CHAPTER-3

MATERIALS & METHODS

3.0. Materials & Methods

3.1. Reagents and chemicals

The whole experimental work was carried out using the animal models (rats) which were taken from the Govt. registered animal provider. Food ingredients for animal feeding were acquired from the neighboring market of Midnapore. Animals were at free access of water *ad libitum*. NAC was bought from Sigma-Aldrich, Catalog number A7250 and purity ≥99%. S D Fine Chem Ltd (SDFCL), India supplied sodium arsenite, hydrogen peroxide, ethanol, methanol and chloroform. From Merck, Mumbai, Maharashtra, India, Potassium ferricyanide, glacial acetic acid, ethidium bromide and TPP were afforded. Agarose, NBT, Tween 20, NAD, sodium acetate, NADP, Tris-base, potassium phosphate and sodium lactate have been availed from SRL, Mumbai, Maharashtra, India. Ray Biotech, Georgia, USA, provided TNF-α and IL-6 kit. From Loba (Mumbai, Maharashtra, India), Riboflavin was acquired. From the reagent provider Tulip group, India, LDH, creatinine, SGOT and SGPT kits were taken. The kits of estradiol, ER-1, LH, FSH, folic acid (B_9), vitamin B_{12}, NF-κB, and MT-1 ELISA were supplied by Wuhan Fine Biological Technology Co., Ltd. (Wuhan, China). From Qualigens, India, Hematoxylin, eosin plus ferric chloride were procured.

3.2. Animal selection and treatment

Female virgin Wistar strain albino rats weighted approx 95 ± 5.0 g were utilized for the whole experimental study. All the experimental animals were procured from the authorized provider. The total experimental work was performed as per the guidelines by the Control and Supervision of Experiments on Animals (CPCSEA),

Ministry of Environment, Forest and Climate (Government of India) with prior approval of Vidyasagar University Ethical Committee. All experimental models were set aside in separate polycarbonate cages and placed in the animal house named Darwin's House of working institution i.e. Vidyasagar University, Midnapore, West Bengal, India. Based on the instruction by the animal ethical committee, all the experimental animals were acclimatized for 10 days in a controlled temperature within 32±2˚C with the maintenance of 12h dark-light cycle and adequate percentage of humidity. During each experimental work the rats were supplied free access of consumable water and standard basal diet. The component of standard basal diet is given below:

- Wheat, flour, atta: 56.42%
- Bengal gram, sattu: 31.56%
- Whole milk powder: 10.52%
- Soyabean oil: 1.00%
- Sodium chloride: 0.5%

3.3. Study of vaginal smear

Detecting the phases of estrous cycle during the experiment, vaginal smear was collected from each rat in the experiment. The smear collection process was performed using 0.9% normal saline with a dropper. The saline containing sterile smooth dropper was inserted into the vagina and then saline was flushed for many times for the vaginal fluid compilation. After that smear was poured on the top of the glass slide, allowed for complete drying. When the slides become completely dehydrated then allocated for Leishman's stain followed by the observation underneath light microscope (magnification 40x).

3.4. Estimation of total protein

The total protein of serum, liver tissue and reproductive organs (uterus and ovary) were determined by Biuret method. A complex is created between the Cu^{2+} and functional group of two peptide bonds in protein. This complex creates a chelate product of violet colour.

Biuret reagent (1.0 ml) was poured in 'Blank', 'Standard' and 'Test' test tubes. Afterwards 10 µl of standard solution and 10 µl of sample were added in 'Standard' and 'Test' test tubes accordingly. The solution mixtures were then allowed for incubation in room temperature for 5 minutes followed by recording of OD at 540 nm against Blank.

3.5. Determination of metabolic toxicity

3.5.1. Estimation of SGPT

SGPT shifts the amino group from L-alanine into α ketogluterate and thereby these two substances become pyruvate and glutamate respectively. In alkali medium the generated pyruvate combined with 2, 4, Dinitrophenyl hydrazine (DNPH) and develop a coloured (brown) complex of hydrazone. The strength of this formed coloured complex was recorded which is proportional with SGPT activity.

