Comparative study on apoptosis in the testes of normal and alcoholic rats

Yalin Bamac, *DVM,* ***Tuncay Colak,*** *PhD,* ***Belgin Bamac,*** *PhD,*
Cengiz Ercin, *MD,* ***Baris Ozturk,*** *MD,* ***Aydin Ozbek,*** *MD.*

ABSTRACT

Objectives: To investigate the tissue injury associated with long-term alcohol consumption in male gonads. To this end, apoptotic testicle tissues of alcoholic rats were compared with the testicle tissues of the control rats.

Methods: This study was conducted in the Department of Anatomy and Division of Pathology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey during the period 2002 to 2003. We used Sprague-Dawley rats as the subject material in the investigation of apoptosis. We divided the rats into 2 groups: alcoholic rats and the control group, with 10 adult male rats in each group. We housed the subjects in each group under controlled temperature (22 ± 3^0C) and humidity (62 ± 7%) and lighting (12 hours darkness and 12 hours daylight per day). We fed the rats in the alcoholic group by ethanol in liquid diet for 12 weeks while the control rats received the normal isocaloric diet. We fixed the testicle tissues of both groups by perfusion of 10% formaldehyde through left ventricle and then removed. We further fixed the tissues in formaldehyde solution for at least 2 days. After dehydration by ethanol, we embedded the tissue in paraffin and used serial paraffin sections (5 µ thickness) for immunohistochemistry. We used Caspase-3 Ab-4 (CPP32) antibody to identify caspase reaction in apoptotic regions. Hence, we observed the stained sections and photographed the apoptotic seminiferous tubules (ST). For comparison of apoptosis in the alcoholic group and control group, we counted the apoptotic germ cells in x 400 magnification under light microscope.

Results: The diameters of seminiferous tubules were measured using light microscope with micrometre. The findings were compared by computer and significant differences in apoptosis between 2 groups (p<0.01) were detected.

Conclusion: Apoptosis is significantly induced in ethanol treated rat related to the overuse of ethanol. The findings indicate the tissue injury of testicles associated with alcohol consumption.

Saudi Med J 2005; Vol. 26 (6): 928-933

It is well-known that chronic alcohol abuse produces sexual dysfunction and impairs sperm production in both humans and animals.[1] Excessive alcohol consumption over long periods of time results in severe cell damages leading to cell death. Cell death occurs via 2 distinct mechanisms: necrosis and apoptosis. Necrosis occurs when exposure to a noxious stimulus such as alcohol causes the loss of the cell's metabolic functions and damage to the cell membrane. In apoptosis, the cell actively participates in the cell death processes by activating a cascade of biomechanical reactions that ultimately lead to cell shrinkage and fragmentation of the nucleus.[2] In any organ, both acute and chronic alcohol exposure induces cell apoptosis. This is also true for the testicular germ cell.[3] Within the testicle, apoptosis of germ cells occurs under physiologic conditions and serves to prevent over production of normal germ cells and maturation of abnormal germ cells.[4] Under pathologic conditions

From the Department of Anatomy (Bamac Y, Colak, Bamac B, Ozturk, Ozbek) and the Department of Pathology (Ercin), School of Medicine, Kocaeli University, Kocaeli, *Turkey.*

Received 24th November 2004. Accepted for publication in final form 26th March 2005.

Address correspondence and reprint request to: Dr. Tuncay Colak, Department of Anatomy, School of Medicine, Kocaeli University, 41900, Derince, Kocaeli, *Turkey.* Tel. +90 (262) 2335980. Fax. +90 (262) 3223041. E-mail: tuncay_colak@mynet.com

such as an exposure to certain environmental toxins, a greater portion of germ cells degenerate through the process of apoptosis leading to impaired spermatogenesis and infertility.[4] In this study, a rat model of chronic alcohol consumption induced testicular injury has been investigated to analyze the apoptosis mechanism in the testicle tissues.

