Effects of Ethanol Treatment on Epididymal Secretory Products and Sperm Maturation in Albino Rats

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ABSTRACT

Alcoholics are often associated with fertility disturbances with low sperm count and impaired sperm motility. Spermatozoa attains forward motility and fertilizing capacity during their transit through the epididymis. Epididymal secretory products form a suitable microenvironment, which favors sperm maturation. To study the effects of ethanol on epididymal sperm maturation, ethanol (3 g/kg body weight as 25%, v/v) was given by gastric intubation twice daily for 30 days, and in another group, rats given treatment for 30 days were withdrawn of treatment for a further period of 30 days to assess the reversibility of ethanol-induced changes. Serum and epididymidal testoster-

tone and dihydrotestosterone (DHT), epididymal tissue and sperm carnitine, acetyl carnitine, glycercyolphosphoryl choline (GPC), and sialic acid were studied along with epididymidal sperm count and cauda epididymidal sperm motility. Ethanol treatment significantly reduced the epididymal tissue/sperm carnitine, acetyl carnitine, GPC, and sialic acid, suggesting its adverse effect on these secretory products. Impaired cauda epididymidal sperm motility and fertility (in vivo) of ethanol-treated rats imply the defective sperm maturation. All these changes were reverted back to normalcy after withdrawal of ethanol treatment, indicating the transient effects of ethanol. In conclusion, it is evident that ethanol has an adverse effect on sperm maturation, which may be affected due to the decrease in serum/epididymidal testosterone and DHT level and epididymal secretory products.

Chronic alcoholics are often associated with impotence, loss of libido, premature or delayed ejaculation, sterility, testicular atrophy and gynecomastia (Boyden and Pamenter, 1983). Both clinical and experimental studies have shown the hampering effects of ethanol on the hypothalamohypophysial-utesticular axis (Valimaki and Ylikahri, 1985). Studies on semen samples of alcoholics have shown decreased sperm count, impaired forward motility of morphologically normal spermatozoa and increased number of teratooospermia (Kucheria et al., 1985; Gomathi et al., 1993; Villalta et al., 1997), which is indicative of impaired epididymal sperm maturation. However, little is known of the impact of ethanol toxicity on epididymal sperm maturation.

Spermatozoa acquire forward motility and fertilizing capacity during their transit through epididymis (Bedford, 1975). Epididymis is the major source of seminal carnitine other than accessory sex organs (Brooks et al., 1974). Carnitine is involved in the mitochondrial transport and β-oxidation of fatty acids (Bremer, 1983). A positive correlation among seminal carnitine with sperm motility, number of motile spermatozoa and fertility rate has been reported (Carter et al., 1980; Menchini-Fabris et al., 1984). Acetyl carnitine serves as a readily accessible energy pool for use in both activation of respiration and motility in mammalian spermatozoa (Harrison, 1977; Hinton et al., 1981). Fatty acids released during glycercyolphosphoryl choline (GPC) synthesis have been proposed to be utilized by spermatozoa as energy source (Scott et al., 1963). Seminal GPC is considered as an index of epididymal secretory activity (Riar et al., 1977) and suggested to involve in maintaining the osmolarity of epididymal luminal fluid (Wales et al., 1966), serve to stabilize spermatozoal membrane (Scott et al., 1963) and play a role in the metabolism of spermatozoa after capacitation (Mitra and Chowdhury, 1994). Sialic acid, another secretory product of epididymis, is bound to the sperm surface as terminal sugars of sialoglycoprotein (Toowicharanont and Chulavatnatol, 1983) during their sojourn through epididy-
mis. Sperm sialic acid contributes net negative surface charge (Yanagimachi et al., 1972), acts as an immunoprotectant (Toshimori et al., 1991) and plays an important role in binding and fertilizing eggs (Lambert and Le, 1984). Because these secretory products form a specific microenvironment, which is essential for sperm maturation, studies on these biochemical parameters were considered to

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ABBREVIATIONS: GPC, glycercyolphosphoryl choline; DHT, dihydrotestosterone; CAT, carnitine acetyl transferase; MDH, malate dehydrogenase; CS, citrate synthase; PEP, phosphoenol pyruvate; LDH, lactate dehydrogenase; CK, choline kinase.
explore the biochemical mechanism or mechanisms by which ethanol induces defective sperm maturation.

