EFFECTS OF FLUORIDE INGESTION WITH PROTEIN DEFICIENT OR PROTEIN ENRICHED DIETS ON SPERM FUNCTION OF MICE
NJ Chinoy, a D Mehta, DD Jhala
Ahmedabad, India

SUMMARY: The effects of ingestion of protein deficient or protein supplemented diets with or without sodium fluoride (NaF, 5, 10, and 20 mg/kg body wt) were studied on sperm function in mice and compared with effects on mice fed a control (standard) protein diet + NaF in the above three dose levels for 30 days. Ingestion of the protein deficient diet together with the three dose levels of NaF caused a significant decrease in sperm motility, sperm count, sperm viability, fertility rate, sperm mitochondrial activity index (SMAI), number of normal spermatozoa, and hyaluronidase activity. However, DNA integrity, assessed by acridine orange fluorescence staining, and total acrosin levels, for acrosome integrity, were comparatively less affected. The control protein diet plus the three dose levels of NaF also caused significant alterations in all these parameters as compared to control Group I. However, mice fed a protein supplemented diet with or without the three doses of NaF did not manifest any changes, and the above parameters in them were almost the same as in control Group I. The decrease in sperm motility appears to be related to decline in SMAI and abnormal sperm counts. The significant decrease in sperm viability along with the above parameters would be expected to affect fertility, whereas inhibition of hyaluronidase and to some extent of acrosin could affect fertilizing capacity of the sperm. The results reveal that dietary factors such as increased protein intake can be especially valuable in curbing fluoride fertility toxicity.

Keywords: Mice; Fluoride and sperm; Protein deficient diet; Protein enriched diet; Sperm function in mice.

INTRODUCTION
Our earlier investigations revealed that fluoride affects the structure and function of several tissues and organs of rats and mice, including liver, muscle, kidney, brain, endocrine glands, reproductive organs in both male and female, and also alters blood/serum parameters in endemic human populations. 1-5 Studies in rodents further indicated that the ingestion of fluoride in different concentrations for 30, 45, or 60 days can interfere with reproduction in both sexes. 1,2,6-7 There is also evidence of fluoride induced oxidative stress in testis and ovary as well as in other organs. 8-14 The disruption of reproductive functions in male mice with ingestion of a protein deficient diet with or without three dose levels of sodium fluoride (NaF) in different groups of mice for 30 days has been reported. 15 That study further elucidated that a protein supplemented diet with or without NaF had a beneficial effect to counteract fluoride induced toxicity.

The present work was undertaken to gain further knowledge about the effects of ingestion of protein deficient and protein supplemented diets alone and together with three dose levels of NaF for 30 days on some specific sperm functional parameters in mice in comparison with mice fed a control protein diet plus NaF at the same three dose levels.

aFor correspondence: Reproductive Endocrinology and Toxicology Unit, Department of Zoology, School of Sciences, Gujarat University, Ahmedabad-380 009, India.
E-mail: zooldeptgu@satyam.net.in
MATERIALS AND METHODS

Animals: Healthy, adult male Swiss strain mice (*Mus musculus*) weighing between 30 and 40 g were obtained from the National Institute of Occupational Health (NIOH), Ahmedabad, Gujarat, India, under the Animal Maintenance and Registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Government of India, and the Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India. The animals were housed in an air conditioned animal unit at 26°C ± 2°C with 10 to 12 hours of daylight/ day. They were fed standard chow and given drinking water containing 0.6–1.0 ppm F *ad libitum*.

Exposure: The animals were divided into twelve groups as shown in the Experimental Protocol table below. Sodium fluoride (NaF, Loba Chemie, Bombay, 99% purity) was administered to mice orally using a feeding tube attached to a hypodermic syringe. The NaF was given to the mice in three dose levels of 5, 10, or 20 mg/kg body weight (bw). These doses were selected on the basis of the LD50 value of fluoride in male mice, which is 54.4 mg F/kg body wt. Oral administration was preferred since water is the main source of fluoride among human populations in endemic areas.

