Chapter 3

Materials and Methods

3.0. MATERIALS AND METHODS

3.1. Chemicals and reagents:

The chemicals which used in the present study were sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), thiobarbituric acid (TBA), tri-chloroacetic acid (TCA), chloroform, methanol, pyrogallol, H_2O_2, guiacol, acrylamide, bis-acrylamide, Tris-HCl, ammonium persulphate (APS), tetra-methyl ethylenediamine (TEMED), riboflavin, nitro blue tetrazolium chloride (NBT), ferric chloride, potassium ferricyanide, glutathione reduced, tris-base, nicotinamide adenine dinucleotide (NAD), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sodium chloride (NaCl), proteinase K, phenol, chloroform, isoamyl alcohol, ethidium bromide, agarose, Trizma base, Triton X-100, DMSO and sodium lauryl sarcosinate, Sodium hydroxide (NaOH), spectroscopic grade glycerol, dehydroepiandrosterone (DHEA), crystalline BSA, testosterone, nicotinamide adenine dinucleotide phosphate (NADP), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), sulfosalicylic acid, hematoxylin, eosin, ethanol.

3.2. Animal selection and treatment:

Selection and treatment procedure for animal:

Wistar strain rats of female sex were considered for the present research work and nurtured in the polycarbonate-cages in the institutional animal house (**IAEC/3/C-8/14, dated 11 March 2014**) as per CPCSEA guidelines, Ministry of Environment, Forest and Climate, Government of India. Maintaining 12-h light-dark cycle at the temperature of 32±2 °C and 50–70% humidity the rats were acclimatized for 10 days in a room. The rats were fed with standard rat chow and water ad libitum.

3.3. Measurement of Lipid Peroxide End Products:

The uterine slices were homogenized (20% w/v) with ice-cold phosphate buffer (0.1 mol/L, pH 7.4) for 3-minute duration and centrifuges at 15,000 × g at 4 °C. The supernatant was separated. MDA was determined from the interaction of thiobarbituric acid with MDA. Measuring the absorbance at 530 nm (ε=1.56×105 mol^{-1} cm^{-1}) the level of MDA was determined (Devasagayam and Boloor, 2003).

For measuring the CD level, the lipids were extracted with chloroform-methanol (2:1) mixture. Then centrifugation was performed for separating the supernatant at 1000×g for 5 minutes. Following the mixing of the lipid residue in 1.5 ml of cyclohexane hydroperoxide was quantified at 233 nm (Kumar, 2012).

3.4. Colorimetric assay of Superoxide Dismutase (SOD) and Catalase (CAT), Peroxidase (Px) and glutathione peroxidase (GPx) activities:

Using 100 mmol / L ice-cold tris HCl buffer that contains 0.16 mol/L KCl (pH 7.4), uterine horns were homogenized to obtain 10% (w/v) tissue concentration. Then centrifugation was executed at 10000 g for 20 minutes at 4°C. The reaction mixture contained a cocktail of 800 µL of TDB (Merck), 40 µL of 7.5 mmol / L NADPH (Sigma), 25 µL of EDTA-$MnCl_2$, and 100 µL of the tissue supernatant. The activity of SOD in this mixture was read at 340 nm from the rate of oxidation of NADPH (Pattichis et al., 1994).

Colorimetrically catalase activity was assessed (Hadwan, 2016). In the existence of H_2O_2 dichromate in acetic acid under heat transform into perchromic acid and finally produced chromic acetate. The chromic acetate was measured at 570 nm. One unit of activity was expressed as a mole of H_2O_2 consumed/min/mg protein.

Following the homogenization of the uterine horn with PBS (0.1 M, pH 7.0) the supernatant was separated for the peroxidase assay colorimetrically. In the presence of the substrate H_2O_2 (12.3 mM) 0.1 ml of supernatant and a cocktail of 20 mM guiacol were read at 436 nm (Sadasivan and Manickam, 1966). According to the procedure of Paglia and Valentine, the activity of uterine GPx was measured, and one unit of GPx activity was expressed in terms of nmol NAD(P)H oxidized/min/mg of protein (Paglia and Valentine, 1967).

