Ginger ameliorates reproductive toxicity of formaldehyde in male mice: Evidences for Bcl-2 and Bax

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**Introduction:** Root of dietary ginger considerably improves the activity of antioxidant enzymes in reproductive system and reduces the signs of cell damage in testis tissues. The present study conducted numerous sperm, hormonal, bio-chemical analysis and gene expression in order to evaluate the reproductive damages caused by exposure to formaldehyde (FA) and to investigate the ameliorative properties of co-administration of FA and Zingiber officinale (Ginger) in mice model.

**Methods:** Forty-eight male NMRI mice were randomized into 6 groups of 8 animals each, including control group, control sham (received distilled water by gavage), FA group (10 mg/kg twice per day), intraperitoneally (i.p) and 3 FA groups (10 mg/kg i.p) + Ginger (500, 1000 and 2000 mg/kg/d by gavage, respectively). Sperm parameters, sexual hormones, antioxidant activity and expression of Bax and Bcl-2 genes were analyzed after 35 days.

**Results:** FA significantly diminished sperm parameters, sexual hormones and antioxidant enzymes (P<0.05). Also, the expression of Bcl-2 and Bax genes had significant (P<0.05) increase and decrease, respectively in FA group. Co-administration of Ginger extract significantly recovered the above parameters.

**Conclusion:** Co-administration of Ginger extract ameliorates reproductive damages of FA by its androgenic, antioxidant and anti-apoptotic properties. Hence, it might be beneficial in these patients.

**Implication for health policy/practice/research/medical education:** Ginger significantly ameliorates oxidative stress induced by formaldehyde toxicity in male reproductive system, therefore, this herb could be used as a natural source for inventing new drugs to cure male infertility.

as a medicinal plant (17). Also, because of the existence of antioxidant compounds such as zingerone, zingibrence, gingerol, glucosides-6-gingerdiol, volatile oils and flavonoid substances in Ginger, it is classified as a strong antioxidant plant. The compounds found in Ginger are able to scavenge free radicals, hence Ginger can prevent peroxidation induced by free radicals in cellular structure (18,19).

Ginger exhibits pro-fertility properties in male animal models (20). Administration of Ginger considerably increases the percentage of sperm viability and motility and also concentration of serum total testosterone. Hence, it can be used to improve sperm quality (19). Root of dietary ginger considerably improves the activity of antioxidant enzymes and malondialdehyde (MDA) level in the reproductive system and also reduces the signs of cell damage in testis tissues (11,18).

The present study conducts numerous sperm, hormonal, bio-chemical analysis and gene expression in order to evaluate the reproductive damages caused by exposure to FA and to investigate the ameliorative properties of co-administration of FA and Ginger in mice model.

Materials and Methods
Ginger extract preparation
Ginger rhizomes were obtained from an herbal store in Urmia, Iran. One hundred grams of ground dried Ginger rhizomes were added to 1000 mL of 96% ethanol and water (50:50) solution for 72 hr. Gauze and Whatman filter (No. 40) was used to filter the extract. Later, using a rotary evaporator, ethanol was evaporated at 40°C under vacuum.

Animals
Adult male NMRI mice at the age of 6-8 weeks old and weighting 30±2 g were obtained from the animal house of Urmia University of Medical Sciences (Urmia, Iran). They received a pellet diet and tap water ad libitum. The animals were kept in polycarbonate cages with woodchip bedding under 12:12 h light/dark cycle. The room temperature and humidity were 22±2°C and 50±10%, respectively. They were kept for 2 weeks to acclimatize before using them for experimental purposes.

Experimental design
Animals were randomly categorized into six groups (n=8). Group I was chosen as the control group. Group II was the sham group and received 0.2 mL distilled water by gavage. Group III intraperitoneally received 10 mg/kg of FA twice a day. Group IV received Ginger (0.5 g/kg/d) by gavage and also FA at dose of 10 mg/kg/d, intraperitoneally. Group V received ginger at dose of 1 g/kg/d, by gavage and FA at dose of 10 mg/kg twice per day, i.p. Group VI received ginger at dose of 2 g/kg/d, by gavage and FA at dose of 10 mg/kg twice per day, i.p. FA and Ginger were simultaneously administrated in groups IV to VI. The doses of Ginger and FA administrated to animals were chosen based on Rong et al (21) and Tajaddini Mahani et al (7) studies, respectively.

