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Article

The Role of Brain-Derived Neurotrophic Factor in Male Subfertility: A Molecular Investigation

Ahmad Bassiouny [1,*], Andrew Mounir [1,2], Eman El-Gendy [1], Rawan Aly [1], Mohamed Abdelmoneim [3]

[1] *Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, 21511, Egypt.*

[2] *European Mediterranean Master of Neuroscience and Biotechnology Program, Alexandria University, Alexandria, Egypt.*

[3] *3Faculty of Medicine, Alexandria University, Alexandria, Egypt.*

[*] *Correspondence Address:*

Ahmad Bassiouny: Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, 21511, Egypt. Email address: arbassiouny@alexu.edu.eg.

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ABSTRACT: Male subfertility represents a significant and escalating global health concern with profound implications for couples worldwide. Emerging evidence has highlighted the potential interplay between several key factors in male reproductive health, with particular emphasis on the interrelationships between Brain-Derived Neurotrophic Factor (BDNF), testosterone, zinc homeostasis, and inflammatory processes. These factors are increasingly recognized as critical modulators of reproductive function. This study is designed to investigate the specific role of BDNF in subfertile males and to elucidate its potential implications for key reproductive pathways, including spermatogenesis, steroidogenesis, and overall reproductive capacity. Twenty-two subfertile male patients, aged between 29 and 40 years, were recruited from the infertility clinic at Al-Madina Fertility Center, Alexandria. Comprehensive evaluations included semen analysis, hormonal assessments, and molecular analyses of BDNF levels in serum and seminal plasma. Significantly lower serum and seminal plasma BDNF levels were observed in all patient categories compared to the control group (n=22 fertile males, $p \leq 0.001$). BDNF levels correlated positively with sperm motility and total sperm count ($r = 0.530$, $p < 0.012$ and $r = 0.449$, $p < 0.045$). Furthermore, a noteworthy inverse relationship was found between BDNF levels and the proportion of abnormal sperm morphology ($r=-0.488$, $p < 0.039$). This study demonstrates that reduced levels of BDNF in subfertile males are associated with impaired semen quality. The findings suggest BDNF's role in male subfertility. Further research is needed to clarify its mechanisms and explore therapeutic potential, ultimately leading to more effective and personalized interventions for men facing challenges with fertility.

1. INTRODCTION

Infertility and subfertility are significant global health issues affecting millions of individuals and couples. The World Health Organization (WHO) defines infertility as the inability to conceive after 12 months of unprotected sexual intercourse, while subfertility refers to a reduced level of fertility characterized by a longer time to conceive [1]. The prevalence of infertility is estimated to affect approximately 15% of reproductive-aged couples worldwide [2]. Infertility is a significant public health issue, affecting approximately 15% of couples of reproductive ages worldwide, with male subfertility contributing to a substantial proportion of these cases [2,3].

The causes of infertility and subfertility are multifaceted, often involving a combination of genetic, hormonal, anatomical, and environmental factors. In women, factors such as age, ovulatory disorders, pelvic inflammatory disease, and endometriosis are commonly implicated [4]. Hormonal factors play a crucial role in regulating fertility in both men and women. Among the various hormones influencing reproductive functions, the Brain-Derived Neurotrophic Factor (BDNF) has emerged as an important player in the reproductive system. Hormones such as estrogen, progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and other endocrine factors are fundamental in regulating the menstrual cycle, ovulation, sperm production, and overall reproductive health. Imbalances in these hormones can lead to various fertility issues in women including:

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- Polycystic Ovary Syndrome (PCOS): This common endocrine disorder is characterized by excess androgen production, irregular menstrual cycles, and ovulatory dysfunction. Women with PCOS often have elevated luteinizing hormone (LH) and low follicle-stimulating hormone (FSH) levels, disrupted normal ovarian function, and resulted in infertility [5].
- Thyroid Disorders: Both hypothyroidism and hyperthyroidism are associated with irregular menstrual cycles and can affect ovulation. Thyroid hormones interact with reproductive hormones, further complicating fertility [6].

On the other hand, male infertility can be due to sperm production issues, hormonal imbalances, and medical conditions such as varicocele and anatomical obstructions [7]. The psychological, social, and economic impacts of infertility cannot be overstated. Couples experiencing infertility often endure significant emotional distress, anxiety, and social stigma [8]. Furthermore, treatment options for infertility, including assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF), can be expensive and may not be accessible to all, thereby intensifying disparities in reproductive health [9]. Infertility affects not only individuals and couples but also has broader psychological, social, and economic implications for society. Understanding these dimensions is essential for developing comprehensive strategies to support those affected and address the societal consequences of infertility.

Male subfertility is a complex condition influenced by a myriad of factors including genetic predispositions, hormonal imbalances, anatomical anomalies, and environmental exposures. The multifactorial nature of male infertility necessitates a comprehensive understanding of its underlying biological mechanisms to inform effective diagnostic and therapeutic strategies [4,10]. Recent advancements in reproductive biology have brought forth an increasing recognition of the role of neurotrophic factors in male fertility. Notably, the BDNF has garnered attention for its pivotal role not only in neuronal survival and function but also in various aspects of reproductive physiology [11,12]. BDNF is a member of the neurotrophin family of growth factors and is widely expressed in the central and peripheral nervous systems as well as in the male reproductive tract, suggesting its potential involvement in spermatogenesis and sperm function.

Emerging research indicates that BDNF may influence key processes in male reproductive biology, such as regulating hormone production, spermatogonial stem cell maintenance, and the modulation of inflammatory responses within the reproductive system [13]. These functions underscore the importance of BDNF in maintaining neuronal health and optimal reproductive performance. Moreover, alterations in BDNF levels have been linked to various forms of reproductive dysfunction, highlighting its potential role as a biomarker for assessing male fertility. Emerging evidence suggests that neurotrophic factors, including BDNF, play critical roles beyond neuronal health; they may also influence reproductive processes [11,12].

