EVALUATION OF SEMINAL FLUID PARAMETERS AFTER IN VITRO SPERM PREPARATION TECHNIQUE IN NON VARICOCELIC AND VARICOCELIC INFERTILE MEN UNDERGOING VARICOCELECTOMY

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ABSTRACT:

This study was designed to evaluate and compare the results of semen parameters, sperm membrane potential integrity and viability for non varicocelic and varicocelic infertile men after varicocelectomy. Thirty semen samples (varicocelic) and twenty normozoospermic men (non varicocelic) as a control group were collected by masturbation in the special semen room collection and prepared by simple layering technique. The semen samples were analyzed and prepared by standard semen parameters. Furthermore, sperm concentration, sperm motility, progressive sperm motility, sperm agglutination, normal sperm morphology, sperm HOST, and sperm Eosin stain were evaluated according to standard WHO criteria (1999). However, direct immunobead assay was used to determine the presence of AS-AB bound on sperm surface. For preparation technique, sperm prepared and incubated for 30 minute in 5% CO2 at 37ºC after in vitro sperm processing. The results of the present study indicate a highly significant ($P<0.001$) differences in all sperm functions parameters of varicocelic infertile men in AS-ABs positive (HOST and Eosin stain; negative) as compare with that noticed in normozoospermic men in AS-AB negative (HOST and Eosin stain; positive). It was concluded that there are a strong positive correlation between varicocele and antisperm antibodies generation and between antisperm antibodies and semen characteristics and HOST-Vitality test. Further studies are recommended to assess the detrimental effect of AS-ABs on DNA damage and embryo quality after in vitro fertilization and embryo transfer in assisted reproductive technologies (IVF-ET-ART).

Key words: Male infertility, Varicocele, Antisperm antibody, Varicocelectomy

INTRODUCTION

Infertility is defined as the inability of couples to achieve pregnancy following one year of unprotected intercourse. By this criterion, infertility affects 13%-18% of couples and male factors account for up to the half of all cases (1). One of male infertility causes is varicocele which is present in 2%-22% of the adult male population (2). Furthermore; varicoceles are the pathological dilatation of venous pampiniform plexus of the spermatic cord and occur more frequently on the left side (3). There are three accepted theories on the causes of varicoceles: First; there are the anatomical differences between the left and right testicular vein; especially that right testicular vein inserts directly into interior vena cava, while the left testicular vein inserts into the left renal vein. The

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different insertion of the left testicular vein is believed to results in an increase in the hydrostatic pressure which is subsequently transmitted to the pampiniform plexus; Second that there is an absence of the competent venous valves resulting in the reflux of venous blood (4). In men with abnormal semen analysis, the prevalence of varicocele reached 25% (5). However; cases of the varicocele have been linked to a serious of events such as: biochemical changes in the epididymal fluid, a stasis of the internal spermatic vein, elevated scrotal temperature, testicular hypoxia, and the retrograde blood flow of renal and adrenal metabolites (6). The varicocelic patient's testis undoubtedly suffers deleterious tissue effect, which sometimes leads to complete atrophy; this may be attributed to prolonged venous stasis and hyperthermia. This tissue injury may induce unshielding of the protected spermatozoa, an antisperm immunologic response and antibody formation (7). As well as, varicocelectomy has been noted to result in an improvement of semen quality in 50%-80% of the cases; pregnancy rates of about 50% have been reported (8). In general, 20%-50% of the patients do not appear to improve significantly after operation. In general, varicocele is thought to be the most common treatable cause of male infertility (9). The some investigators found that varicocele was associated with increased incidence of antisperm antibodies which may be responsible for varicocele-associated infertility (10). Ozen et al. (11) reported that 16 (22.5%) of 71 men with a palpable unilateral varicocele had antisperm antibodies, while Golomb et al. (12) reported that 29 (91%) of 22 men with palpable varicocele were found to have antisperm antibodies by an enzyme-linked immunosorbent assay (ELISA). The increasing number of men showing poor semen quality encouraged the development of a wide array of different laboratory techniques focusing on selection and enrichment of motile and functionally competent spermatozoa from ejaculate (13).The methods were developed to improve sperm functions like motility, protected sperm functions, and reduced detrimental effects from environmental setting like reactive oxygen species (14). The sperm functions parameters results after direct swim-up technique strongly correlated to predict embryo cleavage. Furthermore, it was reported that common laboratory factors like centrifugation, washing, and temperature fluctuation of human spermatozoa both positively and negatively due to direct influence of the laboratory interventions on the cytoskeleton assemblies of sperm function particularly in assisted reproductive technologies (15).

