Collapsed mitochondrial cristae in goat spermatozoa due to mercury result in lethality and compromised motility along with altered kinematic patterns

Bhawna Kushawaha1,4,6*, Rajkumar Singh Yadav2,4, Dilip Kumar Swain3,4, Priyambada Kumari1,4, Akhilesh Kumar1,4, Brijesh Yadav3,4, Mukul Anand3,4, Sarvajeet Yadav3,4, Dipty Singh5 & Satish Kumar Garg2,4

Earlier we have reported mercury-induced alterations in functional dynamics of buck spermatozoa through free radicals-mediated oxidative stress and spontaneous acrosome reaction. Based on our earlier findings, we aimed to investigate the effect of mercury exposure on motility, kinematic patterns, DNA damage, apoptosis and ultra-structural alterations in goat spermatozoa following in vitro exposure to different concentrations (0.031–1.25 µg/ml) of mercuric chloride for 15 min and 3 h. Following exposure of sperm cells to 0.031 µg/ml of mercuric chloride for 3 h, livability and motility of sperms was significantly reduced along with altered kinematic patterns, significant increase in per cent necrotic sperm cells and number of cells showing DNA damage; and this effect was dose- and time-dependent. Contrary to up-regulation of Bax gene after 3 h in control group, there was significant increase in expression of Bcl-2 in mercury-treated groups. Transmission electron microscopy studies revealed rifts and nicks in plasma and acrosomal membrane, mitochondrial sheath, and collapsed mitochondria with loss of helical organization of mitochondria in the middle piece of spermatozoa. Our findings evidently suggest that mercury induces necrosis instead of apoptosis and targets the membrane, acrosome, mid piece of sperms; and the damage to mitochondria seems to be responsible for alterations in functional and kinematic attributes of spermatozoa.

Lead, mercury, cadmium, nickel and arsenic have been reported to adversely affect male fertility including fertilizing competence of spermatozoa1. In-vivo toxicological studies have shown that heavy metals have the tendency to accumulate in testis and disrupt endocrine and regenerative capacity of testicular cells2. Mercury is extensively used in day-to-day life like in dental amalgam, thermometers, batteries, logical gadgets, electrical switches, semiconductors, additives, pesticides, and pharmaceutical formulations3,4. Toxicity of mercury in males, especially its effect on testicular functions, following in vivo exposure of animals has been reported5–7. Certain in-vitro studies have revealed adverse effects of mercury on functional dynamics of spermatozoa of bulls8, humans9, rats10, monkeys11, and fish12. We too have recently reported mercury-induced significant alterations in functional dynamics of goat spermatozoa following in vitro exposure due to significant decrease in total antioxidant defense, increase in intracellular calcium and cAMP levels along with spontaneous acrosomal reaction, inhibition of tyrosine phosphorylation and capacitation like events in sperm cells13.

1College of Biotechnology, Mathura, India. 2Department of Veterinary Pharmacology and Toxicology, Mathura, India. 3Department of Veterinary Physiology, Mathura, India. 4UP Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya (Veterinary University), Mathura 281001, Uttar Pradesh, India. 5ICMR-National Institute for Research in Reproductive Health (NIRRH), Mumbai, India. 6National Institute of Animal Biotechnology (NIAB), Hyderabad, India. *email: bhawnarajput31jan@gmail.com
Various pathways of mercury-induced cell death have been reported in different cells11–18. Biochemical mechanisms of sperm cell death, especially the genes involved in this process are currently under intense study. Ion-deregulation, particularly Ca2+, plays an important role in cell death following either apoptosis or necrosis19. In contrast to apoptosis, accidental cell death leading to necrosis with karyorrhexis, cell shrinkage, dilatation of the endoplasmic reticulum (ER), swelling of cytosol and condensed mitochondria has also been reported20. Necroptosis cell death pathway is mediated by changes in functional status of the mitochondria e.g., change in mitochondrial membrane permeability transition, loss of mitochondrial trans-membrane potential (MTP), ROS generation, Ca2+ overload and release of cytochrome-c15. Intrinsic variability of semen samples as well as individual variations or differences arising as a result of treatments can be better studied by using computer-assisted sperm analysis (CASA) system that allows simultaneous generation of huge data-sets consisting of kinematic trajectories from thousands of spermatozoa21. Therefore, CASA systems have evolved rapidly during the last decade due to major innovations in technology22.

In view of the above and also our own recent findings about the mechanism of mercury-induced alterations in functional dynamics of buck-spermatozoa, we envisaged to unravel the precise target-site and mechanism of mercury-induced alterations in functional dynamics and kinematics of goat sperm cells to substantiate our hypothesis. Therefore, the present study was undertaken to investigate mercury-induced alterations in motility and motion kinematics of goat spermatozoa along with ultra-structural changes and mechanism(s) of sperm cell death. Effect of mercury on motility and kinematic patterns will help also us in determining the quality and its suitability for cryopreservation, and also delineating the molecular mechanism(s) of its toxic effects apart from providing some insights in evolving suitable cyto-protective measures to counter mercury-induced cell damage, and improve sperm competence for successful fertilization.

Material and methods

Experimental animals. Present study was conducted on six healthy adult male goats (bucks) of Barbari breed aging between two and four years and weighing from 25 to 35 kg. Animals were maintained under standard semi-intensive system of management in goat sheds of the Department of Veterinary Physiology of the Institute. Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India were followed and the study was undertaken after approval of the experimental protocols by the Institutional Animal Ethics Committee (IAEC) of U.P. Pandit Deen Dayal Upadhayya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU) (Approval No. 110/IAEC/16).

Semen collection and dilution. Total ninety semen ejaculates were collected from six different bucks during the months of March to May and September to November using artificial vagina (AV) with graduated semen collection cups. The frequency of semen-collection from each buck was twice a week. Immediately after collection, semen samples were kept in CO2 incubator at 37 °C, then processed over thermostatically regulated stage at 37 °C and examined under phase-contrast microscope.

Semen ejaculates of good quality, as manifested by mass motility of ≥ 3, sperm motility of ≥ 80% and abnormal sperms morphology of ≤ 10%, were used for detailed investigation as established earlier in our laboratory23 and rest of the semen samples were discarded. Concentration of spermatozoa in semen samples were determined using hemocytometer (Improved Neubauer’s chamber). Semen samples were diluted using semen dilution medium (phosphate buffer solution containing 0.5% glucose and of pH 7) as described earlier24 to obtain the final working concentration of 50 × 106 spermatozoa/ml.

Experimental design. Stock solution (1 mg/ml) of mercuric chloride (HgCl2 having 73.89% Hg) was prepared in PBS (pH 7.4). The study was undertaken in four treatment groups using four different concentrations of mercury chloride (0.031, 0.125, 0.25 and 1.25 μg/ml) and one control group (PBS + 0.5% glucose). These concentrations of mercuric chloride were 1/40th, 1/10th, 1/5th and equivalent to the LC50 value of HgCl2 for goat spermatozoa13.

Effect on livability. To determine the percentage of live spermatozoa in semen samples following in vitro exposure to different concentrations of mercury chloride (0.031, 0.125, 0.25 and 1.25 μg/ml) for different time intervals (15 min, 1 h and 3 h), smears from semen samples of different groups were prepared, air-dried and then stained with Eosin-Nigrosin employing the method of Hancock24 as briefly described by us in our recent publication25.

Effect on motility and motion kinematics. Effect of mercuric chloride (0.031, 0.125, 0.25 and 1.25 μg/ml) on motility percentage, percent of total motile cells and rapid progressive motility (%), and kinematic patterns of spermatozoa—curvilinear velocity (VCL; μm s−1), straight-line velocity (VSL; μm s−1), average path velocity (VAP; μm s−1), linearity (LIN; %), straightness (STR; %), beat cross frequency (BCF; Hz), wobble (WOB; %), and maximum amplitude of head lateral displacement (maxALH; μm), compared to the control group, was determined using computer-assisted semen analyser (CASA; Biovis-2000, Version V 4.59, developed by Expert Vision Labs. Pvt. Ltd., Mumbai, India, URL:-http://www.expertvisi onlabs.com/BiovisCASA.html), sperm counting chamber, negative phase contrast and 10X objective after different times of in vitro exposure (15 min, 1 h and 3 h) as per the method described by Anand et al.25. The CASA system was programmed using algorithm based on the size, shape, and detection of sperm head as follows: Frames/s—60, number of frames acquired—61, max velocity (μm/s) for tracking 150 motility min, curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), average path velocity (VAP; μm/s) > 10 motility min, straight-line velocity (VSL; μm/s) > 1 min, track length (% of frames) 51, aspect 0–99,999, area 2–20, axis (major) 4–20, axis (minor) 2–10, compactness 0–50, perimeter ratio

Scientific Reports | (2021) 11:646 | https://doi.org/10.1038/s41598-020-08023-y

nature research
Expression of apoptotic and anti-apoptotic genes. After exposure to mercury, semen samples were centrifuged at 2000 rpm for 10 min to remove free mercury present in the media, if any, and then the samples were transferred in DEPC-treated micro-centrifuge tubes (Eppendorf). For lysis of the sperm cells, 1 ml of 0.5% Triton X-100 was added to the pelleted spermatozoa for 10 min and then tubes were briefly vortexed and centrifuged at 3000 rpm for 10 min. After that, pelleted spermatozoa were used for RNA isolation.

