Effects of Chronic Ethanol Consumption in Atherosclerosis-Prone JCR:LA-Corruptent Rat

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Rats of the atherosclerosis-prone JCR:LA-corruptent strain were subjected to long-term low (0.5% wt/vol) or high (4% wt/vol) consumption of ethanol from 1 to 12 months of age. The corruptent rats are hyperphagic, obese, and insulin-resistant; exhibit a marked very low density lipoprotein hyperlipidemia; and develop both vascular and myocardial lesions while eating a normal rat chow. The total lipid profile of the rat sera showed only limited changes with ethanol consumption. There were also no significant effects on high density lipoprotein lipids. Ethanol consumption was associated with elevated fasting glucose concentrations in both lean and corruptent rats and a strong decrease in fasting insulin levels and pancreatic B-cell volume density in the hyperinsulinemic corruptent rats. The relative frequency of myocardial nodules of chronic inflammatory cells was increased in the ethanol-consuming rats, both lean and corruptent. In contrast, old organized lesions (scars) were absent in the ethanol-consuming corruptent rats. Thus, ethanol consumption had no major effect on serum lipids or lipoproteins in the corruptent rat but was associated with a reduction in insulin resistance and tail cell hyperplasia, with an associated decreased incidence of myocardial lesions.

(Arteriosclerosis 9:122–128, January/February 1989)

Epidemiologic studies in man have established that a wide range of risk factors are associated with the development of atherosclerotic disease and its sequelae.1-2 In contrast, few controllable factors lead to a decrease in the extent of the disease. Significant physical activity is associated with a lower incidence of coronary artery disease,3 and there is some evidence that the consumption of ethanol is associated with a protective effect.4 The evidence for the protective effect of ethanol in humans is not uniform, although in balance it is substantial. Thus, Sackett et al.5 found "no statistically significant relation between aortic atherosclerosis and the use of alcohol." Altura and Altura6 suggested that ethanol and particularly binge drinking predispose strongly to cerebral vasospasm and stroke. A complementary clinical study by Gill et al.7 found a reduced risk of stroke in light drinkers compared with nondrinkers and a greatly increased risk in heavy drinkers. Other investigators8,9 found a similar relationship between myocardial infarction and ethanol consumption. The extent of coronary artery occlusion was found to follow the same pattern, with consistency of consumption being associated with a low incidence of disease and a highly variable intake having an association with occlusion.10 In addition to the U-shaped curve of response to ethanol,11 Nanning and French12 have identified a strong association between changes in coronary heart disease and changes in type of beverage consumed. A number of workers have reported increases in the concentration of high density lipoprotein (HDL) cholesterol in man with ethanol consumption.13 These changes, although not dramatic, are similar to those found in individuals engaging in regular vigorous exercise and have been believed to be beneficial.

The rat is not normally prone to develop atherosclerotic disease, although stress14 or intensive breeding15 lead to myocardial lesions. However, Koletsky16,17 isolated a mutant gene that has been designated corruptent (cp) that led to obesity, hyperlipemia, and a fulminant atherosclerosis. Hansen18 incorporated the cp gene into two standard strains of rats, the LA/N and SHR/N, and backcrossed to the parent strain 12 times to create two congenic strains, LA/N-cp and SHR/N-cp. The LA/N-cp strain has been described elsewhere.19 At the fifth backcross to the LA/N a colony was established in our laboratories with stock donated by C.T. Hansen (Veterinary Resources Branch, National Institutes of Health): that colony is now designated JCR:LA-cp. This strain differs to some degree from the fully congenic LA/N-cp rats and has been described previously.20,21 The rats are, if homozygous for the cp gene (cp/cp), hyperphagous, hyperlipemic, insulin-resistant, and obese. If homozygous normal (+/+), the rats are phenotypically lean and not distinguishable from the parent LA/N strain. We have previously reported the presence of atherosclerotic disease in unmanipulated corruptent male rats of the JCR:LA strain together with myocardial lesions that include cell loss and old organized scurs.20,22 These lesions appear to be of ischemic origin. The corruptent rats have a marked very low density lipoprotein (VLDL) hyperlipidemia resulting in greatly raised triglycerides and moderately raised plasma cholesterol.