This study aims to investigate BDNF levels in subfertile males and explore their association with semen quality parameters. By elucidating the relationship between BDNF and semen quality, we aspire to contribute to the growing body of literature that seeks to identify critical biological markers for male infertility. Understanding these associations may pave the way for novel therapeutic interventions and improved clinical management of male subfertility, ultimately enhancing reproductive outcomes for affected couples. The findings will not only enhance our understanding of the biological underpinnings of male infertility but also have significant implications for predictive medicine in this field. Continued rigorous research in this field, employing advanced omics technologies (genomics, proteomics, metabolomics) and sophisticated statistical modeling, is essential to elucidate the intricate interactions among these elements fully and to refine our diagnostic and therapeutic strategies for enhanced fertility management and improved reproductive health outcomes for men. This comprehensive approach will ultimately lead to more effective and personalized interventions for men facing challenges with fertility.

2. Materials and Methods

2.1. Materials

PureSperm 80/40® was obtained from NidaCon International (Mölndal, Sweden). Sperm 360 ^{0} pH Kit was purchased from Sperm Processor (Aurangabad, India). ORIGIO ® Handling ™ media was provided by CooperSurgical (Ballerup, Denmark). Elecsys Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), Prolactin (PRL), Testosterone (T), and C-Reactive Protein (CRP) kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). BIOZOL was purchased from Bioer Technology (Hangzhou, China). RNA Later was purchased from Thermo Fisher Scientific Inc (Dartford, United Kingdom). cDNA synthesis kit and ABT 2X qPCR Mix SYBR® ROX kit were purchased from Applied biotechnology (Abuja, Nigeria). Zinc kit was obtained from cell Biolabs Inc (California, United States).

2.2. Study population

This cross-sectional study included 44 men aged 28-45 years who were recruited from Al-Madina Women's Fertility Center (Alexandria, Egypt). Twenty-two men aged 29-40 years with a diagnosed case of subfertility were defined as the inability to conceive after 12 months of unprotected intercourse. Women were mandated to exhibit regular menstrual cycles, possess a normal hysterosalpingogram, demonstrate normal findings on laparoscopy, and provide a luteal-phase endometrial biopsy specimen that histologically aligned with menstrual dating. Men with known genetic disorders, recent infections, or those who have undergone fertility treatments were excluded [11]. Informed consent was obtained from all participants, and the study protocol was approved (approval number: 0108332) by the Medical Research Ethics Committee, Faculty of Medicine, Alexandria University, Egypt, according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO).

2.3. Semen collection and analysis

The semen samples were collected via masturbation after 2-5 days of abstinence from sexual activity [14]. The study involved selecting samples that met the normal sperm parameters according to the criteria set up by the WHO [15], in which; the

semen volume must be at least 1.5 mL, sperm concentration 15 million sperm/mL or greater, total motility 40% or more, and normal sperm morphology 4% [14]. After ejaculation into the collection vessel, the semen samples were allowed to liquefy for 30 minutes at room temperature (RT). After liquefaction, standard parameters, including volume, pH, concentration, motility, and morphology, were analyzed according to WHO guidelines [15]. The concentration (Count), motility, and morphology of sperm cells were measured using the CASA (computer-assisted sperm analyzer system) (SCA™ motility module, Barcelona, Spain). According to WHO recommendations, the basic assessments of sperm count, motility, and morphology were proceeded using at least 200 thousand sperm cells to assess across various fields [15]. The samples were classified into five groups according to their sperm concentration (count) (Oligo: low sperm count), motility (Astheno: reduced sperm motility), and morphology (Terato: abnormal sperm morphology), as shown in Table 1.

2.4. Blood sample collection

Blood samples were collected from all the participants after 9-12 hours of fasting. After collecting the whole blood, the blood samples were allowed to clot by leaving them undisturbed for 15-30 minutes at RT. The blood clots were removed by centrifugation at 100-390 xg for at -20 °C for 10 minutes using a cooling centrifuge (Hettich Universal 320 Benchtop, Livingston, USA). Following centrifugation, the liquid components (serum) were transferred into a clean microcentrifuge tube. The samples were maintained and stored at –20°C for further use [19].

2.5. Hormonal Analysis

Serum levels of FSH (mIU/mL), LH (mIU/mL), prolactin (PRL) (ng/mL), and total testosterone (T) (ng/mL) were measured using the chemiluminescence technique. The hormonal kits´ principle was based on the reaction of serum hormones with immuno-antibodies and chemiluminescence measurements for the reaction products. The reaction consisted of two incubation steps and a chemiluminescent measurement step via a photomultiplier tube that spanned 18 minutes. Forty microliters of samples were added to monoclonal antibodies attached to biotin and specific antibodies for FSH, LH, PRL, and total T labeled with a complex of ruthenium to form a sandwich complexes. Then, the solid phase became attached to the sandwich complex that formed through reaction between biotin and streptavidin, after the addition of monoclonal antibodies specific for hormones and labeled with a complex of ruthenium. The mixture of the reactions was drawn into cells designed for measurement, in which the micro-particles magnetically retained on the electrode surface. The chemiluminescent emission was induced from voltage electrode and measured by photomultiplier (Wiener Labs CLIA-1000 Special Chemistry Analyzer, Kingston, Jamaica). The manufacturer-provided master curves covered a range of 0.1-200 mIU/mL for FSH and LH, 0.0471-471 ng/mL for PRL, and 0.025-15 ng/mL for total T.

2.6. Zinc Analysis

Serum zinc levels (µg/dL) were measured using the 5-Br-PADAP reagent. This reagent reacted with zinc ions to generate a colorful complex whose absorption (λmax= 560 nm) was proportional to the concentration of zinc in serum. Fifty microliters of samples were added to 200 µL of the reaction reagent to each well containing the sample and mix well. The second step involved the incubation of the reaction plate from 10 to 50 minutes away from light. The third step involved reading the plate with a spectrophotometric microplate reader (Microplate Reader FlexA-200, Hangzhou, China) at 560 nm. According to the master curve provided by the manufacturer, the range of zinc concentration was 0.78-65 µg/dL.