RNA isolation. RNA extraction was carried out using RNA isolation kit (Gene JET K0871, ThermoFischer Scientific, USA) as per the procedure described by manufacturer and stored at ~80 °C for further analysis. Purity of the total RNA was checked by using Biophotometer (Eppendorf, Germany). RNA samples having A260/A280 values equal to or more than 1.8 were used for cDNA synthesis.

Synthesis of cDNA. The cDNA was synthesized from the isolated RNA samples using Reverse Aid First Strand cDNA Synthesis Kit (ThermoFischer Scientific, USA) following manufacturer’s procedure in thermal cycler (Bio-Rad). The cDNA obtained was stored at ~20 °C. End point PCR conditions were optimized to amplify the cDNA Synthesis Kit (ThermoFischer Scientific, USA) as per the procedure described by manufacturer and stored at −80 °C for further analysis. Purity of the total RNA was checked by using Biophotometer (Eppendorf, Germany). RNA samples having A260/A280 values equal to or more than 1.8 were used for cDNA synthesis.

Real-time PCR. Reaction mixture was prepared by adding 1 µl of cDNA template (100 ng/µl), 1 µl each of the forward and reverse primers (10 pmol), 10 µl of PowerUp SYBR Green qPCR Mix (ThermoFischer Scientific, USA), and 7 µl of nuclease-free water to make up the final volume of 20 µl. Initial denaturation was performed at 95 °C for 10 min, annealing at 61 °C for Bcl-2, 60 °C for Bax and β-actin for 30 s, extension at 72 °C for 30 s for 40
cycles, and 72 °C for 5 min for final extension. Light Cycler 480 (Applied Biosystems, USA) was used to analyse the gene expression. Each gene sample was run in duplicate. Non-template control (NTC) was also run simultaneously. Pipetting was done with sterile DEPC-treated tips without creating bubbles to avoid any interference in reading of fluorescence by the instrument. Confirmation of amplification of the specific sequence was done by using agarose gel electrophoresis. 2.5% agarose was mixed with 30 ml of 1X TAE buffer at 60 V/cm. Gene ruler of 50 bp was electrophoresed in parallel to the amplicons (Figs. S1–S6 Supplementary Data).

Electron-microscopic studies. After treatment of semen samples with the lowest (0.031 μg/ml) and highest (1.25 μg/ml) used concentrations of mercuric chloride for 15 min and 3 h, semen samples of the control and mercury-treated groups were centrifuged at 1500 rpm for 5 min in DPBS (twice) to remove the free mercury, if any, from the media. Then the pellets were re-suspended in the mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and kept for 12 h at 4 °C for fixation. Then the samples were again centrifuged at 1500 rpm for 5 min and the supernatant was discarded to remove the fixative. The pellet was suspended in 0.1 M PB, and again centrifuged. Thereafter, the samples (pellets) were fixed for 1 h in 1% osmium tetroxide at 40 °C and then dehydrated in acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK).

Sections (0.5 μm) were cut with an ultra-microtome, mounted on to the glass slides, stained with aqueous toluidine blue and observed under light microscope for gross observation of the area and quality of the tissue fixation.

For scanning electron microscopy, after fixation of samples as mentioned above, the samples were centrifuged at 1000 rpm for 10 min and supernatant was discarded. The pellet was suspended in buffer, centrifuged and washed. It was re-suspended in buffer and a drop of it was spread on a cover slip. The samples were air-dried, sputter-coated with colloidal gold and observed under an EVO 18 Zeiss scanning electron microscope at an operating voltage of 20 kV. Images were digitally acquired by using the SmartSEM software.

Statistical analysis. Data generated were subjected to two-way ANOVA and Tukey’s test using SPSS Version 23.0 where p value of 0 < 0.05 was considered significant. Data presented in tables are mean ± SE of the observations on semen samples of six different male goats. Separate multiple linear regression models were used to examine the effect of different concentrations of mercury and times of exposure on each of the CASA parameters after controlling for potential confounding factors. All the values for kinematic variables were standardized to avoid any scale effect. Correlation was also calculated between the motility and other kinematic patterns.

Analysis of the relative mRNA expression data between different groups was based on the crossing point (Cp) values. The Cp value of each gene was subtracted from the arithmetic mean of Cp value of the β-actin gene to calculate the ΔΔCt. The relative expression of PCR product was determined by the Eq. (2) (− ΔΔCt) as per Livak method26 and the level of significance was set at 0.01% (p < 0.01).

Results

Effect on live spermatozoa count. Compared to the control, there were no significant alterations in per cent count of the live spermatozoa following exposure to different concentrations of mercuric chloride (0.031, 0.125, 0.25 and 1.25 μg/ml) for 15 min. But compared to the control (90.07%), significant (p < 0.05) decrease in live spermatozoa percentage count (80.20 and 79.14%) was observed in semen samples exposed to higher concentrations (0.25 and 1.25 μg/ml) of HgCl₂ after 1 h. After 3 h of exposure to different concentrations of mercury also, compared to the control (85.44%), significant (p < 0.05) reduction in percentage counts of the live spermatozoa (75.65, 72.82, 65.62%) were observed in groups treated with 0.125, 0.25 and 1.25 μg/ml HgCl₂, respectively (Table 2, Fig. 1a).

| Primers sequences | EMBL/reference | Product size (bp) | Annealing temp (°C) |
|-------------------|----------------|------------------|-------------------|
| Bcl-2 F 5′-TGGTTCTGTGTTCTGCTACA-3′ | NM_001164468.1 | 143 | 61 |
| Bcl-2 R 5′-ACACTTTTGATGGTTGCA-3′ | | | |
| Bax F 5′-CATGGAGCTGCAGAGATGA-3′ | XM_002701334.1 | 101 | 60 |
| Bax R 5′-GTGAGATGCTGGGAA-3′ | | | |
| β-actin F 5′-AGTTGGCAGATGATGA-3′ | Dangi et al.41 | 54 | 60 |
| β-actin R 5′-TGCCGGAGCGTTGTT-3′ | | | |

Table 1. Primers sequences of Bcl-2, Bax and β-actin genes.

Electron-microscopic studies. After treatment of semen samples with the lowest (0.031 μg/ml) and highest (1.25 μg/ml) used concentrations of mercuric chloride for 15 min and 3 h, semen samples of the control and mercury-treated groups were centrifuged at 1500 rpm for 5 min in DPBS (twice) to remove the free mercury, if any, from the media. Then the pellets were re-suspended in the mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and kept for 12 h at 4 °C for fixation. Then the samples were again centrifuged at 1500 rpm for 5 min and the supernatant was discarded to remove the fixative. The pellet was suspended in 0.1 M PB, and again centrifuged. Thereafter, the samples (pellets) were fixed for 1 h in 1% osmium tetroxide at 40 °C and then dehydrated in acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK).

Sections (0.5 μm) were cut with an ultra-microtome, mounted on to the glass slides, stained with aqueous toluidine blue and observed under light microscope for gross observation of the area and quality of the tissue fixation.

For scanning electron microscopy, after fixation of samples as mentioned above, the samples were centrifuged at 1000 rpm for 10 min and supernatant was discarded. The pellet was suspended in buffer, centrifuged and washed. It was re-suspended in buffer and a drop of it was spread on a cover slip. The samples were air-dried, sputter-coated with colloidal gold and observed under an EVO 18 Zeiss scanning electron microscope at an operating voltage of 20 kV. Images were digitally acquired by using the SmartSEM software.

