In vitro effects of kraft liquor on testicular cells and sperm motility of Caspian kutum (*Rutilus frisii kutum*)

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Abstract

To identify Kraft liquor effects on spermatogenesis and sperm motility of Caspian kutums by using *in vitro* culture, the fish testis was incubated with 5, 10 and 15 % (V/V) of liquor for 3 and 6 days. The percentage of motile spermatozoa and its total duration were also measured. Adverse effects on testicular cells were more apparent with the increase of liquor concentrations. The mean size of spermatogonia and spermatocytes decreased significantly ($p<0.001$) in dose-dependent manner compared to controls. With the increase of concentration, the number of spermatocytes and spermatids decreased ($p<0.001$) but the number of spermatogonia, increased ($p< 0.05$). The increase of liquor concentration showed a significantly decrease of percentage of motile spermatozoa and total duration of spermatozoa ($p<0.05$); but there was no significant difference in most treatments ($p>0.05$). This study showed the detrimental effects of low levels of kraft liquor on testicular development and sperm quality in the kutums.

Keywords: Kutum, Testis, Kraft liquor, Caspian Sea, Spermatogenesis.

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Introduction

In southern coasts of Caspian Sea in Iran, most factories discharge their effluents into streams that finally end into the sea. Among them, wood and paper industries are under specific attention of the Department of the Environment (DOE) because of using different kinds of chemicals to manufacture their products. Kraft mill effluents, because of composing toxic elements, often lead to change response in aquatic organisms (Kovacs *et al*., 2002; Rana *et al*., 2004). Adverse effects of these kinds of effluents is seen in humans and other animals like Rats (Lee *et al*., 2002; Rana *et al*., 2004). The compounds in these effluents can have endocrine disruption effects that are interfering with steroid hormones function and affecting on growth, immune responses and injurious effects on reproduction (Vos *et al*., 2000) such as, impaired pituitary hormone release, altered sex steroid hormones, suppressed vitellogenin (VTG) levels, abnormal VTG production in male fish, reduced gonad size, abnormal reproductive behavior and spawning delays in various fish species (Munkittrick *et al*., 1998; Rana *et al*., 2004; Fentress *et al*., 2006**;** Rickwood *et al*., 2006). Masculinisation is one of the androgenic responses in female fish because of plant sterols or steroids in these compounds (Howell *et al*., 1980; Ellis *et al*., 2003). These chemicals also increase parasite loads in fish captured in the vicinity of these pollutants (Billiard and Khan, 2003). Because of sufficient sources of forest in the vicinity of southern coasts of Caspian Sea, paper manufacturing factories release a large volume of their wastewater into the rivers and from then into the Sea.

Caspian kutum is one the most famous, valuable and economic fishes that spend their lifelong in the sea, but migrate into rivers in the last days of winter for spawning. The aims of this study were evaluating: 1-the direct effects of kraft liquor on Caspian kutum spermatogenesis in last few days of maturation process by *in vitro* testis culture and 2-the effects of this chemical on sperm motility of this fish.

Materials and method

In vitro tissue culture

During the early period of fish migration for spawning into rivers, four wild maturing Caspian kutum males were caught using gill net, and kept in 13 ppt salinity and 12 °C tanks until transported to the lab. After acclimating (6 days) and anesthetizing, fresh testis were removed, cut into small pieces (10-15 mg) and placed in each wells of 24-well culture dishes (NUNCE, Denmark) containing 1.5 ml Leibovitz (L-15, Gibco, USA) culture medium supplemented with 0.5% bovine serum albumin fraction V (Sigma, USA), 1 mg/l bovine insulin (Miura *et al*., 2005), and 10 mM Hepes, 10000 UL-1 penicillin (Gibco, USA), 5000 mg/µL streptomycin (Gibco, USA), and adjusted to pH 7.5. Kraft liquor was filtered by micro filter 0.2 µm (Wattman) and added with 5, 10 and 15 % (V/V) to the wells and incubated in humidified air at 12°C for 3 and 6 days.