**Materials and Methods**

**Animals.** Healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 200 to 225 g body weight were used. Rats were kept in clean cages in a temperature-controlled room with 12-hr light/dark schedule. Animals were divided into the following groups. Group I included controls (rats received an isocaloric quantity of sucrose in the same volume as experimental rats that received ethanol). Group II included ethanol treated (ethanol was administered twice daily at regular intervals by gastric intubation at a dose of 3 g/kg body weight as 25% w/v aqueous solution for 30 days). Group III includes withdrawal of ethanol treatment (rats treated with ethanol as group II for 30 days were withdrawn of treatment for a further period of 30 days to assess the reversibility of ethanol effect).

Each group consisted of 30 rats. Parallel control groups were maintained for ethanol-treated and ethanol-withdrawn groups. Because there was no significant difference among the controls of ethanol-treated and ethanol-withdrawn groups, a common control group was considered. Body weight was recorded at regular intervals (weekly twice), and the volume of ethanol intubated was adjusted accordingly. Animals were pair-fed by providing the same quantity of pelleted rat feed to control animals (Lipton India Ltd., India) as corresponding experimental animals had consumed the previous day. In addition, the calorie value derived from ethanol (to the experimental animals) was replaced isocalorically by sucrose to control animals. After 15 hr of the respective experimental period, rats were killed by decapitation. Blood was collected, and serum was separated and stored at −20°C until assayed for hormones. Epididymides were dissected out, freed from adhering fat and connective tissues and used.

**Serum and Epididymidal Tissue Hormones.** Diluted serum/homogenized epididymal tissue sample (in distilled water) was vortexed with diethyl ether (twice). Ether extract was collected and allowed to evaporate completely. Residue was dissolved in Tris buffer and used for the estimation of testosterone and dihydrotestosterone (DHT), as described in the procedure enclosed with the kit purchased from Amersham International Plc. (UK).

**Cauda Sperm Forward Motility.** After anesthetizing the rat, epididymis was exposed by scrotal incision, and spermatozoa were expressed out by cutting the distal end of the cauda epididymidal tubule. Spermatozoa with epididymal fluid was diluted with physiological saline and placed on a thin glass slide, and forward motility (rate and percentage) of 100 spermatozoa/rat was observed under microscope using precalibrated ocular micrometer.

**Separation of Epididymyal Sperm and Tissue.** Epididymal spermatozoa were separated as per the method of Brooks (1976) by cutting the epididymal segments approximately into 1.0-mm³ pieces, with a sharp razor blade in Kreb's Ringer's phosphate (KRP) buffer (pH 7.4), after dividing into caput, corpus and cauda regions as per the guidelines of Hamilton (1975). Spermatozoa from the epididymidal pieces were completely removed by vortexing gently in KRP buffer, and the suspension was allowed to settle for 5 min. Spermatozoa released in the buffer were aspirated, centrifuged at 800 × g for 15 min and used for biochemical estimations. All these manipulations were done at 4°C.

**Sperm Count.** Spermatozoa were counted as per the method of Zaneveld and Polakoski (1977). Sperm suspension was placed on both sides of Neubauer's hemocytometer and allowed to settle by keeping in a humid chamber (wet) for 1 hr. The number of spermatozoa in the appropriate squares of the haemocytometer was counted under the microscope at 100× magnification.

**Estimation of Carnitine, Acetyl Carnitine, GPC, and Sialic Acid.** Carnitine and acetyl carnitine were estimated as per the method of Pearson et al. (1974).

Carnitine assay mixture contained Tris (100 mM), DTNB (0.125 mM), acetyl coenzyme A (0.15 mM), EDTA (1.25 mM), sample extract and carnitine acetyl transferase (CAT) (6 mU). Concentration of carnitine was calculated using extinction difference (at 412 nm) noted before and after the addition of CAT as described in the procedure.

