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NOTE

Effects of Ethanol Treatment on Leydig Cellular NADPH-Generating Enzymes and Lipid Profiles

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Abstract. Effects of ethanol treatment on Leydig cell NADPH-generating enzymes and lipid profiles were studied. Ethanol treatment (3.0 g/kg. b.wt.) twice daily as a 25% (v/v) aqueous solution given to adult Wistar rats reduced the body weight, testis weight and relative weights of the seminal vesicles and ventral prostate. Serum LH and testosterone were also decreased. Similarly, the NADPH-generating enzymes such as G-6-PDH, 6-PGDH, $NADP^+$-ICDH were reduced, but malic enzyme was unaltered. Leydig cell total lipid was decreased: neutral lipids such as esterified cholesterol and triacyl glycerol were decreased but free cholesterol and diacyl glycerol were increased. The reduction in total phospholipid was contributed to by fractions such as phosphatidyl inositol, phosphatidyl serine, phosphatidyl choline and phosphatidyl ethanolamine. Withdrawal of ethanol treatment for 30 days restored these to the normal level. The present findings suggest that the ethanol treatment impairs Leydig cellular NADPH generation which may be one of the biochemical mechanisms mediating the direct and indirect effects of ethanol resulting in hypoandrogenization.

Key words: Leydig cell, Ethanol, G-6-PDH, 6-PGDH, $NADP^+$-ICDH, Malic enzyme, Lipids, Neutral lipids, Phospholipids

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CHRONIC male alcoholics and experimental animals exposed to ethanol have subnormal levels of testosterone [1–5]. The Leydig cells are the primary source of testicular androgen [6]. Morphometric analysis at the electron-microscopical level shows reduced cell size, a decreased amount of cytoplasm, smooth endoplasmic reticulum and enhancement of the mitochondrial volume in chronic ethanol treated rat Leydig cells [7, 8]. Moreover, the number of elongated and cup-shaped mitochondria and cytoplasmic pseudopods were increased. Reduction in endoplasmic reticulum was predicted to decrease in steroidogenic activity [9]. Orpana *et al.* [10, 11] have shown that the metabolites involved in substrate shuttles maintaining the NAD(P)H redox state regulate steroidogenesis. The co-factor NADPH is utilized at many steps throughout the steroidogenic pathway starting from acetyl CoA [12]. Alcohol dehydrogenase (ADH) localizes in Leydig cells [13] and ethanol is metabolized in these cells by two mechanisms. Of these, the microsomal ethanol oxidation system (MEOS) was shown to be active, and utilizes NADPH as a co-factor [14]. Apart from steroidogenesis and ethanol oxidation, NADPH is important for lipogenesis as it is essential for malonyl CoA synthesis and fatty acid elongation [15]. Testis stimulated with gonadotropins produces an increased amount of NADPH [16]. The sources of NADPH generation are glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH) of the hexose monophos-

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phate (HMP) shunt pathway, $NADP^+$-isocitrate dehydrogenase ($NADP^+$-ICDH) and malic enzyme [15].

This co-enzyme (NADPH) acting as a hydrogen and electron carrier, and plays an essential role in metabolism. With respect to steroid biosynthesis, it is known that in mitochondria and microsomes there exists an electron transfer system specific for steroids, parallel to the cytochrome chain, whose first link is NADPH. It is therefore likely that any hormone or chemical agent which manipulates the Leydig cellular NADPH generating system would have an effect on the steroidogenic and lipogenic potencies of these specialized cells and thus the reproductive status. In the present study, the effects of ethanol on Leydig cellular enzymes involved in NADPH generation were studied along with the lipid classes.

Materials and Methods

Animals

Healthy adult male albino rats of the Wistar strain weighing 175–225 g were used in the present study. The animals were kept in clean cages and housed in a well ventilated animal house with controlled illumination (12:12, L:D). Water was made available *ad libitum*

The rats were divided into three groups of 20.

Group I: Control animals which received sucrose water (36%) in the same volume as that of the experimental rats through gastric intubation.

Group II: Ethanol treatment [Ethyl alcohol (Reidel-de-Haen, AG D-3016, Seelze-1, Germany) was given orally by gastric intubation as a 25% (v/v) aqueous solution at a dose of 3 g/kg body weight twice daily at about 0930 h and 1630 h, respectively, for 30 days].

