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COMPLEXIN GENE AND MALE INFERTILITY

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Abstract:
Infertility is the failure to have offspring after one year of regular unprotected intercourse. Acrosomal exocytosis is a prerequisite for successful fertilization and it involves numerous proteins. Complexin genes play an essential role in a controlled secretion of acrosome content. The objective of the present study was to determine the expression of the complexin gene in healthy and infertile male spermatozoa by using Real-Time Polymerase Chain Reaction. The demographic parameters like age, semen volume, sperm count, motility and immotile sperm were analyzed. There was no significant difference in the age and semen volume between normal and infertile groups. A significant difference was observed when the sperm count ($p< 0.0000008$) and motility ($p<0.00000001$) were compared between the groups. RT-PCR data analysis showed a fold change difference of 0.0433. From this fold change difference, we determine that our gene of interest is downregulated and the student's t-test was also applied which also showed that there is downregulation of the complexin gene in infertile men. The difference observed in analyzed data indicates that there is a significant role of the complexin gene in male infertility and their expression was inversely proportional to each other in normal and infertile males and complexin is surely playing a major role in male infertility. However, it is highly recommended that future studies investigate the cause of the down-regulation of the complexin gene as it plays an important role in the exocytosis of sperm nuclei.

Keywords: Complexin, Expression, Male Infertility, RT-PCR

1. Introduction:

Male infertility is the incapability of men to cause pregnancy in a fertile female. A thorough reproductive history is the major component for the initial evaluation of the infertile male and should cover frequency of sexual intercourse, infertility duration fertility history childhood disorders including hypospadias, congenital anomalies, the onset of puberty and many other causes [1]. Semen analysis should be performed after 1 to 5 days of abstinence and be spaced at least 7 days apart. Semen analysis is a key step in the determination of male infertility which may be grouped into low

semen volume, oligoasthenoteratospermia, or azoospermia. Data for 42,935 men with samples ranged up to 40 years and had a significant reduction in the number of sperm cells in men in North America, Europe and Australia by 50-60%. The latest discovery caused even more concern about the apparent decline in sperm count in Western men [2]. Male infertility is divided into the following three major groups; i) non Obstructive: it is responsible for almost 60% of cases of total infertility and in this case sperm amount is not sufficient, ii) obstructive: it is responsible for 38% of cases of infertility and sperm production is normal sperm but there will be a blockage somewhere in the genital tract, iii) coital: It is responsible for only 2% of male infertility [3].

As there are very wide causes of male infertility that is the reason the overall prevalence of male fertility and sub-fertility is difficult to determine. The major reason is the lack of population-based studies regarding male infertility. According to NICE guidelines (NICE CG no 156), the overall cases of infertility include almost 30-50% of cases that include the male factor. Low sperm count causes 20% of infertility, but still, many factors of male infertility are unclear [4]. In addition, if a couple is faced with male or female risk factors, they can be assessed before attempting for evaluation. Moreover, other causes include varicocele, endocrinal disorder, ejaculatory disorders, lifestyle, occupational exposure, immunological disorders, genetic causes of male infertility, obesity, sperm capacitation, and acrosomal reactions [5].

An acrosomal reaction is a calcium-dependent complex reaction leading to an exocytic event necessary for the successful fertilization of an egg cell. It was believed that the acrosomal reaction occurred when the sperm was associated with zona pellucida, a viscoelastic layer surrounding the oocyte. Recent studies have shown that the reaction may occur even before the discovery of sperm, possibly mediated by progesterone or another agonist [6]. The interaction of the egg cell and the sperm cell is a specific event of a carbohydrate-mediated species that initiates a cascade of signal transduction leading to the exocytosis of the acrosome spermatozoon content i.e. the acrosomal reaction. This step is considered a necessary condition for the penetration of sperm into the pellucida (ZP) zone and fertilization of the egg. Successful fertilization in mice and many other species, including humans, involves several successive phases. This is (i) sperm capacity in the female genital tract; (ii) the binding of capacitive sperm with the extracellular membrane of the egg, ZP; (iii) induction of the acrosomal reaction (i.e. activation of spermatozoa); (iv) penetration into salary; and (v) melting of the sperm with the yolk membrane [7].

Complexins known as synaphins are small synaptic vesicular proteins that make a small protein family consisting of four members; I–IV but here will focus only on CPLX1 and CPLX2 [8]. For the first time, these were determined as protein co-helper of the SNARE complex. Their name is based on their nature because they bind with the SNARE complex. They are composed of 134 amino acids. Both CPLX1 and CPLX2 have high negative charges. The amino acids homology present in CPLX1 and CPLX2 is almost 84%. This homology is almost high for complexin orthologs in different species. CPLX2 homology is almost 100% in mouse, rat and human. Both Complexins genes have specific regions which help in attaching with the SNARE complex and they are similar in this region but this similarity is limited to some species or one of the two subfamilies [9, 10].

A nuclear magnetic resonance study of the complexin gene has proven that there is no tertiary structure in the Complexin protein except a stable α helix at the N-terminus of the central region that consists of amino acid residues from 29-6 [11]. When the second half of this helix binds to the SNARE complex then it induces the elongation of α helix toward the C-terminal half of the central region resulting in complete interaction between SNARE complexes. This binding does not cause any salient structural changes in the SNARE protein complex. There are hydrophobic as well as ionic interactions and hydrogen bonding in central regions which mediate interaction between complexin and SNARE complex [12, 13].

