Detection of Sperm DNA Integrity and Some Immunological Aspects in Infertile Males

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Abstract—Immunoinfertility caused by anti-sperm antibodies (ASAs) represents about 10–20% of infertility among couples, which interfere with sperm motility and ability to penetrate cervical mucus, sperm-oocyte binding, fertilization, and embryo development. In addition, deoxyribonucleic acid (DNA) damages are increasingly found with infertile cases affecting male reproduction potency and progeny. This study aims to assess the semen, presence of ASAs, and DNA fragmentation index in normozoospermic patients. A total number of 116 cases with an average age of 20–51 years old, and duration of infertility at 4.70 ± 2.77 are classified into 77 and 39 primary and secondary types of infertility, respectively. Mixed agglutination reaction test was used to estimate the ASAs in semen (direct method) and in seminal plasma and blood serum (indirect method), for both immunoglobulins IgG and IgA. Acridine orange test was used to detect DNA fragmentation index. The results showed a significant difference ($P > 0.05$) for those with a secondary type of infertility at means 24.37 and 31.48 for IgG, and 14.46 ± 1.76 and 6.86 ± 0.39 for IgA by both direct and indirect methods, respectively. The direct method showed a significant difference only for the sperm tail, while that for indirect method was in sperm mid-piece. The mean of DFI for all cases was 38.25 ± 2.08, at 41.61 ± 2.19 and 31.63 ± 4.29, for both primary and secondary cases, respectively. The percentage of ASAs revealed no significant difference with DFI, except in some parts of sperm.

Index Terms—Acridine orange test; Anti-sperm antibodies; Deoxyribonucleic acid fragmentation index; Mixed agglutination reaction.

I. Introduction

Sertoli cells forming tight junctions of the blood-testis barrier provide immunologic protection from sperm antigens. The immune response that spermatozoon evokes when exposed to the systemic immune defense system once the barrier is disrupted leads to anti-sperm antibodies (ASAs). However, the presence of naturally occurring ASA is a well-known cause of infertility in men and women, but the antigens for these antibodies are poorly characterized. Antibodies attached to the sperm impair their motility or recognition, and their ability for bounding with the ovum, or might lead to sperm destruction female reproductive tract immune system (El-Sherbiny, et al., 2021). In addition, the detected levels of ASAs within semen samples from infertile men had been associated with specific male genital tract pathology (e.g., testicular trauma, surgery and torsion). Sperm ASAs believed to have an adverse impact on male fertility through two mechanisms of action:

1. Directly interfering with sperm surface interactions (e.g., fertilization) and
2. Indirectly by mediating the release of cytokines that can impair sperm function, possibly including deoxyribonucleic acid (DNA) integrity (Muscianisi, et al., 2021).

DNA within the sperm head characterized by its hypercondensation core due to histones partially being replaced by the protamines during spermiogenesis. The protamines were positively charged DNA proteins that compacted the sperm nucleus into a hydrodynamic form that allowed sperm to move and penetrate egg membranes. There is clinical evidence that damage to sperm DNA integrity of the infertile men results in impaired embryo development and pregnancy in mice and humans, sperm; has shown an elevation in DNA damage aneuploidy, and other genetic abnormalities. Morphological defects in DNA integrity and chromatin organization have been associated with increased DNA fragmentation (Zhang, et al., 2022).

A. The Spermatozoa

Germ cells of the male go on meiosis to begin their multifarious alteration into spermatozoa, highly specialized cells by the initiation of puberty. Spermatogenesis needs 24 days (Houda, et al., 2021). Each ejaculation of fertile men

typically contains from 15 million sperm to over 200 million sperm per milliliter (ml) of semen which is considered a normal sperm count, only one normal sperm precisely enters an ovum. Sperm may live only 44 h in the female genital system. The spermatozoa are characterized by its polarized cell composed of a head and tail or flagellum. The head and tail are covered by a sperm plasma membrane, head contains a nucleus that holds the male genome, in which the DNA condensing core and linker histones has been partially replaced during spermiogenesis by protamines, and the acrosome, a large secretory granule that is oriented over the anterior area of the sperm nucleus, stores enzymes needed to penetrate the egg. In the middle piece, there are mitochondria which are spirally arranged around the first portion of the tail and are responsible for oxidative phosphorylation and provide much of the energy. The first part of the tail is consist of the flagella axoneme that contains nine courses (longitudinally arranged) in the peripheral of two central tubules; the peripheral tubules are arranged as groups of double tubules (Houda, et al., 2021).