2.7. C-reactive protein (CRP) Analysis

Serum levels of C-reactive protein (CRP) (mg/L) were assessed using the immunoturbidimetric technique, in which a specific monoclonal antibody was designed specifically containing anti-C-reactive protein antibody coated with latex particles that agglutinated with C-reactive protein of human forming turbidity and determined via a turbidimeter (2100N Laboratory Turbidimeter, Bath, UK). According to the master curve provided by the manufacturer, the range of CRP concentration was 0.149-20 mg/L.

2.8. Real-Time PCR

Semen samples were segregated using 1 mL PureSperm 80/40® and washed twice in the Origio handling medium at 80 xg at RT for 5 minutes using centrifugation (Benchmark Scientific LC-8 Centrifuge, New Jersey, USA). Subsequently, the pellets were treated with RNA Later to preserve the RNA content of samples for clinical genomic investigation. RNA was extracted using a BIOZOL reagent and applied to reverse transcription into cDNA. The RT-PCR was carried out at a final volume of 20 µL containing 100 ng of cDNA template, 20 µM of primer (Table 2), ABT 2X qPCR mix (SYBR), and RNase free water. The Livak method was used to calculate the mRNA fold change with B-actin as a reference gene [16].

2.9. Statistical Analysis

Data were analyzed using the latest version of IBM SPSS software (NY, USA). Continuous variables were expressed as mean ± standard error of mean (Mean ± SEM). Differences between groups were assessed using one-way ANOVA, and correlations were calculated using the Pearson's correlation coefficient.

3. Results and discussion

3.1. Semen Analysis

Sperm dysfunction is the most common cause of infertility in men. Male infertility is diagnosed based on the presence of "oligozoospermia" (reduced sperm count), "asthenospermia" (decreased sperm motility), and "abnormal sperm" (sperm with an abnormal morphology) [17]. High-end CASA data analysis revealed a significant ($P \leq 0.001$) decrease in the percentages of both sperm motility and Fwd. motility, as well as, sperm concentrations (million/mL) in the subfertile group (mean ± SEM, 21.59 ± 1.157, 2.40 ± 0.414, 10.5366 ± 1.506, respectively) compared to the control group (mean ± SEM, 70.476 ± 0.906, 31.590 ± 0.952, 70.68 ± 7.172, respectively). Meanwhile, the subfertile group showed a significant ($P \leq 0.001$) increase in the percentage of sperm abnormalities (mean ± SEM, 96.66 ± 1.602) compared to the control group (mean ± SEM, 7.68 ± 0.448) (**Figure 1**). Key sperm functions, including motility, viability, capacitation, and acrosome reaction, are contingent upon pH levels.

Table 1. Data of studied groups

Group	Criteria
Normozoospermia (Control) (n = 22)	Conc/mL ≥ 15 million, Motility ≥ 4, and% Abnormality ≤ 96%
Oligoasthenoteratozoospermia (n = 12)	Conc/mL < 15 million, Motility < 40%, and Abnormality > 96%
Asthenoteratozoospermia (n = 2)	Conc/mL ≥ 15 million, Motility < 40%, and Abnormality > 96%
Oligoasthenozoospermia (n =7)	Conc/mL < 15 million, Motility < 40%, and Abnormality ≤ 96%
Oligozoospermia (n = 1)	Conc/mL < 15 million, Motility ≥ 40 %, and Abnormality ≤ 96%

Table 2. The sequences of primers

Target Gene	The sequence of primers (5'– 3')	
B-actin	F = CTTGGCTGCAGTACAAGAGC	R =AGGTCTCAGAAACGCACTCA
BDNF	F =AGCGTGAATGGGCCCAAGGCA	R =TGTGACCGTCCCGCCCGACA
BCL2	F = TCCTCTTTACACTGGCCAGG	R =GAGTATTTGTGCAGCGAGGG
IL6	F =AGGTTGGTCTCGAACTCCTG	R =GAGTATTTGTGCAGCGAGGG

Then, maintaining the optimal pH of semen is vital to ensuring the normal function of spermatozoa [18]. Our pH analysis demonstrated a significant (P ≤ 0.001) reduction in the pH values of the subfertile group (mean ± SEM, 6.114 ± 0.296) compared to the fertile group (mean ± SEM, 7.427 ± 0.034). The data indicated significant (P ≤ 0.05) positive correlations between pH value, sperm motility (%), and Fwd. motility (%), and sperm count (million/mL) (r = 0.532, 0.489, and 0.434, respectively). Additionally, a significant (P ≤ 0.05) negative weak correlation (r = -0.471) was observed between pH and sperm abnormalities (%) (**Figure 2**).

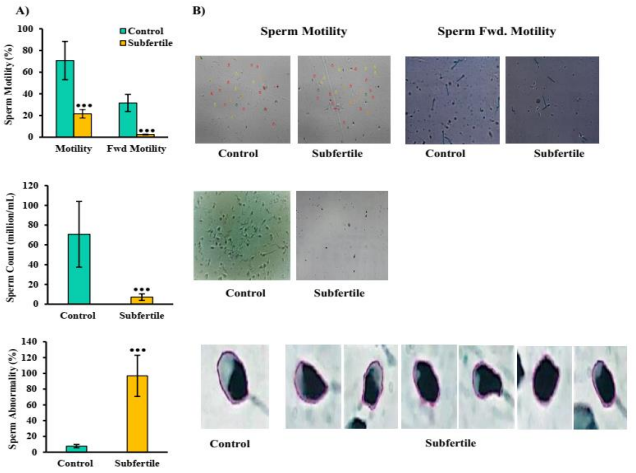

Figure 1. Semen analysis using the CASA system in both fertile and subfertile groups. (A) Bar graphs illustrate the significant (p ≤ 0.001) reduction in the sperm functions; motility (%), Fwd. motility (%), and sperm count (million/mL), alongside a significant (p ≤ 0.001)

increase in sperm abnormalities (%). (B) Represents CASA images for sperm functions at magnifications of 400x and 1000x. Data were represented as Column: mean; bar: SEM. •••P value ≤ 0.001 was considered statistically significant compared to the fertile (control) group.