At the beginning substrate reagent (0.5 ml) was poured into the test tubes and set aside in incubator for 3 minutes at 37˚C. Afterwards distilled water (0.1 ml) was dispensed only in blank tube and test serum (0.1 ml) into the test sample with subsequent incubation for about 30 minutes. Then DNPH (0.5 ml) reagent was discharged into all tubes and considered for standing about 20 minutes in room temperature after proper mixing. Again the total cocktail was allowed to wait about

10 minutes after the mixing of 0.4N NaOH (0.5 ml) and then allocated for absorbance measurement at 505 nm.

3.5.2. Estimation of SGOT

Like SGPT; SGOT facilitates the transamination process and thereby L-aspartate is converted into oxaloacetate and α ketogluterate into glutamate. The generated oxaloacetate caused oxidation of NADH into NAD catalyzed by malate dehydrogenase. After that spectrophotometric absorbance is taken at 340 nm because of oxidation rate of NADH which is reduced during time progression by means of relative SGOT activity in given sample.

Initially the substrate assay was started by adding enzyme reagent (0.8 ml) plus sample (0.2 ml) and then incubation for about 1 min following mixing of starter reagent (0.2 ml). After that initial absorbance was assessed following repetition of the same in every 1 or 2 minutes. The average absorbance was analysed by ΔA/min. For sample assay, a same proceeding was permitted only working reagent (1.0 ml) was poured into sample (0.2 ml) after incubation of 1 minute.

3.5.3. Measurement of serum creatinine

The reaction linking with creatinine and picric acid formed a orange colour complex with alkali picrate in a basic intermediate and then absorbance is taken at 520 nm. The colour intensity is directly related with the magnitude of creatinine remaining in sample.

A mixture blend was prepared with reagent picric acid and sample and centrifuged at about 2500-3000 rpm for approx 10 minutes to acquire a lucid supernatant. Then in blank tube reagent picric acid (1.0 ml), distilled water (0.1 ml) and buffer reagent

(0.1 ml) were blended, whereas in standard same protocol was permitted except distilled water where creatinine standard was poured. In sample test tube only clear supernatant and buffer reagent was discharged. The whole preparation was permitted to stand for accurately 20 minutes in room temperature and then absorbance was quantified against blank.

3.6. Assessment of oxidative stress

3.6.1. Measurement of MDA and CD

Oxidative stress markers were measured in liver, uterine horns and ovarian tissues. Initially these reproductive tissues and liver tissue were taken (20% w/v) for homogenization using the chilled phosphate buffer (0.1 mol/L, pH- 7.4). Homogenate of these tissues was centrifuged at 15,000 g for 3 minutes at 4˚C to get the supernatant. This supernatant part was used for the measurement of MDA and CD.

A mixture solution was prepared with the supernatant and thiobarbituric acid (TBA) and allowed to heat for approx 15 minutes in water bath prior to cooling. After centrifugation the supernatant part was obtained and MDA measurement was done at 530 nm (ε=1.56×105 mol^{-1} cm^{-1}) (Devasagayam and Boloor, 2003).

Measuring of CD the supernatant part was mixed with a solution containing chloroform-methanol in a ratio of 2:1. This mixture was allowed for centrifugation to get the lipid extraction. A drier was used for the evaporation of this lipid residue. In post drying step the rest part of lipid was re-mixed with cyclohexane very well and absorbance is taken at 233 nm (Kumar, 2012).

3.6.2. Measurement of NPSH

Measuring of NPSH, ice cold 0.1 M phosphate buffer (PBS) having pH- 7.4 was applied for homogenization of uterine horn and ovarian tissues at 4˚C. The homogenate utilized for the centrifugation at 4˚C, 10,000 g for 10 minutes to acquire supernatant. Following the technique of DTNB (5, 5″- dithiobis-2-nitrobenzoic acid) this supernatant part was assigned for the estimation of NPSH (Mieyal et al., 2008). Sulfosalicialic acid was employed for the precipitation of protein and then supernatant was collected which was further dissolved with DTNB containing 0.1 M sodium phosphate buffer. At last measurement was recorded at 412 nm of wave length.