Complexin's function is well studied in neuronal transmission release at nerve endings when synaptic vesicle fusion takes place. CPLX1 and CPLX2 are abundantly presented in the brain but with much less in the retina. Neurotransmitter discharge from nerve endings may occur either in response to an action potential or by sudden fusion. The role of CPLX2 binds the SNARE complex and inhibits spontaneous exocytosis by acting as a fusion clamp. Complexin inhibits SNARE function and

neurotransmitters are released when an action potential reaches the synaptic junction [14]. To understand the mechanism of this inhibition, we identified a complex structure associated with the SNARE mimetic primer without the V-SNARE part, which the hinges hold last to cause fusion. The "core of the helix" of the complex is anchored in the SNARE complex, while its "auxiliary helix" stretches to about 45°C and connects with the second complex, occupying the vacant v-SNARE binding site to inhibit fusion. v-SNARE for the t-SNARE bond, but we found that the interaction occurs intermolecularly [15].

Acrosomal exocytosis in spermatozoa and synaptic vesicle exocytosis share many similarities, like intracellular calcium and SNARE complex formation. The state of health in the sperm and the testicle was confirmed by an affinity-purified antibody that detected both proteins in the testicles and sperm. It was adapted for a specific local CPLX2, which binds to amino acid residue 41-91 of the complex and is also found in both CPLX1 and CPLX2 [16]. These results show that CPLX1 and CPLX2 are present in semen but less than in the brain. Spermatozoa with CPLX1 deficiency also respond to calcium signals but are not able to achieve acrosome exocytosis and have a reduced in vitro fertilization capacity. Complexity II is present in I-deficient spermatozoa, and its expression is increased in defective testicular complexes. Consequently, the complexin I functions during exocytosis in two different, but morphologically similar. The main objective of this study was to check the relative expression of the CPLX II gene in normal and infertile males as this gene plays an important role in acrosomal exocytosis during female egg fertilization [17, 18].

2. Methodology

This research was approved by the Ethical Committee of COMSATS University, Islamabad. After that, we collected samples of 200 people (infertility and control). A complete report on sperm analysis was provided by the National Institutes of Health (NIH) Reproductive Physiology Laboratory and the Australian Concept Infertility Medical Center (Islamabad). Patients selected for expression analysis were 20-50 years old and were of the same gender (male). Nearly half of the patients were diagnosed with infertility and the same number of patients belonged to the control group.

Semen samples were collected in sterile semen containers and collected for liquefaction, they were kept in a 37 ° C incubator for 30 minutes, as it was semi-solid. Some quantity of semen samples were used for diagnosis in the infertility centre and the rest were used for our research purposes. These samples were brought to the COMSATS University Laboratory (Functional Genomics and Proteomics Laboratory) in a thermopile box.

2.1 RNA Isolation and Quantification

RNA extraction is complicated by the presence of some somatic cells in sperm. There are some other reasons as well that sperm contains a very minute amount of RNA in the form of fragments of ribosomal RNA (18S and 28S) and the outer covering of sperm which was found to be a major obstacle during RNA extraction from sperm cells, making RNA dissolution even more difficult. Thus, some useful and effective modifications were made using Trizol® (Invitrogen, USA). The major modification that we made for the extraction of RNA using Trizol® was to prepare glass-treated beads (100 g). In the first step, 100 g glass beads were weighed using an electronic weighing balance and transferred to 500 ml Pyrex bottles. The volume of the bead should be one-fifth the volume of the bottle. The glass beads were then washed with 400 ml of a 5.8 M molar concentration of hydrochloric acid (HCl) (Roth, Germany). Then the beads were incubated at room temperature (25˚C) for 1 hour. Then carefully 5.8 M HCl was transferred to another bottle so that some HCl remained with the beads keeping them immersed. The beads treated with acids were then washed with distilled water, and the vial was stirred for 10 minutes so that the mixing and mixing of the beads with the acid solution went well. The distilled water was discarded from the bottle to the sink and this washing step was repeated 4-5 times. The beads were then treated in an autoclave for 1 hour, then removed and transferred to an oven at 70˚C overnight to dry. The beads were then stored in an aluminium foil-covered glass beaker at room temperature.