Group III: Withdrawal of ethanol treatment [rats treated with ethanol in group II for 30 days were withdrawn from treatment for a further period of 30 days to assess the reversibility of the ethanol effect].

Body weight was recorded at regular intervals (twice a week) and the volume of ethanol given was adjusted accordingly. Each day the control animals consumed the same weight of isocaloric Gold Mohur rat feed (Lipton India Ltd., India) as the corresponding experimental animals had eaten by the previous day, and the caloric values derived from the ethanol (to the experimental animals) were replaced isocalorically by sucrose (36%) in the water to control animals for 30 days. After the respective experimental periods, the rats were killed by decapitation, blood was collected, and sera separated and stored at −20 °C until assayed for hormones. The testes and accessory sex organs were removed and weighed.

Chemicals

Collagenase (type I), medium-199, minimum essential medium (MEM), dehydroepiandrosterone (DHEA), androstenedione, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), glucose-6-phosphate, 6-phosphogluconate, isocitric acid, L-malate and lipid standards were purchased from Sigma Chemical Company, USA. All other chemicals (AR) were purchased from local companies. The ethanol estimation kit was purchased from Sigma Diagnostics, USA.

Isolation and identification of Leydig cells

Isolation: The Leydig cells were isolated from the decapsulated testis by incubating with collagenase (Sigma Type-I) in medium-199 following the method of Dufau and Catt [17]. Briefly, the decapsulated testicular tissue was incubated in a sterile polypropylene tube containing 7 m*l* medium-199 supplemented with HEPES (0.1 mg/m*l*), collagenase (0.35 mg/m*l*) and BSA (1 mg/m*l*). The tubes were gassed with 95% O_2 and 5% CO_2 and incubation was preformed by shaking the tubes in a vertical axis in a thermostated shaker water bath (90 stroke/min) at 37 °C for 15 to 20 min and 15 m*l* of medium-199 without collagenase and BSA was added. The supernatant was then filtered through nylon gauze into fresh sterile polypropylene tubes and centrifuged at 100 × g for 10 min. The procedure was standardized to get minimal contamination. The Leydig cell sediment was resuspended in MEM and assessed for contamination.

Identification: Leydig cells were identified by histochemical localization of 3β-HSD staining [18].

Briefly, 1 ml Leydig cell suspension was incubated with 1 ml NAD (8 mg/ml), 0.1 ml nitro blue tetrazolium (NBT) (2 mg/ml) and 0.1 ml DHEA (1 mg/ml) at 37 °C for 60 min in a shaking incubator under an atmosphere of 95% O_2 and 5% CO_2. DHEA was omitted from the control samples. After incubation the cells were evaluated by light microscopy for the presence of dark blue formazen deposits. The cells were counted in a haemocytometer. The dense blue granules were considered to be Leydig cells staining the 3β-HSD enzyme system. The purity of the Leydig cells obtained was 84 ± 4%.

Assessment of Leydig cell viability

The viability of the separated Leydig cell was assessed by trypan blue exclusion test, which was performed by adding 0.1 ml of 0.4% trypan blue solution to 0.1 ml of Leydig cell suspension (5×10^3), containing 0.1% BSA. The cells were exposed to trypan blue for 5 min at room temperature. Leydig cells that are not stained were considered as viable cells and the % viability was determined by the haemocytometer count. The viability was usually 95 ± 2%.

Estimation of blood ethanol concentration

Blood was collected from the orbital vein plexus before and after the administration of ethanol. Ethanol was estimated by means of the Sigma Diagnostic procedure No. 332 UV.

Hormone assays

Serum hormones were measured by radioimmunoassay (RIA). Serum LH was assayed with materials obtained from the National Institute of Arthritis, Diabetes, Digestive and Kidney diseases (NIADDK), National Hormone and Pituitary Programme, Baltimore, Maryland, USA. It was radioiodinated with carrier-free ^{125}I by chloramine-T method, and labelled LH had 32% maximum binding. The sensitivity of the assay was 0.14 ng/ml. Intra- and interassay co-efficient of variation were 4.9% and 8.4%, respectively.

For the assay of serum testosterone, (^3H)-Testosterone, the antisera and standard supplied by the World Health Organization (WHO), Geneva, were used and the WHO protocol was followed. The maximum binding was 40%, the cross reactivity of testosterone antisera with cortisol, DHT, androstenedione and Δ^5-androstenediol was 0.001%, 14%, 0.8% and 6%, respectively. The sensitivity was 0.3 pg/ml and the intra- and interassay variations were 4% and 6%, respectively.

