

IN VITRO INFLUENCE OF SODIUM FLUORIDE ON RAM SEMEN QUALITY AND ENZYME ACTIVITIES

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SUMMARY: The percentage of spermatozoa in ram semen with intact acrosomes and the level of spermatozoa motility decreased significantly after dilution and after 5 hr incubation at 38°C. Both indices decreased significantly in the presence of NaF at concentrations ranging from 20 µmol/L to 0.1 mol/L. The activities of androgen-dependent enzymes—acid phosphatase (ACP), lactate dehydrogenase (LDH), and gamma-glutamyl transferase (γ-GT-10S)—decreased significantly when the ejaculate was treated with NaF at concentrations of 20, 100, 200 µmol/L (0.38; 1.9; 3.8 ppm F⁻), but they returned to the initial value of the control at 0.1 mol/L (1900 ppm F⁻). The activity of aspartate transaminase (AspAT) displayed a large increase with the increasing lower F⁻ concentrations and then showed a substantial decrease in activity at the high F⁻ concentration. These changes undoubtedly affect the physiological functions of the sperm.

Keywords: Acrosomes, Androgen-dependent enzymes, In vitro fluoride, Ram semen, Spermatozoa, Sperm motility.

INTRODUCTION

Long-term high-level fluoride intake can lead to severe damage of the metabolism of many systems and organs.¹ A study by Susheela *et al*² revealed that prolonged exposure to fluoride damages male reproductive organs in rabbits. Data in the world literature indicate there is a close relationship between fluorosis and infertility.¹⁻⁶ Spermatozoa are also known to be particularly susceptible to toxic substances. These findings, however, are still not generally regarded as one of the causes of infertility.

It is possible that the path of fluoride from the blood through the cytoplasm of epithelial cells of the epididymis into its lumen and further with the ejaculate, beside through the digestive and the excretory systems, might be another way of fluoride expulsion from the male body. Skare *et al*⁷ found rats given 84 mg NaF/kg, had plasma fluoride concentrations as high as 12 ppm, but testicular fluoride concentrations were in most cases only 10-20% of those in the plasma. A certain concentration gradient is maintained by the blood-testis barrier, thus keeping the fluoride concentration lower in the testis than in the plasma. This permeability barrier may be crossed during prolonged exposure to fluoride, causing spermatogenic damage resulting in cessation of spermatogenesis.² However, according to Collins *et al*,⁸ animal studies on reproductive toxicity do not provide sufficient data from which to extrapolate to human risk. The aim of this study was to assay the *in vitro*

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influence of various concentrations of sodium fluoride on the quality of ram semen and on the activity of some androgen-dependent enzymes.

MATERIALS AND METHODS

Ejaculated semen samples were collected separately by a standard method⁹ from 25 rams, Suffolk and Laine breed, from livestock of the Agricultural University, Faculty of Biotechnology and Animal Husbandry, and ZZD Kołbacz, Glinna Farm. The time from the previous ejaculation for each ram was kept the same days (3 days) to achieve comparable results among the different rams. Sodium fluoride (NaF) was used in the following concentrations: 20, 100, 200 $\mu\text{mol/L}$ (0.38; 1.9; 3.9 ppm F^-), and the extremely high 0.1 mol/L (1900 ppm F^-), along with a control (no NaF added).

The assay of spermatozoa survival time and acrosomal damage at 38°C during 5 hr was done by microscopy. The sperm survival test was done according to the method of Bielański.⁹ The semen was first analyzed to assess its quality, then diluted with 0.9% NaCl (1 mL semen + 4 mL 0.9% NaCl). Next it was divided into 5 equal parts, to each of which 1 mL of the above fluoride solutions were added. The control semen was treated only with the 0.9% NaCl solution. One sample was kept in water bath at 38°C for 5 hr, and the sperm motility was assessed every hr. At the beginning and the end of the experiments microscopic preparations were taken to assess the degree of the acrosome morphological damage.¹⁰ For this purpose a contrast medium of a 10% solution of nigrosine was used. Beside these analyses, the following androgen-dependent enzyme activities were assayed:

- EC. 3.1.3.2.- acid phosphatase (ACP) activity was determined by the method of Walter *et al*¹¹ and expressed as U/L;
- EC. 1.1.1.27.- lactate dehydrogenase (LDH) activity was assayed by the method of Mathieu *et al*¹² and expressed as U/L;
- EC. 2.3.2.2.- gamma-glutamyl transferase (γ -GT-10S) activity was determined by the method of Szasz¹³ using bioMerieux sa test (Enzyline γ -GT-10S) and expressed as U/L;
- EC. 2.6.1.1.- aspartate transaminase (AspAT) activity was assayed by the method of Thefelt¹⁴ and expressed as U/L.