Methods. ***Animal raising stage.*** This study was conducted in the Department of Anatomy and Division of Pathology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey during the period 2002 to 2003. The subjects of the study were 20 adult Sprague-Dawley rats, reproduced in the laboratories of Kocaeli University Medical Faculty. The rats were divided into 2 groups: 10 alcoholic rats and 10 control rats. The rats in the control group were raised in a room under the temperatures of 22 ± 3^{0}C and relative humidity of 62 ± 7%. Within this process, the room was illuminated for 12 hours (8:00 am to 8:00 pm) and darkened for 12 hours.[5] Alcoholic rats (the study group, n=10) were raised in 2 different cages with 5 rats in each. They were provided with the same type of diets at the same amounts as those of the control group. The only difference in the diet of this group was the alteration in the liquid diet in order to create alcohol dependency. To render the rats in the study group chronic alcoholics, alcohol was added to the liquid diet at a rate of 5%, which was gradually increased to 20% in 12 weeks[6] (**Table 1**).

Determination of blood ethanol volume. At the end of 12th week, 0.5 ml blood samples were obtained (intracardiac) from each rat in the study group while under light ether anesthesia in order to determine plasma alcohol rate. In the light of Technicon opera analysis report, the result revealed that the plasma-alcohol level of alcoholic rats was 49 mg/dl. This value was sufficient to signify the rats in the study group as 'alcoholics'.[7]

Perfusion and tissue fixation stage. The rats were anesthetized in a specially designed glass container (covered with a lid) of 35 cm in diameter which held an ether embedded cotton ball laid on the floor of a special compartment before the rats were subjected to perfusion in order to remove the tissues. To inflict vascular perfusion, a specially designed perfusion model was prepared. The model involved one serum stand, 2 of 0.9% isotonic NaCl solution, 4 liters of 10% formaldehyde solution, 4 serum sets, wire rack for dissections, 10% formaldehyde containers for tissue storage, operation knife, scissors, zephiran solution for instrument sterilization, ether, and a tube with a large opening containing the cotton ball soaked in ether. This method aimed to provide the circulation of the solution infused through left ventricle throughout the entire body and elimination of the recycling (replacement) blood through the right atrium. Thus, by the use of systemic circulation, the isotonic solution could replace the blood in the system. At 5 minutes of formaldehyde infusion, the entire set was turned off. All the tissues contained formaldehyde. A median incision was inflicted on the abdominal cavity, and the pelvic cavity was completely exposed after the displacement of intestines. The testicle was pulled as far as the abdominal cavity, and the funiculus spermaticus was released. Finally, the funiculus spermaticus was cut, and the testicle was removed out of the body. After each testicle was removed by the same method, all were stored in the containers with 10% formaldehyde. The same perfusion process was performed on the rats of the control group.

Preparation of tissues. The testicles of both groups were cleansed with an isotonic solution to eliminate formaldehyde. The testis tissues of both groups were fixed by perfusion of 10% formaldehyde through left ventricle and then removed. These tissues were further fixed in formaldehyde solution for at least 2 days. The cleansed tissue was ready for staining. After dehydration by ethanol, they were embedded in paraffin, and serial paraffin sections (5-µ thickness) were used for immunohistochemistry. Caspase-3 Ab-4 (CPP32) antibody was used to identify the caspase reaction in apoptotic regions. Finally, the tissue samples were immunohistochemically dyed.

Immunohistochemical staining stage. Caspase-3 Ab-4 CPP 32 anticore (1/100 diluted) was dropped onto the tissue samples.[7] Caspase-3 Ab-4 CPP32 is a polyclonal anticore of rabbit origin. It is used in immunochemical staining (formalin/paraffin), Western Blotting, and immunoprecipitation of neomarkers. The cellular localization of this anticore mostly involves some nuclear staining and cytoplasm in particular (Neomarkers Data Sheet Rev. 110602H, USA).

