Chapter 3: Materials And Methods

3.1. SEMEN SAMPLE COLLECTION AND INITIAL ANALYSIS

_Homo sapiens_ (Human) semen samples were collected from the patients, who attended the semen analysis at Andrology department, Bangalore Assisted Conception Centre (BACC) Private Limited Bangalore, Karnataka, India. Samples were collected through masturbation in a clean, sterile and wide-mounted container made up of plastic which is confirmed as nontoxic for spermatozoa. The sample container was kept at 37°C. After collection, the specimen was labeled with name of the donor, identification number, date and time of collection. The semen container was placed in the incubator at 37°C while the semen liquefies.

**Table 3.1: Number of samples used for various objectives**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>OS</th>
<th>OAS</th>
<th>AS</th>
<th>AZ</th>
<th>NO</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen preservation analysis</td>
<td>20</td>
<td>22</td>
<td>24</td>
<td>--</td>
<td>28</td>
<td>09</td>
</tr>
<tr>
<td>Protein Quantification in various fractions</td>
<td>21</td>
<td>32</td>
<td>27</td>
<td>20</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>Analysis of seminal fructose, glucosidase, Zn</td>
<td>21</td>
<td>28</td>
<td>20</td>
<td>12</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>Analysis of antioxidant markers</td>
<td>12</td>
<td>20</td>
<td>32</td>
<td>--</td>
<td>34</td>
<td>18</td>
</tr>
<tr>
<td>Identification of fertility associated proteins</td>
<td>05</td>
<td>05</td>
<td>05</td>
<td>05</td>
<td>05</td>
<td>05</td>
</tr>
<tr>
<td>Artificial neural networks</td>
<td>34</td>
<td>35</td>
<td>35</td>
<td>22</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>92</strong></td>
<td><strong>142</strong></td>
<td><strong>143</strong></td>
<td><strong>59</strong></td>
<td><strong>156</strong></td>
<td><strong>70</strong></td>
</tr>
<tr>
<td><strong>Grand Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OS-oligospermia, OAS-Oligoasthenospermia, AS-Astheno spermia, AZ-Azoospermia, NO-Normospermia, C-Control

**HUMAN ETHICAL COMMITTEE CLEARANCE**

Ethical clearance has been obtained from VIT Human Ethical Committee (Ref. No. VIT/UHEC-3/NO.11) Vellore, for handling human semen samples and performing this research work by us in VIT campus.
3.1.1. SEMEN PARAMETER ANALYSIS USING COMPUTER ASSISTED SEMEN ANALYSIS (CASA)

All the semen samples were subjected to semen analysis for four major semen parameters including sperm concentration, total motility, progressive motility and normal morphology. These parameters were evaluated by using CASA system at BACC, Bangalore.

3.1.2. SEMEN PRESERVATION ANALYSIS (SPA)

About 103 samples were collected used for this study, semen analysis were done for all the samples according to the protocol of WHO, (2010). All the samples were categorized by semen analysis and found to be oligospermia (n = 20), oligoasthenospermia (n = 22), asthenospermia (n = 24), normospermia (n = 28), and control (n = 9). The semen analysis report (before freezing with extenders) was renowned. Four different semen extenders were prepared in our laboratory, (none of them are commercial). Composition of four different semen extenders were as follows,

1. Glycerol Extender (E1)
2. Glycerol-Tris-Egg yolk Extender (E2)
3. Glycerol-Citrate-Egg yolk Extender (E3)
4. Glycerol-Citrate-Egg yolk- Antioxidants Extender (E4)
Table 3.1.1: Composition of extenders: Table 3.1.1. shows the composition of various semen extenders used for semen preservation. E1 was supplemented with only glycerol, the major component of E2 was tris, in E3 the major component tris was replaced by citrate. Extender E4 was supplemented with antioxidants like vitamin E, vitamin C, catalase and taurine with presence of glycerol and citrate. The pH of all the extenders were maintained between 7.0- 7.5. Glucose was the common energy source added to all the extenders which was the substitute for fructose as the semen already contains fructose in a higher concentration. Penicillin and gentamicin were added as an antibiotics to all the extenders.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Extender 1</th>
<th>Extender 2</th>
<th>Extender 3</th>
<th>Extender 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>7%</td>
<td>7%</td>
<td>7%</td>
<td>3%</td>
</tr>
<tr>
<td>Tris</td>
<td>-</td>
<td>2.42 g</td>
<td>-</td>
<td>1.3g</td>
</tr>
<tr>
<td>Citric-Acid</td>
<td>-</td>
<td>1.45 g</td>
<td>-</td>
<td>0.8g</td>
</tr>
<tr>
<td>Monohydrate</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>1g</td>
<td>1g</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Egg Yolk</td>
<td>-</td>
<td>20 ml</td>
<td>20 ml</td>
<td>20 ml</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>-</td>
<td></td>
<td>2.3 g</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>3.7 g</td>
<td>3.45 g</td>
<td>1.3 g</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>-</td>
<td>2.4 g</td>
<td>1.8 g</td>
<td>1.2 g</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>-</td>
<td>0.5 g</td>
<td>0.74 g</td>
<td>0.5 g</td>
</tr>
<tr>
<td>HEPES</td>
<td>-</td>
<td>0.2 g</td>
<td>0.2 g</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td></td>
<td></td>
<td>4.1 g</td>
</tr>
<tr>
<td>Cystine</td>
<td>-</td>
<td>0.1 g</td>
<td>0.1 g</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>0.2 g</td>
<td>0.2 g</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Potassium Acetate</td>
<td>-</td>
<td>0.23 g</td>
<td>0.45 g</td>
<td>0.45 g</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>7.2</td>
<td>7.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>-</td>
<td>25 mg</td>
<td>25 mg</td>
<td>25 mg</td>
</tr>
<tr>
<td>Penicillin</td>
<td>-</td>
<td>50000 IU</td>
<td>50000 IU</td>
<td>50000 IU</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Taurine</td>
<td>2 g</td>
<td>-</td>
<td>-</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.7 g</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.9 g</td>
</tr>
</tbody>
</table>
The composition of each extender was tabulated in the table 3.1.1.