3.5. Assessment of Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (Px) and Glutathione Peroxidase (GPx) in native gel:

Uterine tissue was homogenized (20%; w/v) with cold PBS (1.0 M pH 7.4) followed by centrifugation at 10,000 × *g* for 20 minutes at 4°C. Based on the principle of inhibition of the reduction of NBT the activity of SOD in gel (12.0%) assay system was evaluated and the capacity of O_2 •− to interact with NBT reduced the yellow tetrazolium within the gel to a blue precipitate, will develop a clear area of achromatic bands competing with NBT for the O2•−developed at the active site of SOD (Weydert and Cullen, 2010). Using the supernatant containing 60 μg of proteins on 12.0% native PAGE SOD was separated. The gels were incubated with 2.3 mM NBT, 28 μM riboflavin, and 28 mM TEMED for 20 minutes in the dark. Upon the exposure of the fluorescent light on the gel, the achromatic bands of SOD were visible against a dark blue background. The band strength of SOD was identified by staining the gel with coomassie brilliant blue.

Peroxides were eliminated by the catalase from the area of the gel from where it occupied. Prussian blue precipitate of potassium ferrocyanide and ferric chloride was developed by the reaction with potassium ferricyanide (Lewis et al., 2006). The tissue supernatant comprises of 60 μg proteins were electrophoresed on 8.0% PAGE. Gels were placed in 0.003% H_2O_2 solution for

10 minutes and then shifted to the staining mixture containing 2% potassium ferricyanide and 2% ferric chloride. Bluish yellow bands appeared against a blue-green background.

For the assessment of glutathione peroxidase (GPx) 8.0% native gel was used. GPx level was determined by the removal of peroxide during the conversion of potassium ferricyanide to ferrocyanide. On the blue, green background of native gel, GPx appeared as the achromatic clearing band (Liu et al., 2006). Using Image J software band density strength was analyzed. In terms of density percentage the data was expressed and the control's relative density was considered 100% (Singh et al., 2014).

3.6. Serum total Lactate Dehydrogenase (LDH):

A 1.2% agarose gel was prepared in 50 mM Tris-HCl buffer (pH 8.2). A 20 µL of serum was used for lactate dehydrogenase (LDH) assay. In the presence of H_2O, 1.0 M Tris, tetrazolium blue, phenazine methosulfate, Na-lactate, and nicotinamide adenine dinucleotide (NAD) with slight modification the agarose gel was developed. To acquire a color reaction, the gel was incubated at 37 °C and then water-driven rinsing was done under light (Brandt et al., 1987). Following the Image J software, the densitometric analysis of the gel was performed to highlight the electrozymographic image (Singh et al., 2014).

3.7. DNA Fragmentation analysis:

Before lysis uterine tissue was incubated for 15 minutes at $4^{O}C$ in 500 µL of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) and then cold centrifuged at 12,000 rpm for 20 minutes. The supernatant was mixed with 1:1 mixture of phenol: chloroform with gentle agitation for 5 minutes and precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate and then centrifuged. Using 30 µL of deionized water–RNase solution and 5 µL of loading buffer, the DNA pellet was re-suspended

for 30 minutes at 37°C. The DNA was applied to the lane in 0.8% agarose gel and electrophoresed at 65 V and finally documented in Bio-Rad documentation system in the presence of ethidium bromide (Paoletti et al., 1990).

3.8. Comet assay:

A 1.0 % agarose- precoated glass slide was prepared by a mixture of 75 ml of low melting point agarose (0.6%) in PBS at 37°C. A 25 ml of uterine cell suspension (105 cells) was placed on the agarose-precoated slide. Before electrophoresis, a coverslip was placed above the sample present in the glass slide. The coverslips were separated from the glass slide after solidification of agarose. Followed by washing with PBS for thrice at room temperature the slides were kept in the ice-cold lysis buffer (2.5 mM NaCl, 85 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, and 1% sodium lauryl sarcosinate, at pH 10.0) for 1hr at 4°C. Incubation of slides with coverslips at 37°C for 45 minutes. Followed by washing the slides with water twice for the removal of excess salt if any and finally, transferred to the electrophoresis chamber (Bio-Rad, USA) filled with the alkaline electrophoresis buffer containing 0.3 M NaOH and 1mM EDTA. The electrophoresis was performed at 25 V with a power supply of 300 mA for 30 minutes. Following the neutralization with PBS the slides were stained with a solution of 10 mg/ml ethidium bromide for 5 minutes. The slides were washed with water to remove excess stains and then observed under a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (ImpulsBildanalyse) software (Sing et al., 1988).