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The medium was changed after 3 days. After the culture, testicular fragments were collected and fixed in bouins solution, then were prepared using histotechniques and stained with hematoxylin–eosin (Pearse, 1985). Prepared sections photographed under a light microscope using a digital camera (Nikon Coolpix P6000) and examined for histopathological lesions. The taken data analyzed by image tools software (3.00) for quantitative results. The number and size of germ cells were evaluated in four replicated views ($0.3mm^2$) per histological sections at 1000x magnifications and mean values were used for statistical analysis. Only intact cells were measured in our analysis and dead cells were omitted.

Sperm motility analysis

The semen of eight wild male Caspian kutums were collected separately during their migration into rivers and brought to the laboratory for analysis. Sperm motility was evaluated for total duration of motility (in seconds) when 99% of spermatozoa were immotile (Stoss, 1983) and for the percentage of motile spermatozoa (Rurangwa *et al*., 2004) after activation. To induce initiation of sperm motility, 0.5 µl of semen was placed on a glass slide and then 1 µl of kraft liquor solutions with 0 (as control group), 5, 10 and 15% (V/V) were added. All experiments were performed in five repeated manner using a light microscope under 400X magnification and simultaneously were recorded with digital camera. Semen was stored at 4 °C during motility analysis. 10, 40 and 80 percentages of motile spermatozoa were also calculated.

Statistical analyses

Normal distribution of the data was analyzed with Kolmogorow-Smirnov test. Statistical analyses were determined by ANOVA using SPSS, Version 15. Duncan's Multiple Comparison Test was used to determine significant differences with the control group. Means were calculated and compared using Duncan's multiple range tests via MSTAT-C software (Michigan State University, 1988) and statistical significance was determined at 5% level.

Results

In vitro tissue culture qualitative analysis

Morphological analysis of the control groups showed that testis had a normal histological structure. In the 3rd day groups, lobules contained germinal cells at all stages of spermatogenesis, and in the 6^{th} day groups, clusters of spermatids and spermatozoa were more visible (Fig. 1a). In the 6^{th} day of kraft liquor exposed groups, lumens showed disorganized structures compare to the 3rd day groups and controls. In 15% treatment necroses were clearly visible in germ cells (Fig. 1b), and also in some lumens with no distinct reason lifting of the basal membrane was distinguished (Fig. 1c).

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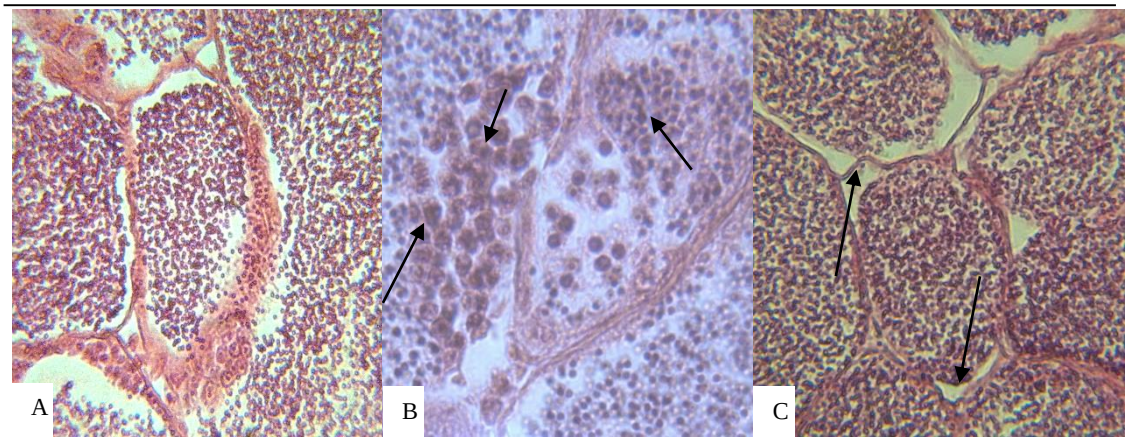

Figure 1: Representative histological sections of kutum testis. a; control, normal clusters of spermatozoa. b; necrotic areas covering spermatocytes and spermatids, 1000X. c; lifting of the basal membrane, 400X.