Extender 1 (E1) was supplemented with only glycerol and antioxidant taurine.

Extender 2 (E2) The major component added to prepare extender 2 (E2) was glycerol (7%), tris (2.42%), egg yolk (3%).

In Extender 3 (E3) preparation tris was replaced by citrate.

In Extender 4 (E4) The major component present in the extender 4 (E4) was supplemented with different antioxidants like vitamin C, vitamin E, catalase and taurine.

In addition to these components, antibiotics like gentamicin (25 mg) and penicillin (50000 IU) were supplemented in all the extenders for preservation and avoiding the contamination. Gentamicin was added to inhibit the protein synthesis of any microbes present in the semen aliquots during semen preservation, while penicillin was added as broad spectrum antibiotic as well as it will inhibit the cell wall synthesis of microbes during the process of semen preservation. Trehalose (0.2%) was added to all the extenders, which will be helpful in high water retention abilities. Trehalose will be the appt component that has the ability to control the water retention during semen preservation.

3.1.3. SEMEN EXTENDER AND PRESERVATION

Four different extenders were prepared in our lab with the above mentioned composition. Each extender was prepared for 100 ml with their respective recipe. All the samples were aliquot into four different straws likewise, equal volume of semen extender were added to their respective straw, named as (E1, E2, E3, E4). All the aliquots (for each sample) were preserved with liquid nitrogen at -196℃ (cryopreservation) for three months from the date of preservation. At the end of three months of preservation, all the aliquots were thawed and semen analysis was carried out four major semen parameters including sperm concentration, total motility, progressive motility and normal morphology. These parameters were evaluated by using CASA system at BACC, Bangalore. The results for the different parameters were compared with the fresh sample analysis
3.2. FRACTIONATION OF HUMAN SEMEN SAMPLE

For this experiment, 137 semen samples were collected in a sterile semen sample collection vial and centrifuged. Initially, the collected samples were subjected to centrifugation at 1500 rpm for 10 min at 4°C. The supernatant (plasma) was transferred to another tube; remaining pellet was stored at -22°C until use for biochemical analysis. The supernatant (plasma) was again subjected to centrifugation at 10000 rpm for 20 min at 4°C. Subsequent to centrifugation, the plasma was transferred to the fresh tube, and the pellet was said to be the material precipitating at 10000 rpm, perhaps it contains only debris, stored in -22°C until use. The plasma was yet again subjected to centrifugation at 35,000 rpm for 3.5 h at -22°C to convalesce prostasomes. The spermatozoa free plasma is well known human seminal plasma, and was stored in -22°C in anticipation of further use. Then there exits four different fractions of human semen regimented as spermatozoa, material precipitating at 10000 rpm, prostasomes and seminal plasma. Spermatozoa and prostasomes were subjected to ultrasonication with phosphate buffer at pH 7.2. Ultimately, the concentrations of protein for each fraction (for all the samples) were assessed with the protocol (Lowry et al., 1951).

3.2.1. SPERM CELL DISRUPTION

After separating spermatozoa from semen samples, the sperm pellet suspensions were supplied with buffers that containing different detergents. 0.5% of nonidet P-40 (NP-40), 0.3% of Tween 20 and 0.1% of Tween 80, 50 mmol/l of N-octyl-β-D- glycopyronoside (NOG), 10 mol/l of urea, 0.9% of triton X-100 and 1.5% of SDS were added in 100 ml of sterile water. 1 ml of this buffer was added to all the sperm pelleted samples. The samples were then ultrasonicated for disruption. The cells were lysed with this buffer for 3 min in an ultrasonicator with parameters were 5 sec pulse, at a frequency of 40 Hz. After sonication was over the samples were kept for centrifugation at 8000 rpm for 10 min. Lowry Protocol (Lowry et al., 1951) was then followed to quantify the protein present in the sperm pellet for all the samples.
3.2.2. PRECIPITATE MATERIAL DISRUPTION:

This particular fraction was easily disrupted by using phosphate buffer at pH 7.4. The pellet was dissolved with 1 ml of phosphate buffer and mixed well. After 10 min of incubation in ice, the samples were vortexed for 2 min. The samples were then centrifuged at 3500 rpm for 8 min. The supernatant was then transferred to another test tube to estimate the protein present in the debris (precipitate at 10000 rpm fraction) by Lowry method (Lowry et al., 1951).