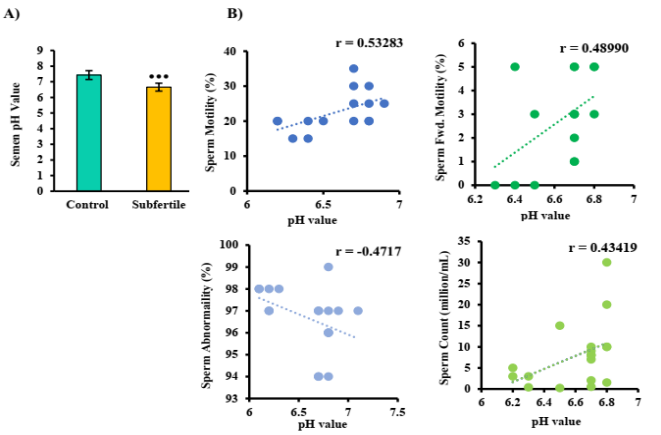

Figure 2. Semen pH analysis and its association with sperm functions. (A) Bar graph indicates the significant (p ≤ 0.001) decrease in pH values of the subfertile group compared to the fertile group. (B) Scatter blot demonstrating pH correlations with sperm functions in subfertility using Pearson correlation test. There was a significant (P ≤ 0.05) positive correlation between pH value, sperm motility (%), Fwd. motility (%), and sperm count (million/mL), while there was a significant (P ≤ 0.05) negative weak correlation between pH and sperm abnormalities (%).

•••P value ≤ 0.001 was considered statistically significant compared to the fertile (control) group.

3.2. Clinical characterization of blood hormones

Hormones such as testosterone, prolactin, FSH, and LH are crucial in regulating male reproductive physiology. Testosterone is essential for spermatogenesis and the maintenance of male secondary sexual characteristics. Low testosterone levels can lead to decreased libido, erectile dysfunction, and impaired spermatogenesis, contributing to subfertility [19]. Conversely,

elevated prolactin levels, a condition known as hyperprolactinemia, can inhibit testosterone production by suppressing gonadotropin-releasing hormone (GnRH), further impairing fertility [20]. Levels of PRL (ng/mL), FSH, and LH (mIU/mL) hormones were markedly ($p \leq 0.001$, 0.05, 0.01, respectively) elevated in the subfertile group (mean ± SEM, 12.044 ± 0.983, 8.635 ± 5.1168, 8.442 ± 2.903, respectively) compared to the control group (mean ± SEM, 5.825 ± 0.296, 6.249 ± 0.3972, 6.515 ± 0.299, respectively). Conversely, both free and total levels of testosterone (U/mL) were significantly ($P \leq 0.001$) diminished in the subfertile group (mean± SEM, 42.94 ±3.155, 2.632± 0.3179, respectively) compared to the control group (mean± SEM, 145.6 ±13.622, 6.507±0.274, respectively) (**Figure 3**). Correlation analysis revealed the negative associations between elevated PRL, FSH, and LH levels and sperm dysfunctions. In contrast, it indicated the positive associations between reduced testosterone levels and sperm dysfunctions, as illustrated in Table 3.

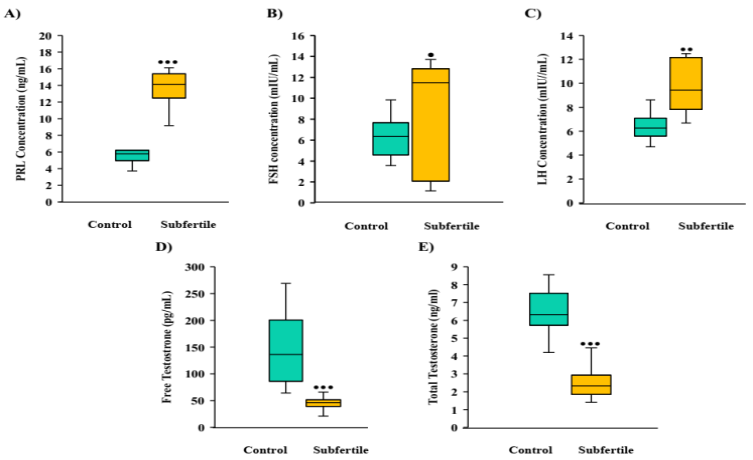

Figure 3. Hormonal profile analysis including prolactin (PRL) (ng/mL), follicle-stimulating hormone (FSH) (mIU/mL), luteinizing hormone (LH) (mIU/mL), and total and free testosterone (ng/mL) and (pg/mL), respectively in fertile and subfertile group using the chemiluminescence technique. (A) Represents the significant ($p \leq 0.001$) increase in the PRL level in the subfertile group compared to the control group. (B) Represents the significant ($p \leq 0.05$) increase in the FSH levels of subfertile group compared to the control group. (C) Represents the significant ($p \leq 0.001$) elevation of LH levels in the subfertile group compared to the fertile group. (D) Represents the significant ($p \leq 0.001$) decrease in the level of total testosterone in subfertile group compared to the control one. (E) Represents the significant ($p \leq 0.001$) decrease in the concentration level of free testosterone in subfertile group compared to the control group. Data were represented as boxplots: mean; SEM.

•••P value ≤ 0.001 was considered statistically significant compared to the fertile (control) group.

Table 3. Hormones correlations with sperm dysfunction

Hormones correlations with sperm dysfunctions	PRL (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	Free T (pg/mL)	Total T (ng/mL)
Sperm motility (%)	r = -0.557(•) (Negative weak)	r =-0.540 (•) (Negative weak)	r = -0.674(•••) (Negative moderate)	r= 0.545(•) (Positive weak)	r = 0.510(•••) (Positive weak)
Sperm Fwd. motility (%)	r = -0.504(•) (Negative weak)	r =-0.602(•) (Negative moderate)	r =-0.581(•) (Negative weak)	r= 0.595(•) (Positive weak)	r = 0.610(•••) (Positive moderate)
Sperm abnormality (%)	r = 0.444(•) (Positive weak)	r = 0.477(•) (Positive weak)	r =0.674(••) (Negative weak)	r= -0.519(•) (Negative weak)	r = -0.413(NS) (Negative weak)
Sperm count (million/mL)	r = -0.484(•) (Negative weak)	r = -0.421(•) (Negative weak)	r =-0.556(••) (Negative weak)	r = 0.456(•) (Positive weak)	r = 0.455(•) (Positive weak)

(•••) P value was significant when judged at ≤ *0.001* level. (••) P value was significant when judged at ≤ *0.01* level. (•) P value was significant when judged at ≤ *0.05* level. (NS): Non-significant. The correlation´s strength was estimated according to [21].