B. Immunological Aspects of Spermatozoa

The risk factors of ASA development are conditions that may disrupt the blood-testis barrier (Maverakis, Moudgil and Sercarz, 2006). Obstruction of the ductal system is associated with the development of ASAs. After vasectomy, approximately 60% of men develop ASAs. Whereas approximately one-third of patients with congenital bilateral absence of vas deferens are found to have ASAs. Thus, most studies have not found testicular torsion to be a risk factor for the presence of ASAs. Conflicting data also exist for cryptorchidism, varicoceles, and testicular biopsy abnormal postcoital tests, particularly when immotile sperm with a shaking motion is noticed, are highly suggestive of the presence of ASAs. Couples with unexplained infertility as well as cases with impaired sperm motility or sperm agglutination have also been to have a higher incidence of ASAs (Rose, 2008; Tahiat, et al., 2021).

Formation of immune markers

There are many assays for investigating the presence of ASAs, for example, the direct assays detect the presence of ASAs on the patient's sperm, and indirect assays measure ASAs in the patient's serum and generally require ASA-negative donor sperm. Because it is the sperm that reaches the female reproductive tract, not serum, direct assays have the advantage of only detecting sperm-bound immunoglobulin (Ig). The presence of ASAs in the serum is not always associated with the presence of these antibodies on sperm. In addition, IgM class antibodies that may be present in serum do not usually make it to the semen. However, the immunobead assay and the mixed agglutination reaction are commonly used for the detection of ASAs. These assays utilize synthetic beads or red blood cells that will bind to antibodies bound to the sperm surface. Scoring is based on the percentage of motile sperm with bead or red blood cell binding (Abu-Raya, et al., 2020).

C. Sperm DNA Chromatin Structure and Fertility

Sperm DNA damage may lead to or considered the cause of infertility elsewhere negative impact on male fertility potential. It may be initiated by a multifactorial etiology such as drugs, chemotherapy, radiation therapy, smoking, and environmental toxins, genital tract inflammation, testicular hyperthermia, varicoceles, and hormonal factors (Selvam, et al., 2021; Mateo-Otero, et al., 2022). Also from paternal DNA damage as well as maternal DNA damage, the fertilization and subsequent embryonic development afflict. The tests for spermatozoon DNA integrity appears to be a threshold of sperm DNA damage beyond which embryo development and pregnancy are impaired, and studies have shown that the spermatozoa of infertile men possess more DNA damage than, and impaired pre-implantation development, increased abortion, and an increased incidence of disease in the offspring, including childhood cancer, with a high percentage of spermatozoa with DNA damage, have a reduced potential for natural fertility (Liu, et al., 2022).

II. Materials and Methods

A. Methodology

The study was carried out at Kirkuk Private Laboratory, Kirkuk, Iraq. This study included two aspects, the first one is the immunological aspect, through which some immunological aspects were measured, which included ASAs (IgA and IgG) in semen and blood serum using agglutination reaction (direct and indirect methods). The second is the molecular aspect, including determination of the integrity of sperm DNA. The DNA fragmentation index (DFI) was also estimated using mixed acridine orange test (AOT).

III. Results

A. Descriptive Parameters and Semen Analysis

The studies involved 116 normozoospermic male cases and were distributed into two groups, primary and secondary, according to the type of infertility. The primary group included 77 individuals with primary infertility representing (66.38%) of total cases, whereas, 39 cases represent (33.62 %) of total cases as the secondary infertility type. The semen of all cases was analyzed to evaluate the sperm integrity based on a number of parameters (Björndahl and Brown, 2022) (Table I). Sperm parameters for a primary and secondary

TABLE I
SPERM PARAMETERS FOR ALL SUBJECTS ACCORDING TO THE WHO CRITERIA (BJÖRNDAHL AND BROWN, 2022)

Parameters	Subjects*	Björndahl and Brown, 2022 reference values
Sperm concentration (million/mL)	46.80±0.6	≥15
Progressive sperm motility (%)	35.70±0.83	≥32%
Non-progressive sperm motility (%)	28.30±0.654	
Immotile sperm (%)	36.0±0.92	
Total progressive motility/*ejaculate*	39.91±1.93	>32
Normal sperm morphology (%)	31.291±0.86	≥4%

Data are Mean±S.E. (Cooper, et al., 2010), (Björndahl and Brown, 2022)

infertile patients were within normal values, except for sperm morphology which showed lower limits than those specified by the WHO (Björndahl and Brown, 2022) (Table II).