Statistical analysis. Data generated were subjected to two-way ANOVA and Tukey’s test using SPSS Version 23.0 where p value of 0 < 0.05 was considered significant. Data presented in tables are mean ± SE of the observations on semen samples of six different male goats. Separate multiple linear regression models were used to examine the effect of different concentrations of mercury and times of exposure on each of the CASA parameters after controlling for potential confounding factors. All the values for kinematic variables were standardized to avoid any scale effect. Correlation was also calculated between the motility and other kinematic patterns.

Analysis of the relative mRNA expression data between different groups was based on the crossing point (Cp) values. The Cp value of each gene was subtracted from the arithmetic mean of Cp value of the β-actin gene to calculate the ΔΔCt. The relative expression of PCR product was determined by the Eq. (2) (− ΔΔCt) as per Livak method26 and the level of significance was set at 0.01% (p < 0.01).

Ethics committee approval. All experiments involved non-invasive procedures on animals including on large animals are approved by the Institutional Animal Ethics Committee (IAEC) of U.P. Pandit Deen Dayal Upadhayya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU) as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Govt of India. (Approval No. 110/IAEC/16, dated: 16-09-2016).

Results

Effect on live spermatozoa count. Compared to the control, there were no significant alterations in per cent count of the live spermatozoa following exposure to different concentrations of mercuric chloride (0.031, 0.125, 0.25 and 1.25 μg/ml) for 15 min. But compared to the control (90.07%), significant (p < 0.05) decrease in live spermatozoa percentage count (80.20 and 79.14%) was observed in semen samples exposed to higher concentrations (0.25 and 1.25 μg/ml) of HgCl₂ after 1 h. After 3 h of exposure to different concentrations of mercury also, compared to the control (85.44%), significant (p < 0.05) reduction in percentage counts of the live spermatozoa (75.65, 72.82, 65.62%) were observed in groups treated with 0.125, 0.25 and 1.25 μg/ml HgCl₂, respectively (Table 2, Fig. 1a).
Effect on total motility and progressive motility. Data presented in Fig. 1b,c and Table 3 revealed that compared to the control, there were no significant (p > 0.05) differences in the percentage count of total and progressive motile spermatozoa in the semen samples exposed to different concentrations of HgCl\(_2\) after 15 min. But significant (p < 0.05) decrease in total and progressive motile spermatozoa percentage count was observed after 1 h in 1.25 µg/ml HgCl\(_2\)-treated group (65.79 and 58.78%, respectively) compared to the control group after 1 h (84.47 and 71.53%, respectively). After 3 h of exposure to HgCl\(_2\) at 0.25 and 1.25 µg/ml, there was further decrease in count of the total motile and progressive spermatozoa as these values were found to be 39.84 and 19.9%, respectively.

Effect on kinematic patterns. Data presented in Table 3 revealed significant differences in kinematic patterns of the spermatozoa in semen samples treated with different concentrations of mercuric chloride (0.031–1.25 µg/ml) compared to the control group. Compared to the control group after 1 h, slow and non-progressive motility in semen samples treated with different concentrations of mercury were found to be significantly (p < 0.05) increased. However, slow and non-progressive motility were found to significantly (p < 0.05) increase in a dose-dependent manner compared to control after 3 h. Different velocity parameters of spermatozoa (VCL, VSL, and VAP) were significantly (p < 0.05) decreased in concentration- and time-dependent manner (Fig. 1d–f). Data presented in Fig. 2a–c showed significant (p < 0.05) increase in values of LIN and STR after 3 h exposure to different concentration of mercury as compared to the control group. WOB percent count was also significantly (p < 0.05) increased as the time of exposure to 0.125, 0.25 and 1.25 µg/ml of HgCl\(_2\).
Table 3. Effect of in-vitro exposure of spermatozoa to different concentrations of mercuric chloride (0.031–1.25 µg/ml) on motile spermatozoa count at different time intervals. Data presented are mean ± SE of the semen samples of six bucks.

| Time (min) | Motility | Rapid progressive | Slow progressive | Non-progressive | VCL | VAP | VSL | LIN% | STR% | WOB% | maxALH | BCF | maxALH |
|-----------|----------|------------------|------------------|----------------|-----|-----|-----|------|------|------|--------|-----|--------|
| 15 min    | Control  | 85.94 ± 1.2a     | 74.98 ± 1.6a     | 6.72 ± 1.45a   | 161.69 ± 0.23a | 129.26 ± 0.17a | 123.6 ± 0.41a | 77.44 ± 0.53a | 95.62 ± 1.36a | 80.74 ± 0.45 | 41.27 ± 0.31a | 3.8 ± 0.28a |
|           | A        | 88.50 ± 1.2a     | 78.39 ± 1.26a    | 6.96 ± 1.23a   | 160.08 ± 0.51a | 128.21 ± 0.48a | 121.3 ± 0.42a | 78.46 ± 1.30a | 94.61 ± 1.32a | 81.51 ± 0.82 | 40.26 ± 0.44a | 3.7 ± 0.22a |
|           | B        | 86.95 ± 0.3a     | 78.18 ± 1.22a    | 4.08 ± 1.7a    | 139.32 ± 0.61a | 125.18 ± 1.29a | 120.02 ± 1.03a | 77.21 ± 1.40a | 93.68 ± 1.44a | 81.42 ± 1.48 | 39.67 ± 1.11a | 3.51 ± 0.41a |
|           | C        | 85.62 ± 0.6a     | 76.20 ± 0.72a    | 6.48 ± 1.02a   | 136.25 ± 1.71a | 122.9 ± 1.20a  | 117.52 ± 1.20a | 76.38 ± 1.58a | 94.04 ± 1.03a | 82.14 ± 1.20 | 37.28 ± 0.49a | 3.3 ± 0.20a |
|           | D        | 84.75 ± 0.3a     | 74.42 ± 0.81a    | 6.44 ± 1.31a   | 150.11 ± 1.43a | 120.1 ± 1.25a  | 111.17 ± 1.25a | 78.01 ± 1.20a | 95.07 ± 1.05a | 82.74 ± 1.06 | 34.25 ± 0.59a | 2.91 ± 0.11a |
| 1 h       | Control  | 84.47 ± 1.7a     | 71.53 ± 0.27a    | 7.47 ± 1.05a   | 140 ± 0.58a    | 129.03 ± 1.49a | 123.03 ± 1.04a | 77.275 ± 1.06a | 95.34 ± 1.30a | 80.64 ± 1.72 | 40.52 ± 0.31a | 3.8 ± 0.21a |
|           | A        | 78.06 ± 0.3b     | 68.02 ± 0.41b    | 6.24 ± 1.36b   | 158.13 ± 1.45a | 125.1 ± 1.48a  | 118.27 ± 0.36a | 78.76 ± 0.91a | 94.34 ± 1.20a | 81.47 ± 1.25 | 39.01 ± 0.32a | 3.2 ± 0.22a |
|           | B        | 77.73 ± 1.2a     | 67.55 ± 1.59a    | 7.81 ± 1.05a   | 152.45 ± 1.62a | 122.11 ± 1.72a | 113.31 ± 0.47a | 79.58 ± 1.22a | 93.33 ± 1.03a | 80.53 ± 1.34 | 39.08 ± 0.33a | 2.81 ± 0.10a |
|           | C        | 72.73 ± 1.0a     | 65.04 ± 0.61a    | 8.18 ± 1.51a   | 150.94 ± 0.93a | 119.42 ± 1.04a | 106.16 ± 0.62a | 80.03 ± 1.42a | 94.7 ± 1.20a  | 82.04 ± 1.44 | 36.28 ± 0.29a | 2.5 ± 0.31a |
|           | D        | 65.79 ± 0.4a     | 58.78 ± 0.39a    | 9.20 ± 0.91a   | 148.7 ± 0.79a  | 116.08 ± 1.81a | 99.32 ± 0.82a  | 80.21 ± 1.39a | 95.37 ± 1.25a | 83.06 ± 1.53 | 33.28 ± 0.71a | 2.4 ± 0.13a |
| 3 h       | Control  | 83.61 ± 0.12a    | 70.37 ± 0.18a    | 8.54 ± 1.61a   | 159.14 ± 1.28a | 128.31 ± 0.10a | 122.17 ± 0.41a | 76.06 ± 1.04a | 95.12 ± 1.74a | 80.27 ± 0.88 | 39.74 ± 0.48a | 3.7 ± 0.29a |
|           | A        | 68.23 ± 1.16a    | 52.29 ± 1.26a    | 10.78 ± 1.29a  | 145.28 ± 1.32a | 120.17 ± 1.51a | 114.07 ± 1.48a | 77.03 ± 1.42a | 97.3 ± 1.52a  | 82.46 ± 0.94 | 60.06 ± 0.29a | 2.8 ± 0.21a |
|           | B        | 63.56 ± 1.1b     | 59.02 ± 1.51a    | 20.59 ± 0.26a  | 132.02 ± 2.01a | 110.28 ± 1.04a | 107.06 ± 1.61a | 78.85 ± 1.81a | 97.19 ± 1.07a | 82.53 ± 1.37 | 35.24 ± 0.71a | 2.4 ± 0.28a |
|           | C        | 47.78 ± 0.3c     | 28.49 ± 1.21a    | 24.67 ± 1.30a  | 129.17 ± 1.41a | 106.02 ± 1.01a | 95.06 ± 1.03a  | 80.92 ± 1.48a | 98.62 ± 1.40a | 83.41 ± 1.40 | 27.93 ± 0.66a | 2 ± 0.41a |
|           | D        | 39.84 ± 0.28c    | 19.9 ± 0.82f     | 29.62 ± 1.49f  | 120.27 ± 0.27a | 97.27 ± 1.06a  | 84.25 ± 0.62a  | 81.19 ± 1.71a | 98.51 ± 1.03a | 84.37 ± 1.29 | 20.61 ± 1.10a | 1.7 ± 0.66 |