Caspases are one of the 3 main components arising from apoptotic reaction. The other 2 components are proteins from Bcl-2 family and Apaf-1/CED-4 proteins. All apoptotic caspases are already present in normal cells in the form of inactive enzymes as analogs of zymogens acting in blood clotting. Whenever the cell tends to be apoptotic, the enzymes are activated by some peptides developing through a 1-2 stage proteolytic mechanism.[8] The anticore used in this study (Caspase-3 Ab-4), reacts to caspases activated in an apoptotic cell, and the cells are stained in the perinuclear or nuclear areas, indicating caspase + cells. After Caspase-3 Ab-4 CPP32 application, the tissue samples were kept for one hour before being washed in distilled water and placed into tris buffer solution for 5 minutes. Each preparation was applied one drop of the chromogen obtained. The chromogen remained on the tissue for 10 minutes,

during which the staining of the tissue was apparent. This indicated reaction of the anticore. Following chromogen application, the preparations were recleansed in distilled water. The preparations were then contrasted dyed with Mayer-hematoxylin-eosin for 3 minutes and washed in distilled water. The tissue was ready for microscopic evaluation.

Evaluation and photographing of preparations. The preparations were studied under Olympus-Optica model (U-DO) micrometer light microscope for Caspase-3 reaction. Apoptotic tissues were identified, and apoptotic and non-apoptotic cells were photographed with Nikon coolpix 995 model digital camera at x 200 and x 400 magnification (**Figures 1- 3**).

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethics committee of our faculty. The results, presented as means ± SD differences between the groups, were calculated using a non-parametric test for independent samples (Mann Whitney-U). The Statistical Package for Social Sciences for personal computer was used for the statisticalanalyses.The*p* value of <0.05 was considered significant.

Results. To collect data for statistical evaluation, seminiferous tubule diameters of both groups were measured with a micrometer microscope at x 100 magnification. The diameters of randomly selected 20 seminiferous tubules per 10 preparations of each group were measured, and a rough table was prepared. A total of 200 measurements were obtained (at x 100 magnification, each 100 unit of a micrometer corresponds to 1 mm) (a value of 30 will correspond to 0.3 mm = 300 µm). The mean diameter of seminiferous tubules was lower in the alcoholic group than that in the control group (**Table 2**), (p<0.005). In randomly selected 10 areas of each preparation, apoptotic cell count was performed under light microscope at x 400 magnification.[9] Positive reaction indicating apoptosis is characterized with brown/black staining

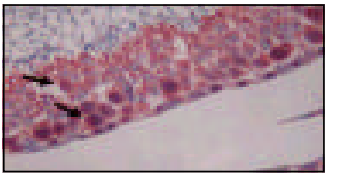

Figure 1 - Arrows show a series of apoptotic germ cells in preparation number 2 from alcoholic rat group at x 400 magnification.

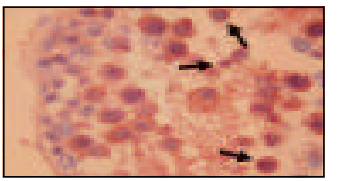

Figure 2 - The photo of preparation number 10 from alcoholic rat group at x 200 magnification. Apoptotic germ cells are pointed by arrows.

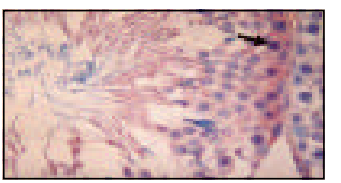

Figure 3 - Preparation number 8 from the control group at x 400 magnification. Apoptotic cells are not observed; thus, a marked staining of germ cells by Caspase is not observed either.

Table 1 - The ratio of alcohol in the liquid diet between 0-12 weeks.

The week of liquid diet	The ratio of alcohol in the liquid diet %
Between 0 - 2 weeks	5
Between 2 - 4 weeks	7.5
Between 4 - 6 weeks	10
Between 6 - 8 weeks	12.5
Between 8 - 10 weeks	15
Between 10 - 12 weeks	20

Table 2 - The mean semiferous tubule (ST) diameters, mean apoptotic germ cell counts, the *p* values and Wilcoxon vales of alcoholic and control group rats in comparison