2.2 RNA extraction:

We extracted RNA from the male sperm sample of humans using Trizol® reagent (Invitrogen USA), following the manufacturer's instructions with slight modifications i.e. using the acid-treated glass beads. First, the semen sample was divided into two Eppendorf tubes. Tubes were then centrifuged at 12,000 rpm (revolutions per minute), at 4˚C for 10 minutes. This centrifugation step allows the solid residual particles of the sperm sample to accumulate in the form of a pellet at the bottom of the tube and residual filtrate. The supernatant was discarded in a small beaker with the bleach in it to prevent contamination. Because the high temperature can degrade the sperm cells of the sample, the whole RNA extraction process must be done at very low temperatures, so the pellets in the tube are stored in ice packs to keep sperm cells intact. Next, the pellet was resuspended in hypotonic solution and the purpose of the solution is to dissolve all the somatic cells in the semen sample leaving only sperm cells. A hypotonic solution was prepared by adding 500 µl of 5 mM Tris HCl (Merck, Germany), 2.5 ml of 50 mM KCl (Merck, Germany), 125 µl of 2.5 mM MgCl 2 (Merck, Germany) and 2 µl of 4 mM β-mercaptoethanol (Merck, Germany), 0.05g of sodium dodecyl sulphate (SDS) (Roth, Germany) and 500 µl of 0.5% Triton X-100 (Roth, Germany). 30 ml of distilled water was added to this solution, and the volume was diluted up to 50 ml. To each tube containing pellet 1 ml of a hypotonic solution was added and the sample was incubated in ice for 10 minutes. After that, the tubes were vortexed for about one minute so that the pellet dissolves in the solution or we can also use a micropipette carefully to move the pellet back and forth so that it decomposes and dissolves in the solution. The next step was the addition of 2 µl of 10 % SDS to the dissolved pellet in the solution. After this, 550 µl of Trizol® with 2-4 beads treated with HCl were added to the sample solution. After this next step was to separate the different components based on their mass, tubes were placed in a centrifuge at 1500 rpm at 4˚C for 30 minutes. After 30 minutes, the tubes were removed and we added 200 µl of chloroform and tubes were left at room temperature for 3 minutes so that the layers settle down. After 3 minutes, tubes were short vortexed for 10-15 seconds at normal room temperature followed by centrifugation at 12,000 rpm at 4˚C for 10 minutes. This centrifugation was subjected to the separation of the suspension into two separate phases: the upper aqueous phase (water) containing RNA, and the lower organic phase with cell debris, genomic DNA and proteins). The aqueous phase was removed with a micropipette and transferred to four new tubes, and the tubes containing the organic phase were discarded.

2.3 RNA Precipitation:

450 µl of acidic PCI (Phenol-Chloroform Isoamylalcohol) (25:24:1) solution was then added to the tubes containing the aqueous phase and tubes were centrifuged at 12,000 rpm, 4˚C for 10 minutes. As a result, two layers were separated, and the aqueous phase was transferred again into four new tubes followed by the addition of 500 µl of Isopropanol (Merck, Germany) and 2 µl of glycogen (Roth, Germany) to allow precipitation. The samples were then incubated overnight at -20˚C. After 22 hours the incubated samples were again centrifuged at 12,000 rpm at 4˚C for 10 minutes, which caused the formation of a pellet. The supernatant was discarded carefully in a disposal beaker containing bleach to prevent contamination. Next, the pellet was washed by adding 1 ml of 75% ethanol (Merck, Germany) to the tubes. Tubes were centrifuged again at 7500 rpm, 4˚C for 10 minutes. The supernatant was again discarded, and the pellet was air-dried by placing the tubes inverted on tissue paper for about 15-20 minutes. When the precipitate (pellet) was completely dried, 30 µl of water without RNase or DEPC water was added to the tubes, the solution was moved up and down several times using a micropipette tip. The RNA samples were then stored at -20 ° C. The integrity of the obtained RNA was then visualized using a 1.2% denaturing agarose gel.

2.4 RNA quantification:

1.2 % denaturing agarose gel was prepared to observe the RNA bands and determine the quality of RNA extracted. First, 0.84 g of agarose (Hydrogen, USA) was measured using an electronic mass balance. 70 ml of 1X TBE (Tris-Boric acid EDTA) buffer solution was prepared by adding 63 ml of distilled water to 7 ml of 10 X-TBE buffers. The buffer solution was transferred to a conical flask and

agarose powder was added into it. This mixture was swirled around till the appearance of the solution turned cloudy and opaque. The solution was then microwaved for about 1-2 minutes so that the cloudy appearance of the solution disappeared completely leaving a clear transparent solution. After the temperature drop of 60 ˚C, 1.4 ml of Clorox® (bleach) and 7 µl of ethidium bromide dye (Roth, Germany) were added and the flask was whirled around so that contents were well mixed. The solution was left to cool down for about 3-4 minutes and then transferred to a gel caster fixed with the comb for solidification. It was assured that while pouring formation of air bubbles does not occur. The gel was left for about 20 minutes to allow solidification. After this caster was carefully transferred to a horizontal gel tank (Cleaver Scientific Ltd. USA), immersed in a buffer solution, and the comb was carefully removed to expose the wells formed in the gel. The caster was placed in such a direction that the wells were closer to the negative cathode so that the RNA which was negatively charged migrated towards the positive anode at the other end of the tank. 2 µl of 6X loading dye was loaded onto a parafilm paper, 5 µl of sample was added to this and mixed by moving the mixture up and down using a micropipette tip. Each sample was then introduced into a separate well formed in the gel and the sequence of the samples was carefully noted in the order they were inserted into the wells. The gel electrophoresis was then started at 100 V, 500 mA for 30 minutes. After 30 minutes, the power supply was switched off and the caster was removed from the tank. The gel was removed carefully from the caster and transferred to a Gel Documentation (Gel Doc) system. In this system, GenoSens Capture software was used to preview and capture an image of the gel, which was viewed under ultraviolet light. The resulting image was saved in the software, and its brightness and sharpness were adjusted by the control settings of the software. In this manner, the RNA bands for each semen sample were obtained and displayed in the image as white bands wherever RNA was present. By observing these bands, it was determined which sample contained RNA, along with the quality of extracted RNA.