Enzyme assays

Ten million Leydig cells were used to extract the enzyme source by the respective methods. The activities of G-6-PDH [19], 6-PGDH [20], $NADP^+$-ICDH [21], and malic enzyme [22] were assayed by the respective spectrophotometric methods.

The increase in extinction at 340 nm due to the formation of NADPH is regarded as the activity of the enzyme. The G-6-PDH assay mixture contains 45 mM triethanolamine buffer (pH 7.5), 0.67 mM Glucose-6-phosphate, 0.5 mM NADP and the enzyme source. After the addition of NADP, the increase in extinction was observed at 2 min intervals for 10 min. The coefficient of variation for this method was 6.0%. The 6-PGDH assay mixture contains 50 mM glycyl glycine buffer (pH 8.6), 20 mM $MgCl_2$, 6.5 mM cystein, 1.5 mM NADP and the enzyme extract. The increase in extinction was recorded at 2 min intervals for 12 to 20 min. The coefficient of variation for the method was 5.0%. The $NADP^+$-ICDH assay system contains 80 mM triethanolamine, 3.7 mM D-L-isocitrate, 42 mM NaCl, 0.32 mM NADP, 3.9 mM Mn^{2+} and the enzyme extract. The increase in extinction was recorded at 60 sec intervals for 3 min. The coefficient of variation for the method was 9.0%. The malic enzyme assay system contains 0.075 mM glycyl glycine buffer (pH 7.4), 0.003 mM $MnCl_2$, 0.0015 mM of L-malate, 0.135 μM NADP and enzyme source. The increase in extinction was recorded at intervals of 15 sec for 1 or 2 min and the increase between 30 and 45 sec after the start of the reaction was used to calculate the enzyme activity. The coefficient of variation for the method was 6.9%. Total protein was estimated according to the method of Lowry *et al.* [23].

Extraction and quantification of lipids

Leydig cells 10^8 in number were homogenized in a Teflon-coated homogenizer containing chloroform: methanol (2:1) [24] containing 0.01% butylated hydroxy toluene as an antioxidant was

centrifuged thrice at 3000 × g for 10 min, the supernatants were concentrated *in vacuo* in a flask rotatory vacuum evaporator (Buchi, Switzerland) at 40–45 °C and the proteolipids were broken by repeating the above process with 0.1 M NaCl [24]. Total lipids were measured with vanillin in a medium of sulphuric acid and phosphoric acid to form chromogen [25], and cholesterol quantification was based on Tachugaeff's colour reaction method [26]. Estimation of glyceride glycerol involved saponification of glycerides, oxidation of glycerol to formaldehyde chromotrophic acid colour complex [27]. Phospholipids [28] were estimated by following the reduction of phosphomolybdic acid with amino-naphtho-sulphonic acid. Neutral and phospholipid fractions were separated by thin layer chromatography on silica gel G plates, with n-hexane: diethyl ether: glacial acetic acid [29] and chloroform: methanol: 7N ammonia solvent systems [30], respectively. The coefficients of variation for assay methods were: total lipid 5.9%, cholesterol 6.4%, glyceride glycerol 6.7% and phospholipid 5.6%.

Statistical analysis

Data were statistically analysed by means of Student's *t*-test.

Results

Mean blood ethanol concentrations 0, 20, 60, 180 and 360 min after ethanol treatment were 0.43, 23.78, 18.94, 8.18 and 1.76 mM, respectively. Ethanol treatment reduced the body weight ($P<0.001$) and the absolute weight of testis ($P<0.001$), whereas there was no change in relative weight. For accessory sex organs such the seminal vesicles ($P<0.05$) and ventral prostate ($P<0.001$), the relative weights were reduced (Table 1). Serum hormones such as LH ($P<0.01$) and testosterone ($P<0.001$) levels were lowered in ethanol treated rats. The specific activities of Leydig cellular enzymes; G-6-PDH ($P<0.001$), 6-PGDH ($P<0.001$) and $NADP^+$-ICDH ($P<0.05$) were also decreased but malic enzyme did not show any appreciable change (Table 2).