Mean	Alcoholic	Controls	*p* value	Wilcoxon
ST diameters (µm)	254.2	316.6	0.001	56.0
Apoptotic germ cell count	97.56	36	0.000	67.5

in the perinuclear and nuclear areas of the cell. For quantification of this kind of cells, 10 randomly selected areas were studies at x 400 magnification and a total of 1000 germ cells were counted (apoptotic and non-apoptotic).[7,10] The nuclear and perinuclear areas of the cells considered apoptotics plus resulted in brown/black staining. Thus, the cells resembling the germ cells indicated with arrows on the figures were counted (**Figures 1-3**). The apoptotic cell count was recorded per 1000 cells. Thus, 1000 cells per 10 preparations from each group (n=20) were counted and numerical values of apoptotic and non-apoptotic germ cells were acquired (**Table 2**). In this study, apoptotic cell evaluations were conducted on the basis of germ cells only. Apoptosis in Leydig cells or Sertoli cells was excluded. The apoptotic germ cell count of the alcoholic group was higher than that of the control group (**Table 2**) ($p<0.005$). Similarly, the percentage of apoptotic cells was higher in the alcoholic group. In the germ cell count, the rate of apoptotic cell to the total germ count was calculated in percentages. The apoptotic cell ratio was 3.6% of the total germ count for the control group and 9.8% of the total germ count for the alcoholic group, which indicates that apoptotic cell count of the control group was lower than that of the alcoholic group. Our results revealed that chronic alcohol use induces apoptosis in germ cells of male gonads; thus, leading to significantly increased percentage of apoptotic germ cells.

Discussion. Research on animals has consistently demonstrated an association between both acute and chronic alcohol consumption and low testosterone.[3] Numerous studies have shown that ethanol exposure impairs spermatogenesis, resulting in testicular atrophy and infertility.[11] In the study of Eamnuelle and Eamnuelle[4] on alcohol and the male reproductive system, the effects of alcohol consumption on male reproductive system have been discussed. The study claims that alcohol consumption affects all 3 components of hipotalamo-hipofizyo-gonadal axis (HPG) that can be considered a common system of male reproductive system hormones and endocrine glands. Furthermore, the study indicates a condition characterized by low testosterone and reproduction hormone secretion associated with alcohol. Chronic alcohol use in rats has led to profound effects on the reproduction and insemination capacity.[3] In our study, the destructive effects of chronic use of alcohol on rat testicles were confirmed through higher counts of apoptotic germ cells and smaller seminiferous tubule diameters in alcoholic rats (the study group). Rats were subjects' choice of subjects for this study because their reproduction system and patterns mimic those of humans. Moreover, earlier studies have shown that both acute and chronic alcohol use are associated with low hypothalamic luteinizing hormone-releasing hormone (LHRH) and hypophysial luteinizing hormone (LH) levels in adult and pubertal rats. Subsequent studies have asserted inhibition of testosterone secretion in testicles by alcohol.[3] In the light of previous studies, a rat model was chosen for this study.

The literature reveals a study investigating whether apoptosis in the germ cells of rat testicles increases with age or diet restrictions.[12] The study involved ad libitum feeding of control rats with 4-13 and 23 months of age, while the study group was subjected to 40% feeding restriction. The samples were evaluated after PAS staining for apoptosis with x 400 magnification. The results indicated that the apoptotic seminiferous tubules count was higher relative to age increase in the rats with ad libitum feeding. However, apoptotic germ cell count in apoptosis-positive tubules had decreased. In the comparison of the 23-month-old groups that were given normal or restricted diet, there was a significant increase in both apoptotic seminiferous tubule percentages and apoptotic cell counts for the group that received restricted diet. Increasing age had caused a significant increase in apoptosis rate of apoptotic germ cells although no diet restriction was inflicted.[12] In our study, not only alcohol but also aging and diet were shown to affect cellular apoptosis, which is in conformity with the aforementioned study. All the subjects of the study were considered to have equal characteristics because they had the same diet, room temperature, age, and living conditions. The rats with similar age were provided with an equal amount of isocaloric diet. Therefore, the effects of age and diet were eliminated. The only responsible agent for apoptosis in the rats that were subjected to ethanol was orally administered alcohol solution.

