

Investigation of In Vitro Effects of Fluoride on Bovine Sperm

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Abstract: The in vitro effects of various concentrations of sodium fluoride on the percentages of motile spermatozoa and abnormal spermatozoa with the hyaluronidase activities of semen were investigated. Holstein bulls (n=20), aged between 4 and 5 years, were used in this study. The semen samples obtained from all animals were divided into five parts and one of them was used as the control sample. Sodium fluoride solutions were prepared at concentrations of 30, 60, 120 and 240mM and then these solutions were mixed 1:1 with semen samples to determine semen hyaluronidase activity, sperm motility and morphological abnormality of spermatozoa at 5, 10, 15, 20 and 30 minutes.

The semen hyaluronidase activities and the percentages of abnormal spermatozoa were determined to increase highly significantly ($p<0.001$) in comparison with control samples at all times. However, significant ($p<0.001$) decreases were established in the percentages of sperm motility. In addition, significant negative correlations ($p<0.001$) were determined between the hyaluronidase activities and sperm motilities of all groups at all times, after the incubation of sodium fluoride with sperm samples.

In conclusion, these results indicated that when sodium fluoride was applied in vitro to bovine sperm, it caused toxic effects. Furthermore, there were significant relationships between hyaluronidase activity and sperm motility.

Key Words: Hyaluronidase, sperm, motility, bull

Sığır Sperması Üzerine İn Vitro Florun Etkilerinin Araştırılması

Özet: Bu çalışmada değişik konsantrasyonlardaki sodyum florürün in vitro olarak sperm motilitesi, anormal spermatozoit oranı ve sperma sıvısı hyaluronidaz düzeyleri üzerine olan etkileri araştırıldı. Araştırmada 4 ve 5 yaşlarında 20 adet Holştayn boğa kullanıldı. Hayvanlardan alınan sperma örnekleri beş kısma ayrıldı ve bunlardan biri kontrol numunesi olarak kullanıldı. Sodyum florür solusyonları 30, 60, 120 ve 240mM'lık konsantrasyonlarda hazırlandı ve sperma örnekleri ile 1/1 oranında karıştırıldı daha sonra 5, 10, 15, 20 ve 30. dakikalardaki sperma sıvısı hyaluronidaz aktivitesi, sperm motilitesi ve anormal spermatozoit oranları belirlendi.

Sperma sıvısı hyaluronidaz aktiviteleri ve anormal spermatozoit oranlarının kontrol grubuna göre oldukça önemli ($p<0.001$) oranda yükseldiği, sperm motilitesi yüzdelерinin ise önemli ($p<0.001$) oranda azaldığı görüldü. Buna ilave olarak, sodyum florür inkübasyonundan sonra, tüm gruplara ait hyaluronidaz aktiviteleri ile sperm motiliteleri arasında önemli ($p<0.001$) korelasyonlar belirlendi.

Sonuç olarak sodyum florürün, sığır sperması üzerine in vitro olarak uygulandığında toksik etkilere neden olduğu ve semen hyaluronidaz aktiviteleriyle sperm motiliteleri arasında önemli ilişkilerin bulunduğu belirlendi.

Anahtar Sözcükler: Hyaluronidaz, sperm, motilite, boğa

Introduction

Fluoride is used for the prophylaxis and treatment of some diseases (especially in stomatology) (1). This element is a highly reactive halogen and has a strong affinity for calcium, aluminium and iron ions (2). In addition, it is a component of some antelmenthics and rodenticides (3). It has been reported that the efflux of calcium ions is required for sperm motility and acrosome reactions (4,5).

Narayana and Chinoy (6) showed that the testosterone levels decreased in rats administered fluoride at a dose of 10mg/kg for 50 days. Furthermore, testosterone levels were reported to decrease in skeletal fluorosis patients (7). It was claimed that fluoride caused significant impairment of egg volume, weights and lengths in owls (8). In addition, it was revealed that fluoride decreased birth rates (9). Hyaluronidase is released from the head of spermatozoa during the

acrosomal reaction (10). Sperm hyaluronidase has been implicated in sperm penetration of the extracellular matrix of the cumulus oophorus and may play an important role in gamete interaction and fertility in mammals (11).

The purpose of this study was to establish the effects of sodium fluoride on the semen hyaluronidase activity, sperm motility and abnormal spermatozoa rates, and whether there is any relationship between semen hyaluronidase activity and sperm motility or not.

Materials and Methods

Materials

Sodium fluoride (Sigma-Aldrich Co.), N-acetylglucosamine (Merck Co.), hyaluronic acid (Merck Co.), potassium tetraborate (Sigma Co.), dimethylaminobenzaldehyde (Sigma-Aldrich Co.) and other chemicals purchased from Sigma.