The mean age of the primary infertility was 34.84 ± 0.644 years, and their duration of infertility was 4.727 ± 0.29 years, at age category 20–48 years. The secondary type had a mean age of 35.82 ± 6.808 years, at 5.0 ± 0.497 years for infertility duration; with age category 23–51 years.

Sperm's concentration, progressive sperm motility, non-progressive sperm motility, total progressive sperm motility/ejaculate, and normal sperm morphology for 20–29 and 30–39 age groups were within normal values, according to the WHO criteria (Björndahl and Brown, 2022). However, normal sperm morphology for age group 40–49 years and percentage of progressive sperm motility and normal sperm morphology for age group $\geq$50 years were less than the lower limits of the WHO values (Björndahl and Brown, 2022) (Table III).

The mean of IgG percentage for 97 positive cases was 23.88 ± 1.75 detected by a direct method, showed no significant difference ($P < 0.05$) with the mean 27.41 ± 2.41 of 71 positive cases for IgG percentage that detected by indirect method (Fig. 1). For the primary infertility subjects, no significant difference ($P < 0.05$) was recorded between the direct and indirect methods of IgG detection (Fig. 2). Whereas a significant difference ($P < 0.05$) was achieved for those with the secondary type of infertility with both methods (Fig. 3).

Surprisingly, the percentage of IgA antibodies by the direct and indirect methods, for all subjects with primary and secondary infertility types showed significant correlation ($P > 0.05$) (Figs. 4-6). Fig. 7 shows an image of positive react sperm with latex particles coated with monoclonal ASAs.

TABLE II
SPERM PARAMETERS FOR PRIMARY AND SECONDARY TYPES OF INFERTILITY

Parameter	Type of infertility		(Björndahl and Brown, 2022) reference values
	Primary*	Secondary**	
Sperm concentration (million/mL)	46.766ª±2.10	44.153ª±2.76	≥15
Progressive sperm motility (%)	36.026ª±0.95	34.692ª±1.63	≥32 (%)
Non-progressive sperm motility (%)	29.402ª±0.75	26.769ᵇ±1.25	
Immotile sperm (%)	34.428ᵇ±0.95	38.641ᵇ±1.86	
Total progressive motility/ *ejaculate*	40.081ª±2.40	36.511ª±3.19	>32
Normal sperm morphology (%)	31.974ª±1.04	29.437ª±1.57	≥4%

(Mean±SE), *no. of primary infertile men=77. **No. of secondary infertile subjects=39. Different superscripts within each row are significantly different ($P<0.05$). Same superscripts within each row are non-significantly different ($P>0.05$)

IV. DISCUSSION

Sperm's normal chromatin structure is essential for the correct transmission of paternal genetic information; as there is a negative correlation between defective sperm chromatin structure (DNA breaks) and fertility, a lower rate of implantation, a higher rate of abortion, and illnesses in offspring that can be explained as multifactorial oxidative stress (Selvam, et al., 2021).

Different results for Igs in several studies, as well as the present study, might be due to different subclasses of Igs (Shibahara, et al., 2021). The genital tract secretions that included IgA have various molecular properties from those of IgA in other body fluids. Containing polymeric (p), dimeric, and tetrameric IgA reflect the dominance of S-IgA, with J chain and other secretory components (SCs) are essential during the selective transepithelial transport of pIgA; and mIgA is present in low quantities. The male and female genital secretions contained relatively similar properties of typical S-IgA, pIgA, and mIgA. The IgA1 and IgA2 subclasses are present in genital secretions in proportions differ from other body fluids (Lavelle and Ward, 2021), whereas in semen, IgA1 percentage dominates and was detected in similar levels in the serum (Woof and Mestecky, 2015). The findings showed no significant difference between the direct and indirect techniques for detecting both classes of IgA according to their distributions on sperm parts in all subjects, including the primary and secondary types of infertility, which have been confirmed by previous studies. ASAs affect virtually all components of sperm, diminished sperm-oocyte binding, faulty zona pellucida penetration cervical mucus penetration, and sperm survival (Silva, Ramalho-Santos and Amaral, 2021). Furthermore, the ASAs linked to abnormal embryo development by retarding the cleavage process, blocking the initiation of the embryo, and most significantly affecting fertilization rates when localized both at the head and the tail tip levels of sperm. Previous data showed an effect of ASAs on actual DNA lack of the sperm cell. In the present study, the IgG and IgA antibodies were detected by the direct method according to their bounding with the sperm head and exhibited no significant difference for all samples and those of secondary type infertility (Chereshnev, et al., 2021). Nevertheless, significant correlations were noticed between the two classes of antibodies, IgG and IgA, which detected bounding to the sperm heads in subjects with primary type infertility (Ata, et al., 2021; Audu, et al., 2021). However, bounded IgG and IgA antibodies to the sperm head directly may inhibit fertilization. It is found that the presence of ASAs has negative effects on sperm-oocyte