2.5 cDNA synthesis

To carry out the synthesis of cDNA (complementary DNA) from the extracted RNA, the first strand of the RevertAid cDNA synthesis kit was used. For the synthesis of cDNA, an appropriate amount of extracted RNA was used, so that the total amount of RNA was in the range from 0.1 ng to 5.0 µg. The optical density (OD) values of RNA obtained from NanoDrop help us to regulate this range of amounts of RNA. This process consists of two important steps, explained as follows.

In the first step, several tubes were labelled as RT+ (reverse transcriptase plus) and RT- (reverse transcriptase minus), and then the following reagents were added to each tube and the final volume was adjusted up to 12 µl and 20 µl respectively.

Table 2.1: component of RT-PCR reaction

Components	Amount
RNA template	5 µl
Random hexamers	1 µl
Autoclaved deionized water	6 µl
Total Volume	**12 µl**

Using all these components of the reaction mixture in the above-mentioned proportions in both RT+ and RT- labelled PCR tubes, all these contents were slightly mixed and then briefly centrifuged. After this short centrifuge spin, all the mixture tubes were run at 65˚C for 5 minutes in the thermal cycler (PCR) machine, and then the tubes were chilled at -20˚C for 5 minutes. In the second phase, different components of the RevertAid First Strand cDNA synthesis kit (Invitrogen, USA) were added according to the following proportions:

Table 2.2: Reagents utilized in cDNA synthesis in RT+ labelled PCR tube

Reagents	Volume
5X Reaction Buffer	4 µl
Roadblock RNase Inhibitor (20 µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid Master Mix µlVRT (200 µl)	1 µl
Total Volume (Master Mix)	8 µl
Total Mixture (Master Mix and RT+ sol.)	**12 µl+ 8 µl = 20 µl**

The Master Mix was prepared according to the number of samples and all the component volumes multiplied by the number of samples among which they were to be distributed. After the preparation of the Master Mix, the mixture was given a short centrifuge spin. Approximately 8 µl of Master Mix was added to each RT+ labelled tube which contained the Phase 1 mixture. Following this addition, the mixture was incubated in the thermal cycler (PCR machine) according to the following sequence: first incubation for 5 minutes at 25˚C, second incubation for 60 minutes at 42˚C and third incubation for 5 minutes at 70˚C. The product obtained at the end of the PCR was stored at -20˚C until further use.

2.6 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a process used in molecular biology for amplification of a single copy or a few copies of DNA across numerous orders of magnitude, generating thousands to millions of copies of DNA sequence. For performing PCR, two oligonucleotide primers are required to hybridize two opposite strands of denatured DNA and allow DNA polymerases to carry out DNA synthesis. The specificity of the PCR reaction depends upon the specificity of the primers used. Any complementarities between the two primers result in the formation of primer dimers, which result in less effective PCR amplification. There are many different genes, which are involved in the regulation of the process of spermatogenesis. A software program known as IDT (Integrated DNA Technologies) was used to design PCR primers for each specific gene. After this, the resulting primers were observed using in silico PCR of UCSC software and we used intron spanning primers to prevent contamination of genomic DNA, were designed. The following parameters were used to develop the required primers:

Table No 2.3: Primer for Complexin genes

Symbol	Forward Primer	Reverse Primer	Amplicon Size
CPLX1	GCTCCGCCGTGAGTATTT	CCTTTCCTCTCCTCTCTGGA	113
CPLX2	CCGAGATAAGGACTGAGGCTTGG	TTCTTCAGCCCATACTGGGGA	103

The primers designed were ordered from IDT and these were then diluted according to the necessary recommended dilutions. For carrying out the PCR reaction, the stock dilution of primers was even further diluted to 1:20 dilution, which involved adding 1 µl primer and 19 µl grade PCR water. The conditions for PCR were optimized for the intron-spanning primers mentioned. This was achieved by adjusting different $MgCl_2$ concentrations, primer concentrations, and annealing temperatures. A 2% agarose gel was prepared using the already described gel preparation protocol and the samples were run on this 2% agarose gel to validate the optimized products. PCR optimization for the CPLX2 and CPLX2 genes involved the denaturing step of 95˚C for 5 minutes, an annealing step at 72˚C for 1 minute and a final extension step at 72˚C for 10 minutes and then a hold at 12˚C.

Table 2.3: Composition of GAPDH PCR reaction mixture

Reagents	Volume/reaction
cDNA template	2.0 µl
PCR buffer	2.5 µl
$MgCl_2$	2.5 µl
dNTPs (10mM)	0.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
PCR water	16 µl
Taq polymerase	0.5 µl
Total Volume	**25 µl**

Above mentioned quantities of reagents were multiplied by the number of samples and a maximum volume of 25ul was added in each PCR tube for amplification.