Acetyl carnitine assay mixture contained Tris (100 mM), L-malate (10 mM), EDTA (1.25 mM), NAD (0.5 mM), coenzyme A (0.125 mM), sample extract, malate dehydrogenase (MDH) (55 mU), citrate synthase (CS) (14 mU) and CAT (6 mU). Concentration of acetyl carnitine was calculated using the extinction differences (at 340 nm) noted before and after the addition of MDH, CS and CAT.

GPC was estimated as per the method of Wirthensohn and Guder (1985) at 340 nm. Assay mixture contained phosphoenol pyruvate (PEP) (0.75 mM), Mg²⁺ (6.2 mM), NADH (0.18 mM), ATP (0.4 mM), lactate dehydrogenase (LDH) (2.0 kU), pyruvate kinase (PK) (1.8 kU), sample extract and choline kinase (CK) (16.4 U). GPC concentration was calculated using the absorbance difference noted before and after the addition of choline kinase as described in the procedure.

Sialic acid was estimated as per the method of Warren (1959). Assay mixture contained 0.2 M sodium metaperiodate in 9 M phosphoric acid, 10% sodium arsenite in 0.5 M sodium sulfate-0.1 N H₂SO₄ and 0.6% thiobarbituric acid in 0.5 M sodium sulfate. Sialic acid concentration was calculated by comparing the standard graph constructed.

**In Vivo Fertility Assessment.** In different set of experiment, two proved fertile proestrus female rats were cohabitated with one male rat and observed for the presence of spermatozoa in the vaginal smear or vaginal plug to confirm mating. Five batches of experiments were conducted and, each batch consisted of five male rats (control/ethanol-treated/ethanol treatment withdrawn). Number of rats that became pregnant were noted and isolated until parturition. The number of rats that became pregnant and litter size (number of pups delivered) were taken into consideration to assess the fertility.
and fecundity of male rats, respectively. Results are also expressed in terms of percentage change in experimental (ethanol-treated or ethanol treatment withdrawn) rats compared with control.

Chemicals. Ethanol (99.9%) was purchased from Riedel-de-Haen, Germany. Fine chemicals like substrates, enzymes, coenzymes, standards and ethanol estimation kit were purchased from Sigma Chemical (St. Louis, MO). Other chemicals and reagents were from Merck and Sisco Research Laboratories, India.

Statistical Analysis. Data were analysed statistically using one-way analysis of variance (ANOVA) (Zar, 1974). When F test was found significant ($P < .05$), the data were further subjected to the Student-Newman-Keul (SNK) test.

**Results**

Structural and functional integrity of epididymis is governed by androgens, derived from peripheral circulation and testicular fluid. Evaluation of serum and epididymal testosterone and DHT provides information on the effects of ethanol on androgen profiles in circulation and at tissue level. Ethanol administration caused a marked fall in both serum (Fig. 1) and epididymal (caput, corpus, cauda) tissue (Fig. 2) testosterone and DHT concentration. These changes were reverted to normal after withdrawal of ethanol treatment.

Sperm count and motility are indices of spermatogenesis and sperm maturation, respectively. Assessment of these parameters provides evidence on the effects of ethanol on male fertility and fecundity. Sperm content in caput, corpus and cauda epididymides was declined due to ethanol treatment. After withdrawal of ethanol treatment, caput sperm content restored only partially, whereas in corpus and cauda, the same was brought back to control level (Fig. 3). The rate of forward motility of morphologically normal spermatozoa and the percentage of forward motile spermatozoa of cauda epididymidis were decreased significantly in ethanol-treated rats. After withdrawal of ethanol treatment, these changes were reverted back to normalcy (Fig. 3). Ethanol treatment decreased the number of rats became pregnant (60%) and number of pups delivered (50%). Withdrawal of ethanol treatment restored fertility and fecundity to normalcy (Fig. 4).

Epididymal secretory products such as carnitine, acetyl carnitine, GPC and sialic acid play an indispensable role in the maturation of spermatozoa. Analysis of epididymal tissue and sperm carnitine, acetyl carnitine, GPC and sialic acid were made to provide an insight on the impact of ethanol toxicity on epididymal synthesis and secretion of these products and on sperm maturation. A marked depletion in carnitine (Fig. 5), acetyl carnitine (Fig. 6) and GPC (Fig. 7) levels was observed in tissues of caput, corpus and cauda epididymides. Carnitine and acetyl carnitine concentration of spermatozoa obtained from these regions also showed a similar trend, whereas GPC was decreased only in corpus and cauda epididymidal spermatozoa. Withdrawal of ethanol treatment restored fertility and fecundity to normalcy (Fig. 4).