3.6.3. Spectrophotometric assessment of SOD, catalase and GPx

All the tissue slices (10% w/v) (uterus, ovary and liver) were taken in 100 mmol/L refrigerated Tris-HCl homogenising buffer (pH- 7.4) for the preparation of tissue slice homogenate and then the whole preparation was centrifuged at 4˚C for 20 minutes at 10,000 g. Afterwards a reaction was introduced by the addition of 100 µl of tissue supernatant, 800 µL of TDB, 40 µL of 7.5 mmol/L, NADPH, 25 µL of EDTA-$MnCl_2$. Finally, based on the oxidation rate of NADPH the absorbance was observed at 340 nm for the estimation of SOD in the mixture (Pattichis et al., 1994).

Catalase activity was determined as per the method proposed by Hadwan, 2016. The dichromate in acetic acid was transformed into per chromic acid and during heating it was finally changed into chromic acetate due to the occurrence of H_2O_2. This chromic acetate was finally used for the assessment of catalase at 570 nm. The catalase functioning was expressed by one part of one mole H_2O_2 consumed per

milligram of protein per minute (Hadwan, 2016). Distilled water was poured in 'Standard' (100 µl) and 'Blank' (1100 µl) test tube followed by addition of sample (100 µl) only in 'Test' test tube. About 1000 µl of H_2O_2 was then dispensed in 'Test' and 'Standard' test tube followed by proper mixing with vortex. The whole mixing was considered to incubate at least 3 minutes at 37˚C. Then 2000 µl of dichromate/acetic acid solution (50 ml of 5% potassium dichromate solution + 150 ml of glacial acetic acid) was discharged in all the test tubes and permitted for heating for 10 minutes at boiling temperature followed by cooling. Afterwards precipitated protein was decanted after centrifugation at 2500 g for 5 minutes and OD was documented at 570 nm against Blank.

The utero-ovarian and hepatic tissues were employed for the judgment of GPx functionality. The changing kinetics of GPx activity was considered as one part of 1 µmol NAD(P)H oxidation per milligram of protein per minute and OD was availed at 340 nm (Paglia and Valentine, 1967). An assay buffer (50 mM phosphate buffer) was made using Titron X-100 (0.1%), pH 7.0. A NADPH reagent solution was also arranged using 24 µmol GSH, glutathione reductase and 4.8 µmol β-NADPH. Then a solution blend was made using 1.0 ml of phosphate buffer, 0.1 ml of NADPH, 0.01 ml of sample plus 0.1 ml of H_2O_2 and OD was availed at 340 nm over an episode of 3 minutes.

3.6.4. Native gel assessment for SOD, catalase and GPx impression

The quantity of 20% w/v reproductive tissues (uterus and ovary) and metabolic tissue (liver) were homogenized with cooled 0.1 M phosphate buffer (pH- 7.4) in the beginning, then centrifugation of homogenate of these tissues was furnished at 10,000 g for few minutes at 4˚C. Afterwards supernatant was considered for

measuring the total protein by Biuret method. The tissue SOD activity was examined in 12% native gel where 50 µg of tissue protein was loaded on it and allowed for electrophoretic run. After that the gels were immerged in a blending of 28 µM riboflavin and 2.3 mM NBT for 20 minutes in a dark state. Thereafter, 28 mmol/L of TEMED was added for the incubation of gels in the identical dark place for 20 minutes. After all, in the dark-blue milieu achromatic bands were visualized when light exposed on the gels (Weydert and Cullen, 2010).

Both catalase plus GPx expression were manifested in 8% native gel wherein 50 µg of protein from tissue supernatant was loaded. After electrophoresis, the gels were placed within a solution containing 0.003% of H_2O_2 in dark condition for few minutes. Then the gels were considered for staining with a decoction of 2% of ferric chloride plus 2% of potassium ferricyanide solution for few seconds. Achromatic bands of catalase were seen against blue-green milieu (Weydert and Cullen, 2010).