2.7 cDNA amplification of infertile and normal samples:

The cDNA samples of infertile and control patients were amplified for their respective genes, using their specific gene primers. The PCR reaction for each sample was carried out in a 25 µl reaction mixture tube which contained the reagents in their appropriate proportions. To perform horizontal gel electrophoresis, 2.5% agarose (HydraGene, USA) gel was used. This gel was prepared by adding 1.75g of agarose powder in 70 ml of 1X TBE buffer, which was prepared by mixing 7 µl of 10X TBE buffer with 63 µl of distilled water. The mixture is then heated for 1-2 minutes, allowing the agarose to dissolve in the solution. After 2 minutes, when the agarose had completely dissolved in the buffer solution, the solution was allowed to cool down so that the temperature dropped down to at least 60˚C, after which 1.4 ml of Clorox® bleach and 7 µl of 5 mg/mL ethidium bromide (Roth, Germany) were mixed in the gel solution and the mixture was swirled to mix its contents. This prepared agarose solution was then poured carefully into a dual comb 16-teethed caster (Cleaver Scientific Ltd. USA), taking caution to avoid the formation of air bubbles. The solution was then left for solidification for about 20 minutes, after which the agarose gel was removed from the caster and transferred to the horizontal gel tank (Cleaver Scientific Ltd. USA), filled with buffer solution. 5 µl of PCR product and 2 µl of 10X loading dye were mixed on parafilm paper, and along with a 100 bp ladder loaded in the first and last wells, all the PCR samples were loaded separately into each well. The agarose gel was then run at 100V, 500 mA for 30 minutes. After 30 minutes, the gel was removed from the tank and the amplified product was visualized using Bio Doc Analyzer (Clinx Science Instruments, China). After this Real time-polymerase chain reaction (qPCR) was set to determine the expression of mRNA in two genes i.e., CPLX2 and CPLX2 in case and control samples. For this step, the CPLX2 gene mentioned earlier was used as an internal control primer. For quantitative PCR reaction, 2X Maxima SYBR Green/ROX qPCR Master Mix (Solis Bio Dyne, Estonia) was used. Real-time PCR was carried out on a Step One Plus™ Real-Time PCR system. The cDNA was amplified by using specifically optimized primers (CPLX2 and CPLX2). For the relative expression of the genes under consideration, three biological replicates were used for each sample. Normalization was achieved here by the Actin gene. The critical values which were obtained from the results of the qPCR were then statistically analyzed. The reagents which were used to prepare the qPCR master mix are described in Table 2.5 below.

Table 2.4: Composition of PCR reaction mixture

Components	Amount
Maxima SYBR Green/ROX qPCR Master Mix	10 µl
cDNA Template	2 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
RNase free water	7 µl
Total Volume	**20 µl**

As shown in the table above, 2 µl of cDNA and 18 µl of MASTER Mix were added to each PCR tube for the quantitative PCR reaction, then the tubes were properly closed and transferred to the QPCR system from step 1 more (Biosystems Applied System, United States America). To determine comparative gene expression. The qPCR and 96-well conditions are set correctly in the Stepone ™ plus program.

Table 2.5: Optimized condition for the PCR

Stage	Temperature	Time	Cycles
Holding	95˚C	12 minute	1
Cycling			
Step 1	95˚C	30 seconds	
Step 2	60˚C (CPLX2)	30 seconds	
Step 3	72˚C	30 seconds	40

3. Results

Extracting RNA from semen samples is a difficult process because RNA is present in degraded form. These are 18S and 28S Ribosomal RNA. Samples were analyzed at the laboratory of NIH and all were diagnosed with primary infertility. All Samples were collected in sterile semen containers and were set at 37^{o}C for liquefaction. After liquefaction analysis was performed under the Microscope. A special Chamber was used for the count and motility of sperm. PH and semen color were also observed. pH was from 7.2-7.8 and the color was whitish grey. The viscosity of some samples was thick and some of them were thin. The volume of semen varied from 1-6 ml but the majority was with 3-4 ml. In this research, our main focus was on sperm count motility and age.

RNA extraction was done by using the TRIzol® reagent (Invitrogen USA) protocol which was previously mentioned. We made some modifications for RNA extraction using ACID-treated glass beads. The RNA extracted using the above-mentioned protocol was run in the denaturing agarose gel. For this purpose, we used prepared 50 ml 1 XTBE buffer and .5g of agarose was added to it. After heating the solution was left for cooling and the 7 µl of 5 mg/mL ethidium bromide dye was added. RNA Band was visualized on denaturing agarose gel. The RNA of both case and control samples were visualized the basic purpose was to determine the presence of gel.

Figure 3.1: Extracted 28S and 18S ribosomal RNA

The modified method of RNA extraction using acid-treated glass beads was found to be very effective. The RNA extracted is shown in the image. In the sample in which sperm cells were in high amounts, there was a large amount of RNA but samples with low sperm count had little amount of RNA.

First strand cDNA from our extracted RNA was prepared using, Reverted Aid First strand cDNA synthesis kit (Invitrogen, USA). We took a suitable volume of our extracted RNA so that the amount of RNA remains very close. Thus cDNA was amplified and for further amplification, GAPDH was used as a positive control to confirm the synthesis of cDNA. The band size of 496 base pairs was observed on the 2% agarose gel.