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This work was supported by the Alberta Heart and Stroke Foundation and the Nova Scotia Heart Foundation.

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Received February 11, 1988; revision accepted August 31, 1988.

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concentrations. The rats exhibit an insulin resistance and impaired glucose tolerance, which like the myocardial lesions is more mild in the female corpulent rats than the males. The vascular and myocardial lesions both develop only in cp/cp males, with lean and female rats being essentially unaffected. Both types of lesions increase in frequency with age. The finding of occlusive thrombi in major arteries and the coronary arteries supports the concept of the ischemic origin of the myocardial lesions. Both vascular and myocardial lesions correlate strongly with the insulin-resistant state. The hyperlipidemia appears to be necessary for lesion development but is not sufficient in itself to create the lesions. The JCR:LA-cp rat is a valuable model for the study of atherogenesis and myocardial damage. It develops the disease state on a normal very low fat diet and mimics the "at risk" human population in many regards. We report here on the effects of long-term low- and high-dose ethanol consumption on the plasma lipids, insulin, and glucose status and disease process in this animal model.

Methods

Male rats cp/cp and +/+ were bred in our established JCR:LA-cp colony. The rats were bred as previously described. They were housed in pairs in 48×26×16 cm polycarbonate cages with stainless steel wire tops. The temperature was maintained at 20°C and the relative humidity at 40% to 50%. The lights were on a 12:12 hour cycle, on at 03:30 and off at 15:30. The food was Wayne Lab Blox (Continental Grain Company, Chicago, IL), a pelleted rat chow containing 4.5% total fat, essentially no cholesterol, and a digestible caloric content of 3.32 kcal/g. It was freely available to the rats at all times. The water supply was available at all times and the glass bottles contained either tap water, or tap water with 0.5% or 4.0% ethanol (wt/vol) (Reagen grade, Reliance Chemicals Limited, Vancouver, BC) added. There were control, low-dose, and high-dose groups for each genotype (+/+ and cp/cp). The rats were maintained in the protocol from 1 month age to sacrifice at 12 months age, without disturbance other than regular cage changing. Food and water consumption were measured once a month.

Serum was obtained from the rats by open chest cardiac puncture following a 12-hour fast and halothane anesthesia. Sera were preserved by addition of 0.01% (wt/vol) sodium azide and 0.01% thimerosal (Sigma Chemical Company, St. Louis, MO) and maintained at 4°C until analyzed. Lipoproteins were isolated by sequential ultracentrifugation at 10°C in a Beckman L5-50B ultracentrifuge using a 50.3 Ti rotor and QuickSeal tubes. Serum density was adjusted by addition of solid sodium bromide and lipoprotein isolated under the following conditions: VLDL at d = 1.006 g/ml for 1.10×10^6 g×min; low density lipoprotein (LDL) at d = 1.030 to 1.063 g/ml for 1.45×10^6 g×min; and HDL at d = 1.063 to 1.21 g/ml for 2.7×10^6 g×min. After ultracentrifugation the lipoproteins were separated from the infranatant by tube slicing.

Lipoprotein and serum lipids were analyzed by the gas chromatographic total lipid profiling technique of Kuksis et al. Electrophoresis and immunoblotting were performed as described previously. Total protein was measured by the method of Lowry et al.

After blood samples were obtained from the heart, the rats were dissected and the pancreas, liver, and heart removed. The hearts were cut transversely into three pieces, base, midlevel, and apex and placed in 10% neutral buffered formalin, as were liver samples. The samples were processed by standard histologic techniques and embedded in paraffin. Two sections were cut serially from each block transversely across the whole heart. These were stained with hematoxylin and eosin and Masson's trichrome, respectively. The slides were examined by an experienced anatomic pathologist without knowledge of the identity of the animals. The lesions were characterized as follows: 1) muscle scar or cell dropout with pigment deposition and inflammatory cell accumulation, 2) necrosis of myocytes with reactive inflammatory cells, 3) nodules of chronic inflammatory cells, or 4) muscle scar without chronic inflammatory cells. Lesions were summed in the three sections from each heart with the two stains used to confirm identification, giving a relative frequency of occurrence.