GPx study was considered by adding of GSH (1 mM) for staining purpose after electrophoresis which was also mandatory for the functional activity of GPx. Following the incubation of gels in a staining solution of cumene hydroperoxide (0.008%) for 10 minutes, 1% of potassium ferricyanide and 1% of ferric chloride in GSH solution were added to initiate the visualization of achromatic band in blue-green background (Weydert and Cullen, 2010). The relative density of each band was assessed by the software image J.

3.7. Spectrophotometric estimation of LDH

As per the proceedings of the manufacturer in kits, LDH activity analysis was performed (Tulip Group, Goa, India). NADH starts the reduction reaction where pyruvate is reduced into lactate and itself becomes NAD.

At first, reagent-A (NADH; 0.02 mmol/l) and reagent-B (pyruvate; 1.6 mmol/l plus sodium chloride; 200 mmol/l) was mixed gently to make a complete solution. In a cuvette 3 ml of this mixed solution was poured and then incubated for few minutes. Then 100 µl of sample was discharged within the cuvette. After 30 seconds the OD was recorded in reducing way by means of NADH oxidation into NAD and that was linked to the LDH function.

3.8. Estimation of LDH on agarose gel

On 8% agarose gel LDH expression was verified. Initially about 20 µl of serum sample was loaded on top of the gel, afterwards allowed for electrophoresis using the Tris-HCl buffer (50 mM), pH-8.2 at 170 Volt. A cocktail was introduced by adding Na-lactate, phenazine- methosulphate, H_2O, tetrazolium-blue, and 1.0 M of Tris and NAD for the gel development following colour reaction of gel on incubation at 37˚C. Then the gel was considered for rinsing for several times with deionized water and recognition of LDH was progressed with light exposure. The image J software was applied for assessing the band density (Brandt et al., 1987).

3.9. Assay of DNA fragmentation

The uterine tissue was advocated for DNA analysis. At the beginning uterine horns were considered for homogenization where 500 µl of lysis buffer (pH 8.0) was needed consisting of 1% SDS, 10 mM NaCl, 50 mM Tris, 0.5 mg/ml proteinase K

and 20 mM EDTA. The above cocktail of reagent was kept for 15 minutes at 4˚C following the centrifugation in low temperature for 20 minutes at 12,000 rpm. Collected supernatant was agitated with a solution comprised of chloroform and phenol at a ratio of 1:1 and allowed for gentle mixing for few minutes. This total solution was now considered for precipitation with mixing of another cocktail of chilled ethanol and sodium acetate followed by further centrifugation. The pellet part was collected after centrifugation and compiled with 5 µl of loading buffer and 30 µl of deionized water–RNAase solution. Following incubation for 30 minutes at 37˚C, electrophoresis was done on 8.0% agarose gel comprising ethidium bromide at 65 Volt. Finally, with the help of Bio-Rad gel documentation system, the gel was observed (Garcia-Martinez et al., 1993).

3.10. Comet assay

Comet test was acting upon as per protocol of Singh et al, 1988 (Singh et al., 1988). The prepared 1.0% agarose solution was emerged for the coating of glass slides. The liver plus uterine cell suspension (about 105-110 cells) with PBS separately and then poured onto the agarose of minimum melting point (0.6%) and allowed for incubation at 37˚C. Prepared 1.0% agarose was covered on the glass slides after proper solidification followed by the placing of cover slips onto the slides very gently and considered for hardening of the agarose. Then the cover slips were taken out from the slides and soaked into the lysis buffer under low temperature containing 10% DMSO, 10 mM Trizma base, 1% SDS, 1% Triton X-100, 85 mM EDTA, and 2.5 mM NaCl (pH- 10) at 4˚C for 1 hour. After lysis, the slides were refreshed thrice with PBS in normal temperature following incubation at 37˚C again for 40-45 minutes. Then the slides were advocated for water washing for several

times to get rid of overloaded salts and allowed for electrophoresis in running buffer (1 mM EDTA and 0.3 M NaOH) at 300 mA and 25 Volt for 30 minutes. After electrophoretic run, slides were considered for neutralization using PBS and then staining was done with the solution of ethidium bromide (10 mg/ml) in dark for few minutes. Then the slides cleared with water to get rid of excess stain. Finally, the slides became ready for visualization under fluorescence microscope (Eclipse LV100 POL, Nikon-Tokyo, Japan) and introduced under Vis Comet software (ImpulsBildanalyse-Amsterdam, Netherlands). The forming comet cells and tail length of comets were also measured.