p-value 0.005 0.0001 0.0123 0.0432 0.0031 0.004 0.022 0.714 0.041 0.844 0.041 0.022

Figure 2. The relationship between different concentrations of HgCl₂ and computer-assisted semen analysis (CASA) kinematic parameters for per cent linearity (LIN; (a)), per cent straightness (STR; (b)), per cent WOB (c), maximum amplitude of head lateral displacement (maxALH; µm; (d)), and beat cross frequency (BCF; Hz; (e)). Linear regression lines are shown along with the coefficients of determination (R²) for 15 min and 3 h. Each data point represents the mean ± SE value of repeated measurements from six male goats.
increased. This suggests that LIN, STR and WOB of spermatozoa were increased and the progressive motility and different velocity parameters (VCL, VAP & VSL) were decreased with increase in exposure time and concentration of mercury; thus, indicating that mercury-induced alterations in sperm progressiveness had relatively greater effect on all the kinematic patterns. However, dose- and time-dependent significant (p < 0.05) decrease in maxALH and BCF, compared to the control group, were observed for up to 3 h (Fig. 2d,e).

### DNA damage.

Data presented in Table 4 and shown in Fig. 3 revealed that mercury even at the lower concentration (0.031 µg/ml) did not induce any DNA damage even after 3 h of exposure. Similarly, compared to the PBS control, even highest used concentration of mercury (1.25 µg/ml) also failed in inflicting any significant DNA damage in sperm cells after 15 min (0.71%). However, compared to the control (0%), significant (p < 0.05) increase in number of sperm cells showing DNA damage (29.04%) was observed after exposure to 1.25 µg/ml HgCl₂ for 3 h.

### Scanning electron microscopy (SEM).

Evaluation of SEM images of the spermatozoa of control group revealed that spermatozoa had normal surface morphology (Fig. 4). Head of the spermatozoa was normal and oval in shape with intact acrosome (Fig. 4). Middle piece showed intact plasma membrane and there were no obvious deformities near the neck and tail joining regions (Fig. 4). Control group spermatozoa had overall homogenous plasma membrane. Following in-vitro exposure of sperm cells to lower concentration of HgCl₂ (0.031 µg/ml) for 15 min, apparently no adverse effect was observed on the surface morphology of spermatozoa (Fig. 5a) and no obvious changes were observed in sperm head, acrosome and tail parts at this time point and it compared well with the spermatozoa of control group (Fig. 5b,c). When spermatozoa were exposed to the same concentration of mercuric chloride (0.031 µg/ml) for longer period of time i.e. 3 h, several defects were noticed (Fig. 5d) as revealed by deformities in the head and middle piece regions of sperm cells at this time-point.

### Table 4. Effect of in-vitro exposure of buck spermatozoa to different concentrations of mercuric chloride (0.031–1.25 µg/ml) on DNA damage in sperm cells at different time intervals. Data presented are mean ± SE of the semen samples of six bucks. Different capital superscripts in the rows indicate significant (p < 0.05) differences between the different time intervals. Different small superscripts in the columns indicate significant (p < 0.05) differences between control and different treatment groups.

| Treatments                      | % spermatozoa showing DNA damage at different time intervals |
|---------------------------------|------------------------------------------------------------|
|                                 | 15 min          | 3 h                     |
| PBS control                     | 0.00 ± 0.00A   | 0.00 ± 0.00A           |
| 0.031 µg/ml mercuric chloride   | 0.00 ± 0.00A   | 0.00 ± 0.00A           |
| 0.125 µg/ml mercuric chloride   | 0.00 ± 0.00A   | 0.00 ± 0.00A           |
| 0.25 µg/ml mercuric chloride    | 0.00 ± 0.00A   | 2.03 ± 1.01A           |
| 1.25 µg/ml mercuric chloride    | 0.71 ± 0.33A   | 29.04 ± 1.06B          |

Figure 3. DNA damage in goats’ spermatozoa. (a) Showing no-fluorescence in spermatozoa (TUNEL-negative) in PBS control and 0.031, 1.25 and 0.25 µg/ml HgCl₂ groups after 3 h. (b) Showing bright green-fluorescence in head of the spermatozoa (TUNEL-positive; arrow) of mercuric chloride (1.25 µg/ml) treated group after 3 h of exposure.
In some of the spermatozoa, wrinkled plasma membrane in the head region (Fig. 5e) and focal areas of membrane rupture at the middle piece were observed (Fig. 5f). However, exposure to higher concentration of HgCl₂ (1.25 µg/ml) resulted in deformities in spermatozoa head plasma membrane and middle piece even after 15 min of exposure (Fig. 6a). The SEM analysis also showed that at this time point, 1.25 µg/ml HgCl₂ hampered the head plasma membrane integrity and there were focal points of membrane damage (Fig. 6b). Middle piece of sperm cells harbouring mitochondria also appeared wavy depicting the harmful effects of higher concentration of mercury.
of mercury on sperm cells (Fig. 6a,c). Further, the morphological defects in sperm cells were more pronounced after 3 h of exposure to 1.25 µg/ml of HgCl₂ (Fig. 6d) as the head plasma membrane was found to be wrinkled and acrosome was swollen (Fig. 6e). Tail part of the sperm cell also appeared damaged and the neck appeared to have irregular plasma membrane (Fig. 6f).

Transmission electron microscopy (TEM). Evaluation of TEM images of the spermatozoa of control group did not show any notable abnormalities in sperm cells after 15 min or 3 h. Spermatozoa of the control group (15 min) showed intact plasma membrane and well-organized axonemal components (Fig. 7a,b). The longitudinal section of spermatozoa tail showed normal axoneme arrangements and intact mitochondrial ultrastructure at the same time point (Fig. 7c). Even after 3 h, it showed intact membrane, axonemal components (*) and well-organized mitochondria (Fig. 7a,b), intact head (Fig. 7d), mitochondria and axonemal arrangements (Fig. 7e). Spermatozoa of 0.031 µg/ml HgCl₂ treated group after 15 min of exposure showed almost normal ultra-structures (Fig. 8a–c) and the TEM observations were in accordance with the SEM observations. However, few spermatozoa at this time point showed irregular head plasma membrane (Fig. 8a). But no notable changes were observed in the longitudinal sections of sperm tail at this time point (Fig. 8c). But following exposure to HgCl₂ (0.031 µg/ml) for 3 h, certain adverse effects were observed on ultra-structures of the buck spermatozoa as revealed by ruptured plasma membrane and premature acrosome reaction in longitudinal sections of spermatozoa (Fig. 8d). The longitudinal sections of the middle piece revealed disorganized and swollen mitochondria having collapsed cristae in the middle piece of some of the spermatozoa (Fig. 8a,c). Disorganized outer dense fibres were also observed at this time point in few of the spermatozoa (Fig. 8f). However, higher concentration (1.25 µg/ml) of HgCl₂ damaged spermatozoa head and tail integrity even after 15 min of exposure (Fig. 9a–c). Some of the spermatozoa showed disorganized axonemal components and bent neck (Fig. 9b). The distorted outer dense fibres in tail part were also observed in few of the spermatozoa (Fig. 9c). Compared to the ultrastructural damages observed in sperm cells after exposure to 1.25 µg/ml HgCl₂ for 15 min, more severe and adverse ultra-structural defects were observed after 3 h of exposure (Fig. 9d–f) and these were in agreement with the SEM observations. Longitudinal sections of the spermatozoa head showed fragmented plasma membrane and focal areas of lysis (Fig. 9d). The middle piece region had swollen mitochondria with collapsed cristae (Fig. 9e,f). Additionally, disorganized axoneme and disorganised outer dense fibres were also noticed at this time point in some of the spermatozoa (Fig. 9f). Based on the observed lesions observed in TEM images, it is apparent that the toxic effect of mercury on spermatozoa was both time- and concentration-dependent and mercury damaged almost all parts of the sperm cells including mitochondria.