The tail of the pancreas was fixed for 2 hours in Bouin's solution, further fixed in 10% neutral buffered formalin, processed by standard histologic techniques, and embedded in paraffin. Sections were cut, and stained for insulin and glucagon according to the methods of Stemberger with modifications. Sections were incubated with guinea pig antisera to porcine pancreatic insulin (Dako, Cedarlane Laboratories, Hornby, Ontario, Canada). Link reagents were protein-A (E-Y Laboratories, San Mateo, CA) and goat antirabbit IgG (Zymed, Cedarlane Laboratories), and developing reagent was rabbit antiperoxidase and peroxidase (Dako, Cedarlane Laboratories). Demonstration of immunolocalized peroxidase was performed according to Graham and Karnovsky.

Morphometric analysis was performed as previously described by using an automatic image analyzer (Image Plus+, Dapples System, Sunnyvale, CA). The insulin-stained areas were measured together with the total parenchymal area. The B-cell volume density was calculated for each pancreatic sample based on four random independent fields of view to give 24 observations for each experimental group.

All results are expressed as means ± standard deviation. Statistical analysis was by analysis of variance and unpaired t test, with p<0.05 considered significant.

All care, treatment, and handling of the animals was in accordance with the guidelines of the Canadian Council on Animal Care. These procedures were subject, as specified in the guidelines, to prior approval by the Health Sciences Animal Welfare Committee of the University of Alberta.

Results

The rats were maintained to 12 months of age with no losses. The animals drank the water containing either 0.5% or 4.0% ethanol readily and at a rate that was insignificantly different from the control rats drinking tap water (Table 1). The only clinical sign of the alcohol
Table 1. Body Weights and Food and Water Consumption in JCR:LA-cp Male Rats

| Genotype group | Body weight at 12 months (g) | Food consumptions (g/day) | Water consumptions (g/day) | Caloric intake (kcal/day) |
|---------------|-----------------------------|---------------------------|---------------------------|--------------------------|
|               |                             |                           |                           |                          |
| cp/cp Control | 816 ± 32                    | 28.7 ± 3.3                | 36.2 ± 1.8                | 0                        | 90.8 ± 10.8              |
| 0.5% ethanol  | 826 ± 34                    | 27.0 ± 4.0                | 37.5 ± 1.2                | 1.33 ± 0.04              | 85.5 ± 5.3               |
| 4% ethanol    | 820 ± 48                    | 23.4 ± 1.1*               | 35.2 ± 1.5                | 9.4 ± 0.4                | 85.4 ± 4.0               |
| ++/+ Control  | 408 ± 36                    | 19.3 ± 2.4                | 24.8 ± 2.0                | 0                        | 62.3 ± 7.7               |
| 0.5% ethanol  | 428 ± 38                    | 19.2 ± 0.2                | 27.1 ± 0.4                | 0.96 ± 0.01              | 63.1 ± 6.6               |
| 4% ethanol    | 430 ± 40                    | 17.8 ± 0.8                | 25.6 ± 0.8                | 7.3 ± 0.22               | 64.7 ± 2.6               |

*With the exception of body weight, values are means ± SD of the monthly observations from months 6 to 12; n=6 in all groups.

Table 2. Whole Serum Lipid Concentrations in 12-Month-Old JCR:LA Rats

| Lipid               | Control1 (n=6) | 0.5% Ethanol (n=6) | 4% Ethanol (n=6) |
|---------------------|---------------|-------------------|-----------------|
| Cholesterol         | 10.7 ± 0.6    | 20.6 ± 3.1        | 17.8 ± 4.3      |
| Phospholipids       | 55.6 ± 6.8    | 177.5 ± 26.3      | 162.2 ± 26.2    |
| Triglycerides       | 17.7 ± 5.8    | 214.7 ± 51.7      | 236 ± 41.2      |
| Esterified cholesterol | 42.7 ± 5.2   | 108.1 ± 16.7      | 105 ± 21.4      |
| Total cholesterol   | 53.3 ± 5.7    | 128.7 ± 19.6      | 123 ± 25.6      |

*P<0.005 vs. control.