Diets: The control protein diet and the protein enriched and protein deficient diets were prepared according to the protocol of the National Institute of Occupational Health (NIOH), Ahmedabad, India. The diets had the following composition:

<table>
<thead>
<tr>
<th>Diet</th>
<th>Protein (%)</th>
<th>Casein (%)</th>
<th>Starch powder (%)</th>
<th>Salt mixture (%)</th>
<th>Vitamin mixture (%)</th>
<th>Ground nut oil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>20</td>
<td>23.53</td>
<td>63.47</td>
<td>4</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Protein deficient diet</td>
<td>5</td>
<td>5.88</td>
<td>81.12</td>
<td>4</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Protein enriched diet</td>
<td>40</td>
<td>47.06</td>
<td>39.94</td>
<td>4</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

Experimental protocol:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment and dose (10-15 animals used in each group)</th>
<th>Duration (days)</th>
<th>Day of autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control protein diet (20% protein)</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>II</td>
<td>Protein deficient diet (5% protein)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>III</td>
<td>Protein enriched diet (40% protein)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>IV</td>
<td>Control protein diet + NaF (5 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>V</td>
<td>Protein deficient diet + NaF (5 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>VI</td>
<td>Protein enriched diet + NaF (5 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>VII</td>
<td>Control protein diet + NaF (10 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>VIII</td>
<td>Protein deficient diet + NaF (10 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>IX</td>
<td>Protein enriched diet + NaF (10 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>X</td>
<td>Control protein diet + NaF (20 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>XI</td>
<td>Protein deficient diet + NaF (20 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
<tr>
<td>XII</td>
<td>Protein enriched diet + NaF (20 mg/kg bw)</td>
<td>30</td>
<td>31st</td>
</tr>
</tbody>
</table>

* Sacrificed with treated group; bw = body weight.

Data collection: The control and treated animals were weighed on an animal weighing balance (Ohaus, USA) and sacrificed by cervical dislocation after the respective treatments. The cauda epididymis was dissected out carefully, blotted free of blood, and used for the study. The sperm suspension in buffered saline was employed for the sperm studies.
Acridine orange staining,\textsuperscript{17} sperm motility and count,\textsuperscript{18} sperm viability,\textsuperscript{19} sperm mitochondrial activity index (SMAI),\textsuperscript{20} sperm morphology,\textsuperscript{21} total acrosin,\textsuperscript{22} and fertility rate\textsuperscript{24} were determined according to the methods cited.

\textbf{Statistical analysis:} For all the parameters, a minimum of five or six replicates were made and the data were analyzed by Student’s t test and ANOVA.