3.3. BDNF mRNA downregulation and Zinc deficiency influence testosterone levels and sperm functionality

BDNF is a neurotrophin that has been found to play a significant role in reproductive physiology**.** BDNF is also involved in spermatogenesis. Researches indicate that BDNF is critical for the survival and function of spermatogonial stem cells [13,22,23]. Mice lacking BDNF exhibit impaired sperm production, highlighting its vital role in male fertility. Our findings indicated the significant ($p \leq 0.001$) downregulation of BDNF mRNA levels in subfertile group (mean±SEM, 0.155 ± 0.0217) compared to the control group (mean ± SEM, 1 ± 0.010) (**Figure 4A**). The Pearson correlation analysis revealed a significant ($p \leq 0.01$, 0.05) positive relationship ($r = 0.530$, 0.466, 0.449) between downregulated BDNF mRNA level and reduced sperm motility (%), Fwd. motility (%) and count (million/mL), respectively. Furthermore, a significant ($p \leq 0.05$) negative association (r=-0.467) was observed between the downregulated BDNF mRNA level and the prevalence of sperm abnormalities (%) (**Figure 4B**). Testosterone levels are influenced by and can influence BDNF levels, with lower testosterone levels often associated with reduced BDNF expression [13]. This was consistent with our results as downregulated BDNF mRNA levels showed significant ($p \leq 0.05$) positive correlations with lower levels of total and free testosterone ($r = 0.443$ and 0.479, respectively) (**Figure 4C**).

Zinc is an important factor affecting sperm formation and regulates DNA replication, transcription, packaging, protein biosynthesis, cell differentiation, and proliferation. Deficiency in this element can lead to poor sperm quality and male infertility by increasing oxidative stress [24]. Our data showed a notable ($p \leq 0.001$) decrease in zinc level (µg/dL) in the subfertile group (mean ± SEM, 42 ± 2.188) compared to the control group (mean±SEM, 96.9± 2.141) (**Figure 5A**). Low level of zinc was significantly ($p \leq 0.05$) positively correlated ($r = 0.560$, 0.405, 0.530, respectively) with reduced sperm motility (%), Fwd. motility (%) and concentration (million/mL). Supplementation with zinc has been shown to increase testosterone levels in men with zinc deficiency [25]. By supporting the enzymatic processes involved in testosterone synthesis, zinc is vital for maintaining optimal reproductive function. A significant ($p \leq 0.05$) negative correlation ($r = -0.5130$) was found between low zinc levels and high sperm abnormalities (%) (**Figure 5B**). Furthermore, low zinc level was significantly ($p \leq 0.01$, 0.05) positively correlated ($r = 0.618$, 0.464, respectively) with low levels of total and free testosterone (**Figure 5C**). There was a significant (($p \leq 0.05$) strong positive correlation (r=0.734) between zinc deficiency and downregulated BDNF mRNA levels.

Zinc has been shown to have a neuroprotective effect and can influence BDNF signaling pathways. Zinc deficiency could impair BDNF signaling, potentially affecting neurogenesis and neuronal survival [26]. Since BDNF is crucial for the development and maturation of reproductive cells, an adequate zinc supply may indirectly support BDNF-related reproductive functions. The interplay among BDNF, testosterone, and zinc highlights a multifaceted relationship that is crucial for maintaining reproductive health and overall well-being. BDNF influences testosterone production and spermatogenesis, while testosterone promotes the expression of BDNF in the brain. Zinc is essential for maintaining testosterone levels and may also interact with BDNF signaling pathways.

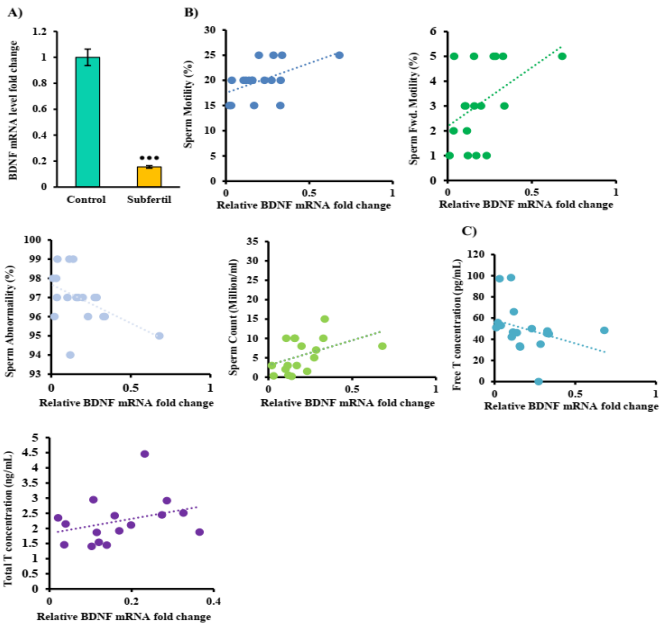

Figure 4. BDNF mRNA levels correlations with testosterone levels and sperm functions. (A) Illustrates the significant ($p \leq 0.001$) downregulation of BDNF mRNA levels in subfertile group compared to the control group using the Real-Time PCR technique. (B) Illustrates the significant ($p \leq 0.05$) positive relationship between downregulated BDNF mRNA level and reduced sperm motility (%), Fwd. motility (%) and count (million/mL), conversely, it showed the significant ($p \leq 0.05$) negatively correlation between downregulated BDNF mRNA level and high sperm abnormalities (%) in subfertility. (C) Represents the significant ($p \leq 0.05$) positively correlations between downregulated BDNF mRNA levels and low levels of total and free testosterone.

•••P value ≤ 0.001 was considered statistically significant compared to fertile (control) group.