3.11. Estimation of ovarian Δ^5, 3β-HSD and 17β-HSD activities

Frozen ovarian tissues (10mg/ml) were placed for homogenization at 4°C with low temperature buffer containing 20% spectroscopic-grade glycerol, 1.0 mM EDTA and 5.0 mM potassium phosphate followed by centrifugation for 40 minutes at 10,000 g. The obtained supernatant was employed for assessing Δ^5, 3β-HSD. Then a solution was prepared with 30 μg of DHEA, 0.5 μM of NAD and supernatant and allowed for proper mixing for few minutes. After that the OD was recorded at 340 nm with respect to blank without NAD (Talalay, 1962).

Determining the activity of 17β-HSD, the ovarian tissue homogenate was combined with testosterone (Conc. 0.3 μM), NADP (Conc. 1.1 μM), and crystalline BSA (Conc. 25 mg) and mixed thoroughly for few seconds. Matching with the reagent blank, the absorbance was estimated at 340 nm without NADP (Jarabak et al., 1962). The change of absorbance at the velocity of 0.001/minute was considered as the equivalent of one unit of enzyme activity.

3.12. Estimation of the components of SAM pool by ELISA

The competitive ELISA method was applied for evaluating Vit. B_{12}, B_9 and Hcy in serum. The protocols were pursued as per the manual of manufacturer (Wuhan Fine test kit from China).

The pre-coated microtiter plates of ELISA (with B_{12}, B_9 and Hcy) were deemed for a competitive reaction when standards or test samples (50 µl) were poured on it and simultaneously 50 µl of HRP-Streptavidin (SABC) was loaded in every microtiter well and permitted for incubation for 60 minutes in room temperature. These could go for a competition between each other for their attachment for capturing biotinylated recognition specific antibody which was also particular for Vit. B_{12}, B_9 and Hcy. The excess standards or samples were then rinsed with washing buffer (300 µl) for three times. Then a solution of TMB substrate (100 µl) was poured into all well and allowed to react in dark room temperature for 20 minutes. Then 100 µl of stop solution was added in each microtitre well prepared with sulphuric acid (0.5 M) to stop the enzyme and substrate reaction. In spectrophotometer, the colour altering response was evaluated at 450 nm. Finally by plotting the sample's OD against standard curve, the quantity of Vit. B_{12}, B_9 and Hcy remain in the samples were estimated.

3.13. Estimation of Hormones in serum by ELISA

Consistent with the competitive ELISA process (Wuhan Fine test kit from China), the level of estradiol, LH and FSH were estimated in serum following the procedure suggested by the manufacturer.

The microtiter wells of ELISA were pre-layered with estradiol, LH and FSH. The test samples (50 µl) and standards (50 µl) were then added and allowed for combining with biotinylated recognition antibody specifically in favor of estradiol, LH and FSH respectively. The subtraction of excess unbound standards and samples were followed thorough rinsing and then HRP-streptavidin (50 µl) (SABC) and TMB substrate (100 µl) were added and considered for incubation for few minutes. Finally, a stop solution (100 µl) of sulphuric acid was given to cease the reaction between enzyme and substrate. At last, the changing of colour in the wells was documented at 450 nm and plotted the sample's reading with standard curve.

3.14. Estimation of MT-1, TNF-α, IL-6, ER-α and NF-κB, by ELISA

The guidelines derived by the manufacturer, the sandwich ELISA technique was performed for assessing MT-1 in liver, TNF-α and IL-6 in serum and ER-α and NF-κB in uterus.