3.2.3. PROSTASOMES PELLET DISRUPTION

This fraction was too sensitive to heat and disruption. For disrupting this particular fraction, the pellet was mixed with 1 ml of buffer containing 0.9% of triton X-100 and 1.5% of SDS in 100 ml. Ultrasonication was done for all the prostasomes pelleted samples for 1 min only. The samples were then centrifuged at 3000 rpm for 6 min and then the supernatant were carefully transferred to another test tube to carry out the quantification of protein in prostasomes by Lowry method (Lowry et al., 1951).

3.2.4. QUANTIFICATION OF PROTEIN BY LOWRY METHOD

The Lowry assay (protein mixture with Folin reaction) yields the blue colour present in the sample (Lowry et al., 1951).

**Principle:** The principle of Lowry’s method was first the samples were first pre-treated with the alkaline solution which consists of copper ions; later the aromatic acids present in the treated samples reduce the phosphomolybdate to phosphotungstic acid in the presence of the Folin reagent. The substrate and reagent mixed and yield a blue colour which was absorbed at 660 nm.

**Solution Preparation:** Alkaline solution (Solution A): 2.98 gms of sodium hydroxide and 14.73 gms of sodium carbonate was mixed with 500 ml of sterile water. Copper solution (Solution B): 1.45 gms of copper sulphate was mixed well with 100 ml of sterile water. This solution was mixed well and kept in 4°C until the assay start. Tartarate (Solution C): 2.95 gms of sodium potassium tartarate was mixed in 100 ml of sterile water. For the preparation of Lowry reagent, solutions A, B, and C were mixed with the ratio of 100:1:1 (volume/volume). This solution was prepared freshly before use. Folin Reagent: For the preparation of 1 N solution of
Folin and Ciocalteu’s phenol reagent was added with 6 ml of sterile water. This particular solution was very light sensitive solution and it should be prepared during the final incubation of all the samples. This solution should be kept in an amber container.

**Procedure**: Bovine serum albumin (BSA) was taken as a standard (1 mg/ml of stock solution). Human semen samples were separated into four main fractions by centrifugation and were kept in -20°C until the start of assay. The samples were taken in an order like seminal plasma set, spermatozoa set, precipitate set and finally the prostasomes set, standard set and blank. 100 μl from each samples for each set were taken and the work was carried out with triplicate. Lowry reagent was prepared according to the below mentioned ratio and from that 0.7 ml of reagent was added to all the samples serially. The samples were sealed and allowed for incubation in dark for 30 min at RT. During the last time of incubation Folin reagent was prepared. 0.2 ml of diluted Folin phenol reagent was added and mixed well and then incubated for 30 min at RT. After incubation was over the samples were vortexed and mixed well. 200 μl of samples were transferred to 96 ELISA plate and mixed well. Immediately after transferring the samples were taken absorbance at 650 nm with ELISA reader. The amount of protein in the each sample was directly proportional to the intensity of the blue colour of each sample. Finally the concentration of each sample from each set was calculated for 1 ml solution. The concentration was expressed as mg/ml of protein.

**3.3. EVALUATION OF HUMAN SEMINAL PLASMA FRUCTOSE CONCENTRATION**

For this experiment, 120 semen samples were collected in a collection vial. The population of each category was found to be (after evaluating the parameters based on the standardized protocol of WHO (2010)). In this experiment, the number of semen samples under each category are oligospermia (n=21), oligoasthenospermia (n=28), asthenospermia (n=20), azoospermia (n=12), normospermia (n=28) and control (n=11). All the semen samples were subjected to centrifugation at 1500 rpm and – 4°C for 10 min. The spermatozoa free seminal plasma was lay up in a fresh tube and preserved in -22°C until use to estimate seminal fructose concentration.
The protocol was followed by the WHO, 2010 with minor modifications. First the seminal plasma samples were diluted with purified, sterile water (to 50 µl, 5 µl of seminal plasma) was added and mixed. 12.5 µl of deproteinizing agent (67 µmol/l Zn So₄) was added, assorted with a vortex mixer. Additionally 12.5 µl of deproteinizing agent 2 (0.5 mol/l Na OH) was added. The samples were then allowed to stand in at RT for 20 min. Subsequently, the samples were centrifuged at 9500 rpm for 7 min. 50 µl of sample was transferred and 50 µl freshly prepared indole reagent was added and assorted. Afterwards, 500 µl of H Cl (32 % v/v, concentrated) was added. The samples were prone to heat at 55°C for 15 min in a water bath, subsequently cooled with ice for 10 min. Only 200 µl of the total sample was transferred to the 96 well plate, absorbance was read at 490 nm wavelength with the help of ELISA reader.