Size of germ cells

The size of spermatogonia (Spg) and spermatocytes (Spc), after exposure to different concentrations of kraft liquor, became significantly smaller in dose dependent manner compare to control groups (p<0.001, Fig. 2). The mean areas of Spg in the 3rd and 6th days were 0.475±0.06 mm^2 and 0.445±0.03 mm^2, and the mean areas of Spcs were 0.222±0.03 mm^2 and 0.195±0.02 mm^2 respectively. There were no significant interaction between kraft liquor and length of exposure on germ cells size (p>0.05).

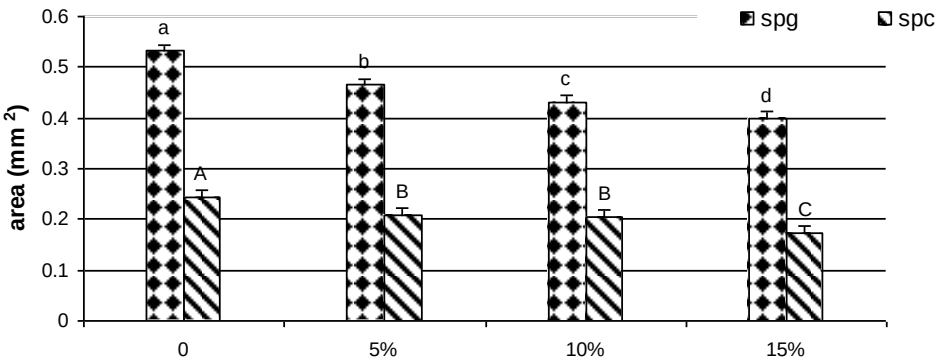

Figure 2: Mean area of germ cells (±SE) exposed to different concentrations of kraft liquor (p<0.001); Spg: spermatogonia; Spc: spermatocytes. The same cell types are compared and highlighted with the same error bars. Small letters are related to Spgs and the Capitals for Spc cells.

Number of germ cells

After more exposure time, mean number of Spg was increased from 21.333±2.24 at the 3rd day to 26.750±2.68 at the 6th day (p<0.05). Mean numbers of Spc decreased in a dose and time dependant manner (p<0.001), but in 5% treatment it increased a little compare to controls and the spermatid cells which decreased (p<0.05). The mean numbers of Spc and

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spermatids were 54.111±4.73 and 107.833±5.67 in the 3rd day and 38.417±3.84 and 70.250±3.09 in the 6th day, respectively. There was significant interaction between kraft liquor and length of exposure on spermatid number (p<0.001) but in Spg and Spc cells; there were no significant interaction (p> 0.05).

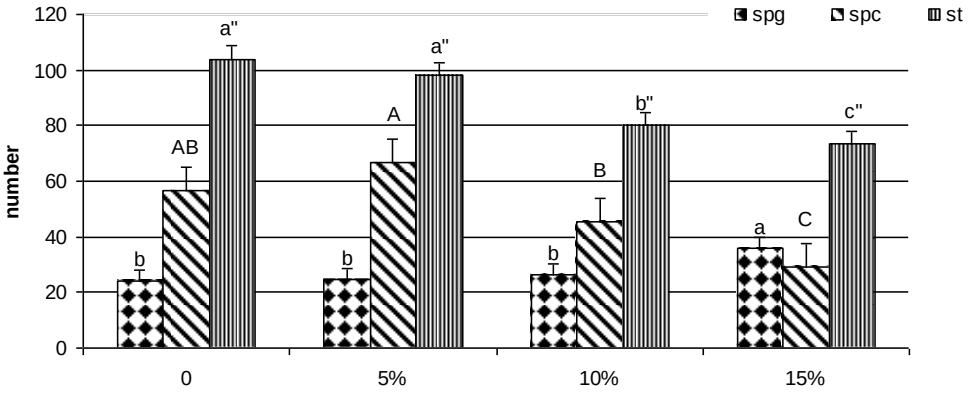

Figure 3: Mean numbers of germ cells (± SE) exposed to different concentrations of Kraft liquor (p<0.001; n=36). Spg: spermatogonia, Spc: Spermatocyte, St: spermatid. The same error bars are related to the same type of cells comparison.