The estimation of above said parameters involved a sequence of orderly steps. The microtiter wells of S-ELISA were remaining pre-covered with anti-MT-1, anti-TNF-α and anti-IL-6, anti-ER-α and anti-NF-κB antibody. In every microtiter well the test samples (100 µl), standard (100 µl) along with biotin conjugated detection specific antibody (50 µl) were discharged and considered for incubation followed by decanting with washing buffer. Afterwards, both HRP-streptavidin (50 µl) plus TMB substrates (100 µl) were included in all well and then incubated. A colour reaction was noted because of HRP on TMB and produced a colour (blue) product. This blue colour was changed into yellow colour after inclusion of acidic stop solution (100 µl). Finally, the OD was evaluated in ELISA reader at 450 nm to determine the concentration of above said parameter.

3.15. Histopathology of uterus and ovary

After sacrifice of the experimental animal, ovarian-uterine tissues were collected and preserved in formalin. Prior to embedding these tissues were dried out with ethanol in increasing order (30%, 50%, 70%, 90% and 95% respectively) and cleaned with xylene. After that the ovarian-uterine tissues were placed in paraffin and then sectioned at 5µm thickness and kept on top of glass slides for staining with hematoxylin and eosin (Harris). Then slides were visualized under microscope (Olympus, CX21i, magnification x40) to highlight the tissue architectural changes.

The ovarian folliculogenesis stages were assessed through the counting of follicular numbers and based on their breadth the follicles were assigned in different groups like 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) (Patil et al, 1998). Simultaneously, quantification of atretic follicles (ATF) was exempted too. The thickness of uterine endometrium and myometrium were also assessed.

3.16. Semi-quantitative analysis of pro-inflammatory and apoptotic markers through PCR

RNA isolation kit (Promega) was applied for study through PCR. At first mRNA was pulled out from the uterine tissue (95 mg tissue). It was then dissolved in RNase-free water and employed for cDNA synthesis (Himedia). After that cDNA was considered for PCR amplification in which it acted as template by using PCR

master mix (Qiagen, Germany) for 5 minutes at 94˚C. The primer sequences are the following:

NF-κB: F: 5′CAGACACCTTTGCACTTGGC3′

R: 5′GCCTCCACCAGCTCTTTGAT3′

P^{53}: F: 5′CTACTTCCCAGCAGGGTGT3′

R: 5′AAAGTCTGCCTGTCGTCCAG3′

BAX: F: 5′GATCGAGCAGAGAGGATGGC3′

R: 5′CAGTCCAAGGCAGCAGCAGGAA3′

Bcl-2: F: 5′TGGCATCTTCTCCTTCCAGC 3′

R: 5′ATCCCAGCCTCCGTTATCCT 3′

GAPDH: F: 5′GGGAAACCCATCACCATC 3′

R: 5′CCCTGTTGCTGTAGCCAT3′

Then a mixture was arranged with every PCR product (5 µl) and the loading buffer (5 µl). Afterwards this mixture was loaded in the electrophoretic tank on agarose gel (2% w/v) for electrophoresis. In assistance with Molecular Analyst software (version 1.5; Bio-Rad) and Model GS-700 Imaging Densitometer, the DNA bands were examined. Here GAPDH was served as reference or control (Tan et al., 2014; Kang et al., 2013; Seo et al., 2011).

3.17. Assessment of ER-α through immunohistochemistry

The quantity of ER-α was studied in ovarian-uterine tissue. These reproductive tissues were placed and fixed in paraffin and sectioned at 4 µm diameter and incubated afterwards for antigen reclamation. Then with the help of regular goat serum the slides were placed at 37˚C and then incubation with primary antibody at 4˚C, polyperoxidase anti mouse IgG was applied on slides for 30 minutes. Finally, the DAB chromagen was exposed for staining purpose which was again stained with heamatoxylene and ultimately the glass slides allowed for examination with a microscope (magnification of x100) (Ward et al., 2006).

3.18. Statistical analysis

The organo-somatic index (%) was calculated as weight of the organ by dividing it by body weight of the rats and multiplied by 100.

The outcome of entire experiment were presented here in terms of mean ± SE, the sample size in every group was 6 (n=6). The statistical significance between the vehicle control and sodium arsenite treated group as well as between the arsenite treated and supplemented group were determined with the application of one-way-ANOVA following Dunnett's test (post-hoc). Statistically, $p<0.05$ was implicated as the least level of significance.