Apoptosis and necrosis. No apoptosis was observed in the spermatozoa of PBS control and mercury-treated groups after 15 min of exposure. After 3 h, compared to just 0.67% apoptotic sperm cells in semen samples of the control group, 41.67% apoptotic spermatozoa were observed in the positive control group (Camptothecin) as evident from the data summarized in Table 5 and shown in Fig. 10. But compared to the untreated control or positive control (Camptothecin) groups, no apoptosis was observed in sperm cells of any of the mer-
Figure 7. Transmission electron micrographs of the goat spermatozoa of control group after 15 min and 3 h showing intact ultrastructures. (a) Longitudinal section of the sperm nucleus; head (arrow; (a)); axonemal components (star; (a,b)), (b) intact sperm head (arrow) and intact acrosome (AC; (b)), organized mitochondria (arrow; (c)), sperm head (arrow; (d,e)), axonemal components (star; (e)), organized mitochondria (triangle; (e)), intact plasma membrane (arrow; (e)).

Figure 8. Transmission electron micrographs of goat spermatozoa exposed to 0.031 µg/ml HgCl₂ showing ultra-structural defects. (a) sperm head (arrow); damage membrane (triangle), (b) sperm head (arrow); axonemal components (star), (c) outer dense fibers (arrow), (d) reacted acrosome (arrow), (e) mitochondria (arrow); collapsed mitochondria (arrow), (f) disorganized outer dense fiber (star); collapsed mitochondria (arrow) after 15 min and 3 h.
cury-treated groups even after 3 h of incubation. Rather, large number of sperm cells in mercury-treated groups were found to be necrotic (clearly visible under red filter; Fig. 10d) and their counts were 18.31, 23.45, 32.13 and 39% in 0.031, 0.125, 0.25 and 1.25 µg/ml HgCl₂-treated groups, respectively after 3 h of exposure (Table 5).

Correlation between DNA damage, apoptosis and necrosis. Data presented in Table 6 although indicated negative correlation between apoptosis and necrosis after 15 min of exposure to mercury, but it was statistically insignificant. Likewise, apoptosis was negatively correlated with necrosis in HgCl₂-treated groups after 3 h of exposure as well. But DNA damage was significantly correlated with necrosis while negatively correlated with apoptosis, although it was statistically insignificant.
Comparative expression of apoptotic and anti-apoptotic genes. Data presented in Fig. 11 revealed that compared to the control group, no significant (p > 0.01) change was observed in the relative mRNA expression of Bcl-2 gene in semen samples exposed to different concentrations of HgCl₂ (0.031, 0.125, 0.25 and 1.25 µg/ml) for 15 min. But after 3 h, compared to the control, there was significant (p < 0.01) increase in the relative mRNA expression of Bcl-2 in 0.25 and 1.25 µg/ml mercuric chloride-treated groups. Compared to β-actin and Bcl-2 genes, relative mRNA expression of Bax gene was not altered either in the control or in HgCl₂ treated groups after 15 min of exposure. But after 3 h, although significant (p < 0.01) increase in the relative expression of Bax gene was observed in the control group but there was no change in the relative mRNA expression of Bax gene in any of the mercury-treated groups even after 3 h. Rather significant (p < 0.01) increase in the relative mRNA expression of Bcl-2 gene was observed in HgCl₂ (0.25 and 1.25 µg/ml) treated groups.

Table 6. Correlation between necrosis, apoptosis and DNA damage of different concentrations of mercuric chloride (0.031–1.25 µg/ml) at different time intervals (*p ≤ 0.05).

|          | 15 min Necrosis | Apoptosis | DNA damage |
|----------|-----------------|-----------|------------|
| Necrosis | 1               |           |            |
| Apoptosis| −0.42           | 1         |            |
| DNA damage| 0.53*          | −0.149    | 1          |

|          | 3 h Necrosis | Apoptosis | DNA damage |
|----------|-------------|-----------|------------|
| Necrosis | 1           |           |            |
| Apoptosis| −0.86*      | 1         |            |
| DNA damage| 0.75*       | −0.27     | 1          |

Figure 10. Detection of apoptosis and necrosis using Annexin-V staining in goat spermatozoa. (a) Spermatozoa with no sign of phosphatidylserine translocation [Annexin-V-FITC (negative) and PI (negative)] in control group. (b) Annexin-V-FITC (green) and PI (red) positive spermatozoa in positive control (Camptothecin 10 µM). (c) Annexin-V-FITC (green; negative) and PI (positive; red) spermatozoa in mercuric chloride treatment groups after 3 h (0.031–1.25 µg/ml) in green filter. (d) Annexin-V-FITC (green; negative) and PI (positive; red) spermatozoa in mercuric chloride treatment groups after 3 h (0.031–1.25 µg/ml) in red filter. (e) DIC image of (c,d); mercury treated groups.
Discussion

According to U.S. Geological Survey and Harvard University, mercury levels in the northern Pacific Ocean have increased by about 30 per cent over the past 20 years and it is expected to rise further by 50 per cent by 2050 with increase in industrial mercury emissions. Mercury levels in air are in the range 2–10 ng/m³. Although naturally occurring levels of mercury in groundwater and surface water are less than 0.5 µg/l, but local mineral deposits may result in higher levels of mercury in groundwater. The permissible limit for mercury in drinking water is 0.01 ppm. Average daily intake of mercury from food is in the range 2–20 µg, but may be much higher in regions where ambient waters are more contaminated with mercury and where fish constitute major proportion of human’s diet. Fish mostly contain varying levels of mercury like 0.031 ppm in pollock, 0.126 ppm in tuna, 0.144 ppm in fresh/frozen skipjack, 0.150 ppm in freshwater perch, and 1.123 ppm in tilefish in Gulf of Mexico. United Nations Environment Programme’s (UNEP) Chemicals Working Group has pointed out that mercury contamination in India is reaching at alarming levels largely due to the discharge of mercury-bearing industrial effluents ranging from 0.058 to 0.268 mg/l, and this is several times more than the prescribed Indian and WHO standards for drinking water and for industrial effluents. Thus, all the above-mentioned reports suggest that human beings and animals in India and other countries are being exposed to higher concentrations of mercury.

In our recent publication, we have reported mercury-induced significant increase in ROS and MDA, significant decrease in TAC and SOD activity, significant decrease in spontaneous acrosome reaction, and inhibited capacitation in sperm cells despite increase in cAMP and intracellular Ca²⁺ levels even at 0.031 µg/ml compared to the control group after 15 min exposure. Based on these findings, we proposed that oxidative stress causes alterations in sperm functions possibly by affecting sperm membrane as well as sub-cellular structures. To substantiate our proposed mechanism of mercury-induced alterations in functional dynamics of spermatozoa, present study was undertaken to understand how cell death takes place and what changes take place in sperms at molecular and ultra-microscopic levels, especially at organelle level, that affect the motility and kinematic patterns in an endeavour to give a fillip to our understanding about mercury-induced cell death and alterations in functional dynamics of spermatozoa.

Mercury has been associated with compromised sperm functions and reproductive failures. Men having mean mercury level of 8.1 ng/ml have been reported to be infertile compared to those having 6.3 ng/ml in control. Infertile males have been reported to have higher level of mercury (0.048 µg/l) in their seminal plasma compared to the fertile males having 0.032 µg/l in seminal plasma. Decreased sperm motility, sperm swimming speed, and increased abnormal sperm tail morphology have been reported in monkeys having blood mercury levels around 2000 ng/ml. Rats having mean blood mercury levels of 30.8 ng/ml and 94.3 and 176.5 ng/ml have also been reported to have significant variations in testosterone levels and adverse effects on male reproduction. In all these studies, spermatozoa were exposed to comparatively higher concentrations of mercury than used by us in the present study. Several reports on toxic effects of mercury on semen quality and functional dynamics of sperm cells in humans and experimental animal are also available. But the specific target site(s) for action of mercury and its mechanistic pathway(s) are yet to be precisely delineated.