Erkkila et al[13] studied the regulation of apoptosis by testosterone in adult seminiferous tubules and developed an in vitro model to investigate apoptosis development and identification in adult human testicles. The samples were received from individuals who had undergone orchidectomy due to the prostate or testicle cancer. Seminiferous tubules were isolated from these samples and kept under serum free conditions. They were then left in testosterone medium. Following testosterone incubation in 0.000001 and 0.0001 mol/L concentrations for 4 hours, a significant inhibition of apoptosis was noted. In the immunohistochemical analysis for apoptosis evaluation, anti-DIG-AB, Boehringe Mannheim: 1:10000 solution was used and the samples were evaluated under light and electron microscopes.[13] Our evaluations revealed apoptosis in interstitial tissue resembling that in Leydig cells. Nevertheless, the differences in the apoptotic tendencies of these cells were not studied. Miura et al[14] evaluated the apoptosis, and apoptosis

related genes after heat application on rat testicles. The study aimed to define apoptosis regulators after heat application on rat testicles and describe, the molecular mechanism of germ cell apoptosis associated with heat stress. They used anti-Bcl-2 (1:1000), anti-Bcl-xl (1:1000) rabbit polyclonal anticores and anti-fas (1:200) for apoptosis evaluation. At Bcl-xl level, no significant difference was observed; however, 7 days after heat application at Bcl-2 level, a significant decrease was noted. In 1-3 days of Fas-l heat application, there was a significant increase. On general evaluation, 3 days after the heat application, a significant decrease was observed in the bilateral testicle weights. They also found a significant increase in apoptotic cell counts and apoptotic tubule surfaces in one day. In our study, however, all the rats were raised under the same room temperature, humidity, and in the same size of cages. In this way, the effect of heat on the germ cells of rat testicles was ruled out. Some authors have elucidated the type of localization tendency in apoptotic cells by Caspase-3 and described the visualization of an apoptotic cell reactive to Caspase-3 under the microscope.[14] As in the study of Keith et al, in our study, apoptotic cells were evaluated on the basis of 1000-cell count with x 400 magnification. When compared with normal tissues, a significant count of apoptotic cells and Caspase-3 reaction was observed. To identify apoptosis immuno-histochemically, Caspase-3 (CPP32) anticore was used as in earlier studies and discovered a similar image of intracellular localization of Caspase-3.[10]

Wright et al[15] in their study of "the effect of alcohol on male reproductive system" stated that alcohol could lead to loss of libido, impotency, and sterility in males as well as account for the sexual dysfunctions associated with the direct damage to testicular cells and control centers in the brain. The relation between alcohol consumption and loss of reproductive functions has long been established. Deficient sexual function (hypogonadism), sterility, libido loss, impotency, decreased prostate gland volume, and lower sperm production are associated with alcohol consumption. Hypogonadism has been connected to the direct effect of alcohol on the testicles in addition to its effects on the parts of the brain that regulate gonadal functions of the brain. The cells responsible with sperm production in the testicle comprise 95% of all the testicle volume. Thus, a malfunction in sperm production may cause a clinical picture characterized with testicle atrophy.[15,16] Martincic et al[17] defined spermatogenesis as a dynamic process of germ cell proliferation and differentiation and maintained that throughout normal spermatogenesis numerous testicular germ cells undergo apoptosis. Our results were compatible with the results of Martincic et al[17] in that self-raised control group rats were expected to present normal spermatogenesis; however, they had an apoptotic germ cell count of 3.9%.

In the light of literature review, the evaluation of our results showed that alcohol use leads to considerable damage in the testicles. The side products of alcohol metabolization clearly drive testicular germ cells to apoptosis, at the end of which sperm production is inhibited. Another consensus of the previous and current study is that alcohol metabolites impel seminiferous tubules to apoptosis and reduce their diameters. Our results revealed that chronic alcohol use induces apoptosis in germ cells of male gonads; thus, leading to significantly increased percentage of apoptotic germ cells. Due to decreased counts of healthy germ cells, the sperm production of males deteriorates with regard to sperm quality and counts. This study also showed that seminiferous tubules, the most important building stone of lobular structure of the testicle, undergo significant decreases in diameter due to alcohol use. This finding along with increased apoptosis in germ cells indicate that alcohol consumption can lead to considerable deterioration in the long-term reproductive ability of males.