Comparison of the mean numbers of spermatids in testis exposed to different concentration of kraft liquor showed that with increasing liquor concentration in the 3rd day after culture, the numbers of spermatids decreased significantly, and there were no statistical difference between D2T3 and D3T3 treatments (Table 1). Spermatids decreased significantly in the 6th day, but there were no significant difference between D1T1, D1T2 and D2T2 treatments (p>0.05). Maximum number of spermatids is observed in D2T2= 115 and the minimum number was 38.67 in D4T2.

Table 1: Comparison of mean numbers of spermatids.

Treatment	Concentration	Day	Time	Spermatid Number	
D1	0	3	T1	110	(A)
		6	T2	108	(A)
D_2	5 %	3	T1	98	(B)
		6	T2	115	(A)
D_3	10 %	3	T1	98	(B)
		6	T2	81.67	(C)
D_4	15 %	3	T1	62.67	(D)
		6	T2	38.67	(E)

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Diameter of seminiferous tubules in testis decreased significantly (p<0.05) (Fig. 4) but there were no significant difference among D2, D3 and D4. Also, there was not significant correlation between time and tubules diameter, and even between liquor and time on diameter of seminiferous tubules (p>0.05).

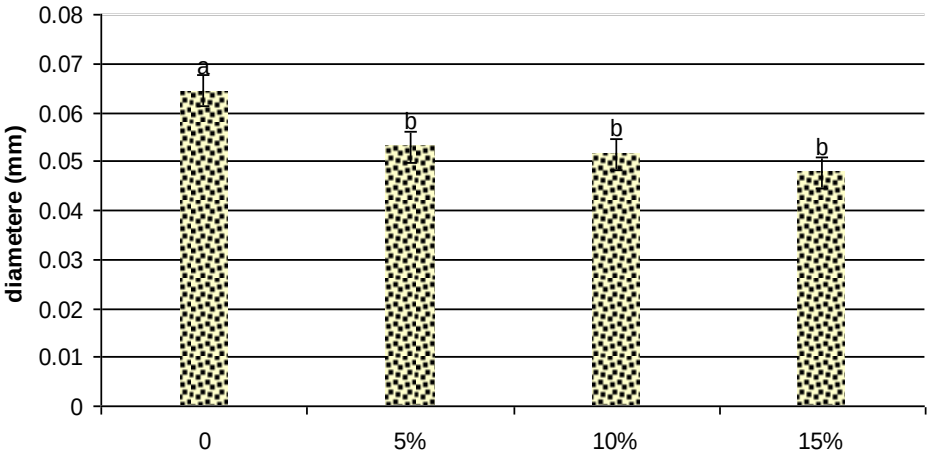

Figure 4: Mean diameter of seminiferous tubules (± SE) exposed to different concentration of kraft liquor.

Sperm motility analysis

Decreasing the effect of liquor on total duration of spermatozoa motility showed no significant difference among 5, 10, 15 % concentrations, but they had significant differences compared to the control group (p<0.05; Fig. 5a).

Comparison of the mean motility duration of different percentages of motile spermatozoa that were affected by liquor indicated that the observed decreased sperm motility duration have resulted in negative significant correlation between liquor concentrations and the percentage of motile spermatozoa (p<0.05; Fig. 5b).