3.9. Ovarian Steroidogenic Enzyme Assay:

Ovarian tissue was separated from the visible fat body present in it. By transferring the ovary into the ice-cold homogenizing buffer and produces the tissue concentration at 10 mg/ml Ovarian tissue (10 mg/ml) was homogenized with ice-cold buffer containing 5.0 mM potassium

phosphate and 1.0 mM EDTA and 20% spectroscopic grade glycerol and centrifuged at 10,000×g for 45 minutes at 4 °C subsequently. Then the supernatant was collected for the assay of delta-5, 3-beta-hydroxysteroid dehydrogenase (Δ5, 3β-HSD). Adding 0.5 μM of NAD and 30 μg of dehydroepiandrosterone (DHEA) to the supernatant the absorbance was read at 340 nm against a reagent blank (without NAD) (Talalay, 1962).

25 mg of crystalline BSA, 0.3 μM of testosterone, and 1.1 μM of NADP were mixed with the supernatant for measuring the activity of the 17-beta-hydroxysteroid dehydrogenase (17β- HSD) at 340 nm in a spectrophotometer against a blank (without NADP) to promote the conversion of testosterone to androstenedione (Jarabak et al., 1962). At 340 nm one unit of enzyme activity was equal to a change in absorbance of 0.001/min.

3.10. Serum Gonadotrophin and Estradiol analysis:

The level of LH, FSH, and estradiol was determined through the ELISA kits as suggested by the manufacturers (Wuhan Fine Test, China). The ELISA assay of LH, FSH, and estradiol was based on the competitive-ELISA method.

In LH, FSH, and Estradiol ELISA assay the microtiter plates are pre-coated with LH, FSH, and Estradiol. The tested LH, FSH, and Estradiol (sample and standard) and LH, FSH, and Estradiol on the solid phase supporter can compete with each other for binding with biotinylated detection antibody which is specific for LH, FSH, and Estradiol. Washing of the plate removes the unbound samples, standards, and excess conjugates. Following the incubation, with HRP-Streptavidin (SABC) TMB substrate is added. Finally, sulphuric acid terminates the enzyme-substrate reaction. At 450 nm wavelength, the colour change reaction was recorded spectrophotometrically and compared with the OD of the samples to the standard curve.

3.11. Estimation of NPSH:

With a slight modification, the level of NPSH was measured by the standard DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) method and followed by the centrifugation at 10,000 × g the uterine tissue was homogenized using phosphate buffer (0.1 M, pH 7.4). The protein was precipitated by the use of sulfosalicylic acid. In 0.1 M sodium phosphate buffer which contains 5 μM of DTNB clear cytosol was added. The level of NPSH was determined against a reduced glutathione (GSH) standard curve (Forman et al., 2009).

3.12. Determination of Esr 1, NF-κ B, Metallothionein-1 (MT-1), TNF-α and IL-6:

Following the method recommended by the manufacturers (Wunhan Fine test, China and RayBio) the level of ovarian Esr 1, uterine NF-κ B, liver Metallothionein-1, serum TNF- α, and serum IL-6 were measured by sandwich ELISA.

In Esr 1, NF-κ B, MT-1, TNF- α, and IL-6 assay technology the microtiter plates are pre-coated with anti-Esr 1, anti NF-κ B, anti MT-1, anti TNF- α, and anti IL-6 antibody. In each well, the standards, test samples, and biotin conjugated detection antibody are incorporated and then followed the washing step with wash buffer. Next, HRP-Streptavidin is added to each well and incubated. The washing procedure is followed to eradicate the unbound conjugates with wash buffer. To observe the HRP enzymatic reaction, TMB substrates are added. Blue colour product is formed after catalyzing the TMB by HRP. After giving the acidic stop solution the blue colour product is transformed into a yellow colour product. The concentration of Esr 1, NF-κ B, MT-1, TNF- α, and IL-6 were evaluated after taking the absorbance of OD at 450 nm in an ELISA reader.

3.13. Serum Vitamins and Homocysteine assay:

To analyze the presence of vitamins in serum C_{18} column of reverse phase HPLC was used. Upon mixing with 5.0 ml of 1.0% acetate buffer solution (pH 4.6) vitamin B_{12} was extracted from the 5.0 ml of serum and following a thorough mixing the extract was kept for 30 minutes in a boiling water bath. A clear supernatant solution (pH 6.9) was taken after the centrifugation (Stefova et al., 1997).