RESULTS

The progressive decrease in spermatozoa motility and undamaged acrosomes as affected by NaF is presented in Table 1. With NaF concentrations of 20 to 200 $\mu\text{mol/L}$ the sperm motility decreased stepwise from 74.5% by more than 10% at every concentration increase. At the highest fluoride concentration (0.1 mol/L) the sperm motility was only 23.3%, and after 5 hr in-

cubation there was no movement. The number of intact acrosomes decreased from 78.8 to 50.6%, and after 5 hr incubation from 65.4 to 33.0%, following the treatment with the increasing fluoride concentrations.

Table 1. Mean percentage changes \pm SD in the motility of spermatozoa and intact acrosomes at 38°C in separate semen samples from 25 rams

NaF Treatment	Sperm motility (%)		Spermatozoa with intact acrosomes (%)	
	After dilution	After 5 hr	After dilution	After 5 hr
1: Control	75.4 \pm 6.4	28.1 \pm 15.5	78.8 \pm 6.2	65.4 \pm 6.1
2: 20 μ mol/L	65.0 \pm 11.7	10.2 \pm 8.9	72.6 \pm 6.6	54.5 \pm 7.6
3: 100 μ mol/L	56.7 \pm 14.1	2.7 \pm 3.3	65.1 \pm 7.9	45.9 \pm 9.6
4: 200 μ mol/L	48.4 \pm 14.0	0.3 \pm 0.7	59.3 \pm 7.4	39.2 \pm 8.3
5: 0.1 mol/L	23.3 \pm 17.3	0.0 \pm 0.0	50.6 \pm 11.7	33.0 \pm 6.2
Significance at $p \leq 0.05$	1 – 2,3,4 2 – 3,4	1 – 2,3,4,5 2 – 3,4,5	1 – 2,3,4,5 2 – 3,4,5 3 – 4	1 – 2,3,4,5 2 – 3,4,5 3 – 4

The activity of the enzymes analyzed is given in Table 2. Lower fluoride concentrations—up to 200 μ mol/L—had a statistically significant inhibitory action in comparison with the control. An anomaly was found at the extremely high concentration of 0.1 mol/L: the activity of acid phosphatase equaled that of the 200 μ mol/L treated samples, whereas those of lactate dehydrogenase and aspartate transaminase were statistically higher than those of the control. The activity of gamma-glutamyl transferase had only a small tendency to decrease, but at the highest NaF concentration it returned to the initial value of the control.

Table 2. Influence of NaF on mean *in vitro* enzyme activities (U/L \pm SD) at 38°C in separate semen samples from 25 rams

NaF treatment	Acid phosphatase	Lactate dehydrogenase	γ - Glutamyl transferase	Aspartate transaminase
1: Control	64.9 \pm 14.6	551.0 \pm 112.8	430.9 \pm 130.3	177.4 \pm 53.7
2: 20 μ mol/L	31.3 \pm 7.0	62.3 \pm 18.4	347.9 \pm 80.9	329.9 \pm 91.9
3: 100 μ mol/L	36.8 \pm 8.5	67.5 \pm 15.1	333.2 \pm 68.5	325.5 \pm 76.6
4: 200 μ mol/L	51.9 \pm 20.1	61.2 \pm 21.3	322.0 \pm 70.0	337.5 \pm 83.9
5: 0.1 mol/L	59.9 \pm 14.8	770.8 \pm 123.2	460.7 \pm 126.9	239.8 \pm 35.9
Significance at $p \leq 0.05$	1 – 2,3,4 2 – 4,5	1 – 2,3,4,5 2 – 5	1 – 2,3,4 2 – 5	1 – 2,3,4,5 2 – 5

DISCUSSION

The affinity of fluoride for hard tissues is well documented,^{15,16} but its influence on the reproductive system is less understood. Fluoride appears to have a great influence on reproduction,^{1,3-6} and there is evidence that it can penetrate the placental barrier of humans and animals.¹⁷⁻¹⁹ Thus fluoride could be one of the causes of infertility. In our study we found a statistically significant decrease of the spermatozoa motility after treatment with various concentrations of fluoride. Similar results were reported by Schoff *et al*, who reported that bull sperm treated with 30 mM fluoride became immobile within 2 min at 30°C.²⁰ Chinoy *et al*²¹ found a significant decrease in the motility of cauda epididymal spermatozoa of NaF-treated rabbits.