Sampling of serum and collecting tissue
The mice were decapitated under anesthesia after 35 days. Their blood was transferred into laboratory tubes and centrifuged for 10 minutes at the rate of 3000 rpm. The serum was obtained and frozen at -20°C for biomedical experiments after aliquoting into micro tubes. The right testes of the mice were obtained from all groups and stored at -20°C in order to analyze the testicular enzyme activity.

Epididymal sperm analysis
In order to obtain the epididymal sperm content of the mice, the tails of epididymes were excised and minced to small pieces and put into a Petri dish contained 1 mL of pre-heated HTF (human tubal fluid) medium (pH=7.4). The Petri dishes were put in incubator for 20 minutes at 37°C to let the sperms swim out from epididymis. HTF medium was used to dilute the sperm suspension (1:20). Sperm parameters were analyzed based on WHO laboratory manual for the examination and processing of human semen (22).

Sperm motility
A phase contrast microscope (BC41, Olympus Co., Japan) was utilized in order to analyze a drop of sperm suspension. A minimum of ten fields were noticed in 400X magnification in order to determine the sperm motility percentage.

Sperm count
Hemocytometer (HBG, Germany) was used to specify the sperm concentration. About 10 µL of sperm solution was put in the chambers of hemocytometer and left for 5 minutes in order to impede drying. The settled sperms were moved to a phase contrast microscope and analyzed under 400X magnification and the sperm count was stated by “n x 50000 x d” formula, in which n is the count and d is the dilution solution.

Sperm morphology and viability
Eosin nigro sine stain and the suspension of diluted sperm were combined in an equal volume. The stained mixture was spread on clean slides and air-dried. The obtained sperms were analyzed by a phase contrast microscope (400X). The viability percentage calculation was conducted by scoring 200 sperm cells per mouse. The unstained sperms were alive and the color of the dead ones turned red. The experiment was conducted in a minimum of 10 fields in order to determine the live sperm percentage. The slides were also observed under 400X magnification in order to determine the morphological anomalies of sperms. The anomalies included amorphous shape, not having hook, bicephalic, coiled or abnormal
tails. About 200 sperms were inspected in each mouse via the microscope (23).

**Teratozoospermia index**

Teratozoospermia index (TZI) expresses the number of anomalies per each abnormal sperm. Depending on the numbers of anomalies of each sperm in different parts (head, neck and/or tail), values of TZI are between 1-3. To obtain TZI, total of anomalies were divided by the number of abnormal spermatozoa as described by Krassas et al. (24).

**DNA fragmentation**

Staining with acridine orange (AO) was utilized to assess sperms DNA fragmentation. Carnoy’s fixative (methanol/acetate acid 3:1) was used to fix smears and they were placed in the fixative for two hours. After removing from the fixative, they were left intact at room temperature in order to be dried. Then, slides were put in a stock solution containing 1mg AO and 1000 mL distilled water. The solution was placed in a dark place with the temperature of 4°C. Meanwhile, the stained solution was prepared containing a mixture of 10 mL stock and 40 mL of 0.3 M NaHPO4.7H2O solution. Fluorescent microscope was used for the analysis of the sperms following staining for 5 minutes. Normal sperms were green and the yellow-red sperms were classified as anomalous. Finally, the percentage of sperms having green and orange/red fluorescence were determined (25).

**Hormonal assays**

Testosterone’s serum levels were analyzed by ELISA based on the manual provided by the manufacturer’s kit (Diablast Co., USA). Commercial kits (Amersham, Buckinghamshire, UK) were used to determine serum LH and FSH by ELISA according to previous studies (26).

**Antioxidant activity and lipid peroxidation**

Ferric reduction antioxidant power assay (FRAP) was used to determine the total antioxidant capacity of the semen (27). One microliter of FRAP reagent (Tritipiridyl triazine; Merck, Germany) was mixed with 100 μL of cellular supernatant and incubated for 10 minutes at 37°C in dark environment. The blue-colored reagent was read at 595 nm in 20 seconds interval for 10 minutes. FeII (FeSO4.7H2O) solution was selected as blank solution. Fluorescent microscope was used for the analysis of the sperms following staining for 5 minutes. Normal sperms were green and the yellow-red sperms were classified as anomalous. Finally, the percentage of sperms having green and orange/red fluorescence were determined (25).