Animals and semen collection

In this study, 20 healthy Holstein bulls, aged between 4 and 5 years, were used. The bulls were fed on grass supplemented with lucerne hay and corn silage. Drinking water was provided ad libitum. Semen samples were obtained from all animals (n=20) and spermatological examinations of the samples were carried out immediately. The bull ejaculates had a sperm concentration of $1.5 \pm 0.3 \times 10^9$ spermatozoa ml⁻¹ (mean \pm SEM) and a volume of 6 ± 0.2 ml (n=20). Semen samples were collected by artificial vagina (12).

The preparation of sodium fluoride solutions and addition of this solution to semen samples

Sodium fluoride (NaF) solutions were prepared at doses of 30mM, 60mM, 120mM and 240mM in distilled water. The semen samples were divided into five parts and one of them was used as the control sample. Each sodium fluoride solution was mixed 1:1 with semen samples and incubated at 37°C in air. Control spermatozoa were treated with distilled water at the same rate and incubated at 37°C in air. After the addition of sodium fluoride solutions, the semen hyaluronidase activities, the percentage of sperm motility and abnormal sperm rates were determined at 5, 10, 15, 20 and 30 minutes. The motility and abnormality of the spermatozoa were monitored by light microscopic observation (13).

The measurement of hyaluronidase activity

N-acetylglucosamine (NAG) was weighed into 50, 100 and 200 mg quantities and dissolved in 1l of water. This reagent was used to prepare the calibration curve. The semen hyaluronidase activities were measured by the method described by Wilkinson et al. (14). The semen samples of the control and experiment groups were diluted 1 in 5 with 0.15 mol/l sodium chloride before assaying. One millilitre of each diluted sample was added to 0.1ml acetate buffer (0.3mol/l, containing 0.45mol/l sodium chloride) and 0.1ml of hyaluronic acid substrate (4mg of hyaluronic acid was dissolved in 1l of water) was added to these mixtures, which were then incubated for 24 h at 37°C in a thermostatically controlled room. After the reaction mixtures were taken, 60µl of potassium tetraborate (0.8mol/l in water, pH:10) was added and the reaction was terminated by heating block for 5min. Then, the mixtures were cooled in an ice-water bath before the addition of 2ml of dimethylaminobenzaldehyde (Stock DMAB reagent-10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid: Stock reagent diluted 1 in 10 with glacial acetic acid before use) and then they were incubated for 20 min at 37°C in a water bath. The reaction mixtures were centrifuged immediately at 1500g for 10min and the absorbance of the supernatant read at 582nm within 30min using a spectrophotometer. Hyaluronidase activity was expressed as the mean (\pm SEM) µmol NAG/min/l.

Statistical analyses

The means (\pm SEM) and the differences between the control and experiment groups were calculated by independent t-test. The correlation test was carried out to establish the relations between sperm motility and semen hyaluronidase activity. All tests were realized by the SPSS program (Win 6.0).

Results

The sperm motility and morphological abnormality of spermatozoa after incubation with sodium fluoride

After the treatment of semen samples with sodium fluoride at doses of 30, 60, 120 and 240 mM, the values of sperm motility were estimated to decrease highly significantly ($p < 0.001$) in comparison with control samples at all times (Figure 1). The sodium fluoride solutions were mixed with semen and then all spermatozoa were observed to be immobile at 30, 20, 15

and 10 minutes, respectively (Figure 1). The percentage of morphological abnormality of the control samples was $7\pm 0.4\%$. After incubation of sperm with sodium fluoride at doses of 30, 60, 120 and 240 mM, the values of morphological abnormality were determined to increase rather significantly ($p<0.001$) in comparison with the control samples. The means (\pm SEM) of morphological abnormality were $33\pm 1.09\%$, $34\pm 1.16\%$, $34\pm 1.01\%$ and $35\pm 1.03\%$ respectively, at all times. All spermatozoa treated with sodium fluoride showed deflagellation, loss of head, coiling of tail and shoehook tail.

The semen hyaluronidase activities after incubation of sodium fluoride

The hyaluronidase activities of semen samples were established to increase rather significantly ($p<0.001$) when compared with the control samples at all times. (Figure 2). Although all spermatozoa were immobile at different times (Figure 1), the hyaluronidase activities were estimated to increase in all groups (Figure 2). Significant correlations were calculated between the

hyaluronidase activities and sperm motilities at 5, 10, 20 and 30 minutes, after incubation with sodium fluoride at doses of 30, 60, 120 and 240mM (Table).