Computer assisted semen analyser CASA provides quantitative data of sperms motility (rapid-, slow- and non-progressive) and specific kinematic measures. Our data provides quantitative evidence in favour of mercury-induced reduction in proportion of the motile sperms and their straight-line motion. Major effects of mercury on motility parameters (rapid, slow and non-progressive) and kinematic motion parameters (VCL, VSL, VAP and LIN) were highly correlated. Total motility, rapid and non-progressive were associated with the exposure level of mercuric chloride (0.31 to 1.25 µg/ml) and time of exposure. The VCL, VSL, VAP and LIN of sperms are bioindicators of the fertilizing ability and have been correlated with in-vivo fertilization rates in humans. After 3 h, we observed dose-dependent decline (~ 31% shorter) in VSL of the sperms of 1.25 µg/ml HgCl₂ exposed...
group as the spermatozoa travelled at the rate of 84.25 µm/s compared to 122.17 µm/s in the control group. The VAP and VCL data also suggest that sperm of 1.25 µg/ml HgCl₂ treated group moved ~ 24% slowly along their average path and point to point path of travel compared to control (Table 3). The concentration-dependent significant decline in ALH and BCF suggest that mercury alters the frequency at which sperms cross the average path (~ 25 times per second) and maximum amplitude of deviation around that average path (~ 3.8 µm) in dose- and time-dependent manner. Taken together, with mercury concentration-dependent increase in LIN, STR and WOB trend, our findings suggest that sperms following exposure to higher concentration of mercury follow more linear path (i.e. less curved) and possess less beat cross frequency than the sperms of control group.

The ALH measures the vigour of flagellar beating in conjunction with the frequency of cell rotation 42, and both these are associated with the ability of sperm to penetrate cervical mucus and fuse with oocytes. Therefore, ALH is often used to assess the outcome of in vitro fertilization programmes 41. Higher concentration (1.25 µg/ml) of HgCl₂ drastically reduced the sperm population that is likely to reach the oocyte as a consequence of the reduced percentage of rapid motile sperms and reduced BCF and maxALH motion kinetic in the remaining motile sperm cells. Thus, our CASA data evidentially suggests that mercury directly diminishes the ability of sperms to penetrate and fuse with oocytes. Our observations of the kinematic patterns also validate our earlier finding 13 in which we did not observe any capacitation in sperms following exposure to mercuric chloride at 1.25 µg/ml for 15 min and 3 h.

Mercury (217.216 ppm; 800 µM) has been reported to significantly (p < 0.001) increase (12.0% compared to 2% in control) percentage of DNA breaks in human sperms 42. Positive correlation between sperm DNA fragmentation and levels of ROS has been reported in testicular tissue of mice 43 and semen of humans 44,45. TUNEL assay in the present study revealed that mercuric chloride at lower concentration (0.031 µg/ml) did not induce any DNA damage in sperm cells even after 3 h of exposure. But DNA damage was observed in 2.03 ± 1.01 and 29.04 ± 1.06% sperm cells following exposure to 0.125 and 1.25 µg/ml, respectively, for 3 h. Our findings on DNA damage at higher concentrations are in agreement with the effect of 150, 350 and 550 µM HgCl₂ induced DNA breaks in bull sperm nuclei where interestingly 92% of the DNA breaks were double-stranded 51. Hayati et al. 46 also reported mercury-induced increase in DNA fragmentation in fish sperms after in-vitro exposure to different concentrations of HgCl₂ (0.5, 1, 2.5 and 5 ppm) for 5 s. DNA damage observed in sperm cells in the present study can be attributed to mercury-induced significant increase in oxidative stress biomarkers in goat semen 13. But possibly such a low concentration of inorganic mercury (0.031 µg/ml) was not sufficient to induce DNA damage in goat spermatozoa despite significant oxidative stress and viability losses in goat spermatozoa 15. It seems interesting and but difficult to put forth any plausible explanation for the same.

Arabi et al. 1 reported 64% viability losses in bull sperm cells after exposure to mercuric chloride (13.576 µg/ml) for 2 h. Similar viability loss in of goat spermatozoa was also observed and it was found to be reduced by 50% after 3 h of exposure but at comparatively much lower concentration (1.25 µg/ml). Thus, goat sperm cells seem to be much more sensitive to mercury-induced cellular insult than bull spermatozoa. Higher vulnerability of goat sperms vis-à-vis bull sperms at the moment can be only attributed to species-difference, however, to unravel the precise possible reason for such a variability in response to mercury requires comparative studies on sperm cells of both these species.

Scanning and transmission electron microscopy images of mercury treated spermatozoa revealed loss of plasma membrane integrity along with acrosomal membrane damage, damage of the head region, damaged outer dense mitochondrial sheath along with collapsed cristae, disorganized and swollen mitochondria, reacted acrosome, disorganized axonemal components with altered base 42. These defects in mitochondria seem to be responsible for reduced mitochondrial transmembrane potential and motility of the spermatozoa. Abnormalities in head and neck region may be responsible for detachment of the head and tail leading to instant death of some spermatozoa. Our observations on ultra-structural alterations in goat spermatozoa are almost similar to those reported in rabbit sperms after treatment with higher than 1 µM mercury, arsenic, cadmium, and platinum as in rabbit sperms also damage to sperm head membranes and acrosome breakeage with formation of various sized micro-vesicles was observed 47.

In the present study, we observed that mercury did not induce apoptosis even at the highest used concentration (1.25 µg/ml); rather it induced necrosis even at the lowest used concentration (0.031 µg/ml). Generally, little percentage of labelling with annexin is observed in every basal sample but in the present study, compared to the control, none of the spermatozoa from mercury-treated groups showed any exteriorization of phosphatidyler serine. This also substantiates that mercury did not induce early apoptosis like changes in mercury-treated sperm cells. Rather, mercury resulted in instant cell death as substantiated by propidium iodide positive (PI+) cells data during Annexin V assay as the sperm cells were mainly labelled with PI and very less/no with annexin which indicates that sperm membrane ruptures as indicated by electron microscope images might be responsible for necrosis. Rather mercury appears to be protective against P-serine externalization, however, to substantiate the protective role of mercury against P-serine externalization, further studies are required specifically taking spermatozoa of some other species as well from comparative perspective.

Theory of apoptosis-induced sperm DNA damage has been challenged by several researchers 48–50. Apoptosis-independent DNA damage observed in the present study is in agreement with the similar observations of Pereira et al. 51 who have reported mercury induced DNA damage in blood cells by a nonapoptotic mechanism in wild and caged fish. On U-937 cells also, mercury was found to damage DNA, but apoptosis was not involved. Similarly, Muratori et al. 48 have also reported that sperm DNA fragmentation did not correspond to the apoptosis-like phenomenon and the impaired motility of sperms in human ejaculate was associated with ultrastructural damages. Sakkas et al. 49 also observed that although DNA damage was initiated in some of the spermatozoa by mercury but subsequently this escaped apoptosis, and they termed it as “abortive apoptosis”. Lack of mercury-induced apoptosis in goat spermatozoa is also substantiated by our real time quantification data of the expression of pre-apoptotic (Bax) and anti-apoptotic (Bcl-2) genes where the former gene was expressed only in sperm
samples of the control group after 3 h but not at all in the mercury-treated groups; thus implying routine and
time-dependent apoptosis in sperm cells of control group while inhibition of Bax gene and significant concen -
tration- and time-dependent increase in expression of Bcl-2 gene in mercury-treated groups. Thus, our findings
are evidently indicative of the anti-apoptotic effect of mercury.

Mammalian Bcl-2 and related anti-apoptotic family member (Bcl-xL, Bcl-w, A1, Mcl-1, Boo/DIVA/Bcl-2,
L-10, Bcl-B) proteins are localized on the cytoplasmic aspect of nuclear envelope, endoplasmic reticulum and
outer mitochondrial membrane. The fate of a cell depends on the balance of Bcl-2 family protein levels through
a dynamic process by which cellular signals modulate the expression of pro- or anti-apoptotic proteins to tip the
equilibrium towards survival or death. When the balance favours death, the oligomerization of pro-apoptotic
effector proteins on outer mitochondrial membrane leads to mitochondrial outer membrane permeabilization
and release of cytochrome-c. This triggers activation of the cascade of caspases which cleave downstream sub-
strates and ultimately cell death. Tissue homeostasis in eukaryotes requires multiple level regulation in tissue
specific manner for fine-tuning of the apoptotic pathway. However, functional dynamics of Bax and Bcl-2 family
members makes it extremely difficult to establish the physiological relevance of different modes of regulation
reported either in cultured cell lines or in animal models.