Table 3. Distribution of Serum Lipids by Density Fraction in cp/cp Rats

| Lipid               | VLDL (d=1.006-1.006 mg/ml) | LDL (d=1.030-1.063 mg/ml) | HDL (d=1.063-1.21 mg/ml) |
|---------------------|----------------------------|---------------------------|--------------------------|
| Cholesterol         | 6.2 ± 1.1                  | 6.8 ± 1.2                 | 6.8 ± 0.7                |
| Phospholipids       | 40.0 ± 10.2                | 43.1 ± 3.6                | 43.1 ± 3.3               |
| Triglycerides       | 196 ± 38                   | 227.5 ± 34.5              | 13.5 ± 0.35*            |
| Esterified cholesterol | 7.5 ± 2.7                | 5.4 ± 1.0                 | 9.8 ± 2.5†              |
| Total cholesterol   | 13.7 ± 3.7                 | 12.3 ± 2.1                | 15.5 ± 3.9               |

*P<0.005 vs. control; †P<0.02 vs. control.

consumption was a tendency to sleep more than the control rats and to become irritable if deprived of the water bottle for any lengthy period. Food consumption varied by genotype, as shown in Table 1. Food consumption of the control rats was greater than that of the lean, as is normal for these hyperphagous animals. The control rats on low-dose ethanol ate slightly less food than the control animals did, but this difference was not significant. High-dose control rats ate less food than the control animals. The lean rats on high-dose ethanol also ate less food than the lean controls, but this difference was not significant (P>0.05). Table 1 shows the average caloric value of the ethanol consumed by the rats between 6 and 12 months age, together with the total caloric intake/day (food and ethanol). Although the high-dose control rats consumed approximately 10 kcal/day as ethanol, the total caloric intake dropped slightly and nonsignificantly (5 kcal/day) in both high- and low-dose rats. Similarly, the total caloric intake did not change significantly between the groups of lean rats. This is reflected in the essentially identical weights of the rats of the same genotype, regardless of group.

Table 2 shows the concentration of lipids in whole serum from lean and corpulent rats on no ethanol and on low- and high-dose ethanol. The results for the control groups are consistent with those previously reported for 3-month-old male JCR:LA rats, lean and corpulent.24 The triglycerides and phospholipids showed no significant changes between groups. In the high-dose corpulent rats the plasma total cholesterol content was significantly reduced. Although free cholesterol was unchanged, esterified cholesterol showed significant decreases. The lean and corpulent rats on low-dose ethanol showed no significant differences between control and ethanol-consuming groups.

Table 3 shows the distribution of the serum lipids by density fraction for the corpulent rats, control and high-dose ethanol. The only significant differences between the two groups are in the LDL fraction. Free cholesterol was not significantly altered by ethanol consumption, but esterified cholesterol and consequently total cholesterol
Table 4. Glucose, Insulin, and Glucagon Concentrations and Insulin Cell Density in 12-Month-Old JCR:LA Rats

| Genotype: group | No. | Plasma glucose (mg/dl) | Insulin (mU/l) | Glucagon (ng/l) | Insulin-secreting cells (volume density %) |
|-----------------|-----|------------------------|---------------|----------------|------------------------------------------|
| cp/cp:          |     |                        |               |                |                                          |
| Control         | 6   | 118±7.8                | 326±130       | 314±45         | 10.3±9.4                                 |
| 0.5% ethanol    | 6   | 150±18*                | 287±110       | 309±39         | 5.39±5.0*                                |
| 4% ethanol      | 6   | 129±3.5*               | 189±70†       | 278±28         | 6.29±5.54*                               |
| +/+:            |     |                        |               |                |                                          |
| Control         | 6   | 127±14                 | 18±8          | —              | 1.25±1.5                                 |
| 0.5% ethanol    | 6   | 154±17*                | 35±16         | 288±28         | 1.27±2.66*                               |
| 4% ethanol      | 6   | 160±24*                | 40±9          | 292±48         | 101±1.41                                 |

Values are means±SD, with the volume density results reflecting 24 observations per group. Significance of difference vs. control: *p<0.01, †p<0.025.