Discussion

Schoff and Lardy (15) claimed that bovine sperm incubated with 30mM fluoride became immobile within 2 min. In this study, after sodium fluoride was incubated with spermatozoa at doses of 30, 60, 120 and 240mM, all spermatozoa lost their motilities gradually and were immobile at 30, 20, 15 and 10 minutes, respectively. The decrease in sperm motility can be explained by the fact that sodium fluoride caused loss of the head, coiling of tail, deflagellation and shoehook tail.

It was reported that the release of hyaluronidase is used as an index of acrosomal membrane damage during cold shock of sperm (16). In our experiment, it is clearly demonstrated that after the incubation of sodium fluoride at doses of 30, 60, 120 and 240 mM, semen hyaluronidase activities were significantly increased

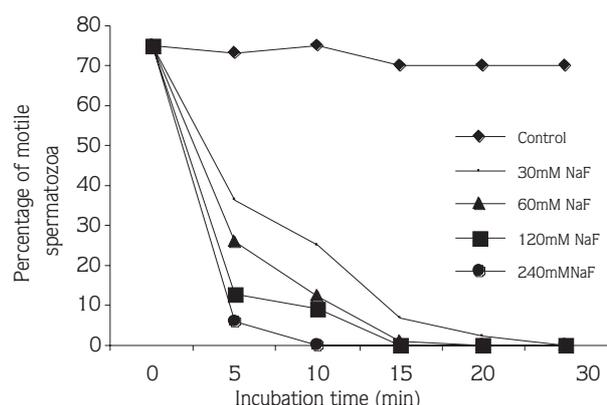


Figure 1. The percentage (mean \pm SEM) of motile spermatozoa of Holstein bulls after incubation with sodium fluoride. The values of sperm motility were observed to decrease highly significant ($P<0.001$) when compared with control samples ($n=20$).

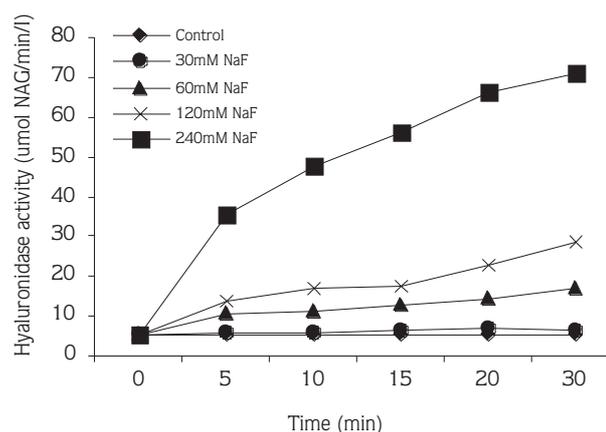


Figure 2. The hyaluronidase activities (mean \pm SEM) of semen samples of Holstein bulls, after incubation with sodium fluoride. The semen hyaluronidase activities were observed to increase highly significantly ($p<0.001$) when compared with control samples at all times ($n=20$).

The Comparisons	NaF (mM)			
	30	60	120	240
Motility-Hyaluronidase Activity	-0.91***	-0.90***	-0.96***	-0.94***

Table. The correlation coefficients between the values of sperm motility and semen hyaluronidase activities at all times (5, 10, 15, 20 and 30 minutes).

***: $p<0.001$

($p < 0.001$) in comparison with the control samples. The rise in hyaluronidase activity in semen can be explained by the fact that by the release of hyaluronidase from the head of spermatozoa to seminal plasma due to acrosomal damage and this damage might cause an accumulation of fluoride at the acrosomal membrane of spermatozoa.

In this study, significant ($p < 0.001$) correlations were determined between sperm motilities and semen hyaluronidase activities at all times. While the hyaluronidase activity increased according to doses of sodium fluoride, sperm motility was observed to decrease. Therefore, the disorders of motility may be caused by the impairment of hyaluronidase activity in semen.

It was revealed that mouse spermatozoa did not show any abnormalities and hence fluoride toxicity (17,18).

These findings indicate that after incubation of sodium fluoride at doses of 30, 60, 120 and 240mM, the percentages of motile spermatozoa decreased highly significantly ($p < 0.001$) when compared with control samples. In addition, the rates of morphological abnormality increased rather significantly ($p < 0.001$) in comparison with the control samples. These contradictions can be explained by the use of in vivo sodium fluoride and mouse spermatozoa in the other study.

The findings of this study show that sodium fluoride has toxic effects on sperm motility, sperm morphology and semen hyaluronidase activity in vitro. Furthermore, there are significant correlations between the hyaluronidase activities of semen and sperm motilities. To understand the in vitro and in vivo toxic effects of fluoride as more detailed studies are required.

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