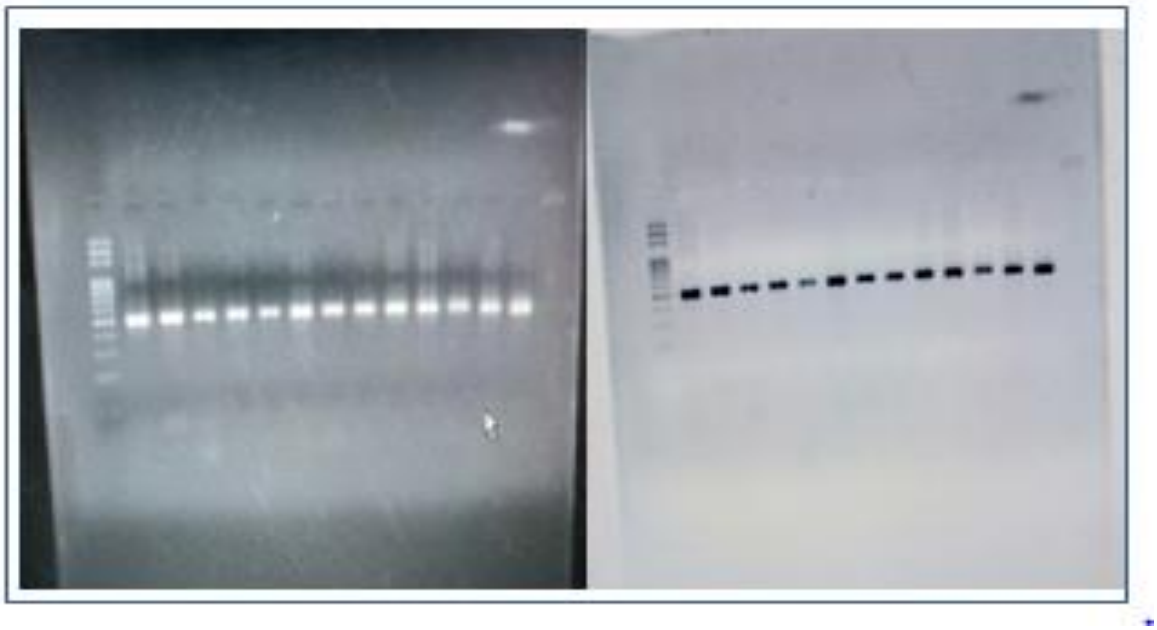

Figure 3.2: Amplified Result for GAPDH

RNA quantification was done using a NanoDrop spectrophotometer (ND-1000, USA). The surface of NanoDrop was cleaned with distilled water using Watman wipes. To eliminate the systematic error a blank reading was set. A zero value indicates that the reading is fine. After that 1ul sample on the sensor of NanoDrop and the lid factor of 50 concentrations along with the 260/280 ratio was loaded to quantify the amount of RNA. The amount of RNA measured in ng/ul is mentioned below. The 260/280 ratio of more than 1.50 indicates that RNA quality is good.

3.1 Statistical Data Analysis for the Demographic Data of Infertile and Fertile

Table 3.1: Statistical analysis of Control and Healthy sample

Variables	Sample	No	Mean	S. D	SEM	95% CI of Mean		P-Value
						Lower	Upper	
Age	Fertile	30	33.8	7.78327	1.42102	-3.58065	3.12937	0.89361
	Infertile	39	34.02	6.18354	0.99016			
Abstinence	Normal	33	6.39394	5.76053	1.00278	-0.80329	3.02707	0.25084
	Infertile	39	5.28205	1.55511	0.24902			
Semen Volume	Normal	33	3.63939	1.34721	0.23452	-0.4742	0.88632	0.5477
	Infertile	39	3.43333	1.51733	0.24297			
Sperm Count/Million	Normal	33	126.2989	69.58155	12.11259	51.38802	111.42802	8.36154E-7
	Infertile	39	44.89077	58.16278	9.3135			
Total Motility (%)	Normal	33	77.78788	15.14669	2.63674	29.33196	50.70534	1.73441E-10
	Infertile	39	37.76923	27.42594	4.39167			
Immotile	Normal	33	22.212	15.147	4.3916	36.8034	50.9743	0.0001
	Infertile	39	62.231	27.426	2.6367			

3.2 Age, Immotile Spermatozoa, Semen Volume, Sperm Count, Total Motility, and Relative expression of Complexin II gene In Normal and Infertile male

The semen parameters of all subjects (both diseased and healthy) were analyzed by WHO 2010 criteria. The subjects are age-matched. Hence, the difference between the two groups was statistically insignificant as given in Figure 3.3 A. The percentage of dead spermatozoa was analyzed and the number of dead sperm was higher in infertile subjects compared to the normal subjects. Thus, the difference between the groups was statistically significant (62.23 ± 4.39 vs 22.21 ± 2.64; $P < 0.0001$), provided in Figure 3.3 B. The semen volume of all subjects was assessed based on the WHO 2010 guidelines for semen analysis. Low volume of semen is one of the alterations associated with semen parameters in diseased subjects. However, the difference between the groups enrolled in this study was not significant (given in Figure 3.3 C). Sperm count is another essential parameter in semen analysis. Thus, the sperm count was assessed and the count of the infertile group reduced drastically compared to the normal group (Figure 3.3 D). The total motility of spermatozoa in the infertile group

reduced significantly compared to the normal group (Figure 3.3 E). As such, the difference between the groups was statistically significant at $P \leq 0.05$.