Figure 1. Section of pancreas from a +/+ control rat at 12 months of age showing small discrete islets of Langerhans. Immunostained for insulin as described in the methods. ×36

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were significantly lower in the LDL fraction. The very small amount of triglyceride present in this density fraction was significantly raised, but this effect is probably trivial.

Only very limited changes were found in the apolipoproteins in the sera from the ethanol-consuming rats. No significant changes occurred in the lean rats whatsoever. In the cp/cp rats, whole serum apo E concentrations in the rats consuming 4% ethanol were 26.1±5.2 mg/dl, compared with 36.2±6.4 mg/dl for the control group (p<0.01). This decrease largely reflected a very significant (p<0.01) decrease in the apo E levels in the LDL fraction (1.54±0.82 vs. 3.60±0.50 mg/100 ml). There were no significant changes in the concentrations of apo A-1 or apo B in any of the groups.

The plasma glucose, insulin, and glucagon concentrations in fasted rats of all groups are shown in Table 4. Ethanol consumption, both high- and low-dose, significantly raised the fasting glucose concentrations of both corpulent and lean rats. High-dose ethanol in the corpulent rats was associated with markedly lower insulin concentrations. There were no significant differences in the glucagon levels between the groups or genotypes. Histologic examination of the liver showed no significant lesions in any of the groups. There was an occasional very minor steatosis among the alcohol-consuming rats. Examination of the pancreatic tissue of control rats gave results similar to those reported previously, with normal islets of Langerhans in lean rats and massive hyperplasia of the islets in the corpulent rats. Figures 1 and 2 show typical islets from lean and corpulent control rats, respectively. The islets of lean rats that consumed alcohol were not distinguishably different from those of the control rats. However, the islets of corpulent rats that consumed ethanol showed a reduced degree of staining for insulin and a diffuse character with loss of organized islet structure. Figure 3 shows a typical example of such islets from
a cp/cp rat that consumed 4% ethanol. Table 4 shows the insulin cell volume density in the experimental groups. Islet size could not be measured in these 12-month-old rats as the islets of corpulent rats have become diffuse and the distinction between individual islets thus becomes partially lost. The results in Table 4 show a significant decrease in B-cell density and, therefore, of B-cell hyperplasia between the alcohol-consuming and control groups of corpulent rats and no effect on the normal islets of lean rats.

The relative frequency of occurrence of myocardial lesions of the four types defined in the methods is shown in Table 5. The incidence of type 1 lesions was not significantly affected by ethanol consumption, being very
rare in the lean rats and common in the corpulent rats. Type 2 lesions were rare to absent in the corpulent rats and absent in the lean rats of all groups. The incidence of type 3 lesions (nodules of chronic inflammatory cells) was significantly increased in all alcohol-consuming groups. In the corpulent rats the incidence more than doubled, and the lesions, while not present in the lean control rats, appeared in the alcohol groups. In contrast, type 4 lesions (old scars) were absent in the corpulent rats consuming ethanol, but appeared occasionally in the lean rats consuming ethanol while they were absent in the control rats.

### Discussion

The rats maintained their normal state of health while consuming up to 3.5 g of ethanol per day. The additional caloric content of the ethanol was significant, at some 10 kcal/day, compared with the caloric content of 28 g of food per day (approximately 90 kcal). The rats, including the corpulent animals, maintained a common weight for the genotype regardless of the ethanol intake. This suggests that the rats, including the corpulent individuals, regulate their weight to a target value and must adjust food intake and/or metabolism to accomplish this. The data in Table 5 suggest that both mechanisms were being used. These data are consistent with the finding that the corpulent rat has at least as strong a thermogenic capacity in response to cold, norepinephrine, or amphetamine as does its lean counterpart.