**Isolation of RNA**

Based on the manual provided by the manufacturer, RNX plus solution (Cinnagen, Iran) was used to isolate the total RNA for the testis. One microliter of RNX solution was added to homogenize the tissue and left intact in the room temperature for 5 minutes. Later, 200 μL chloroform was added to the tube and centrifuged for 15 minutes at the rate of 12000 g at 4P o PC. RNA was obtained from the solution and an equal amount of isopropanol was added to it. The resulting compound was placed in the centrifuge with the rate of 12000 g, at 4P o PC for 10 minutes. 75% ethanol was used to rinse the pellet and suspended again in 50 μL of diethylpyrocarbonate (DEPC) treated water. Absorbance ratio A260/A280 nm was used to evaluate RNA quantity and its integrity was measured by 1% agarose gel electrophoresis.

**cDNA synthesis for real-time RT-PCR**

cDNA Synthesis Kit Revert Aid was obtained from the Fermentas Corporation (Germany). One microgram of RNA was reverse transcribed with 5X Reaction Buffer, 20 U/μL Ribolock RNase inhibitor, 10 mM dNTP , 200 U/μL MMLV reverse transcriptase, and oligo (dt) 18 primer in a 20 μL reaction in order to synthesize cDNA. The resulting combination was incubated for 60 minutes in 42°C. Later, the enzyme was inactivated for 5 minutes at 70°C.

**Real-time RT-PCR**

RT-PCR was used to specify the levels of Bcl-2 and Bax transcripts using SinaClon (Tehran, Iran). In order to normalize the input load of cDNA, 18SrRNA RA was chosen as a house-keeping gene. Nucleotide sequence of GenBank was used as the basis of PCR primers for finding the Bax, 18SrRNA and Bcl-2 genes in the mitochondrial genome of mice. The nucleotide sequences of the primer pair chosen for 18SrRNA were as follows: 5’- TGCGGAAGGATCATTAACGGA-3’ and reverse, 5’-GAGTGGAGAGGAGGCAGACC-3’ with amplified product of expected 300 bp. The sequences selected for Bcl-2 were as follows: forward, 5’-TACCGTCGTGACTTCCAGAG-3’ and reverse, 5’-GCGACGCTGAGGAGGTTT-3’ with amplified product of expected 350 bp. The nucleotide sequences of the primer pair selected for Bax were as follows: forward, 5’- CCGGGAATTGGGATGAACCTGA-3’ and reverse, 5’- GCCAAAGTAGAAGAGGCAACC-3’. These
primers yielded a 160 bp product. Hot Taq (cat# BT 11101- SinaClon, Iran) was used for conducting PCR (RT- qPCR) analysis. One microliter of cDNA was combined with a mixture of 1 μM of each primer and 4 μL of Hot Taq Eva Green Ready Mix (SinaClon, Iran) and the total amount reached 25 μL. 1.5% agarose gel electrophoresis was conducted on an aliquot of each reaction mixture. Reaction condition was 95˚C for 15 minutes, 40 cycles of 95˚C for 15 seconds, 62˚C for 60 seconds, and 70˚C for 20 seconds. The amplification of PCR was conducted for 3 times for each sample by using Bcl-2, Bax, and 18SrRNA (32).

The obtained standard curve data were used to measure the efficacy of PCR amplification of Bcl-2, Bax and 18SrRNA. The value of cycle threshold (CT) for the target genes (Bcl-2 and Bax) were normalized according to the reference gene (18SrRNA), and ΔΔCT model was used to conduct relative quantification. Melting curve analysis was done following the RT-PCR in order to ensure the homogeneity of the product.

Statistical analysis

All values were presented as the mean ± standard deviation (SD). Differences between mean values were compared using SPSS 17.0 (Chicago, USA) by one-way analysis of variance (ANOVA) test followed by Tukey post hoc. P < 0.05 was considered as statistically significant.

Results

Sperm parameters

Table 1 shows parameters including sperm count, viability, motility, morphology, TZI, and DNA fragmentation. A significant decrease was seen in FA group in comparison to other groups. Simultaneous administration of Ginger along with Ginger reduced the decrease in parameters of sperm in comparison to FA group. Hence, using Ginger leads to a decrease in sperm parameters but it is not comparable to that of control sham and sham groups (Figures 1 and 2).

Hormonal assays

Mean serum concentration of sexual hormones (testosterone, luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) are shown in Table 2. FA group exhibited a significant decrease in hormonal concentration in comparison to other groups. It should be noted that the group receiving Ginger had a considerable increase dependent on the dosage, but it could not reach to control sham and control groups.