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**Fig. 2.** Effects of ethanol treatment on the epididymal testosterone and dihydrotestosterone (DHT). Each value is mean $\pm$ S.E.M. of 10 (caput, cauda) and 5 (corpus) estimations. $^a P < .05$ versus control; $^b P < .05$ versus ethanol treated.

**Fig. 3.** Effects of ethanol treatment on the epididymal sperm content and cauda sperm motility. Each value is mean $\pm$ S.E.M. of 20 animals. $^a P < .05$ versus control; $^b P < .05$ versus ethanol treated.

**Fig. 4.**
The data on serum testosterone suggest the suppressive effect of ethanol and is consistent with the earlier reports (van Thiel and Gavaler, 1990). Epididymis derives testosterone from circulation and testicular fluid (Robaire and Hermo, 1988). The low epididymal testosterone could be due to the decreased serum and testicular (Adams et al., 1991) testosterone concentration. The decrease in serum and epididymal DHT may be due to low testosterone available for 5\(\alpha\)-reduction and increased conversion of DHT to 5\(\alpha\)-androsten-3\(\alpha\),17\(\beta\)-diol and 5\(\alpha\)-androstan-3\(\beta\),17\(\beta\)-diol, as reported in liver (Murono and Fisher-Simpson, 1985; Chung, 1990) and in Leydig cells (Murono and Fisher-Simpson, 1984).

The reduced sperm content in caput, corpus and cauda epididymides of ethanol-treated rats implies an adverse effect of ethanol on spermatogenesis. In support of this, a number of clinical and experimental studies have shown impaired spermatogenesis under chronic ethanol consumption (Willis et al., 1983; Haider et al., 1985). Anderson et al. (1983) also observed low sperm content in cauda epididymis of ethanol (5% for 20 weeks and 6% for 5 weeks)-consumed rats. After withdrawal of ethanol treatment, caput spermatozoa content was not completely restored to control level. The reversibility of caput sperm content after withdrawal of ethanol treatment for a period of 60 days (data not included) suggests that 30 days of withdrawal period is insufficient for resumption of spermatogenesis to normalcy.

Impaired sperm motility in ethanol-treated rats is indicative of a defect in the acquisition or maintenance of motility. Previous report from our laboratory and other’s have shown impaired forward motility of human spermatozoa (Kucheria et al., 1985; Gomathi et al., 1993). Anderson et al. (1983) reported a similar decrease in average sperm velocity in ethanol-consumed C57B1/6J mice. After spermatozoa leave the testis in an immature state, they undergo maturational changes and acquire the potential for progressive motility during passage through the caput and corpus epididymides (Bedford, 1975). Epididymal secretory products furnish a microenvironment that is considered vital for acquisition of motility and viability of sperm.

In the present study, mating was confirmed by the presence of vaginal plug or spermatozoa in the vaginal smear. The data revealed that only 40% of the proestrus female rats were not mated successfully. The failure of mating may be due to the adverse impact of chronic ethanol ingestion on sexual behavior of male rats. The impaired forward motility, altered epididymal milieu due to modified composition of secretory products, leading to defective sperm maturation, may contribute to the reduced fertility in ethanol-treated rats.