The changes in the myocardial lesion frequency in response to ethanol are important. The type 1 lesions appear to represent a relatively recent injury to the myocardium, showing loss of viable cells and reparative and consolidative processes in progress. The frequency of these lesions in the corpulent rat was not altered by the ethanol, suggesting that at the time of sacrifice lesions were being generated. The increase in type 3 lesions (chronic inflammatory cell infiltration) would tend to support this. The clear-cut decrease in type 4 lesions in the corpulent rats, in contrast, indicates that there were far fewer old matured lesions present at sacrifice. The two changes are consistent with a lower incidence of lesion formation at younger ages, with an increase in lesion formation in these middle-aged rats. The data in Table 5 suggest that high-dose ethanol not only increased the incidence of type 3 lesions in lean rats, but had resulted in the generation of type 4 lesions as well. This would represent a major change, as such lesions normally are not seen in lean rats. Thus, it may be that ethanol consumption reduces myocardial damage in the rats in early to middle adulthood, but leads to increase in disease processes in advanced middle age (about 1 year in the rat). Such a pattern would be very similar to that reported in man.

The changes in serum lipids in the lean rats were very small and unlikely to have any influence on lesion frequency. The changes in the lipids in the corpulent rats were beneficial in direction, and essentially confined to decreases in the LDL cholesterol concentrations. Importantly, there was no significant change in the HDL fraction of any of the rats. The lipoprotein changes were confined to the corpulent rats and a reduction in apo E concentration in the LDL fraction (d=1.030 to 1.063 g/ml). This latter change is consistent with the lower concentrations of esterified cholesterol in the LDL fraction. Overall, the results do not support the concept that ethanol consumption has any major effect on the serum lipids or lipoproteins, at least in this strain of rat.

Other studies have suggested that the impaired glucose tolerance or insulin resistance is required for atherogenesis and myocardial damage in the JCR:LA-cp rat. Ethanol consumption was associated with increased fasting glucose concentrations and decreased insulin levels. Taken with the substantially smaller B-cell hyperplasia, this suggests an inhibition of the development of the insulin resistance. Despite the fact that insulin has been demonstrated to promote triglyceride synthesis and VLDL release, the lower insulin levels were not accompanied by any changes in these lipids. Firm conclusions regarding this effect and the implications regarding metabolism in this abnormal strain of rat will have to depend on detailed studies of animals at the younger ages when the pathologic processes are first active. Such studies include precise determinations of glucose tolerance, insulin metabolism, and hepatic lipid secretion rates. The strong effect of ethanol feeding on the myocardial lesion frequency in

### Table 5. Myocardial Lesion Frequency in 12-Month-Old Male Rats

| Genotype group | No. | Type of lesion | 1 | 2 | 3 | 4 |
|---------------|-----|---------------|---|---|---|---|
| cp/cp Control | 6   | 1.5±0.54 1.0±0.41 0.83±0.88 1.00±0.89 |
| 0.5% ethanol  | 6   | 2.0±1.4 0.5±0.55 1.83±0.75 1.0±0.75 |
| 4% ethanol    | 6   | 1.3±0.81 0      1.83±0.75 1.0±0.75 |
| +/+ Control   | 6   | 0.17±0.41 0      0      0      0      |
| 0.5% ethanol  | 6   | 0      0      0.03±0.05 0.17±0.05 |
| 4% ethanol    | 6   | 0.17±0.41 0      0.5±0.55 0.3±0.55 |

Values are means±SD of the number of lesions observed in the three sections from each rat. Lesion types: (1) muscle scar or cell dropout with pigment deposition and inflammatory cell accumulation; (2) necrosis of myocytes with reactive inflammatory cells; (3) nodules of chronic inflammatory cells; and (4) old scars. Significance of difference vs. control: *p<0.05, †p<0.02.
the JCR:LA-cp rat suggests that such work will be revealing of elements of both normal and abnormal metabolism.

The present results do establish the strain as a valuable animal model for the study of the effects of ethanol consumption, as well as study of the mechanisms of atherogenesis. The myocardial lesions correlate strongly with the atherosclerosis in cp/cp male rats. The reduction in myocardial lesions caused by ethanol suggests that atherogenesis was also inhibited. This, taken with the decrease in insulin resistance, both indicates that the abnormal metabolism of these rats can be altered and provides further evidence for a link between hyperinsulinemia and atherogenesis.

Acknowledgments

Dorothy Kossiaia rendered invaluable technical services and managed the breeding colony of JCR:LA-cp rats, with the assistance of Sandra Husberg.

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Index Terms: myocardial lesions • hypertendipidemia • insulin resistance • JCR:LA-cp rat • ethanol
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