The mechanism by which fluoride affects sperm motility has not been elucidated. It has been postulated that fluoride acts directly on the motile apparatus without affecting other metabolic systems.²⁰ Opinions on the influence of the fluoride upon reproduction processes also differ. This could be due to the fact that the authors had used different means of intoxication – like oral, intravenous and intratesticular, or varying length of exposure to fluoride or amounts of it. Species sensitivity also plays a major role in the differing effects observed on male reproductive function in sodium fluoride treated animals.²² Considering studies done by Chinoy *et al*,²³⁻²⁶ who found that toxic effects of fluoride disappeared after removal of the intoxication source, it is reasonable to conclude that the toxic effect of fluoride on reproduction is due to inhibition of enzyme activity, particularly of enzymes whose cofactor is the cation of a bivalent metal.²⁷

Fluoride is a well-known inhibitor of many enzymes.²⁸ Inhibition of Mitochondrial enzymes by fluoride manifests itself in two ways. The first mode of action pertains to enzymes whose cofactors are Mg, Ca, Zn, and Se. The high affinity of fluoride toward these elements can impair enzyme activity,²⁷ thus inhibiting glycolysis, respiration, and motility of bull spermatozoa, or it may form insoluble complexes with magnesium or phosphates in enolase-type enzymes and acid and alkaline phosphatase.²⁹ Another mode of action is believed to involve blocking the thiol-groups in free sulfhydryl groups of amino acids at central points of the enzyme active centers, thus making the formation of S-S bridges impossible. Fluoride can also form hydrogen bonds to amides that are stronger than normal -N-H-O- hydrogen bonds. This is of paramount importance for the formation and maintenance of steric structure of proteins in the catalytic center.³⁰ Thus Sullivan³¹ reported an inhibitory effect of fluoride on the activity of succinate dehydrogenase (SDH), and Chinoy *et al*²¹ observed a decrease of activity of SDH, ATPase, and ACP in sperm of rabbits exposed to fluoride.

Srivastava *et al*³² discovered that seminal plasma exhibits high activities of acid and alkaline phosphatase, the acid form being predominant. The se-

men acid phosphatase activity of cocks was inhibited by 53% by fluoride.³³ Acid phosphatase assayed in the spermatozoa of rabbits has shown a tendency to decrease as a result of exposure to fluoride.²¹ In our study we found a statistically significant decrease of semen acid phosphatase activity at fluoride concentrations of 20, 100, and 200 $\mu\text{mol/L}$, whereas after application of 0.1 mol/L the enzyme activity returned to its initial level. Every enzyme has an optimum pH, but by our applied procedure the pH solution did not change much.

Most of the energy needed by spermatozoa for motility comes from fructose oxidation in the process of anaerobic glycolysis, whose product is lactic acid, and in its passage through the cell membrane, lactate dehydrogenase plays a role. The LDH activity in our sperm samples treated with the three lower concentrations of fluoride decreased nearly nine-fold but increased significantly with 0.1 mol/L. As of now it is difficult to explain these results. One can speculate that the high concentration of fluoride damages the sperm plasma membranes, which is connected with an outflow of the acrosome content. Bogin *et al*³⁴ reported declines in LDH levels in the liver, kidney, heart, and skeletal muscles of mice treated with 100 ppm NaF. Similarly, Chitra *et al*³⁵ found decreased LDH activity in muscle and liver of NaF-treated *Channa punctatus*.

The activity of gamma-glutamyl transferase also decreased significantly with 20 to 200 $\mu\text{mol/L}$ of fluoride, but it was back to the initial level after application of 0.1 mol/L. The main source of $\gamma\text{-G-10S}$ in sperm plasma is excretion from the testicles and epididymis or, according to Dubiel,³⁶ only from the epididymis. In Dubiel's study a ligature of the boar epididymis prevented the flow of its secreted fluid thus inhibiting the activity of $\gamma\text{-GT-10S}$ drastically. Disturbances of the activity of that enzyme in animal sperm analyzed *in vivo* could indicate damage to the epididymis.

The doubling of AspAT activity from that of the control to that at 20 $\mu\text{mol/L}$ might be due to some structural alterations in the mitochondria in the midpiece region, thereby causing leakage of this enzyme into the ejaculate.³⁷ At present it is hard to explain why the activity of AspAT decreased at the 0.1 mol/L fluoride concentration.

From this study it can be concluded that sodium fluoride at concentrations of 20, 100, 200 $\mu\text{mol/L}$, and 0.1 mol/L causes a statistically significant decrease in the motility of spermatozoa and the number of intact acrosomes. The lower concentrations significantly decreased the activity of ACP, LDH, and $\gamma\text{-GT-10S}$ in comparison with the control. At the extremely high concentration of sodium fluoride the activities of ACP and $\gamma\text{-GT-10S}$ returned to initial control levels and in the case of LDH even significantly surpassed the initial value. Equally remarkable, the activity of AspAT increased significantly with increasing concentrations of NaF, although it decreased

slightly at 0.1 mol/L. Such anomalies are examples of paradoxical concentration effects of fluoride³⁸ apparently resulting from destructive or stimulatory changes within the spermatozoa or the seminal fluid.

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