Ethanol treatment reduced Leydig cellular total lipid ($P<0.05$). Among neutral lipids, esterified cholesterol ($P<0.01$) and triacyl glycerol ($P<0.001$) were decreased, but free cholesterol and diacyl glycerol ($P<0.001$) were increased. A marginal decrease was observed in total phospholipid ($P<0.05$) due to ethanol treatment, which was mainly contributed by its fractions such as phosphatidyl inositol ($P<0.05$), phosphatidyl serine ($P<0.05$), phosphatidyl choline ($P<0.001$) and phosphatidyl ethanolamine ($P<0.01$) (Table 3).

The withdrawal of ethanol treatment restored the parameters studied to the control levels.

Discussion

A significant reduction in body weight was observed in ethanol treated rats, reflecting the overall effects of ethanol on various organs [31]. The tes-

Table 1. Effects of ethanol treatment on body, testis and accessory sex organ weights

Parameters	Control	Ethanol treated	Withdrawal of ethanol
Body weight (g)	247.3 ± 6.01	206.00 ± 4.55***	247.60 ± 5.96
Testis absolute weight (g)	2.50 ± 0.02	2.16 ± 0.04***	2.49 ± 0.02
Testis relative weight (g/100g b wt)	1.04 ± 0.02	1.02 ± 0.01	1.02 ± 0.01
Accessory sex organ weights (mg/100 g body weight)			
Seminal vesicles	217.16 ± 2.36	193.30 ± 7.70*	209.50 ± 2.85
Ventral prostate	89.89 ± 1.86	72.38 ± 2.12***	89.35 ± 2.76

Each value is expressed as the mean ± SEM for 20 animals. *$P<0.05$; ***$P<0.001$; Control *vs.* Ethanol treated and Withdrawal of ethanol.

Table 2. Effects of ethanol treatment on serum hormones, Leydig cellular NADPH-generating enzymes

Parameters	Control	Ethanol treated	Withdrawal of ethanol
Serum hormones (ng/ml)			
LH	30.77 ± 2.94	12.28 ± 5.14**	29.73 ± 1.29
Testosterone	4.16 ± 0.25	2.02 ± 0.12***	3.43 ± 0.38
NADPH-generation enzymes (Units/mg protein)			
G-6-PDH	0.679 ± 0.022	0.454 ± 0.015***	0.619 ± 0.033
6-PGDH	0.911 ± 0.016	0.671 ± 0.009***	0.834 ± 0.044
$NADP^+$-ICDH	0.044 ± 0.002	0.037 ± 0.002*	0.043 ± 0.003
Malic enzyme	1.035 ± 0.02	1.039 ± 0.09	1.035 ± 0.06

Each value is expressed as the mean ± SEM for 10 estimations. *$P<0.05$, **$P<0.01$, ***$P<0.001$; Control *vs.* Ethanol treated and Withdrawal of ethanol.

Table 3. Effects of ethanol treatment on Leydig cellular lipid profiles (mg/10^9 Leydig cells)

Parameters	Control	Ethanol treated	Withdrawal of ethanol
Total lipid	5.56 ± 0.32	4.32 ± 0.34*	5.06 ± 0.62
Neutral lipids			
Total cholesterol	0.31 ± 0.02	0.32 ± 0.01	0.30 ± 0.02
Esterified cholesterol	0.16 ± 0.01	0.12 ± 0.001**	0.15 ± 0.01
Free cholesterol	0.11 ± 0.01	0.17 ± 0.01***	0.12 ± 0.01
Total glyceride glycerol	1.30 ± 0.03	1.10 ± 0.03***	1.27 ± 0.03
Monoacyl glycerol	0.29 ± 0.01	0.28 ± 0.02	0.29 ± 0.03
Diacyl glycerol	0.23 ± 0.01	0.31 ± 0.01***	0.23 ± 0.01
Triacyl glycerol	0.57 ± 0.01	0.38 ± 0.02***	0.58 ± 0.02
Phospholipids			
Total phospholipid	2.85 ± 0.25	2.12 ± 0.12*	2.68 ± 0.50
Phosphatidyl inositol	0.18 ± 0.02	0.13 ± 0.01*	0.15 ± 0.04
Phosphatidyl serine	0.11 ± 0.01	0.05 ± 0.02*	0.09 ± 0.02
Phosphatidyl choline	0.74 ± 0.05	0.42 ± 0.05***	0.82 ± 0.09
Phosphatidyl ethanolamine	0.53 ± 0.05	0.32 ± 0.03**	0.49 ± 0.06
Sphingomyelin	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.04
Cardiolipin	0.13 ± 0.02	0.09 ± 0.02	0.12 ± 0.04
Phosphatidic acid	0.20 ± 0.01	0.18 ± 0.02	0.22 ± 0.04