References

1. Yen SSC, Jafe RB, editors. Reproductive Endocrinology. 3rd ed. Philadelphia: WB Saunders Co; 1991.
2. Nanji AA, Hiller-Sturmhofel S. Apoptosis and necrosis: two types of cell death in alcoholic liver disease. ***Alcohol Health Res World*** 1997; 21: 325-330.
3. Emanuele MA, Emanuele N. Alcohol and the male reproductive system. ***Alcohol Res Health*** 2001; 25: 282-287.
4. Bartke A. Apoptosis of male germ cells a generalized or a cell type-specific phenomenon? ***Endocrinology*** 1995; 136: 3-4.
5. Utkan T, Erden F, Yildiz F, Ozdemirci S, Ulak G, Gacar MN. Chronic ethanol consumption impairs adrenoceptor- and purinoceptor-mediated relaxations of isolated rat detrusor smooth muscle. ***BJU Int*** 2001; 88: 278-283.
6. Chan TC, Sutter MC. Ethanol consumption and blood pressure. ***Life Sci*** 1983; 33: 1965-1973.
7. Eid NA, Shibata MA, Ito Y, Kusakabe K, Hammad H, Otsuki Y. Involvement of Fas system and active caspases in apoptotic signalling in testicular germ cells of ethanol-treated rats. ***Int J Androl*** 2002; 25: 159-167.
8. Imawati B, Holt O, Lutter M, Xu Luo, Xiodong W. Biochemical pathways of Caspase activation during apoptosis. ***Annu Rev Cell Dev Biol*** 1999; 15: 269-290.
9. Mallat Z, Ohan J, Leseche G, Tedgui A. Colocalization of CPP-32 with apoptotic cells in human atherosclerotic plaques. ***Circulation*** 1997; 96: 424-428.
10. Izban KF, Wrone-Smith T, Hsi ED, Schnitzer B, Quevedo ME, Alkan S. Characterization of the interleukin-1beta-converting enzyme/ced-3-family protease, caspase-3/CPP32, in Hodgkin's disease: lack of caspase-3 expression in nodular lymphocyte predominance Hodgkin's disease. ***Am J Pathol*** 1999; 154: 1439-1447.
11. Van Thiel DH, Gavaler JS, Lester R, Goodman MD. Alcohol-induced testicular atrophy. An experimental model for hypogonadism occurring in chronic alcoholic. ***Gastroenterology*** 1975; 69: 326-332.

12. Barnes CJ, Covington BW 4th, Lee M. Effect of aging and dietary restriction on rat testicular germ cell apoptosis. ***J Gerontol A Biol Sci Med Sci*** 1999; 54: B199-204; discussion B205-B206.
13. Erkkila K, Henriksen K, Hirvonen V, Rannikko S, Salo J, Parvinen M, et al. Testosterone regulates apoptosis in adult human seminiferous tubules in vitro. ***J Clin Endocrinol Metab*** 1997; 82: 2314-2321.
14. Miura M, Sasagawa I, Suzuki Y, Nakada T, Fujii J. Apoptosis and expression of apoptosis-related genes in the mouse testis following heat exposure. ***Fertil Steril*** 2002; 77: 787-793.
15. Wright Harlan I, Gavaler Judith S. Effects of alcohol on the male reproductive system. ***Alcohol Health Res World*** 1991; 15: 110-115.
16. Zhu Q, Meisinger J, Emanuelle NV, Emanuelle MA, La Paglia N, Van Thiel DH. Ethanol exposure changes apoptosis within testis. ***Alcohol Clin Exp Res*** 2000; 24: 1550-1556.
17. Martincic DS, Klun IV, Zorn B, Vrtovec HM. Germ cell apoptosis in the human testis. ***Pflugers Arch*** 2001; 442 (6 Suppl 1): R159-R160.