Mann-Whitney test was performed by using OriginPro software and the relative expression difference was found to be significant. From our result, the P value was detected as not greater than 0.05 between normal and infertile. The maximum expression difference between normal and infertile males was 6.03934 and 0.71776. These results indicate that there is a significant role of the complexin II gene during acrosomal exocytosis provided in Figure 3.3 F. Further studies are required to investigate the downregulation causes of male infertility.

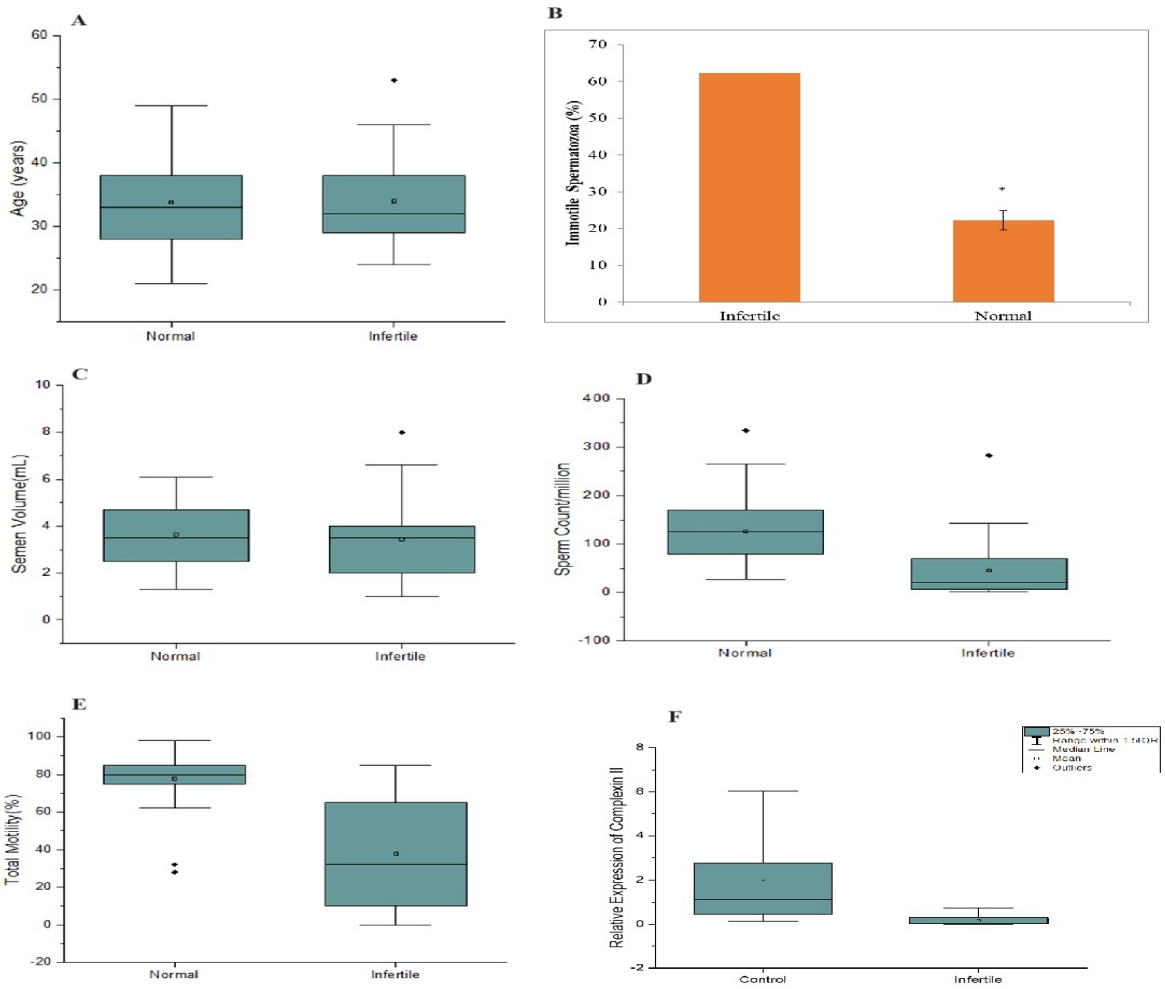

Figure 3.3: **A**) The age (Mean ± SD) of study subjects, compared using student's t-test, **B**) Percentage (Mean ± SEM) of immotile spermatozoa compared using student's t-test, **C**) Semen volume (Mean ± SD) of normal and infertile subjects compared using student's t-test, **D**) Semen count (Mean ± SD) of normal and infertile subjects compared using student's t-test, **E**) Total motile spermatozoa (Mean ± SD) of normal and infertile subjects compared using student's t-test, **F**) Box graph Showing Relative expression of the Complexin II gene between normal and infertile males using Mann-Whitney test.

*$P \leq 0.05$ was considered significant.

