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COMBINED EFFECTS OF PROTEIN DEFICIENCY AND CHRONIC ETHANOL CONSUMPTION ON THE ULTRASTRUCTURE OF RAT NEUTROPHILS. A MORPHOMETRIC STUDY

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This study was performed in order to delineate the combined effects of protein deficiency and chronic ethanol ingestionon on the ultrastructural morphology of rat polymorphonuclear neutrophils (PMNs).Morphometric methods have been used to show that chronic ethanol feeding decreased cell profile area, cytoplasm profile area, and the total number of cytoplasmic granules per cell profile. In addition, it was found that both protein deficiency and ethanol administration reduced the total number of cytoplasmic granules per cell profile and that their effects were aditive. Also, reduction of the volume density of granules was observed in PMNs from both protein-deficient animals and animals fed ethanol in combination with a protein-deficient diet. These animals contained cytoplasmic granules with a smaller mean diameter, whereas alcohol ingestion with a protein adequate diet was associated with normal mean diameter of the granules. The volume density of mitochondria and endoplasmic reticulum nearly doubled during ethanol intake, and an interactive effect of ethanol consumption and protein deficiency was observed. The results indicated some ultrastructural abnormalities of PMNSs in chronic experimental alcoholism regardless of whether alcohol was administered as part of a nutritionally adequate or a protein-deficient diet.

Key words: alcohol, protein deficiency, neutrophils, ultrastructure, morphometry.

INTRODUCTION

Consumption of large amounts of ethanol has been associated with various hematologic and immunologic abnormalities (reviewed by MacGregor, 1986; reviewed by Ballard, 1989). Although numerous authors have commented on the toxic effect of alcohol on polymorphonuclear neutrophil (PMN) function (MacFarland and Libre, 1963; Hallengren and Forsgren, 1978; MacGregor et al., 1988; Corberand et al., 1989; MacGregor, 1990; Todorovic et al., 1994b) their findgins are inconsistent and to some extent contradictory. However, whether the altered immune response is due to the effect of alcohol per se, or to the other

frequently associated complications of alcoholism (i. e. nutritional deficiencies, liver dysfunction, lower socioeconomic lifestyle, ect.) has not been determined.

On the other hand, poor nutrition (especially hypoprotein) may be reflected by many metabolic parameters in the granulocytes, which are associated with a large number of enzyme and functional disturbances (Avila et al., 1973; Douglas and Schopfer, 1974; Fensenfeld and Gyr, 1977; Todorovic et al., 1985; 1986; 1988).

To the best of our knowledge, quantitative analysis of the fine structure of the peripheral blood PMNs has not been made in combined chronic alcoholism and protein deficiency.

The present study described our observations on the ultrastructural features of those cells utilizing morphometric measurements in a model of experimental chronic alcoholism and protein malnutrition of rats.

MATERIAL AND METHODS

A total of forty 60-day-old male Wistar rats were divided into four groups of 10 animals each. Each group was offered one of the following diets: I, a nutritionally adequate diet without ethanol; II, a protein-deficient diet without ethanol; III, a nutritionally adequate diet with ethanol; and IV, a protein-deficient diet with ethanol. The composition of the diets was described previously (Todorovic et al., 1986; Todorovic et al., 1994a). The ethanol groups were given free access to an aqueous solution of 24% sucrose-32% ethanol as recommended by Hartroft (1971). The amount of food fed to animals in groups I and II was adjusted daily to the energy intake of the animals receiving alcohol (groups III and IV), i. e. all groups were pair-fed.

For preparing buffy coat specimens, 5 ml of blood was drawn into heparinized syringes from each rat after cardiac puncture. Leukocytes were separated by a sedimentation method. They were then pelleted into beam capsules, fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed in 2% osmium tetroxide at 4^0C for 1 hr. Following that the pellets were dehydrated in a graded series of ethanol, and embedded in Epoxy resin. Ultrathin sections cut from the embedded cell suspension (using an LKB ultratome II) were contrasted with lead citrate and uranyl acetate prior to viewing with an Opton 109 eletron microscope. Cytochemical staining for ultracytochemical presentation of leukocyte myeloperoxidase was performed essentially as descibed by Graham-Karnovsky and Hayhoe (Hayhoe and Quaglino, 1980), using the peroxidative activity of myeloperoxidase against diaminobenzidine. Blood was collected and leukocytes were fixed with 1.5% glutaraldehyde in 0.1M cacudylate buffer with 1% sucrose at pH 7.2 for 30 min, incubated in DAB-medium for 10 min, osmicated, dehydrated, and embedded in Epon using a standard procedure. Ultrathin sections were contrasted with lead citrate before examination in an electron microscope.