3.4. Downregulated expression levels of anti-apoptotic gene BCL2 correlates positively with male subfertility

Studies have shown that there is a significant correlation between BCL2 expression levels and various parameters of sperm quality, including motility and morphology. Evaluating BCL2 can thus provide insights into the overall function of sperm and their ability to fertilize an oocyte. [12,27,28]. Understanding BCL2 expression in spermatozoa may help identify patients at risk of infertility due to increased apoptotic rates. Our data showed the significant ($p \leq 0.001$) downregulation of BCL2 mRNA levels in subfertile group (mean±SEM, 0.183± 0.03179) compared to the control group (mean± SEM, 1±0.0074) **(Figure 6A).** The Pearson correlation analysis showed the significant ($p \leq 0.01$) positively relationship ($r = 0.562$, 0.4707, 0.539) between downregulated BCL2 mRNA level and reduced sperm motility (%), Fwd. motility,

and count (million/mL), respectively. While demonstrating the significant ($p \leq 0.05$) negatively weak relationship ($r = -0.4236$) between downregulated BCL2 mRNA level and high sperm abnormalities (%) **(Figure 6B).**

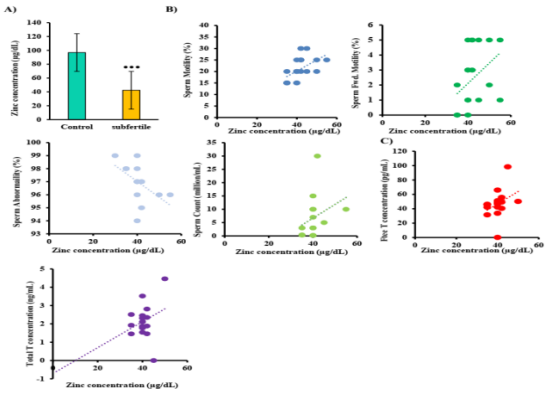

Figure 5. Zinc levels (µg/dL) correlations with testosterone levels and sperm functions. (A) Represents the significant ($p \leq 0.001$) decrease in zinc level (µg/dL) in subfertile group compared to the control group. (B) Represents the significant ($p \leq 0.05$) positively correlations between low levels of zinc and reduced sperm motility (%), Fwd. motility (%), and count (million/L), while illustrating the significant ($p \leq 0.05$) negatively correlation of low zinc levels with high sperm abnormalities (%) in subfertility. (C) Represents the significant ($p \leq 0.01$, 0.05) positively correlated of low levels of zinc with low levels of total and free testosterone.

•••P value ≤ 0.001 was considered statistically significant compared to fertile (control) group.

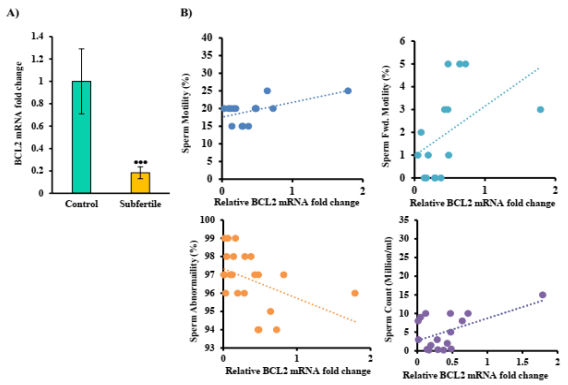

Figure 6. BCL2 mRNA levels correlations with sperm functions. (A) Represents the significant ($p \leq 0.001$) downregulation of BCL2 mRNA levels in subfertile group compared to the control group using the Real-Time PCR technique. (B) Represents the significant ($p \leq 0.01$) positive relationship between downregulated BCL2 mRNA level and reduced sperm motility (%), Fwd. motility (%) and count (million/mL), as well as, the significant ($p \leq 0.05$) negatively correlation between downregulated BDNF mRNA level and high sperm abnormalities (%) in subfertility.

3.5. Upregulation of IL6 mRNA and CRP levels enhances the inflammatory pathway and sperm dysfunction.

Inflammation plays a significant role in male reproductive health. Cytokines such as IL-6 are linked to inflammatory processes that can adversely affect fertility. Elevated IL6 levels may indicate chronic inflammation, which has been associated with impaired spermatogenesis and reduced sperm quality [29]. Likewise, CRP, an acute-phase protein, serves as a systemic marker of inflammation and has been linked to reduced fecundity in men [30]. Increased CRP levels indicate ongoing inflammation, which can interfere with the hypothalamic-pituitary-gonadal (HPG) axis. Specifically, inflammatory cytokines can lead to disruptions in the secretion of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), ultimately impacting testosterone production by Leydig cells in the testes [31]. Our results showed the significant ($p \leq 0.001$) upregulation of IL6 mRNA levels and c-reactive protein levels (mg/L) in the subfertile group (mean ± SEM, 21.261 ±1.805, 19.708 ± 0.3004, respectively) compared to the control group (mean± SEM, 1± 0.005, 3.183±0.174, respectively) (**Figure 7A & C**).

The correlations analysis showed a significant ($p \leq 0.05$) negative relationship ($r = -0.4154588$, -0.30314, -0.38987) between upregulated IL6 mRNA level and reduced sperm motility (%), Fwd. motility (%), and count (million/mL), respectively. While demonstrating the significant ($p \leq 0.05$) positive relationship ($r = 0.441$) between upregulated IL6 mRNA level and high sperm abnormalities (%) (**Figure 7B**). On the other hand, high levels of CRP significantly ($p \leq 0.05$) correlated negatively with reduced sperm motility (%), Fwd. motility (%) and count (million/L) ($r = -0.453$, -0.478, -0.476, respectively) and positively ($r = 0.325$) with high sperm abnormalities (%) (**Figure 7D**). The data showed the significant (($p \leq 0.001$) positive correlations ($r = 0.845$) between the upregulated expression level of IL6 and CRP levels (**Figure 7E**).