3.3 Quantitative PCR Result Analysis

Relative expression analysis of Complexin I and Complexin II genes we performed qPCR. Analysis was performed in both normal and infertile males. For this purpose comparison of two target genes β-actin was used as standard. We used Step One PlusTM Real-Time PCR was used to calculate the CT values for Normal and infertile males. After that, we calculated the Δ CT values by subtracting

the CT values of the housekeeping gene from our gene of interest. Average Δ CT values of both normal and infertile males were taken to calculate ΔΔCT values and later fold change difference was determined by applying the following formula ΔΔCT. We also calculated the percentage decrease of complexin II in normal male % infertile which was found to be 76.39%.

Table 3.2: Calculation of fold change difference and relative expression

Sample	CplxII C_T	**GAPDH** C_T	**D**C_T (Avg. CplxII C_T 2 Avg. GAPDH C_T	**DD**C_T (Avg. DC_T	**Normalized** CplxII amount relative to control 2^{-DDCT}
				2 Avg. $DC_{T,\ control}$)	
Control	23.11957741	24.3104248	-1.190847397	-2.594391505	6.039342551
	24.97335243	25.04141426	-0.068061829	-1.471605937	2.773304324
	23.67296028	21.0640049	2.608955383	1.205411275	0.433645705
	28.38532257	24.06904411	4.316278458	2.91273435	0.132794348
	20.64979935	18.67705727	1.972742081	0.569197973	0.673991372
	21.38598061	20.60378265	0.782197952	-0.621346156	1.538309884
Average	23.69783211	22.294288	1.403544108	0	1
Infertile	26.28676796	18.92466354	7.362104416	5.958560308	0.016080317
	27.6968174	19.50538635	8.191431046	6.787886937	0.009049872
	32.31587219	25.04226685	7.273605347	5.870061239	0.017097613
	31.66786003	22.66465187	9.00320816	7.599664052	0.005155528
	22.69108391	19.15806007	3.533023834	2.129479726	0.228540265
	23.23460388	20.06697655	3.167627335	1.764083227	0.294413713
	25.75878334	18.66924858	7.08953476	5.685990651	0.019424339
	22.37306023	20.49109077	1.881969452	0.478425344	0.71776061
	20.59438896	17.92727089	2.667118073	1.263573964	0.416510865
	29.32476044	20.18763924	9.137121201	7.733577092	0.004698523
Average	26.19439983	20.26372547	5.930674362	4.527130254	0.043370857
	23.69783211			-3.55271E-15	1
				-3.55271E-15	1.931898031
					0.161100227

4. Discussion and conclusion

Infertility is the inability of couples to reproduce their offspring after regular and unprotected intercourse for one year [19]. The epidemiology of infertility is widely spread around the universe still a lot of research is needed to be done in this field. 50% of infertility is related to females and a certain percentage is associated with male factor and some of the causes are still unknown. In Pakistan, the infertility rate is almost 21% with 18% of secondary infertility and 3% of primary infertility [20]. Male infertility is quite complex, multifactorial disease and heterogeneous groups of genes are involved [21].

Many biological, environmental and physiological factors play a role in male infertility. Risk factors like smoking drinking obesity, some gonadotoxic medicine STDs and occupational exposure can also have their part in infertility [22]. Male infertility is usually diagnosed based on semen analysis parameters like sperm motility, count in millions and sperm shape. This evaluation of male infertility which depends only on semen analysis provides us only general features of sperm but with this, we can’t recognize the exact fertilizing and functioning ability of sperm. This method of diagnosis

primarily depends on alteration in one or more of the semen parameters. Infertility is usually associated with a decrease in motility, volume and count. Also, an increase in abnormal morphology and immotile spermatozoa were reported [21, 23, 24].

The results of this study also show significant differences in sperm count, immotile and motility between the infertile and normal groups. However, the difference in semen volume between the infertile and normal groups was not significant. Several modern techniques like assisted reproductive technique (ART) like In vitro fertilization and ICSI are helpful in the treatment/ management of infertility, although these techniques also have their drawbacks.

This current study was designed to investigate the expression and role of complexin genes in infertility at the genomic level. The complexin genes played a role in the fusion of sperm and egg through the SNARE proteins. Both CPLX1 and CPLX2 genes play an important role in the exocytosis of sperm acrosome present at the tip of sperm which is a modified form of Golgi bodies acting hydrolytic enzyme, rupturing the sperm membrane and egg zona pellucida fusion of their nuclei [25]. From demographic data it was concluded that there is a significant difference in sperm count, motility and immotile was observed between the groups.

Real-time PCR data analysis showed a fold change difference of 0.043280741. From this fold change difference, we determined that our gene of interest is downregulated and a Student t-test was also applied which also showed that there is downregulation of our gene of interest in infertile men. The probability value for the qPCR data was also ≤ 0.05 which means our data was significant. The difference observed in analyzed data indicates that there is a significant role of complexing genes in male infertility and their expression was inversely proportional to each other in Normal and infertile males and complexin is surely playing a major role in male infertility. However, it is highly recommended that future studies investigate the cause of the down-regulation of the Complexin gene as it plays an important role in the exocytosis of sperm nuclei.

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