3.3.1. EVALUATION OF HUMAN SEMINAL PLASMA NEUTRAL α-GLUCOSIDASE (PAQUIN et al., 1984)

Two types of glucosidase isoenzyme were present in human seminal plasma, the neutral and acid α-glucosidase, which originates from epididymis and prostate respectively. The acid α-glucosidase was inhibited selectively by SDS (Paquin et al., 1984). This will be very much helpful in measuring only the concentration of neutral α-glucosidase, which directly impacts the functions of epididymis. Castanospermine was used to restrain the breakdown of non-glucosidase interrelated substrate there in the seminal plasma.

**Principle:** Glucosidase in any form will be converted into the synthetic glucopyranoside (substrate) to product called para nitrophenol (PNP).

All the semen samples were subjected to centrifugation at 1500 rpm and −4°C for 10 min. The spermatozoa free seminal plasma was lay up in a fresh tube and preserved in −22°C until use to estimate seminal neutral α-glucosidase concentration. 25 µl of seminal plasma sample was added to test tube, diluted with 75 µl of pure and sterile water. 10 µl of 5 mmol/l of castanospermine was added, to inhibit the acid α-glucosidase. 50 µl of PNPG (p-nitrophenol glucopyranoside) substrate was added and mixed well through vortex mixer. All the samples were subjected to incubation at 37°C for accurately 2 h. The reaction was clogged by adding 1 ml of 0.25 mol/l of sodium carbonate with 0.1 g of SDS. For acquiring standard curve, 5 mmol/l of PNP
(70 mg of PNP in 100 ml of sterile water) was prepared. 250 µl of sample was finally transferred into the 96 well plates and the assay was read at the wavelength of 400 nm.

3.3.2. EVALUATION OF TRACE ELEMENTS IN SEMINAL FLUID

Initially, semen samples were centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was transferred to another Eppendorf tube and centrifuged at 1500 rpm for 10 min to completely remove any debris or spermatozoa. Subsequently, the samples were taken for the estimation of trace elements like Zn, Mg, Ca, and Na using atomic absorption spectroscopy. Standards used for the analysis were purchased from Hi-Media, Mumbai, India. ZnCl$_2$ (H$_2$O)$_4$ at 1–25 mg/100 ml, MgCl (H$_2$O)$_4$ at 1–15 mg/100 ml, Ca$_2$ Cl (H$_2$O)$_2$ at 5–25 mg/100 ml, NaCl (H$_2$O)$_4$ at 90–180 mg/100 ml, and the standard curve were plotted. The seminal plasma was diluted 10 times using Millipore distilled water. Trace element concentrations were estimated using the standard curve.

3.4. ANTIOXIDANTS ASSAY

For this experiment, 116 samples were collected from the outpatients who attended the Andrology lab of BACC, for semen analysis. For comparing the semen quality with seminal antioxidant markers, the samples collected were categorized by the number (sperm count in millions/ml). The different categories by evaluating the sperm count were cut off with 0 to >100 millions/ml, 0-20 (n=20), 20-40 (n=18), 40-60 (n=12), 60-80 (n=18), 80-100 (n=32), >100 (n=16).

All the semen samples were subjected to centrifugation at 1500 rpm and –4 °C for 10 min. The spermatozoa free seminal plasma was lay up in a fresh tube and preserved in -22 °C until further assay of all antioxidant markers.

3.4.1. SUPEROXIDE DISMUTASE (SOD) ASSAY (MARKLUND AND MARKLUND, 1974).

SOD assay was carried out with the protocol (Marklund and Marklund, 1974). The principle of this assay is based on the capability of the SOD to inhibit the autooxidant of the standard pyrogallol. 200 µl of seminal plasma sample was mixed with 3 ml of phosphate buffer (0.25 M with pH 8.4). The standard pyrogallol was purchased
and 9 mM was prepared for this sensitive assay. With the 200 µl of sample, 100 µl of pyrogallol was added and was made miscellaneous. Instantaneously, the absorbance for all these solution mixture was taken (recorded) for 5 min at the wavelength of 450 nm. For the standard curve, auto-oxidation of pyrogallol was deliberated by adding 3 ml of phosphate buffer (pH 8.4) and 100 µl of standard pyrogallol. The readings were documented for 3 min at 450 nm. The early absorbance (A₁) at Zero th min and the absolute (final) absorbance (A₂) at third min were deliberated for both the standard and the samples. The activity of SOD was deliberated by using the calibration curve (percentage of inhibition of each standard against log₁₀). Ultimately, the activity of SOD was measured and expressed as U/ml.