Prints were made from negatives of nucleated neutrophils (7,000 time original magnification) at a final magnification of 17,000 times. About 6 prints were analyzed from each animal (total of 60, 56, 67 and 52 prints for groups I, II, III and IV respectively). For stereological analysis (excluding granular radius and relative ratio of azurophilic to other granules) test micrographs were covered with a

transparent lattice point-counting grid with lattice ratio r=9 (1:9, 690:210 the number of coarse to fine points; 0.9:0.3 the length in centimeters of one of the three linear probes associated with each of the coarse and fine points), 20x29 cm, using the method of Veibel et al., and Weibel and Bolender as described by Aherne and Dunnill (1982). The test grids were used to obtain "hits" on test organelles of interest (coarse points for nucleus and cytoplasm, and fine for mitochondria, Golgi complexes, endoplasmic reticulum and grannules). The following morphometric (stereologic) parameters were obtained from these prints: **for the whole cell,** the profile area; **for the nucleus,** the profile area and volume dennsity; **for the mitochondria,** the number of profiles per section and volume density; and **for the granules,** the profile area, volume density, number of profiles per section, and number of μm^2 of cytoplasm. The mean granular radius was measuring using Kotron MOP AMO 3 semiautomatic analyzer. Test micrographs were placed on the measuring tablet and a light-weight cursor used to mark four radii of each graule. The number of granules outlined was then available in printed form.

Data were expressed as means+SEM and were analyzed by two-way analysis of variance (AVNOVA) using 2x2 classification (ethanol, no ethanol vs low-protein, regular protein). When indicated by analysis of variance, individual means were compared using the Student-Newman-Keuls post hoc test.

RESULTS

A summary of the quantitative analysis of ultrastructural morphometry of peripheral blood neutrophils is hown in Table 1. Chronic ethanol feeding decreased the cell profile area and cytoplasm profile area, as well as the total number of cytoplasmic granules per cell profile. In addition, it was found that both proteindeficiency and ethanol administration reduced the total number of cytoplasmic granules per cell profile and that their effects were additive. Also, reduction of the volume density of granules was observed in PMNs from both protein-deficient animals and animals fed ethanol in combination with a protein-deficient diet. These animals contained cytoplasmic granules with lower mean diameter, whereas alcohol ingestion with a protein adequate diet was associated with normal mean diameter of the granules. The volume density of mitochondria and endoplasmic reticulum nearly doubled during ethanol abuse, and an interactive effect of ethanol consumption and protein-deficiency was observed. There were no significant differences between the results for the examined groups with respect to any of the following parameters studied: profile area and volume density of the nucleus; volume density of the cytoplasm; number of mitochondria per cell profile; volume density of the Golgy system, as well as azurophilic to specific granule ratio.

In addition, our observation in ethanol-fed rats indicated alterations in the mitochondria such as clumping, elongation, swelling and cristae disruption, and changes in the topographic distribution of granules such as registration of cytoplasmic areas with numerous granules and areas with a smaller number of granules or without them. Some cells obtained from ethanol-fed rats had autophagic vacuoles.