Our Bcl-2 gene expression data is in agreement with the observation of up-regulation of Bcl-2 mRNA expres-
sion in rat kidney cells and in neuronal and glial cells following exposure to mercury. Mercury-induced necrosis and death of spermatozoa seems to involve two independent pathways; mercury results in increase in levels of ROS and MDA and reduces total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity and these ultimately result in decreased membrane intactness and cell death. Oxidative stress also results in lowered mitochondrial transmembrane potential (MTP) that may lead to increase in cAMP and increased intracellular Ca²⁺ release from spermatozoa that increase spontaneous acrosome reactions (AR) and reduction of capacitation which ultimately lead to reduction of sperm fertilizing ability. Altogether, mercury leads ROS-induced cell death via necrosis rather than apoptosis pathway. Necrosis seems to be the dominant signaling pathway in mercury-induced sperm death, and it may be due to major damage to ultra-structures of sperm cells.

Figure 12. Proposed mechanistic pathways of mercury-induced toxicity to spermatozoa. Mercury-induced
necrosis and death of spermatozoa seems to involve two independent pathways; mercury results in increase in
levels of ROS and MDA and reduces total antioxidant capacity (TAC) and superoxide dismutase (SOD) activity
and these ultimately result in decreased membrane intactness and cell death. Oxidative stress also results in
lowered mitochondrial transmembrane potential (MTP) that may lead to increase in cAMP and increased intracellular Ca²⁺ release from spermatozoa that increase spontaneous acrosome reactions (AR) and reduction of capacitation which ultimately lead to reduction of sperm fertilizing ability. Altogether, mercury leads ROS-induced cell death via necrosis rather than apoptosis pathway. Necrosis seems to be the dominant signaling pathway in mercury-induced sperm death, and it may be due to major damage to ultra-structures of sperm cells.
mercury-induced sperm cell death by necrosis along with inhibition of Bax and upregulation of expression of Bcl-2, further studies are warranted. Since ultra-structural damage in sperm cells was observed even after 15 min of exposure to mercury, therefore, changes in motility and kinematic patterns, and necrosis in sperm cells may be due to abrupt stoppage of the functional machinery of sperm cell, especially mitochondria. Sperm DNA damage and apoptosis do not seem to be the main cause of instant sperm cell death as DNA damage was observed only after 3 h of exposure. DNA fragmentation coupled with severe ultrastructural damage seem to be the important mechanisms for delayed toxic effects including viability and altered functional dynamics of goat spermatozoa. Accordingly, based on our findings in the present study and those reported by us earlier, we propose the possible mechanistic pathways of mercury-induced alterations in functional dynamics and death of goat spermatozoa (Fig. 12). However, possibility of involvement of certain other pathway(s) cannot be ruled out which may include cytochrome-c or TNF-α dependent and most likely direct activation of the caspase(s) that lead to necrosis.

Conclusions
Exposure of goat spermatozoa to mercury resulted in instant damage to sperm plasma membrane, acrosome cap, mitochondrial sheath and microtubules of spermatozoa and thus cell death while DNA damage was observed only after 3 h. Mercury seems to primarily target sperm mitochondria and plasma membrane and thereby results in spontaneous cell death independent to apoptosis. Mercury-induced alterations in cellular integrity and functionality of sperm cells will affect the fertilizing competence of mercury-exposed animals. Therefore, to ensure the quality control of cryopreserved semen, it should be ensured that the semen of bucks does not contain mercury or other toxic metals.

Received: 24 August 2020; Accepted: 18 December 2020
Published online: 12 January 2021

References
1. Arabi, M. The role of mercury in the etiology of sperm dysfunction in holstein bulls. Asian-Austral. J. Anim. Sci. 19, 335–340 (2006).
2. Hayati, A. et al. Effects of in vitro exposure of mercury on sperm quality and fertility of tropical fish Cyprinus carpio. Egypt. J. Aquat. Res. 45, 189–195 (2019).
3. Bagger, S., Birgitte, R. B. & Breddam, K. Binding of mercury (II) to protein thiol groups: A study of proteinase K and carboxy-peptidase Y. J. Inorg. Biochem. 42, 97–103 (1991).
4. Rice, K. et al. Environmental mercury and its toxic effects. J. Prev. Med. Public Health 47, 74 (2014).
5. El-Desoky, G. E. et al. Improvement of mercuric chloride-induced testis injuries and sperm quality deteriorations by Spirulina platensis in rats. PLoS ONE 8, e59177 (2013).
6. Heath, J. C., Abdelmageed, Y., Braden, T. D. & Goyal, H. O. The effects of chronic ingestion of mercuric chloride on fertility and testosterone levels in male Sprague Dawley rats. J. Biomed. Biotechnol. 2012, 1–9 (2012).
7. Shikovka, J. et al. Concentration of trace elements in human semen and relation to spermatozoa quality. J. Environ. Sci. Health A 44, 370–379 (2009).
8. Alahmar, A. T. Role of oxidative stress in male infertility: An updated review. J. Hum. Reprod. Sci. 12, 1 (2019).
9. Lachaud, C., Tesarik, J., Canadas, M. L. & Mendoza, C. Apoptosis and necrosis in human ejaculated spermatozoa. Mol. Hum. Reprod. 19, 607–610 (2004).
10. Martinez, C. S. et al. 60-Day chronic exposure to low concentrations of HgCl2 impairs sperm quality: Hormonal imbalance and oxidative stress as potential routes for reproductive dysfunction in rats. PLoS ONE 9, e11202 (2014).
11. Mohamed, M. K., Lee, W. I., Mottet, N. K. & Burbacher, T. M. Laser light-scattering study of the toxic effects of methylmercury on sperm motility. J. Androl. 7, 11–15 (1966).
12. Van Loo, K. J. W. & Kime, D. E. Automated sperm morphology analysis in fishes: The effect of mercury on goldfish sperm. J. Fish Biol. 63, 1020–1033 (2003).
13. Kushawaha, B., Yadav, R. S., Swain, D. K., Rai, P. K. & Garg, S. K. Mercury-induced inhibition of tyrosine phosphorylation of sperm proteins and altered functional dynamics of buck spermatozoa: An in vitro study. Biol. Trace Elem. Res. 198, 1–15 (2020).
14. Castellini, C. et al. In vitro toxic effects of metal compounds on kinetic traits and ultrastructure of rabbit spermatozoa. Reprod. Toxicol. 27, 46–54 (2009).
15. Kim, S. H. & Raghubir, P. S. Mercury-induced apoptosis and necrosis in murine macrophages: Role of calcium-induced reactive oxygen species and p38 mitogen-activated protein kinase signaling. Toxicol. Appl. Pharmacol. 196, 47–57 (2004).
16. Saeko, A. et al. Mercuric chloride induces apoptosis via a mitochondrial-dependent pathway in human leukemia cells. Toxicology 184, 1–9 (2003).
17. Shenker, B. J., Tai, L. G. & Irving, M. S. Mercury-induced apoptosis in human lymphoid cells: Evidence that the apoptotic pathway is not cell type specific. Environ. Res. 84, 89–99 (2000).
18. Verstreeten, S. V. Participation of reactive oxygen species in the toxicity of cobalt, nickel, cadmium and mercury. In Reactive Oxygen Species, Lipid Peroxidation and Protein Oxidation, Vol. 95 (2014).
19. Trump, B. F. & Berezesky, I. K. The role of altered [Ca2+]i regulation in apoptosis, oncosis, and necrosis. Biochim. Biophys. Acta Mol. Cell Res. 1313, 173–178 (1996).
20. Weersinghe, P. & Buja, L. M. Oncosis: an important non-apoptotic mode of cell death. Exp. Mol. Pathol. 93, 302 (2012).
21. Martinez-Pastor, F., Tizado, E. J., Garde, J. J., Anel, L. & de Paz, P. Statistical series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783–795 (2011).
22. Van der Horst, G., Maree, L. & du Plessis, S. S. Current perspectives of CASA applications in diverse mammalian spermatozoa. Reprod. Fertil. Dev. 30, 875–888 (2018).
23. Swain, D. K., Yadav, S. & Singh, S. K. Effect of four different in vitro incubation temperatures on functional dynamics, process of capacitation and apoptosis in goat spermatozoa. Small Rumin. Res. 147, 120–124 (2017).
24. Hancock, J. L. The morphology of bull spermatozoa. J. exp. biol. 29, 445–453 (1952).
25. Anand, M., Yadav, S., Kumar, A., Vaswani, S. & Shukla, P. Effect of dilution and sperm concentration on post thaw semen quality in Barbari BuckIndian. J. Anim. Res. 8, 587–592 (2018).
26. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25, 402–408 (2001).
27. Sunderland, E. M., Krabbenhøft, D. P., Moreau, J. W., Strode, S. A. & Landing, W. M. Mercury sources, distribution, and bioavailability in the north pacific ocean-insights from data and models. Glob. Biogeochem. Cycle 23, 1–14. https://doi.org/10.1029/2008G

28. World Health Organization (WHO) report – Policy paper. http://www.who.int/water_sanitation_health/dwq/chemicals/mercuryfinal.pdf. Accessed 5 Apr 2018 (2005).