3.4.2. CATALASE ASSAY (AEBI, 1984)

Catalse assay was conceded with the standardized protocol (Aebi, 1984), with slight adaption according to our lab conditions (which was standardized with almost 10 test samples). 2 ml of 110 mM phosphate buffer (pH 7.0) was prepared only just each time previous to starting the assay. 1 ml of H₂O₂ (50 mm) was taken and mixed well with the phosphate buffer. 50 µl of seminal plasma was mixed with the solution and stirred it well. The decomposition of H₂O₂ was pursued frankly by the decline in extinction at 240 nm which was recorded after every 50 s for 5 min. The divergence in disappearance per unit time was quantified as the catalase activity. Catalase activity (one unit) was defined as the amount of catalase obligatory to decompose 1 M H₂O₂ per 1 min. Finally, catalase activity was expressed in specific activity, U/ml.

3.4.3. GLUTATHIONE ASSAY

Glutathione assay was conceded with the standardized protocol (Aebi, 1984). Volume of 50 µl of trichloro-acetic acid (22%) was very well mixed with 5 ml of phosphate buffer (0.5 M). 1000 µl of DTNB (5-5 dithio bis 2-nitrobenzoic acid, 0.1% w/v) was added and made miscellaneous. 200 µl of plasma (seminal) was further added to the tube. A chain of known concentrations of glutathione were prepared and the standard curve was developed at 420 nm with ELISA plate. Finally, the blank was prepared by adding TCA, DTNB, and phosphate buffer and made prepared. The readings were recorded at 420 nm for all the test as well as standard samples.
3.4.4. LIPID PEROXIDATION ASSAY (BUEGE AND AUST, 1978)

Seminal plasma lipid peroxide level was monitored using TBARS (thiobarbituric acid reactive substances assay), according to the procedure Buege and Aust (1978) with some modifications which standardized in our laboratory. This assay was standardized with monitoring the malonaldehyde production. On the account to start the assay, 100 µl of seminal plasma was added to a fresh tube, 100 µl of (10% SDS) was added to it. 4 ml of coloring reagent solution which was prepared by adding, 530 mg of TBA, 100 ml of acetic acid, and 50 ml of Na OH. All the samples were kept in 100°C in boiling water for 1 h. Following that, instantly the samples were subjected to incubation in ice for more than 10 min. All the samples were centrifuged at 4500 rpm for 10 min at 4°C. After centrifugation, 250 µl of the sample were transferred to 96 well plates and were made ready for recoding the readings. In the intervening time, standard malonaldehyde were prepared in different concentrations ranging from 0-5 µM/ml. The standard cure was built with different concentrations by the same protocol followed for the seminal plasma samples. At last, the reading was recorded at 560 nm with the assistance of ELISA reader.

3.5. IDENTIFICATION OF FERTILITY ASSOCIATED PROTEINS IN SEMINAL PLASMA

For this experiment, 30 samples were collected from Andrology department, BACC, Bangalore, Karnataka, . The major parameters considered for analysis were total volume ejaculated in ml, pH of the semen, sperm concentration in millions/ml, total motility in percentage, total progressive motility in %, total morphologically normal sperm in %.

Remaining semen samples after semen analysis were kept n -196 °C in a liquid nitrogen cryocan for three days. After that, the samples were kept in a cryo box and transferred to VIT University with dry ice. Immediately the semen samples were prepared for elucidating the protein profile by SDS-PAGE.
3.5.1. ACQUISITION OF SEMINAL PLASMA

All the semen samples were centrifuged at 3000 rpm for 10 min at 4 °C to get the seminal plasma. The plasma was transferred to the other tube. These particular sets were again centrifuged to get the pure seminal plasma that does not contain any spermatozoa or cell debris.

3.5.2. SAMPLE PROCESSING FOR SDS-PAGE

Before stating the protocol for SDS-PAGE, gel loading dye has to be prepared. **Gel loading dye preparation**: 2.5 ml of freshly prepared tris buffer with pH 6.8 (altering the pH with 0.1 N HCl solutions) was added to a beaker. Alongside 2 ml of concentrated glycerol with 100 μl of β-mecracaptomethanol was added. Finally 0.1 mg of bromophenol blue (an indicator dye) was added to the beaker. The total volume was makeup to 10 ml with the help of sterile milliq water. Before adding SDS, the solution was mixed well and kept in ice for 10 min. Later 0.25 g of SDS was added without disturbing the solution. SDS was automatically dissolved in the presence of ice.

3.5.2.1. Separating gel preparation

Before starting to caste the gel, the plates were cleaned well with the detergent SDS and then rinsed several times with water. The plates were then cleaned with 100% ethanol, later with 70% ethanol. The plates were sealed with 0.8% agarose. Separating gel beaker was rinsed and cleaned well and kept ready for making separating gel solution. 2.5 ml of acrylamide and bis-acrylamide (29:1) was added in separating gel beaker. Tris buffer (1.8 ml) with pH 8.8 was added to it and then 3 ml of distilled water was added and mixed well without making any air bubbles in the solution. Later 60 μl of freshly prepared 10 % of APS (Ammonium per sulphate) was added, finally 25 μl TEMED was added and instantly poured into the plates without bubbles. The set was kept undisturbed for more than 30 min.