Antioxidant activity and lipid peroxidation

FA group had experienced a considerable decrease in antioxidant activity. The groups receiving Ginger and FA had higher antioxidant activity in comparison to FA group (Table 3). FA groups also showed a considerable increase of MDA but it decreased in Ginger + FA groups dependent on the dose (Table 4).

Real-Time PCR

As it is obvious in Table 5, Bcl-2 expression was considerably lower in comparison to control and other groups, but it had incremental in Ginger + FA groups. FA group showed a considerable increase in Bax gene expression while Ginger + FA groups had significant decreases (Figure 3).

Discussion

In this study damages caused by exposure to FA and ameliorative effects of co-administration of Ginger with FA were demonstrated by evaluation of sperm parameters,

<table>
<thead>
<tr>
<th>Table 1. Comparison of sperm parameters in different groups</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>Sperm count (10⁶)</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
</tr>
<tr>
<td>Sperm morphology (%)</td>
</tr>
<tr>
<td>DNA fragmentation (%)</td>
</tr>
<tr>
<td>Teratozoospermia index</td>
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</tbody>
</table>

Abbreviation: Fa, formaldehyde.
Different letters indicate significant differences (P < 0.05) between groups. Values represent means ± SEM (N = 8).

<table>
<thead>
<tr>
<th>Table 2. The serum mean concentrations of sexual hormones in different groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>Testosterone (μmol/L)</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
</tr>
</tbody>
</table>

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Different letters indicate significant differences (P < 0.05) between groups. Values represent means ± SEM (N = 8).
Ginger ameliorates reproductive toxicity of formaldehyde

Figure 1. Sperm viability; Dead sperm stained red (White arrow) and viable sperms are colorless (Black arrows); (Eosin/nigrosin, 1000×).

Figure 2. Mice spermatozoa; Normal sperm stained green (Black arrow) and damaged DNA stained yellow (White arrow); (AO, 400×).

Figure 3. Effects of Ginger/FA on the expression of: (Lane 1-2) 18SrRNA (300 bp), (Lane 3-4) Bax (160 bp) and (Lane 5-6) Bcl-2 (350 bp) at mRNA level.

sexual hormones, antioxidant activity, lipid peroxidation and gene expression of apoptotic and anti-apoptotic factors.

The reduction of sperm quality in the present study indicates negative effects of oxidative stress induced by FA on male reproductive system. Several other studies have indicated the adverse effects of FA on sperm parameters (6,7,33,34) and our study is in line with them. FA has ability to cross the blood-testis barrier and induces oxidative stress by producing ROS which leads to degeneration of germ cells and disruption of spermatogenesis (6,33).

Exposure to FA also initiates autophagy in testis which is related to spermatogenetic failure and germ cells death (35). FA decreased Tsga10 which is correlated with quality of sperm parameters, especially morphology and motility rates (7). Exposure to FA also decreases number of Leydig cells in testes (6,13). Henkel et al (36) demonstrated a direct correlation between sperm motility and decline in Leydig cells. A direct relationship between the FA exposure and dispersion of DNA chromatin of sperm was found by Betancourt-Martínez et al (34).

In this research, Ginger extract improved sperm parameters when administrated simultaneously with FA. This results confirm previous reports (19,20,37–39). It might be due to Ginger could prevent from apoptosis or/and due to decrease of ROS in testis tissue. Mohammadi et al (38) indicated that Ginger extract could cause a significant increase in Sertoli cells. Therefore, the increase in Sertoli cells might be one reason for the significant improvement of spermatogenesis.

Similar to other studies (6,8,11,13), in this study FA exposure reduced levels of testosterone in serum. It might be consequence of LH decrement, because LH