29. Hall, R. A., Zook, E. G., & Meaburn, G. M. National Marine Fisheries Service Survey of Trace Elements in the Fishery Resources, 721 (Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, 1976).

30. Johnson, A. K., Rediako, B. & Wirth, E. Metal concentrations in monkfish, Lophius americanus, from the northeastern USA. Environ. Monit. Assess. 177, 385–397 (2011).

31. IPCS. Methylmercury. Geneva, World Health Organization, International Programme on Chemical Safety. In Environ Health Criteria (1990), 101.

32. Tan, S. W., Meuller, J. C. & Mahaffey, K. R. The endocrine effects of mercury in humans and wildlife. Crit. Rev. Toxicol. 39, 228–269 (2009).

33. Chen, Y. et al. Inorganic mercury causes pancreatic b-cell death via the oxidative stress-induced apoptotic and necrotic pathways. Toxicol. Appl. Pharmacol. 243, 323–331 (2010).

34. Mendiola, J. et al. Relationships between heavy metal concentrations in three different body fluids and male reproductive parameters: A pilot study. J. Environ. Health. 10, 6 (2011).

35. Mohamed, M. K., Evans, T. C., Motte, N. K. & Burkbar, T. M. Effects of methyl mercury on sperm oxygen consumption. Acta. Pharmacol. Toxicol. (Copenh) 58, 219–224 (1986).

36. Moussa, H., Hachfi, L., Trimeche, M., Najjar, M. F. & Sakly, R. Accumulation of mercury and its effects on testicular functions in rats intoxicated orally by methylmercury. Andrologia. 43, 33–37 (2011).

37. Berlin, M. & Zafars, R. Mercury. In Handbook on the Toxicology of Metals (eds Nordberg, G. F. et al.) (Elsevier, New York, 2007).

38. Mohamed, M. K., Burkbar, T. M. & Motte, N. K. Effects of methyl mercury on testicular functions in Macaca fascicularis monkeys. Pharmacol. Toxicol. 60, 29–36 (1987).

39. Hirano, Y. et al. Relationships between sperm motility characteristics assessed by the computer-aided sperm analysis (CASA) and fertilization rates in vitro. J. Assist. Reprod. Genet. 18, 213–218 (2001).

40. Verstegen, J., Iguez-Ouza, M. & Onclin, K. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149–179 (2002).

41. Barlow, P. et al. Predictive value of classical and automated sperm analysis for in-vitro fertilization. Hum. Reprod. 6, 1119–1124 (1991).

42. Arabi, M. & Heydarnejad, M. S. In-vitro mercury exposure on spermatozoa from normospermic individuals. Pak. J. Biol. Sci. 10, 2448–2453 (2007).

43. Kamar, T. R., Doreswamy, K. & Shrilatha, B. Oxidative stress associated DNA damage in testis of mice: Induction of abnormal sperms and effects on fertility. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 513, 103–111 (2002).

44. Alahmar, A. T., Calogero, A. E., Sen Gupta, P. & Dutta, S. Coenzyme Q10 improves sperm parameters, oxidative stress markers and sperm DNA fragmentation in infertile patients with idiopathic oligo asthenozoospermia. World J. Mens Health. https://doi.org/10.5534/wjmh.190145 (2020).

45. Henkel, R. et al. DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod. Biomed. Online 7, 477–483 (2003).

46. Hayati, A. et al. Effects of in vitro exposure of mercury on sperm quality and fertility of tropical fish Cyprinus carpio L.. Egypt. J. Aquat. Res. 45, 189–195 (2019).

47. Catto, S. W. & Michael, A. Mitochondrial redox dysfunction and environmental exposures. Antioxid. Redox Signal. 23, 578–595 (2015).

48. Muratori, M., Piombo, P. & Baldi, E. Functional and ultrastructural features of DNA-fragmented human sperm. J. Androl. 21, 903–912 (2000).

49. Sakkas, D., Gellis, D., Tzouzou, N. & Mani, G. C. Abnormal spermatozoa in the ejaculate: Abortive apoptosis and faulty nuclear remodelling during spermatogenesis. Reprod. Biomed. Online 7, 428–432 (2003).

50. Zini, A. & Libman, J. Sperm DNA damage: Importance in the era of assisted reproduction. Andrologia. 101, 103–111 (2002).

51. Pereira Carla, S. A. et al. Evaluation of DNA damage induced by environmental exposure to mercury in Liza aurata using the comet assay. Arch. Environ. Contam. Toxicol. 58, 112–122 (2010).

52. Puthalakath, H. & Strasser, A. Keeping killers on a tight leash: Transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 9, 505–512 (2002).

53. Joseph, T. C. & Kothari, A. Anti-apoptotic Bcl-2 family members in development. Cell Death Differ. 25, 37–45 (2018).

54. Mason, K. D. et al. Programmed anuclear cell death delimits platelet life span. Cell. 128, 1173–1186 (2007).

55. Nath, K. A., Anthony, J. C., Scott, L., Tim, W. B. & David, W. Renal oxidant injury and oxidant response induced by mercury. Kidney Int. 50, 1032–1043 (1996).

56. Lohren, H. et al. Toxicity of organic and inorganic mercury species in differentiated human neurons and human astrocytes. J. Trace. Elem. Med. Biol. 32, 200–208 (2015).

57. Schwartz, M. & Vissing, J. Paternal inheritance of mitochondrial DNA. N. Engl. J. Med. 347, 576–580 (2002).

58. Badr, F. M. & Ola, E. H. Heavy metal toxicity affecting fertility and reproduction of males. In Bioenvironmental Issues Affecting Men’s Reproductive and Sexual Health (eds Sikka, S. C. & Hellstrom, W.) 293–304 (Academic Press, Cambridge, 2018).

59. Boghila, M. A. et al. Testicular toxicity in mercuric chloride treated rats: Association with oxidative stress. Reprod. toxicol. 28, 81–89 (2009).

60. Jan, A. et al. Heavy metals and human health: Mechanistic insight into toxicity and counter defense system of antioxidants. Int. J. Mol. Sci. 16, 29599–29630 (2015).

61. Dangi, S. S. et al. Expression profile of HSP genes during different seasons in goats (Capra hircus). Trop. Anim. Health Pro. 44, 1905–1912 (2012).

Acknowledgements
We are thankful to Head, Department of Veterinary Physiology for providing the necessary laboratory facilities to carry out this research. We are also thankful to Professor and Head, Department of Pharmacology and Toxicology for providing the necessary facilities in ICAR Niche Area of Excellence Laboratories. We are thankful to Dr Deepak Modi, Scientist-F, National Institute for Research in Reproductive Health, Mumbai for allowing and helping us in interpretation of the electron microscopy data.

Author contributions
Conceptualization—S.K.G. and D.K.S.; Methodology—B.K., R.S.Y., P.K. and A.K.; Software—B.K. and M.A.; Validation—S.K.G. and D.K.S.; Electron microscopy—D.S.; Data Curation—B.K. and S.K.G.; Writing of MS: Original
Draft Preparation—B.K.; Review & Editing—S.K.G.; Supervision—S.K.G.; Lab: S.Y. and B.Y.; Resources—College of Biotechnology; Funding Acquisition—University funding.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-020-80235-y.

Correspondence and requests for materials should be addressed to B.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021