3.5.2.2. Stacking gel preparation

After 30 min of waiting for the gel to solidify, meanwhile staking solution was prepared. 0.6 ml of 29:1 acrylamide and bisacrylamide was added with 1.2 ml of tris buffer 6.8 pH. 3 ml of sterile water was added and mixed well without making
bubbles. Later 35 μl freshly prepared 10% of APS and 25 μl of TEMED was added and instantly poured on the top of the separating gel and instantly the comb was fixed. The setup was remains undisturbed for 30 min.

3.5.2.3. Sample preparation

Different categories of semen samples were marked and centrifuged during the process of SDS PAGE. The centrifugation was done at 1000 rpm for 10 min at 4 °C. From that only 10 μl of seminal plasma was transferred and equal volume of gel loading dye was added and mixed well. The standard marker varies from 29 kD to 205 kD was also prepared as like as samples. All the samples were mixed and boiled at 100 °C for 3 min exactly. The samples were then kept in ice until loading.

3.5.2.4. Sample loading and running

Gel was kept in a tank containing running buffer (glysine, tris and SDS). The comb was carefully removed and then the various samples were added from well to well including standard marker. After loading the setup was allowed to run for 30 min at 50 V, then the voltage was increased to 100 V and kept for running until the indicator touches the buffer solution.

3.5.2.5. Staining the gel

After running time was over, the gel was carefully removed from the gel plates and then kept in a sterile box with vast surface area. Then the gel was stained with silver staining protocol followed by the steps which explained in the table 5.1. After staining was over the gel was photographed and visualized.

3.5.2.6. Coomassie brilliant blue staining

For Coomassie brilliant blue staining, the gel was stained with 13 ml of acetic acid, 07 ml of methanol and 80 ml of distilled water and 50 mg of CBB R-250 for overnight. The next day the gel was destained with 13 ml of acetic acid, 10 ml of methanol and the volume was making up to 100 ml with the distilled water. The bands was visualized and photographed.
### Table 3.5.1: Silver staining protocol

<table>
<thead>
<tr>
<th>Steps</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>60 ml of 50% acetone, 1.5 ml of 50%TCA, 25 ml of 3% CH₂O.</td>
<td>4 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>DD.H₂O</td>
<td>3X5 S</td>
</tr>
<tr>
<td>Wash</td>
<td>DD.H₂O</td>
<td>5 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>DD.H₂O</td>
<td>3X5 S</td>
</tr>
<tr>
<td>Pre-treat</td>
<td>60 ml of 50% acetone</td>
<td>5 min</td>
</tr>
<tr>
<td>Pre-treat</td>
<td>100 ml of 10% Na₂S₂O₃ with 60 ml of dd.H₂O</td>
<td>30 S</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.8 ml of 20% silver nitrate, 9 0.6 ml of 3% CH₂O, 60 ml of dd.H₂O</td>
<td>6 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>DD.H₂O</td>
<td>2X5 S</td>
</tr>
<tr>
<td>Development</td>
<td>1.2 g of Na₂CO₃, 25 µl of CH₂O, 25 ml of 10% Na₂S₂O₃ in 150 ml of dd.H₂O</td>
<td>10-20 S</td>
</tr>
<tr>
<td>Storage</td>
<td>1 % glacial acetic acid</td>
<td></td>
</tr>
</tbody>
</table>
3.6. ARTIFICIAL NEURAL NETWORKS

3.6.1. BACK PROPAGATION NEURAL NETWORK (BPNN)

Back-propagation networks were poised of layers of neurons. Each layer consists of neurons. The input and output layer were connected. The BPNN consists of an input layer, one or two hidden layers, and an output layer. A schematic diagram of a BPNN with \( n \) input nodes, \( r \) output nodes and a single hidden layer of \( m \) nodes were shown in Fig 6.1. All the connections have multiplying weights associated with them. Therefore, the output \( o_k \) can be expressed as:

\[
  o_k(x) = \sum_{j=1}^{m} w_{2kj} f \left( \sum_{i=1}^{n} W_{1ij} x_i + b_{1j} \right) + b_{2k}, \tag{1}
\]

where function \( f \) is the transfer function or activation function, \( W_{1ij} \) is the weight between the \( i \)th input node and \( j \)th hidden, \( W_{2kj} \) is the weight between the \( j \)th hidden node and \( k \)th output node, \( b_{1j} \) is the bias at \( j \)th hidden node, and \( b_{2k} \) is the bias at \( k \)th output node.