Table 3. The mean amounts of antioxidant activity in different groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FA</th>
<th>Sham</th>
<th>Ginger 0.5 + FA</th>
<th>Ginger 1 + FA</th>
<th>Ginger 2+ FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAC (µmol/L)</td>
<td>1.95 ± 0.36a</td>
<td>0.77 ± 0.23b</td>
<td>1.85 ± 0.08c</td>
<td>0.95 ± 0.13d</td>
<td>1.37 ± 0.24e</td>
<td>1.40 ± 0.19e</td>
</tr>
<tr>
<td>SOD (U/mg of tissue proteins)</td>
<td>1519 ± 1.48a</td>
<td>1078 ± 1.08b</td>
<td>1510 ± 1.33c</td>
<td>1120 ± 0.94d</td>
<td>1193 ± 1.62e</td>
<td>1276 ± 1.29e</td>
</tr>
<tr>
<td>GPx(U/mg of tissue proteins)</td>
<td>6.19 ± 1.35a</td>
<td>2.88 ± 1.20b</td>
<td>6.08 ± 1.76c</td>
<td>3.27 ± 1.28e</td>
<td>4.09 ± 1.13e</td>
<td>4.73 ± 1.41e</td>
</tr>
<tr>
<td>CAT (U/mg of tissue proteins)</td>
<td>1.07 ± 0.54a</td>
<td>0.54 ± 0.33b</td>
<td>1.04 ± 0.49c</td>
<td>0.62 ± 0.23d</td>
<td>0.86 ± 0.41e</td>
<td>1.01 ± 0.35e</td>
</tr>
<tr>
<td>GSH (U/mg of tissue proteins)</td>
<td>73.30 ±0.89a</td>
<td>48.66 ±1.01b</td>
<td>72.38 ±0.77c</td>
<td>55.72 ±0.89c</td>
<td>56.30 ±0.63e</td>
<td>62.08 ±1.06e</td>
</tr>
</tbody>
</table>

Abbreviations: Fa, formaldehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase
Different letters indicate significant differences (P< 0.05) between groups. Values represent means ± SEM (N = 8).

Table 4. Comparison of MDA in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>FA</th>
<th>Sham</th>
<th>Ginger 0.5 + FA</th>
<th>Ginger 1 + FA</th>
<th>Ginger 2+ FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol.mg⁻¹)</td>
<td>2.74 ± 1.22a</td>
<td>7.23 ± 1.37b</td>
<td>2.81 ± 1.09c</td>
<td>6.85 ± 1.30d</td>
<td>5.79 ± 0.89e</td>
<td>4.38 ± 1.32e</td>
</tr>
</tbody>
</table>

Abbreviation: MDA, malondialdehyde.
Different letters indicate significant differences (P < 0.05) between groups. Values represent means ± SEM (N = 8).
Therefore, it inhibits release of 11-CAT, GPX, and GSH activity. Furthermore, it led to the reduction of lipid peroxidation in the testes. These findings have been proved by previous studies (11,18,38,47). As a pro-apoptotic protein, Bax leads to mitochondrial membrane release of cytochrome C into cytoplasm which results in activation of caspase and starting of apoptosis process. Bcl-2 prevents the insertion of Bax into the mitochondrial membrane. Therefore, it inhibits release of cytochrome C and as a result prevents apoptosis process (9). Real-time PCR delineated that FA decreased Bcl-2 gene expression and increased expression of Bax gene in mice testes. These findings support the results obtained by Özen et al (9) which showed exposure to FA induces apoptosis in the spermatogenic and Leydig cells. Thereby, it can be concluded that FA reduced spermatogenesis probably by induction of apoptosis in spermatogenic germ cells. Co-administration of Ginger with FA down-regulated Bax and up-regulated Bcl-2 genes expression. These results confirm anti-apoptotic effects of Ginger which reported in previous studies (47-49).

**Conclusion**

This paper investigated the ameliorative effects of Ginger on FA induced oxidative stress in male mice reproductive system. It is recommended that more researches be conducted on the relationship between the co-administration of Ginger with FA and gene expression such as apoptotic and testis-specific genes in male fertility. Based on this study, we suggest powerful anti-oxidants such as Ginger be added to the daily diet of people who are at risk of exposure to environmental toxic contaminants which cause oxidative stress in order to decrease the chance of infertility.

**Acknowledgements**

The authors would like to sincerely thank the members of the Faculty of Veterinary Medicine and Urmia University Research Council for the approval and support of this research protocol.

**Authors’ contributions**

AS contributed in conception, design, data collection, statistical analysis and drafting of the manuscript. AS, MP, ND and MK, contributed in conception, design, supervision of the study and drafting of the manuscript. All authors approved the final version for submission.

**Conflict of interests**

The authors declare that there is no conflict of interest.

**Ethical Considerations**

The study was approved by Animal Ethics Committee in Urmia University, Urmia, Iran (AECVU-163-2018) and conducted under the regulations of this committee.

**Funding/Support**

This research has been financially supported by Research Council of Urmia University.

**References**

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