The intricate interplay between inflammatory markers, hormonal milieu, and male subfertility underscores the multifactorial nature of this complex condition **(Figure 8)** [32,33]. This intricate relationship presents both a challenge and an opportunity: while the complexity demands rigorous investigation, it also holds considerable promise for the development of innovative, targeted therapeutic interventions to address male reproductive health challenges [34]. A comprehensive understanding that integrates hormonal balance, inflammatory status, and cellular health at the molecular level is paramount for advancing effective diagnostic and therapeutic strategies.

The panorama sketch (**Figure 8**) offers an overview of the biological mechanisms associated with male infertility, emphasizing the role of Brain-Derived Neurotrophic Factor (BDNF) and its receptors, TrkB and p75, in normal male reproductive health. It highlights various genetic and non-genetic factors contributing to infertility and underscores the importance of nutritional assessments, particularly zinc, in improving sperm quality. The sketch illustrates the interplay between neurotrophic signaling, physiological development, and the multifaceted nature of male infertility, where genetic predispositions and environmental influences interact with

neurotrophic pathways. Ultimately, this analysis suggests that a deeper understanding of these factors can inform personalized treatment strategies to enhance reproductive health.

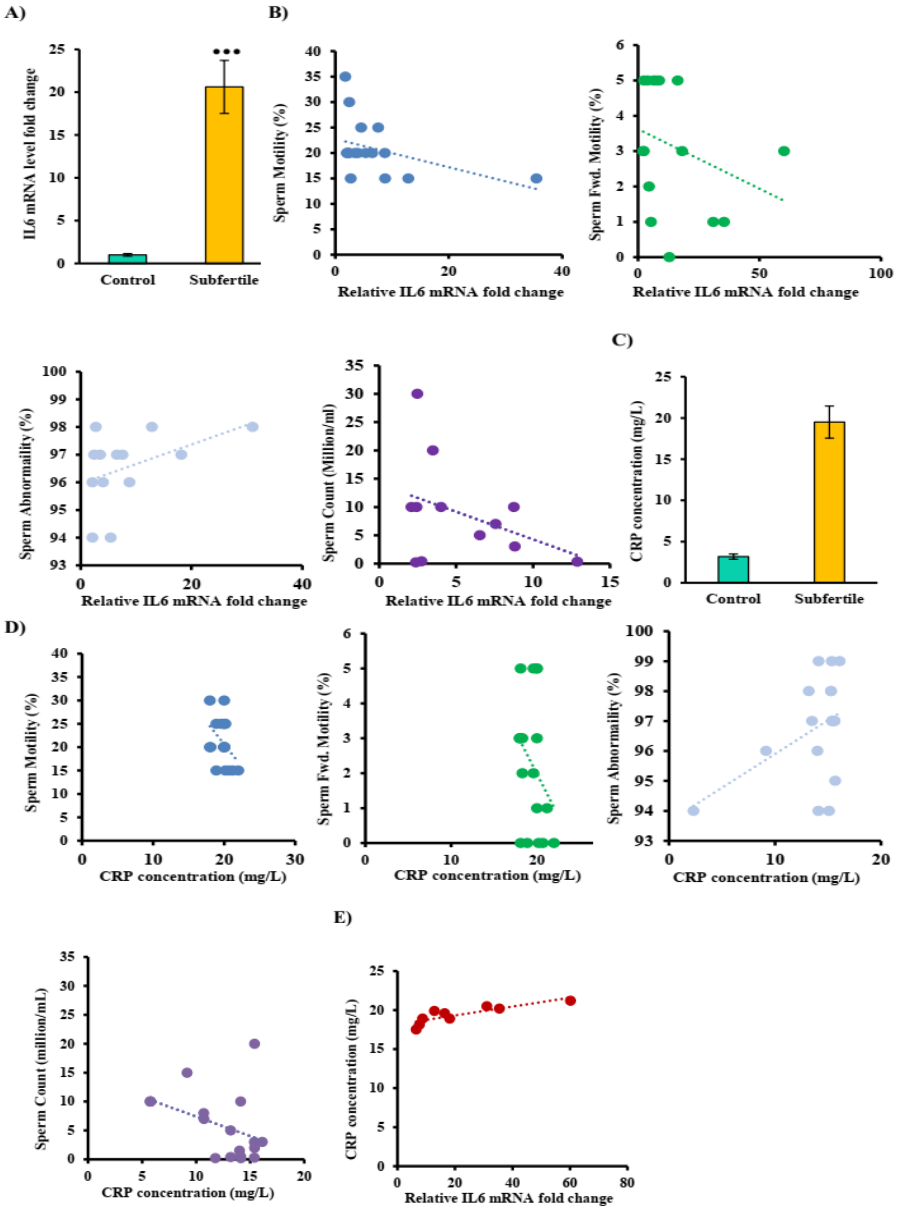

Figure 7. IL6 mRNA levels and CRP levels (mg/L) correlations with sperm functions. (A) Illustrates the significant ($p \leq 0.001$) upregulation of IL6 mRNA levels in subfertile group compared to the control group using Real-Time PCR. (B) Illustrates the significant ($p \leq 0.05$) negative relationship between upregulated IL6 mRNA level and reduced sperm motility (%), Fwd. motility (%) and count (million/mL), while showing the significant ($p \leq 0.05$) positively correlation between upregulated IL6 mRNA level and high sperm abnormalities (%) in subfertility. (C) Represents the significant ($p \leq 0.001$) increase in CRP level (mg/L) in subfertile group compared to the control group using the immunoturbidimetric technique. (D) Represents the significant ($p \leq 0.05$) negative correlations between high levels of CRP and reduced sperm motility (%), Fwd. motility (%), and count (million/L), while illustrating the significant ($p \leq 0.05$) positive correlation of high CRP levels with high sperm abnormalities (%) in subfertility. (E) Represents the significant ($p \leq 0.05$) positively correlated of increased mRNA levels of IL6 with high levels of CRP.

•••P value ≤ 0.001 was considered statistically significant compared to fertile (control) group.