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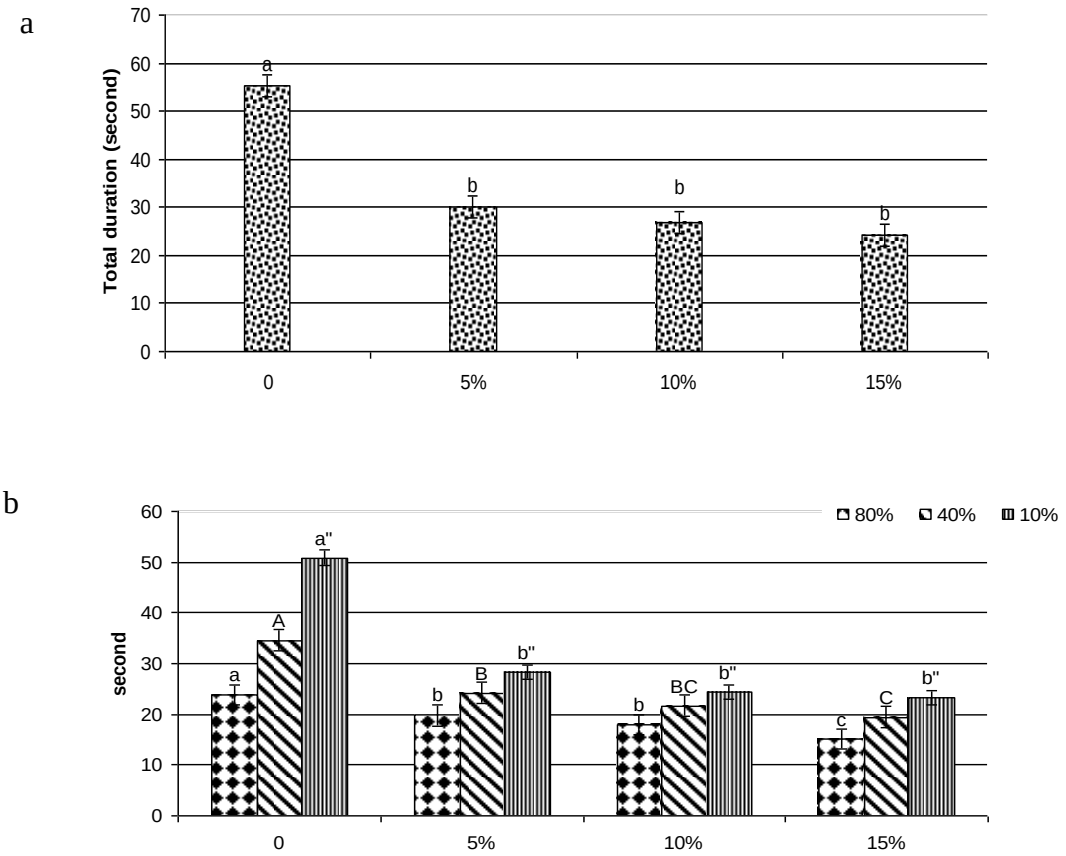

Figure 5: Mean total duration of spermatozoa motility (±SE); (a) percentage of motile spermatozoa (±SE), (b) exposed to different concentrations of kraft liquor, a, A and a" are related to 80%, 40% and 10% of motile sperms comparison, respectively.

Discussion

Paper and pulp mill effluents are composed of a complex mixture of different wood extractives such as plant sterols, resin acids, fatty acids, dioxins, tannins and terpenes (Ali and Sreekrishnan, 2001; Kostamo *et al*., 2003) which are capable of altering the endocrine system of fish (Mercure and Van Der Kraak 1995). These effluents have also direct effects on gonads to make low quality gametes or affecting sex behaviors like spawning. Significant reduction levels of sex steroids synthesis and dysfunction of hormonal control in gold fish (*Carassius auratus*), largemouth bass (*Micropterus salmoides*), white sucker (*Catostomus commersoni*), longnose suckers *(Catostomus catostomus)* has been reported earlier (Owens 1991; Sepulveda *et al*., 2003). Also, other adverse effects like declining gonad development, VTG and GSI levels have revealed this kind of chemicals as largely reproductive function inhibitors.

Apparent reduction in germ cell size and number in current study showed that these concentrations were sufficient to cause disorganization on testis but in some treatments there were no