\section*{RESULTS}

The cauda epididymal sperm motility, count, viability, SMAI, activities of hyaluronidase and acrosin and fertility rate in the mice decreased significantly in Groups IV, VII, X (Control protein diet + 5, 10, and 20 mg NaF/kg body weight) and in Groups II, V, VIII and XI (Protein deficient diet with or without three doses of NaF (Tables 1 and 2).)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Treatment} & \textbf{Motility} & \textbf{Count} & \textbf{Viability} & \textbf{Fertility rate} \\
\hline
I & Control protein diet (20% protein) & 80.45±1.08 & 44.62±0.20 & 78.83±1.60 & 95-100% \\
II & Protein deficient diet (5% protein) & 45.08±0.74$^\S$ & 30.51±0.42$^\S$ & 54.00±2.09$^\S$ & 10% \\
III & Protein enriched diet (40% protein) & 81.32±1.87 & 44.69±0.39 & 78.85±1.86 & 95-100% \\
IV & Control protein diet + NaF (5 mg/kg bw) & 45.37±0.97$^\S$ & 30.60±0.39$^\S$ & 58.83±3.12$^\S$ & 20% \\
V & Protein deficient diet + NaF (5 mg/kg bw) & 45.31±0.38 & 29.99±0.51 & 52.50±2.16 & Nil \\
VI & Protein enriched diet + NaF (5 mg/kg bw) & 80.44±0.56 & 40.94±0.40 & 79.00±1.79 & 95% \\
VII & Control protein diet + NaF (10 mg/kg bw) & 32.47±0.61$^\S$ & 28.21±0.48$^\S$ & 47.00±1.09$^\S$ & 10% \\
VIII & Protein deficient diet + NaF (10 mg/kg bw) & 31.68±0.81$^\S$ & 27.54±0.75$^\S$ & 50.00±1.21$^\S$ & Nil \\
IX & Protein enriched diet + NaF (10 mg/kg bw) & 80.27±0.75 & 42.54±1.14 & 78.00±1.36 & 90% \\
XI & Protein deficient diet + NaF (10 mg/kg bw) & 31.87±0.91$^\S$ & 27.15±0.82$^\S$ & 43.00±2.90$^\S$ & Nil \\
XII & Protein enriched diet + NaF (20 mg/kg bw) & 79.25±1.01 & 40.07±0.25 & 74.00±1.82 & 85% \\
\hline
\end{tabular}
\caption{Cauda epididymal sperm motility, sperm count, sperm viability and fertility rate in mice of Groups I to XII.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Treatment} & \textbf{Sperm hyaluronidase activity (HYALU, nm-N-acetyl glucosamine liberated/hr/10^6 sperm)} & \textbf{Sperm mitochondrial activity index (SMAI, %)} & \textbf{Abnormal sperm forms (\%)} & \textbf{Total acrosin (\mu moles of benzoylarginine ethyl ester (BAEE) hydrolysed/min/10^6 sperms)} \\
\hline
I & Control protein diet (20% protein) & 4.56±0.14 & 41.70±0.13 & 12.65±0.19 & 5.45±0.06 \\
II & Protein deficient diet (5% protein) & 3.25±0.42$^\S$ & 33.06±0.40$^\S$ & 26.08±1.36$^\S$ & 4.38±0.03$^\S$ \\
III & Protein enriched diet (40% protein) & 4.25±0.39 & 41.88±0.21 & 12.55±0.13 & 5.38±0.08 \\
IV & Control protein diet + NaF (5 mg/kg bw) & 3.32±0.39$^*$ & 29.53±0.70$^*$ & 43.69±0.57$^*$ & 42.22±0.12$^*$ \\
V & Protein deficient diet + NaF (5 mg/kg bw) & 2.63±0.18$^\S$ & 28.26±1.07$^\S$ & 34.83±3.60$^\S$ & 4.15±0.04 \\
VI & Protein enriched diet + NaF (5 mg/kg bw) & 4.17±0.24 & 40.41±0.63 & 12.69±0.24 & 5.34±0.07 \\
VII & Control protein diet + NaF (10 mg/kg bw) & 1.96±0.24$^S$ & 22.53±0.38$^S$ & 49.55±1.05$^S$ & 4.23±0.02$^S$ \\
VIII & Protein deficient diet + NaF (10 mg/kg bw) & 1.55±0.77$^S$ & 21.69±0.94$^S$ & 50.27±10.9$^S$ & 4.26±0.03 \\
IX & Protein enriched diet + NaF (10 mg/kg bw) & 4.21±0.37 & 29.98±10.60 & 12.81±0.37 & 5.28±0.05 \\
X & Control protein diet + NaF (20 mg/kg bw) & 1.38±0.10$^\S$ & 21.26±0.61$^\S$ & 59.97±1.89$^\S$ & 4.28±0.04$^\S$ \\
XI & Protein deficient diet + NaF (20 mg/kg bw) & 1.27±0.15$\S$ & 20.07±0.61$\S$ & 57.18±1.32$\S$ & 4.21±0.06 \\
XII & Protein enriched diet + NaF (20 mg/kg bw) & 4.57±0.24 & 39.98±0.42 & 13.36±0.57 & 5.24±0.14 \\
\hline
\end{tabular}
\caption{Cauda epididymal sperm hyaluronidase activity (HYALU, nm-N-acetyl glucosamine liberated/hr/10^6 sperm), sperm mitochondrial activity index (SMAI, %), sperm abnormal forms (%), and total acrosin (\mu moles of benzoylarginine ethyl ester (BAEE) hydrolysed/min/10^6 sperms) in mice of Groups I to XII.}
\end{table}

\textsuperscript{a}Data are expressed as mean ± S.E. $^*P<0.05; ^\S P<0.02; ^\S P<0.01; ^\S P<0.001; \text{no sign}=\text{not significant. Comparison between Group I with II or III or IV or VII or X; Group II with V or VIII or XI; Group III with VI or IX or XII. bw}=\text{body weight.}$
On the other hand, as also seen in Tables 1 and 2, the percentage of abnormal forms of sperm increased significantly, whereas DNA integrity (as determined by acridine orange staining) was affected less in all the above mentioned groups. However, mice in Groups III, VI, IX and XII who were supplied with a protein enriched diet alone or with the three dose levels of NaF showed no changes in any of the parameters as compared to control.