In Equation (1), function \( f \) is a kind of mapping rule to convert neuron weighted input to output and also a kind of design to commence non-linear influence into the BPNN. The sigmoid function is used to process the network. Our method is based on the same protocol of the current sigmoid function shown as

\[
  f(x) = \frac{1}{1 + e^{-x}} \tag{2}
\]

In BPNN, each unit within the same layer is not connected to one another, and the connection between the adjacent layers is expressed based on the weighted coefficient. Then the difference between the expected output and actual output is used as adjusting parameter for weighted coefficient. The process is repeated to the minimum of such difference. The optimum parameter is obtained through adjusting the weighted values in the neural network, and optimum means that the squared deviation between the network output \( o_k \)'s and the actual values as target values \( t_k \)'s achieve a minimum, i.e.,
Figure 3.6.1. Back Propagation neural network model (BPNN)

The whole process is continued for each of the study cases, then back to the first case again, and so on. The cycle is repeated until the overall error value drops below some predetermined threshold. At this point, we say that the network has learnt the problem “well enough” - the network will never exactly learn the ideal function, but rather it will asymptotically approach the ideal function.

3.6.2. RADIAL BASIS FUNCTION NETWORK (RBFN)

RBFN is a three layer feed-forward network that contains of one input layer, one middle layer and one output layer as shown in the model. First layer is input layer for inputting data and is acting as the node for connecting to the network. Second layer is a singular hidden layer that is different from the hidden layer of BPNN which has multiple layers.

The input nodes pass to incoming input vector to the hidden nodes. The connection between the hidden nodes and the input nodes are not weighted. But, the connection between hidden nodes and output nodes are weighted. Hidden layer is

\[
E = \frac{1}{2} \sum_{k=1}^{l} (o_k - t_k)^2
\]  

(3)
acting as an input space for non-linear mapping to hidden space. Third layer is an output layer for linear mapping to obtain output value. A radial function is real valued function whose value depends only on the distance from the origin. Their characteristics feature is that their response decreases (or increases) monotonically with distance from central point.

A Gaussian RBFN monotonically decreases with distance from the centre. In contrast, a multi-quadric RBFN which, in the case of scalar input monotonically increases with distance from the centre. Gaussian-like RBFNs are local (give a significant response only in a neighborhood near the centre) and are more commonly used than multiquadric-type RBFNs, which have a global response. The Gaussian basis function $\Phi(\cdot)$ is used for the hidden units. Each hidden unit’s output is obtained by calculating the closeness of the input $x$ to an $n$ dimensional parameter vector $\mu_j$ associated with the $j^{th}$ hidden units.

Referring to Fig. 6.2, the net input to the $j^{th}$ radial basis neuron is given by

$$y_j(x) = b_1^j \|x - \mu_j\|$$  \hspace{1cm} (1)

Where the bias $b_1^j$ is a fixed function of the width of the receptive field $\sigma_j$ that follows the sensitivity of the $j^{th}$ radial basis neuron to be adjusted and is described below,

$$b_1^j = \sqrt{-\log(0.5)}/\sigma_j$$  \hspace{1cm} (2)

The output of the $j^{th}$ radial basis neuron is described as:

$$z_j(x) = \exp(-y_j(x)^2)$$  \hspace{1cm} (3)

Given an input vector $x$, the output, value $o_k(x)$ of the $k^{th}$ output node is equal to the sum of the weighted outputs of the hidden nodes and the bias of the $k^{th}$ output node and is described by.

$$o_k(x) = \sum_{j=1}^{m} w_{jk} z_j + b_{2k}$$  \hspace{1cm} (4)
Where $w_{kj}$ is the weight between the $j^{th}$ hidden node and $k^{th}$ output node.

It may be noted that here that the choice of $\Phi(\cdot)$ and $\mu_j$ be made carefully so that the RBFNs be able to match closely to the performance of the two-layer back propagation neural networks. The RBFNs employ a hybrid two-stage training scheme which decouples the learning task from both hidden and outputs layers and thus eliminates the need for slow back error propagation. In the training process, the sum of the mean-squared error (MSE) criterion function is considered as the error function, and it is minimized over the given training data sets by adaptively updating the free parameters of the RBFN. These parameters are the RBF centers $\left(\mu_j, s\right)$ their widths $\left(\sigma_j, s\right)$ and the second layer weights $\left(w_{kj}, s\right)$. The RBFN is trained in three steps. Firstly, the hidden node (RBFN) centers are determined, secondly hidden widths were determined, and thirdly the second layer connection weights were determined.

**Figure 3.6.2. Radial neural basis function network model (RBFN)**

In this section, widths of all Radial basis function units were taken as to be equal, which is known as the spread factor (SF) of the RBFN. If SF is too small, over fitting can occur, while under fitting may occur if SF were too large. Therefore, it is of utmost importance to choose a proper value for SF in order to achieve better
generalization ability of the RBFN. Finally, once the hidden units are synthesized, the second layer weights are computed by using the supervised least-square rule.

3.6.3. SIMPLE RECURRENT NEURAL NETWORK (SRNN)

Simple recurrent neural network follows the same algorithm as BPNN algorithm for which the procedure and theory as mentioned above. The only change with SRNN is having one extra layer called simple recurrent layer which has been shown in model. In this algorithm, first the input nodes were connected the hidden layer. Again the hidden layer was connected to an extra layer called context layer or recurrent layer, where it got again weight and connected to hidden layer through another route as clearly shown in model.