TABLE III
SPERM PARAMETERS ACCORDING TO THE AGE GROUP

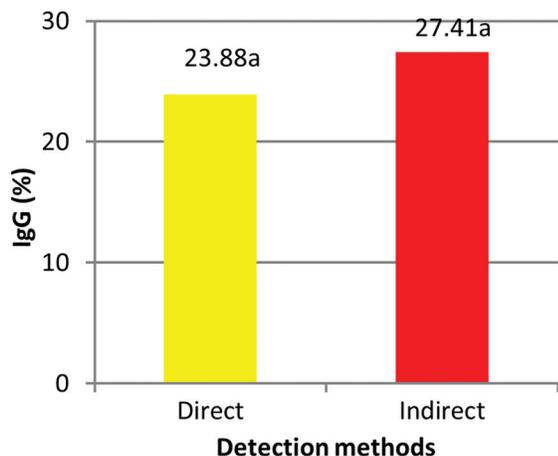| Age groups (years)/sperm parameters	20–29	30–39	40–49	≥50	Björndahl and Brown, 2022
Sperm concentration (million/mL)	39.882±4.03	50.4533±2.18	40.727±2.80	35.50±9.50	≥15
Progressive sperm motility (%)	34.079±2.80	37.035±0.99	33.181±1.34	26.50±1.55	≥32%
Non-progressive sperm motility (%)	30.173±1.81	27.120±0.78	31.41±1.38	27.50±1.00	
Immotile (%)	35.748±2.79	35.845±1.18	35.409±1.51	46.0±1.00	
Total progressive motility/ejaculate	36.444±4.66	43.678±2.60	30.870±2.22	26.89±2.03	>32
Normal morphology (%)	30.823±2.60	32.442±1.06	29.300±1.48	14.00±1.00	≥4%

Fig. 1. Means of IgG by direct and indirect detection methods for primary and secondary subjects. Same superscripts above the column indicate non-significant difference ($P < 0.05$). No. of positive cases using direct method = 97. No. of positive cases using indirect method = 71.
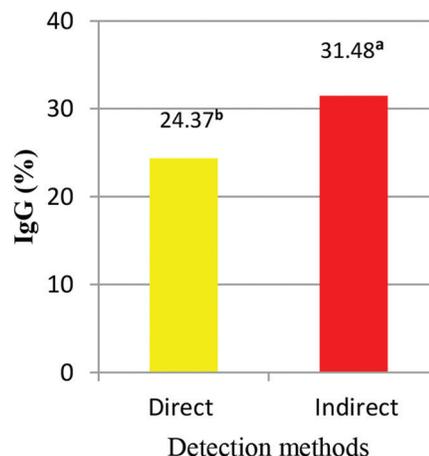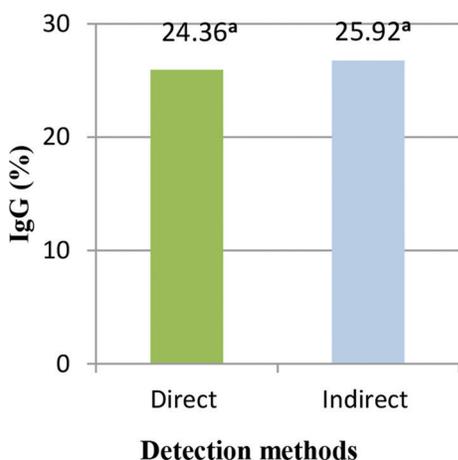

Fig. 3. The IgG means detected by direct and indirect methods for secondary infertility subjects. Different superscripts within the column are significantly different ($P < 0.05$). No. of positive cases using direct method = 27. No. of positive cases using indirect method = 19.