Table 1. Ultrastructural morphometry of PMNs: summary of quantitative analysis

	Results (mean ± SEM)			
	Control (I)	Protein deficient (II	Alcohol (III)	Alcohol and protein deficient (IV)
CELL				
No of cells analysed	60	56	67	52
Profile area (μm^2)[a]	380 ± 9.2	372 ± 8.0	323 ± 10.3	325 ± 10.4
NUCLEUS				
Profile area (μm^2)	116 ± 2.9	114 ± 2.3	110 ± 4.4	109± 4.1
Volume density (μm^3)	0.34 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.34 ± 0.01
Ratio of nuclear to cell profile area	0.30 ± 0.01	0.31 ± 0.01	0.34 ± 0.01	0.30 ± 0.01
CYTOPLASM				
Profile area (μm^2)[a]	246 ± 9.8	258 ± 9.0	213 ± 8.4	216 ± 8.2
Volume density ($\mu\mu m^9/\mu m^9$)	0.66 ± 0.01	0.66 ± 0.01	0.65 ± 0.01	0.66 ± 0.01
MITOCHONDRIA				
No per cell profile	5.45 ± 0.17	5.55 ± 0.40	4. 78 ± 0.31	4.92 ± 36
Volume density (% of cytoplasm)[a]	2.44 ± 0.18	2.00 ± 0.23	4.20 ± 0.43	2.52 ± 0.20
GOLGI SYSTEM				
Volume density (% of cytoplasm)	0.10 ± 0.03	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.03
ENDOPLASMIC RETICULUM				
Volume density (% of cytoplasm)[a,ab]	0.14 ± 0.03	0.12± 0.03	0.22 ± 0.04	0.30 ± 0.02
CYTOPLASMIC GRANULES				
No per cell profile[a, b, ab]	108 ± 4.7	79 ± 3.2	84 ± 3.6	70 ± 5.6
No per μm^2 of cytoplasm	0.43 ± 0.02	0.31 ± 0.01	0.42±0.02	0.32 ± 0.02
Volume dennsity (% of cytoplasm)[b, ab]	6.25 ± 0.23	4.72 ± 0.32	5.59 ± 0.27	3.99 ± 0.31
Azurophilic to specific granule ratio	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.01
Mean diameter (nm)[b, ab]	359 ± 6.2	326 ± 7.2	348 ± 10.9	301 ± 6.7

a = ethanol effect; b = hypoprotein nutrition effect; ab = interactive effect

DISCUSSION

The morphometric ultrastructural results for rat PMNs showed that the cell profile area, the cytoplasm profile and the total number of cytoplasmic granules per cell profile were decreased, but the volume density of mitochondria and endoplasmic reticulum nearly doubled during alcohol consumption without protein deficiency. Stereological analyses were similar to those reported by Todorovic et al., (1994a) previously. The results indicated some ultrastructural abnormalities of PMNs in chronic experimental alcoholism and may be related to polymorphonuclear metabolic and functional dysfunction (Todorovic et al., 1994b). Reduction of the volume density and total number of cytoplasmic granules per cell profile, as well as the mean diameter of granules, were observed in PMNs from both protein-deficient animals and animals fed ethanol in combination with the protein-deficiennt

diet. Douglas and Schopfer (1974) reported that electron microscopy of resting and phagocytosing phagocytes showed no qualitative differences in the granules, mitochondria, phagocytic vacuoles or extent of degranulation in children with kwashiorkor. Our previous studies indicated that the activity of many enzymes and antibacterial and bactericidal systems were altered in PMNs during protein malnutrition (Todorovic et al., 1985; 1986; 1988), and we speculated that altrations of those enzymes and antibacterial systems which are predominantly localized in granules may be associated with morphological changes of cytoplasmic granules.

The results indicated many ultrastructural abnormalities of PMNs in chronic experimental alcoholism regardless of whether alcohol was administered as part of a nutritionally adequate or a proteinn-deficient diet.

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KOMBINOVANI EFEKAT PROTEINSKOG POTHRANJIVANJA I HRONIČNOG UNOŠENJA ETANOLA NA ULTRASTRUKTURNE KARAKTERISTIKE NEUTROFILA PACOVA. A -MORFOMETRIJSKA ANALIZA

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SADRŽAJ

Ova studija urađena je s ciljem da se razjasni kombinovani efekat proteinskog pothranjivanja i hroničnog unosa etanola na subcelularne karakteristike polimorfonuklearnih leukocita (PMNs) periferne krvi pacova. Primenjene morfometrijske metode pokazale su da hronični unos etanola dovodi do redukcije površine ćelijskog profila i citoplasme kao i do redukcije broja citoplazmatskih granula po ćelijskom profilu. Takođe je pokazano da redukciju broja granula po ćelijskom profilu uzrokuje kako ishrana siromašna u proteinima tako i kombinovan unos etanola uz proteinsko energetsko pothranjivanje. Životinje koje su bile na hipoproteinskoj dijeti ili su pak uz hipoproteinsku dijetu konzumirale alkohol, imale su redukovanu volumensku gustinu granula a same granule manji dijametar. Volumenska gustina mitohondrija i endoplazmatičnog retikuluma bila je duplirana u životinja koje su konzumirale alkohol. Rezultati ukazuju da pod delovajem alkohola dolazi do značajnog narušavanja subcelularne organizacije granulocita bez obzira da li se alkohol administrira kao sastavni deo proteinski adekvatne ili neadekvatne dijete.