A 0.2 ml of serum was used to extract folic acid in presence of 1.2 ml of 50 mM potassium tetraborate/L and 1.0% sodium ascorbate (pH 9.0) at 4^0C and vortex and boiled for 30 minutes and then kept at 4 °C in the dark for overnight. Synthetic ethyltetrahydrofolate (eTHF) is referred as an internal standard. Extracted samples were filtered and then analyzed in an HPLC (Kalmbach et al., 2011).

The separation of serum was performed through centrifugation (4 °C) at 2740 g for 8 minutes prior to analyzing vitamin C. The supernatant was collected and mixed with 10% (w/v) metaphosphoric acid (MPA) in 2.0 mmol/L disodium EDTA and kept at ice for 5 minutes. Then the mixture was centrifuged (4 °C) at 16,000g for 10 minutes (Robitaille and John, 2015). Before HPLC analysis, the extracted protein-free ascorbic acid kept on dry ice and then stored at −80 °C. Finally, using a C_{18} column in reverse phase HPLC, the serum level of vitamin C was measured in serum.

The level of B_{12}, folic acid, and homocysteine in serum was also measured using an ELISA kit as suggested by the manufacturers (Wuhan Fine Test, China). The ELISA assay of B_{12}, folic acid, and homocysteine was based on the competitive ELISA assay technique.

Here, the microtiter plates are pre-coated with vitamin B_{12}, folic acid, and homocysteine. The tested vitamin B_{12}, folic acid, and homocysteine in samples or standards and vitamin B_{12}, folic

acid, and homocysteine on the solid phase supporter can compete with each other for binding with biotinylated detection antibody that is specific for vitamin B_{12}, folic acid and homocysteine. The plates are washed for removing the excess conjugate, unbound sample, or standard. In each well, the HRP-Streptavidin (SABC) is added and then the incubation step is followed. In each well, TMB substrate solution is added. The sulphuric acid solution is added to stop the enzyme-substrate reaction. At 450 nm wavelength, the colour change reaction was determined spectrophotometrically. The concentration of vitamin B_{12}, folic acid, and homocysteine of the samples was measured by matching the sample OD with the standard curve.

3.14. Determination of serum SGPT and SGOT level:

According to the procedures recommended by the manufactures (Coral), the serum SGPT and SGOT levels were determined using a biochemical assay kit.

L-alanine and α-ketoglutarate are transformed into pyruvate and glutamate by SGPT. To generate hydrazone derivative; pyruvate reacts with 2,4, dinitrophenyl hydrazine. In an alkaline medium brown coloured compound is created by hydrazone derivative. The colour complex was measured at 505 nm. The activity of SGPT was determined through the calibration curve that was plotted using a pyruvate standard.

The transfer of the amino group between L-aspartate and α-ketoglutarate is broken through SGOT and produced oxaloacetate and glutamate. In presence of malate dehydrogenase; oxaloacetate reacts with NADH and generates NAD. The rate of formation of NADH to NAD was measured at 340 nm. The activity of SGOT in the sample was proportional to the absorbance.

3.15. Ovarian and uterine histopathology:

Prior to sectioning at 5μm thickness, the ovarian and uterine tissues were embedded with paraffin. Using hematoxylin (Harris) and eosin, the staining procedure was performed and then examined under a microscope (Olympus, CX21i, magnification x400).

Stages of folliculogenesis in the ovary were analyzed by follicular quantification and diameter measurement (Patil et al., 1998). The follicles were classified as small preantral follicles (SPAF) (< 94 μm), large preantral follicles (LPAF) (94–260 μm), small antral follicles (SAF) (261–350 μm), medium antral follicles (MAF) (351–430 μm), large antral follicles (LAF) (431–490 μm), graafian follicles (> 491 μm) on the basis of their diameter and morphology. Then the numbers of atretic follicles (ATF) were counted. The uterine breadth and the thickness of different uterine layers (μm) (i.e. endometrium, myometrium, and perimetrium) were also measured.

3.16. Statistical analysis:

Using ANOVA the statistical significance of the differences in these variables between treated cases and controls was assessed followed by the post hoc Dunnett test (McHugh, 2011). Differences of data (Mean ± SE, N= 6), $p<0.05$ were considered significant statistically.