	0.1503	49	5	48	7.7218	0.1427	65	5	67
2	7.2422	0.4465	560	8	563	8.6215	1.8490	110	5	110
3	7.0500	1.0057	353	5	354	6.6475	0.1532	41	3	41
4	9.5156	0.1248	240	9	241	9.3261	0.2726	17	3	18
5	7.8781	0.3681	158	4	156	8.6777	0.2407	0	0	0
6	9.3959	0.4568	187	1	187	9.1984	1.1290	93	7	89
7	9.5716	0.1898	156	5	154	9.3787	0.2683	50	9	47
8	10.1800	0.4789	162	2	162	9.9505	0.2149	57	2	58
9	7.2510	0.2458	266	2	265	9.9220	0.1732	140	1	140
10	9.9487	0.3400	88	4	90	6.7612	0.2529	118	10	115
11	7.9555	0.2707	369	9	374	7.2629	0.1760	0	0	0
12	6.7754	0.1754	342	0	342	7.8050	0.1227	0	0	0
Mean for group + SEM			244 + 41					58 + 14		

CFU, colony forming units.
*Ethanol group values significantly different from pair-fed groups values at $P < 0.01$.
**Pieces that were homogenized in 1.0 ml of sterile saline to obtain a viable count.

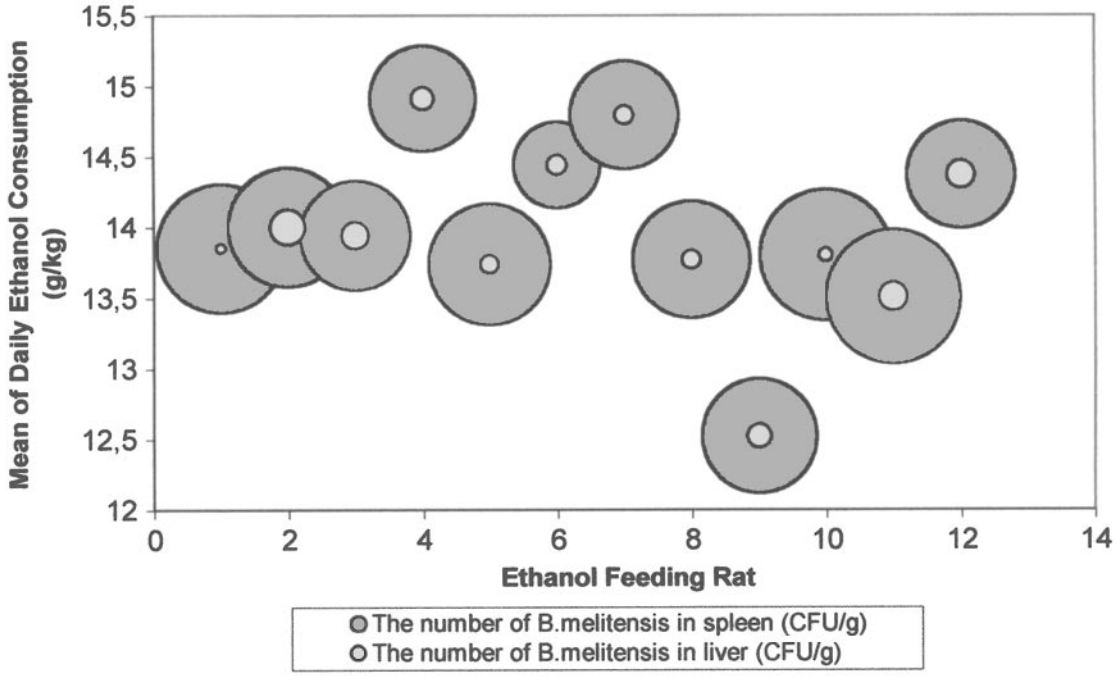

Figure 1. Relationship of long-term ethanol feeding and *B. melitensis* infection.
The number of *B. melitensis* in spleen ($r = –0.48$, $P > 0.05$) and liver ($r = –0.062$, $P > 0.05$) varies independently from daily ethanol consumption. CFU, colony forming units.

consumption and the characteristics of *B. melitensis* infection.

The disease spectrum of brucellosis depends on the infecting organism. *B. abortus* and *B. canis* tend to produce mild disease with rare suppurative complications. *B. melitensis*, the most common cause of brucellosis, also causes severe disease with a high incidence of serious complications (Koneman *et al*., 1997). However, in this study, there were no physical signs of infection observed. Furthermore, the number of *B. melitensis* among the ethanol-receiving rats seemed not to be affected by the amount of ethanol consumption. In the literature, the adverse effects of ethanol on intracellular bacteria such as *L. monocytogenes* (Saad *et al*., 1993), *Streptococcus pneumonia* (Davis *et al*., 1991; Lister *et al*., 1993), *Legionella pneumophilia* (Yamamoto *et al*., 1993), and *M. avium* (Bermudez and Young, 1991), have been reported, but that on *B. melitensis* has not been studied.

The animal model, which mimics human brucellosis, was developed and used to study the efficacy of various antibiotics in its treatment. The criteria for therapeutic efficacy in brucellosis animal models are a cure documented by the sterilization of the animals' spleen or reduction of viable counts of brucella

cultured from the homogenized spleens (Shasa *et al*., 1994). Studies have employed a similar model using mice. However, studies of the effects of ethanol on host defence mechanisms in mice are difficult for several reasons. When ethanol is administered parenterally or intragastrically once before experiment, elevated blood-ethanol levels are achieved for only a short time. In addition, they do not address the adaptive changes that occur with chronic ethanol ingestion or the effects of ethanol on host defence mechanisms (Davis *et al*., 1991). In fact, the mechanisms of impaired resistance to *B. melitensis* infection might be more appropriately studied in an animal model of chronic ethanol feeding. Therefore, in this study rats were used. The influence of ethanol consumption on *B. melitensis* infection in rats was estimated by a method similar to that used to measure the efficacy of antibiotics, and the criterion for the course of infection in this model was determined to be the number of *B. melitensis* in rats' spleen and liver.

Despite extensive studies over the past 15 years, the optimum antibiotic therapy for brucellosis is still in dispute (Corbel, 1997). In chronic alcoholism, the use of antibiotics for brucellosis treatment might not be effective. In this study a new model of *B. melitensis* infection in the setting of chronic ethanol administration was used. Further use of this model may provide new insights into the therapy of human brucellosis.

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