The reduced concentration of carnitine in epididymidal tissues may be due to the low uptake of carnitine from circulation (James et al., 1981) or reduced concentrating capacity of epididymal epithelial cells (Brooks, 1980), which may...
be secondary to the consequence of low level of androgens (Marquis and Fritz, 1965) induced by ethanol treatment. At the epididymal level, spermatozoa acetylates carnitine by carnitine acetyltransferase (Marquis and Fritz, 1965; Jeulin et al., 1994). The low level of acetyl carnitine in both epididymal tissue and sperm may be due to the low availability of carnitine. Accumulation of carnitine in the epididymis (Hinton et al., 1981; Lenzi et al., 1992) and secretion of acetyl carnitine in epididymal fluid (Hinton et al., 1979) are shown to be associated with the acquisition of flagellar movement and sperm motility. The low levels of carnitine and acetyl carnitine in spermatozoa may be responsible for the impaired motility and thus fertility of ethanol-treated rats. After withdrawal of ethanol treatment, although carnitine and acetyl carnitine levels were restored to normal in the caput epididymal tissue, the effect was partial in caput spermatozoa. This may probably be due to the reduced uptake of carnitine from luminal fluid by spermatozoa and its subsequent acetylation to acetyl carnitine or due to enhanced use of carnitine in β-oxidation.

In control rats, the concentration of GPC is higher in the tissue and sperm of caput and corpus regions than in cauda, and this finding agrees with previous studies (Hoffmann and Killian, 1981; Wang et al., 1981). Atreja and Anand (1985) reported that the activities of lysophospholipase and phospholipase enzymes involved in the synthesis of GPC are more active in spermatozoa from caput than in cauda epididymidis of goat. The decrease in tissue GPC concentration after ethanol treatment may be due to the impaired synthesis of the same. Because the synthesis and secretion of GPC are under androgenic control (Dawson and Rowlands, 1959; Brooks et al., 1974), the decrease in epididymal testosterone and DHT is attributed to the decreased tissue GPC concentration. Further, the activity of phospholipase A₂, which is responsible for the first hydrolytic step in the conversion of lecithin to GPC, also is androgen dependent (Beck, 1980). The normal flow of fluid and sperm concentration have been suggested to promote the synthesis of GPC (Wang et al., 1981). Therefore, in the present study, the fall in sperm concentration observed after ethanol treatment may also be correlated to the reduced concentration of GPC. Because GPC also plays a role in maintaining epididymal luminal fluid osmolarity (Wales et al., 1966) as well as in stabilizing spermatozoal membrane (Scott et al., 1963), the low level of GPC observed after ethanol treatment may have an adverse effect in creating the peculiar epididymal milieu conducive for sperm maturation. The changes in GPC levels are transient as they are reversed to normalcy after withdrawal of ethanol treatment.

In control, a maximum level of sialic acid was noticed in corpus tissue and a fall in cauda epididymidis occurred whereas in sperm, an increase in sialic acid was observed from caput to cauda epididymidis. After ethanol treatment, the low levels of sialic acid observed in caput, corpus and cauda epididymidal tissues suggest an impairment in sialic acid synthesis. The low levels of sialic acid in spermatozoa obtained from all the three regions of epididymis of ethanol-treated rats indicate the reduced adsorbance of sialic acid from the luminal fluid. Sperm sialic acid contributes net negative surface charge (Yanagimachi et al., 1972; Nicolson et al., 1977) acts as an
immunoprotectant (Toshimori et al., 1991; Lassalle and Testart, 1994) and plays an important role in binding and fertilizing eggs (Lambert and Le, 1984). The impaired fertility of ethanol treated rats may partly be due to low levels of GPC and sialic acid and the associated changes in sperm maturation. The reversal of sialic acid to normal level in both tissue and sperm after withdrawal of ethanol treatment suggests that the toxic effect of ethanol is transient.

The impaired synthesis or uptake of a number of secretory products such as carnitine, acetyl carnitine, GPC and sialic acid as well as defective sperm motility is a clear manifestation of altered epididymal milieu and impaired sperm maturation, which may be a plausible reason for the diminished fertility of ethanol-treated rats. Further, few studies have shown the presence of ethanol in epididymis (Salonen and Eriksson, 1989), suggesting the possible direct action of ethanol on sperm motility. Supportive evidence can be drawn from the study of von Deeperman and Hinckers (1966), who have shown that ethanol (21.7 mmol/l) exposure to human spermatozoa in vitro decreased the percentage of motile spermatozoa and their average speed.

Apart from the indirect effects of ethanol on epididymal testosterone, DHT concentration and secretory products, the direct action of ethanol on epididymal tissue/spermatozoa could also be the reason for the impaired sperm maturation events.

References