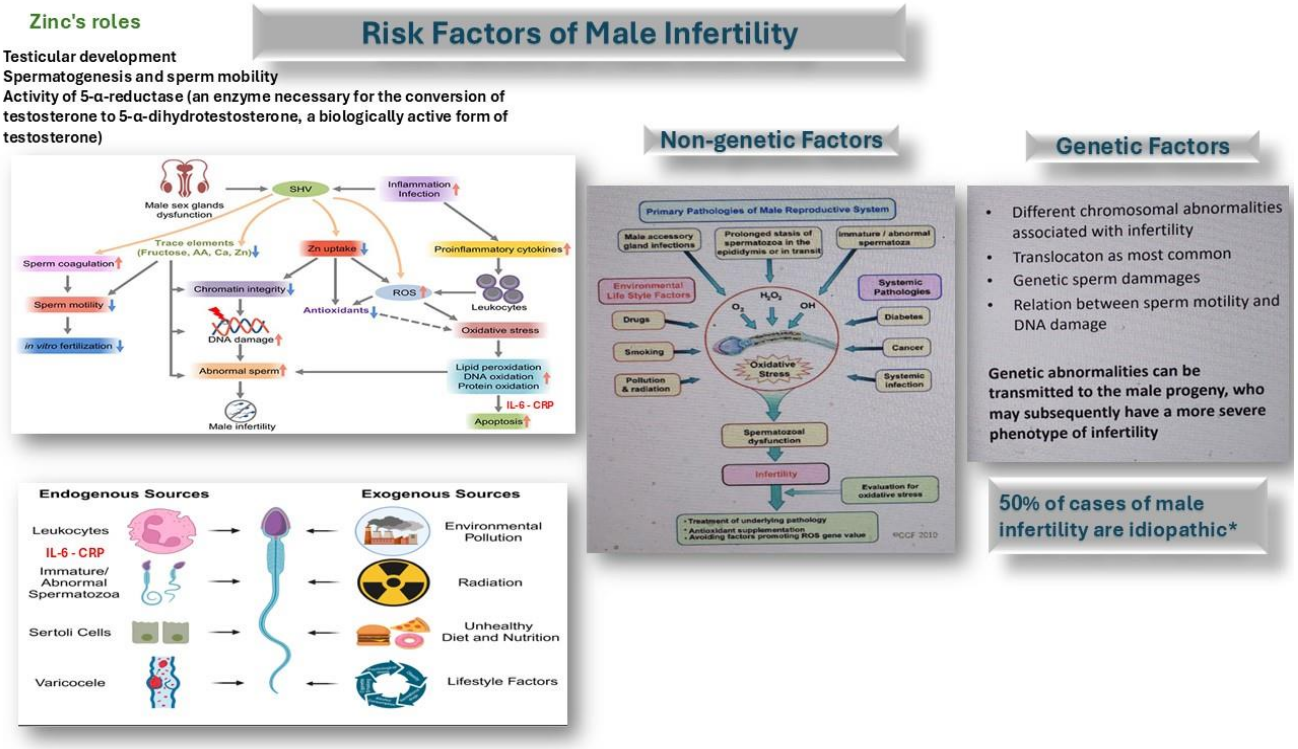

Figure 8. Panorama sketch provides a comprehensive overview of the complex biological mechanisms associated with male infertility. SHV= semen Hyperviscosity. In the male genitourinary system, BDNF and its receptors TrkB and p75 participate in normal physiological activities, such as the maturation and morphogenesis of testes and epididymis and the maintenance of isolated sperm motility. We reasonably speculate that the absence or low secretion of BDNF will lead to a disorder of the paracrine regulation between Sertoli cells and spermatogonial stem cells and the mechanism of sperm defense against oxidative stress, leading to oligospermia or weak sperm, which may be the cause of some male infertility. BDNF has protective effects against oxidative stress in spermatozoa and could improve sperm functions essential for sperm-egg fusion and subsequent fertilization.

4. Conclusion

In conclusion, our results showed a significant ($P \leq 0.001$) downregulation of BDNF mRNA levels in all subfertile patients, which was accompanied by a significant ($P \leq 0.05$) reduction in sperm functions including sperm motility and concentration, as well as an increase of sperm abnormalities. Our results indicated that hormones such as testosterone, prolactin, FSH, and LH are crucial in regulating male reproductive physiology. Testosterone is essential for spermatogenesis and the maintenance of male secondary sexual characteristics. C-reactive protein (CRP) and Interleukin-6 (IL6) are identified as critical biomarkers of inflammation in male subfertility. Elevated levels of these inflammatory mediators are associated with impaired spermatogenesis and decreased sperm quality and quantity. Our results also indicated a significant ($P \leq 0.001$) deficiency in zinc levels in subfertile patients. Moreover, it confirmed the positive correlation between zinc deficiency and low testosterone-level sperm dysfunctions. Understanding these interactions could lead to the development of targeted interventions that improve fertility outcomes for men facing subfertility. As research evolves, the potential for personalized treatments that consider these factors will likely enhance our approach to male reproductive health and fertility management. This comprehensive approach will ultimately lead to more effective and personalized interventions for men facing challenges with fertility, and effectively addressing male subfertility requires a holistic and integrative perspective considering the complex interplay of biological (genetic, hormonal, cellular), environmental (exposures to toxins, pollutants), and lifestyle factors. Continued rigorous research in this field, employing advanced omics technologies (genomics, proteomics, metabolomics) and sophisticated statistical modeling, is essential to elucidate the intricate interactions among these elements fully and to refine our diagnostic and therapeutic strategies for enhanced fertility management and improved reproductive health outcomes for men. This comprehensive approach will ultimately lead to more effective and personalized interventions for men facing challenges with fertility.

ART	Assisted Reproductive Technologies
BCL2	B-cell lymphoma 2
BDNF	Brain-Derived Neurotrophic Factor
CASA	Computer-Assisted Sperm Analyzer System
CIOMS	Council for International Organizations of Medical Sciences
CRP	C-reactive protein
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HPG	Hypothalamic-Pituitary-Gonadal Axis
IL6	Interleukin 6
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
PCOS	Polycystic Ovary Syndrome
PRL	Prolactin
RT	Room Temperature
T	Testosterone
TrkB	Tropomyosin Receptor Kinase B
WHO	World Health Organization

Abbreviations

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