Each value is expressed as the mean ± SEM for 10 estimations. *$P<0.05$, **$P<0.01$, ***$P<0.001$; Control *vs.* Ethanol treated and Withdrawal of ethanol.

ticular absolute weight was reduced, but the relative weight did not change. Several other reports [32–34] are in agreement with the results of the present study and the reduced testis weight could be due to a decrease in intra-testicular fluid volume [35], altered testicular structure and atrophy [32, 36].

In agreement with the present study, other reports also showed reduced serum LH and testosterone in ethanol treated rats [1–4]. The reduced serum testosterone level may be due to defects in various parts of the hypothalamo-hypophyseal-testicular hormone axis, such as the inhibition of LHRH release [36] and thus low LH [1, 2], and at the level of the Leydig cells by reducing LH receptors [37–39] and steroidogenic enzyme [3]. In general, the reduced levels of both LH and testosterone are suggestive of hypogonadotropic hypogonadism and an impaired feed back system due to ethanol treatment.

G-6-PDH, 6-PGDH, $NADP^+$-ICDH and malic enzyme are known to provide NADPH, the essential

co-factor for steroidogenesis and lipogenesis in testis [41]. The decreased serum LH and its receptors in Leydig cells [38, 39] may be responsible for the decreased activity of NADPH generating enzymes, as LH is the principal regulating factor in Leydig cellular structure and function [41]. *In vitro* studies by Akane *et al.* [3] have suggested that ethanol (1, 5, 20 and 100 mM) has a direct inhibitory effect on Leydig cellular 3β-HSD and 17, 20 lyase. It is therefore suggested that the changes in NADPH generating enzymes could also be due to the direct effect of ethanol.

Among the NADPH generating enzymes studied, the ethanol-induced inhibition was much pronounced in HMP shunt pathway enzymes, suggesting that these enzymes are more vulnerable than the other enzymes.

Cholesterol plays a pivotal role in Leydig cells, as it is the precursor for steroids [42]. Even though the total cholesterol was unchanged, its esterified form showed a reduction and free cholesterol was found to be increased. These findings may be due to decreased shunting towards steroidogenesis and/or increased conversion of esterified cholesterol to free cholesterol. In essence, the ethanol treatment could have an adverse effect on Leydig cellular cholesterol turnover which may be due to the direct effect and/or indirect effects through impaired LH action.

The reduction in total lipid was due to decreased glyceride glycerol and phospholipids. Triacyl glycerol serves as the source of energy [15] and the ethanol-induced decrease in triacyl glycerol is suggestive of impaired availability of energy to Leydig cells. The increased diacyl glycerol may be the result of decreased conversion of diacyl glycerol to phospholipids [15]. Phospholipids and cholesterol are the main constituents of mammalian cell membrane, and the acyl chain of the phospholipids maintains the fluidity of the same [43]. An increase in (synaptosomal) membrane cholesterol with unaltered phospholipids has been reported due to ethanol treatment [43], but in our study we have observed a significant reduction in total phospholipid with unaltered levels of total cholesterol. Nevertheless, the cholesterol: phospholipid ratio was increased in both the studies.

The increased cholesterol: phospholipid ratio observed in the present study may alter the membrane characteristics and functions. Since receptors for peptide hormones such as LH, prolactin and insulin are on the plasma membrane, changes in membrane characteristics may ultimately modify their binding due to ethanol treatment. Analysis of membrane phospholipids would provide a better picture in this regard.

Withdrawal of ethanol showed replenishment of depleted LH receptors as well as plasma testosterone [34, 37, 38]. In the present study, withdrawal of treatment for 30 days brought back the parameters studied to normal, showing that the effects of ethanol on the parameters studied are transient and reversible.

From the above observations, it is inferred that NADPH generation is depleted by ethanol treatment through LH, which ultimately results in reduced serum testosterone. In addition, a correlation does exist between impaired NADPH generation and Leydig cellular lipid profiles which registered a parallel decrease. It is concluded that the reduction of NADPH generation could be one of the vital factors and the biochemical mechanism responsible for the ethanol-induced changes in the functional integrity of Leydig cells.

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