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significant differences, as the number of Spg cells incubated in 5% and 10% treatments showed no significant difference with the controls. The seminiferous tubules diameter slightly decreased, with increasing of liquor concentrations compared to the controls, but they did not differ among each other. These results showed that there was a direct correlation between germ cells size, dose and length of exposure. Large numbers of cells were negatively correlated with kraft liquor concentrations suggesting dose-dependent inhibitory effects of liquor on spermatogenesis. Spg cells in D4, because of stopping regressive effects on cell division with statistically significant differences, were more than other groups (p<0.001). In histopathological survey, in testis some germ cells were dead and necrosis was obviously recognized, so in these sections spermatogenesis seemed to be negatively affected (Spermatogenesis is a functional aspect and could not be recognized in sections). As further result indicated numbers of Spcs and spermatids were decreased because of low Spg and Spc proliferation rate. Undoubtedly, reduction in germ cells number and size in testis weakened its normal functions and fish spermatozoa quality were declined. However, these adverse effects are related to fish species, age, dose and length of exposure (Rana *et al*., 2004). Fish exposed to kraft mill effluents showed lower gonad size and smaller eggs than those in reference sites (McMaster *et al*., 1996; Munkittrick *et al*., 1998). It is not well known that which specific compounds in these effluents are responsible for these impacts but it is clarified that some parts of these effluents like sterols, lignans, and resin acids have weak estrogenic activity (Van Der Kraak *et al*., 1998; Chamorro *et al*., 2010), and some are antiestrogenic (Zacharewski *et al*, 1995). Sometimes effect of pollutants on fish is gender specific (Cajaraville *et al*., 2003), so the study of kraft liquor toxicity on different kutum sexes must be reviewed.

Sperm motility is a key factor that determines fertilization success in fish (Alavi *et al*., 2010) and there is highly significant correlations between motility and fertilization (Moccia and Munkittrick, 1987). Bleached kraft mill effluent caused reduction of sex behaviors and sperm motility in White sucker but milt volume did not decrease (McMaster *et al*., 1992). The data from sperm motility analysis demonstrated that, liquor has adverse effect on the percentage of motile spermatozoa and this was related to exposure time. Except the controls, 80% of motile spermatozoa in the treatments observed just in <20 sec after activation. Spermatozoa motility stopped in nearly 56 seconds following exposure to distilled water. Total duration of spermatozoa motility declined with no statistically significant differences among all groups compare to controls, but significant decrease in sperm motility duration was observed when spermatozoa were in >10% of movement. In other word, in early seconds after initiation of motility, when percentage of sperm motility was at

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highest values, they were more sensitive to any concentration of liquor and their significant differences were more elucidated. These results indicated that there is direct relationship between liquor concentration and sperm quality with regard to motility and its total motility duration. Sufficient time to fertilize eggs in kutum is 15 seconds after initiation of sperm cells motility (Gage *et al*., 2004), so each toxicant with decreasing effect on sperm movement, could have an adverse effect on fertilization efficiency. Spermatozoa motility of spawning fish expose to bleached kraft mill effluents in Jackfish Bay decreased significantly (McMaster, 1991) but in white fish, unbleached kraft mill effluents declined milt production and decreased sperm motility (McMaster, 1991; McMaster *et al*., 1992). Some of the major constituents of pulp mill effluent, inhibited sperm motility in sea urchin (*Strongylocentrotus purpuratus)* and echinoderm (Cherr *et al*., 1987; Higashi *et al*., 1992). Pulp and paper mill effluents decreased total sperm count and number of motile spermatozoa in rats, too (Rana *et al*., 2004). So in aquatic ecosystems, transitive levels of toxic elements like kraft liquor act as reproductive toxicant either interfering with endocrine system, hormonal regulation and sperm production or spermatozoa activity after releasing in water environments. Undoubtedly adverse effects of these effluents on reproduction can occur in female fish (Davis and Bortone, 1992; Cody and Bortone, 1997; Sepulveda *et al*., 2003). The egg production of fathead minnow (*Pimephales promelas*) exposed to different mills effluent in laboratory became lower in comparison with the controls (Kovacs *et al*., 2011). Also, damaged gametes can produce less viable offspring and it is found that it can affect on fish communities (Greenfield and Bart, 2005).

In conclusion, this study confirmed that kraft mill effluents are testicular toxicant in male kutums and its effect on testicular dysfunction is dose and time dependent manner. Kraft mill also affect the sperm motility which can affect its potency for natural reproduction. Also additional studies are needed in order to determine other harmful effects of black liquor on reproduction covering *in vivo* experiments, fry growth and survival of kutums to express the exact harmful reproductive effects.

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