Table 3. ANOVA of various parameters of mice sperm and fertility rate

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-Cal</th>
<th>F-Tab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm motility</td>
<td>Between groups</td>
<td>34374.3</td>
<td>11</td>
<td>3124.936</td>
<td>3569.658</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>52.5251</td>
<td>60</td>
<td>0.875419</td>
<td>885.4975</td>
<td>1.9522</td>
</tr>
<tr>
<td>Sperm count</td>
<td>Between groups</td>
<td>3616.886</td>
<td>11</td>
<td>328.8079</td>
<td>379.5707</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>22.2795</td>
<td>60</td>
<td>0.371326</td>
<td>4.002778</td>
<td></td>
</tr>
<tr>
<td>Sperm viability</td>
<td>Between groups</td>
<td>16712.71</td>
<td>11</td>
<td>1519.337</td>
<td>37.95707</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>240.1667</td>
<td>60</td>
<td>0.400277</td>
<td>1.9522</td>
<td></td>
</tr>
<tr>
<td>Fertility rate</td>
<td>Between groups</td>
<td>110617.8</td>
<td>11</td>
<td>10065.16</td>
<td>4333.767</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>1112.8</td>
<td>60</td>
<td>23.18333</td>
<td>0.371326</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Between groups</td>
<td>111.7161</td>
<td>11</td>
<td>10.15601</td>
<td>154.4085</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>3.9464</td>
<td>60</td>
<td>0.065774</td>
<td>0.065774</td>
<td></td>
</tr>
<tr>
<td>SMAI</td>
<td>Between groups</td>
<td>4308.53</td>
<td>11</td>
<td>391.6846</td>
<td>1135.929</td>
<td>1.9946</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>16.5511</td>
<td>48</td>
<td>0.344814</td>
<td>0.344814</td>
<td></td>
</tr>
<tr>
<td>Abnormal sperm forms</td>
<td>Between groups</td>
<td>20382.9</td>
<td>11</td>
<td>1852.991</td>
<td>1094.393</td>
<td>1.9946</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>81.2721</td>
<td>48</td>
<td>1.693163</td>
<td>1.693163</td>
<td></td>
</tr>
<tr>
<td>Total acrosin</td>
<td>Between groups</td>
<td>15.6778</td>
<td>11</td>
<td>1.425257</td>
<td>62.65305</td>
<td>1.9522</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>1.09192</td>
<td>60</td>
<td>0.022748</td>
<td>0.022748</td>
<td></td>
</tr>
</tbody>
</table>

SS=Sum of squares; df=degree of freedom; MS=Mean sum of squares; F-cal=Fisher calculated; F-tab=Fisher tabulated.

DISCUSSION

The present study revealed a reduction in sperm motility especially in mice treated with NaF and fed a control protein diet as well as a protein deficient diet. These results could be due to the lowered metabolic activity caused by fluoride.\textsuperscript{1,2} Chinoy and Narayana\textsuperscript{25} reported that human spermatozoa lost their motility \textit{in vitro} in the presence of 0.25 mM NaF within 20 min after incubation, while Schoff and Lardy\textsuperscript{26} demonstrated that bovine sperm treated with 30 mM fluoride became immobile within two minutes. Earlier studies\textsuperscript{27-35} also elicited similar effects on spermatozoa of mice, rats, rabbits, and guinea pigs by NaF treatment. The sperm mitochondrial activity index (SMAI) was also inhibited, thereby confirming the low metabolic activity of the spermatozoa as well as the significant decline in the percentage of live spermatozoa or the increase in abnormal spermatozoa after treatment.

The decrease in cauda epididymal sperm count in NaF treated mice ingesting a control protein diet and a protein deficient diet could be correlated with testicular spermatogenic arrest after NaF treatment.\textsuperscript{31, 36-41} Similar reduction in sperm density in mice, rats, and rabbits has been reported following fluoride ingestion.\textsuperscript{27,29,32,34,37}
It is noteworthy that the treatments did not affect the nuclear integrity as observed by acridine orange staining of spermatozoa. The sperm acrosomal membrane bound enzymes, viz., hyaluronidase and acrosin, are involved in the acrosome reaction before fertilization. Hyaluronidase, a lysosomal enzyme, has a role in the dispersion of cumulus oophorus and thus facilitates sperm penetration. The reduction in hyaluronidase and acrosin activities obtained after treatment might therefore be associated with lower penetrating and fertilizing ability of the sperm resulting in decreased fertility rate. Neelam et al. have also observed infertility among men in a higher fluoride endemic area in India.

The above mentioned alterations in sperm morphology, viability, and metabolism might be the outcome of the altered and hostile internal milieu of the epididymis of treated mice, since the epididymal microenvironment is important for sperm maturation and maintenance in a viable, motile state. This interpretation is corroborated by other studies. This investigation demonstrated that fluoride has adverse effects on several sperm functional parameters, especially when fed to mice along with a standard control protein diet and a protein deficient diet. On the other hand, ingestion of a protein rich diet overcame these effects, thus emphasizing the importance of increased levels of protein to offset or reduce fluoride toxicity.

This paper was presented at the XXVIth Conference of the International Society for Fluoride Research, Wiesbaden, Germany, September 26–29, 2005.

ACKNOWLEDGEMENTS

We are grateful to the Director, National Institute of Occupational Health (NIOH), Ahmedabad, India, for supplying the protocols for preparing the different protein diets.

REFERENCES

41 Chinoy NJ, Tewari K, Jhala DD. Fluoride and/or arsenic toxicity in mice testis with formation of giant cells and subsequent recovery by some antidotes. Fluoride 2004;37(3):172-84.