Figure 3.6.3. Simple recurrent neural network model (SRNN)
3.7. COMPOSITION OF REAGENTS AND SOLUTION

Sperm cell disruption detergent preparation:

0.5% of nonidet P-40 was prepared by dissolving 0.5 g of NP-40 in 100 ml of sterile distilled water.

50 mmol/l of N-octyl-β-D- glyccopyronoside was prepared by dissolving 0.1921 g of N-octyl-β-D- glyccopyronoside in 100 of sterile dissteled water.

100 mM/l of urea was prepared by dissolving 0.6006 g of urea in 100 ml of sterile distilled water.

0.9% of triton X-100 was prepared by dissolving 0.72 ml of triton X-100 in 100 ml of sterile distilled water.

1.5% of SDS was prepared by dissolving 1.5 g of SDS in 100 ml of sterile distilled water.

Lowry Reagent Preparation:

Alkaline solution (Solution A): 2.98 g of sodium hydroxide and 14.73 g of sodium carbonate was mixed with 500 ml of sterile water.

Copper solution (Solution B): 1.45 g of copper sulphate was mixed well with 100 ml of sterile water.

Tartarate (Solution C): 2.95 g of sodium potassium tartarate was mixed in 100 ml of sterile water.

Lowry Reagent: Solutions A, B, and C were mixed with the ratio of 100:1:1 (volume/volume).

Folin Reagent: For the preparation of 1 N solution of Folin and Ciocalteu’s phenol reagent was added with 6 ml of sterile water.

Fructose estimation reagents:

Deproteinizing agent 1: 67 µmol/l ZnSO₄ was prepared by dissolving 0.1614 g of ZnSO₄ in 100 ml of sterile distilled water.
Deproteinizing agent 2: 0.5 mol/l of NaOH was prepared by dissolving 2.0 g of NaOH in 100 ml of sterile distilled water.

**Glucosidase estimation reagents:**

5 mmol/l of castanospermine was prepared by dissolving 0.0946 g of castanospermine in 100 ml of sterile distilled water.

0.25 mol/l of sodium carbonate was prepared by dissolving 2.6497 g of 0.25 mol/l of sodium carbonate in 100 ml of sterile distilled water.

**SOD assay:**

0.25 M phosphate buffer was prepared by dissolving 0.0978 g of monosodium phosphate, monohydrate and 6.5101 g of disodium phosphate, heptahydrate in 100 ml of sterile distilled water. The pH was adjusted with 0.1 M of NaOH for 8.4.

9 mM of pyrogallol was prepared by dissolving 0.126 g of pyrogallol in 100 ml of sterile distilled water.

**Catalase assay:**

110 mM phosphate buffer was prepared by dissolving 0.642 g of monosodium phosphate monohydrate and 1.7013 g of disodium phosphate heptahydrate was dissolved in 100 ml of sterile distilled water. The pH was adjusted to 7.0 by using 0.1 N of HCl.

50 mM of H₂O₂ was prepared by dissolving 0.7 ml of H₂O₂ in 100 ml of distilled water.

**Glutathione assay:**

22% of trichloro acetic acid was prepared by dissolving 22 g of trichloro acetic acid in 100 ml of sterile distilled water.

500 mM phosphate buffer was prepared by dissolving 1.5584 g of monosodium phosphate monohydrate and 10.3736 g of disodium phosphate heptahydrate was dissolved in 100 ml of sterile distilled water. The pH was adjusted to 7.4 by using 0.1 N of HCl.
0.1% of DTNB was prepared by dissolving 0.1 g of DTNB in 100 ml of distilled water.

**SDS-PAGE:**

30% of Acrylamide solution was prepared by adding 29 g of acrylamide and 1 g of N, N methyl, bis-acrylamide in 100 ml of sterile distilled water.

4 X Tris SDS buffer (pH 6.8): 6.05 g of tris base was dissolved in 100 ml of distilled water. The pH was adjusted to 6.8 with 0.1 N HCl. 0.4 g of SDS was added finally and stored in 4 °C.

4X Tris SDS buffer (pH 8.8): 18.2 g of tris base was dissolved in 100 ml of distilled water. The pH was adjusted to 8.8 with 0.1 N HCl. 0.4 g of SDS was added finally and stored in 4 °C.

1% APS was prepared by dissolving 100mg of APS in 1 ml of distilled water and made every time freshly.

5X SDS tank buffer: 15.1 g of tris, 72 g of glycine and 5.0 g of SDS was added and dissolved in 1 litre of sterile distilled water.

**SDS gel loading dye:**

2.5 ml of 4X tris buffer pH 6.8, 2 ml of glycerol, 0.4 g of SDS, 0.2 ml of 2-mercapto-ethanol and 0.1 mg of bromophenol blue was added in 10 ml of sterile distilled water.