Fig. 2. The IgG means detected by direct and indirect methods for primary infertility cases. Same superscript above the bars indicates non-significant difference ($P < 0.05$). No. of positive cases using direct method = 70. No. of positive cases using indirect method = 57.
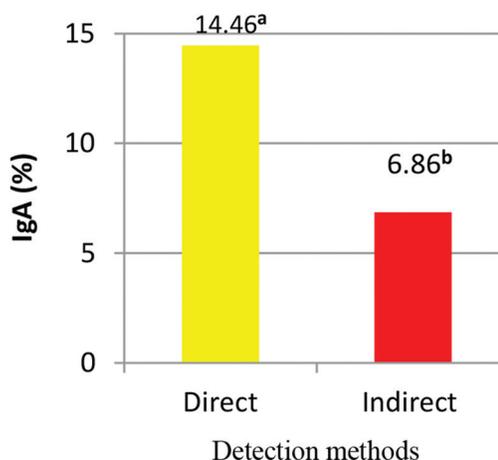

Fig. 4. The IgA means detected by direct and indirect methods for primary and secondary subjects. Different superscripts above the column indicate no significant ($P < 0.05$). No. of positive cases using direct method = 88. No. of positive cases using indirect method = 27.

binding, penetration, fertilization, and post-fertilization events (Vickram, et al., 2019). IgA isotype of ASA demonstrated the lowest pregnancy rate. Moreover, the antibodies directed on sperm mid-piece show significance in percentage in all subjects and those with primary and secondary types of infertility that disagreed with a previous study. Whereas only subjects with secondary type infertility showed a significant differences as related to detected IgG and IgA by indirect methods were bounded to the sperm tail, all subjects and those with primary type showed no significant difference (Barbonetti, et al., 2019).

The results showed significant differences between detected IgG in serum and the round cells count. Furthermore, the IgG bound the sperm mid-piece had a highly significant correlation with the round cells. Regarding the DFI and IgA, there was a significant correlation, because ASAs may promote the release of cytokines and may be associated with increased semen leukocyte concentration (Tennakoon,

Yasawardene and Weerasekera, 2012). As related to the sperm parameters of subjects with primary infertility and those with secondary infertility, many studies reported large and overlapping differences, whereas this study showed no significant difference in sperm parameters in exception to the non-progressive sperm motility, which had a significant difference between the two types of infertility that agreed by other studies. The studied cases with primary type of infertility showed an increasing level of DNA fragmentation compared to other cases with a secondary type of infertility which are in agreement with similar studies and reported that the incidence of sperm DNA damage is higher in primary infertile patients who may harm fertility potential (Garolla, et al., 2021). All outcomes of involved cases and those with primary type infertility indicated a significant difference between the IgG and IgA detected by a direct method according to sperm tail bound. These findings were in disagreement with a study that investigated the impact of different Ig classes based on their
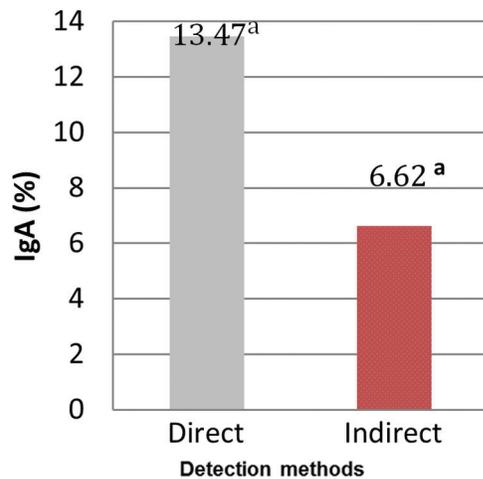
Fig. 5. The IgA means detected by direct and in direct method for primary infertility subjects. Same script above the bars indicates no significant ($P < 0.05$). No. of positive cases using direct method = 64. No. of positive cases using indirect method = 21.
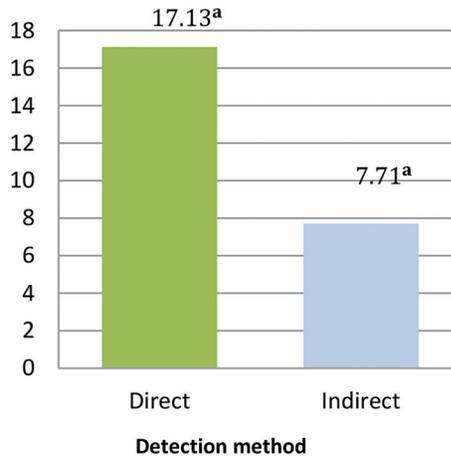

Fig. 6. The IgA means detected by direct and indirect method for secondary infertility subjects. Same script above the bars indicates no significant ($P < 0.05$). No. of positive cases using direct method = 24. No. of positive cases using indirect method = 6.
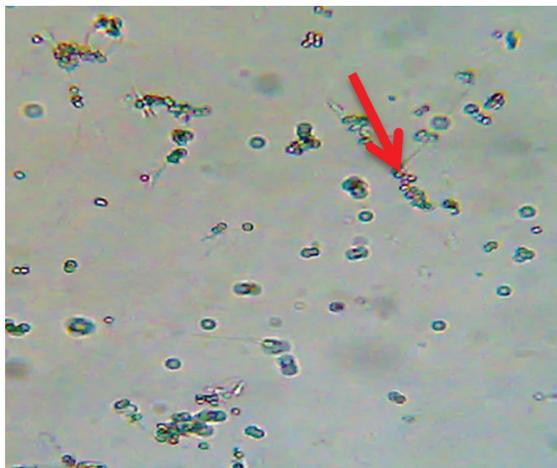

Fig. 7. Spermatozoa attached to latex particles (red arrow) ×60.

bounded site. The concentrations of IgA and IgG in semen (detected by direct method) were not correlated; however, the results were in contradiction with the fact that IgA may be because of its secretory origin. Other studies found that prostate and vesicle infection and subclinical reproductive tract infection might lead to dysfunction of sperm and changes in semen parameters, and the latter lead to infertility (Tennakoon, Yasawardene and Weerasekera, 2012). Some possible mechanisms of the development of infertility are linked either to inhibition of spermatogenesis resulting from testicular damage or an autoimmune process. The IgA detected in seminal plasma (indirect method) and according to its bound with sperm (mid-piece and tail) had a significant correlation with round cells count. Whereas the direct method of IgA detection showed a significant correlation between the bounding site of sperm (head and tail) and the count of round cells. The detected IgG by an indirect method that is bound to the mid-piece and tail showed a significant difference with normal sperm morphology but had no significant difference with the direct method. Whereas IgA detection by indirect method bound to the sperm head and mid-piece showed a significant difference compared to normal sperm morphology. On the other hand, no significant difference was recorded with the direct method that is bound to different sites of the sperm.

The origin of IgA present in the sperm and pre-ejaculate has yet to be confirmed. However, it appears that both local syntheses, primarily in the penile urethra and circulation, contribute to the Ig pool in these fluids, based on the molecular characteristics (Chantler, Sharma and Sharman, 1989; Sadecki, et al., 2022). The sperm parameters such as total progressive sperm motility/ejaculate and round cells count were significantly correlated with DFI that was agreed by a previous study, whereas the other sperm parameters such as normal sperm morphology and the sperm count did not correlate with DFI. According to the age category, the studied cases demonstrated lower records of sperm morphology in the age group 40–49 and ≥50 years. These outcomes were in agreement with several investigation studies of aging effect on fertility (Ahmed, et al., 2019; Silva, Ramalho-Santos and Amaral, 2021). In addition, the progressive sperm motility for the age category ≥50 years was below the reference value of the WHO (Björndahl and Brown, 2022) that was in agreement with similar studies (Stewart and Kim, 2011). No correlation was found between DFI and IgG detected by both direct and indirect methods, which are compatible with the results of another study (Mateo-Otero, et al., 2022). As related to correlation within sperm parts, however; significant results were recorded that were agreed by several studies investigating the correlation between ASA presence and DNA damages (Selvam, et al., 2021). Conflicting evidence was observed on the relationship between the presence of ASAs and sperm characteristics including concentration, motility, and morphology in the sperm (Dacheux and Dacheux, 2014). The majority of published studies found no link between the presence of ASAs and sperm concentration or motility and morphology (Chereshnev, et al., 2021). There were

significant correlations between the sites of sperm to which the IgA bound detected in semen plasma indirectly and on the sperm directly with DNA fragmentation.

V. Conclusions

The findings of the study led to the following conclusion:
1. The ASAs interfere in different manners with sperm parameters
2. The ASAs detected indirectly in seminal plasma (IgA) class and in serum (IgG) have strong correlation with total progressive sperm motility and with round cells counts
3. Sperm parts (head, mid-piece, and tail) attached to the different ASAs class correlate and with sperm parameters in different manners, by which showed no significant difference except for the sperm tail. However, the indirect method for ASAs showed a significant difference in sperm mid-piece
4. There was no correlation between the levels of the ASA and the percentage of DFI, except in some parts of sperm.

VI. Acknowledgment

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