2. Materials & Methods

2.1. Subjects and semen collection

Fifty infertile couples were enrolled in this study and semen samples were obtained from Al-Hussein Teaching Hospital /Thi-qar health directorate/laboratories section. The mean age of infertile subjects was 31.35±0.66 years old with range from 18-49 years and duration of infertility was 5.66±0.33 years with range from 2-16 years. The semen samples were collected by masturbation after 3-5 days abstinence and allow liquefying at 37ºC in 5% CO_2 for 30 minutes and evaluated before and after *in vitro* sperm preparation. Sperm function tests including sperm concentration, sperm motility, progressive sperm motility, sperm agglutination, normal sperm morphology, sperm membrane integrity, sperm Eosin staining, and antisperm antibodies assay were evaluated according to WHO criteria.

2.2. Semen preparation techniques

2.2.1. Conventional layering technique (CLT)

The semen was prepared by using 1ml of prepared culture medium was added to test tube, and then 1ml of liquefied semen was layered beneath a culture medium. After incubation for 30 minute in 5% CO_2 at 37ºC, 10µl. of the mixture was aspirated by pasture pipette and examined under light microscope at 400X magnification for assessment parameters of sperm functions in infertile patients.

2.2.2. Centrifugation swim-up technique (CSUT)

One of the two portions of liquefied semen (1ml) was diluted and mixed gently with (1ml) of culture medium by a Pasteur pipette for a several times and run in a centrifuge at 2250 rpm for 6 minute. Then after supernatant was discarded and 1ml of culture medium was added to pellet with care and again put in the incubators for 30 minute. Then, a drop (10µl.) was taken and put on a slide and cover with a cover slip and examined at a microscope under 400X objective for assessment of sperm functions.

2.2.3. Hypo-osmotic swelling test (HOST)

The HOS test was performed after examination of standard semen parameters by mixing 0.1 ml of semen with 1.0 ml of a 150 mOsm/ kg NaCl as a hypo-osmotic solution. The mixture was incubated for 30 minute at 37 ºC in 5% CO_2. Then, 10 µl of the mixture was placed on a slide and mounted with a cover and examined immediately at a magnification of 40X objective under a light microscope. A total of 100 spermatozoa were counted in at least ten different fields, and sperm tails were classified into seven distinct subtype of coiling in various regions. The percentage of HOS reacted spermatozoa (with coiled and swollen tail) and non-reacted spermatozoa (with straight or non swollen tails) were calculated.

2.2.4. Antisperm antibodies assay (ASAs)

A direct immunobead test IBT (Polyacrylamide; BioRad Laboratories, Richmond, CA) was performed for each semen samples and prepared with covalently bound rabbit antibody to human IgG and IgA were used for the performance of the immunobead test. The percentage of sperm with ASA was noted. Therefore, the washed sperm were mixed with IgG or IgA beads and read microscopically for the percentage and attachment sites of sperm binding to the head. However, At least three beads had to be attached to be considered positive. A level of $\geq$ 50% was considered positive and $\geq$ 20% to 49% weakly positive.

2.2.5. Statistical Analysis

Statistical analysis was performed with the SPSS version 12.00 by Statistical Package for Social Sciences Software. The data analysis was done using paired sample t-test to assess statistical differences in results of SFTs. Mean and standard error of mean (S.E.M) obtained from crude data to compare between seminal fluid analysis parameters. P-value < 0.05 was used as a level of statistically significant.

3. Results

The results of present study indicate that after sperm processing with conventional layering and centrifugation swim-up technique; sperm concentration and sperm agglutination were significantly ($P<0.001$) decreased as compared to the pre-processing, while sperm motility (%), progressive sperm motility (%), normal sperm morphology (%), sperm HOST and Eosin staining test were significantly ($P<0.001$) increased post-*in vitro* sperm processing as compared to the pre-processing. Also, the percentage of HOST score in antisperm antibodies positive

(68.20 ± 6.3) with varicocele in (CLT) compared with (60.12 ± 7.3) in (CSUT) sperm samples was significantly lower than that noticed in antisperm antibodies negative (72.23 ± 5.3) in (CLT) non varicocele compared with (66.10 ± 6.2) in (CSUT) sperm samples (P<000.1). While, results of Eosin stain for sperm viability significantly correlated with the results of sperm plasma membrane integrity (HOST) from towards the increase and decrease values.

DISCUSSION

The superlative improvement in sperm function parameters was achieved by using simple layer technique as compared to centrifugation swim-up techniques. The selection of sperm preparation methods depend on quality of ejaculates. The ejaculates with ROS production by spermatozoa and leukocytes should not be separated by centrifugation method due to the severely damage to the spermatozoa (22). When semen samples prepared by centrifugation, functional spermatozoa can come into close cell-to-cell contact with defective sperm, leukocytes, and cell debris contained by centrifugation force causing massive oxidative damages of sperm plasma membrane via produce very high levels of ROS by pelleting of semen with impairment of sperm functions and decrease in normally chromatin-condensed spermatozoa (23). It was reported that common laboratory factors like centrifugation, washing, temperature fluctuation, and processing delay harmfully affect response pattern of human spermatozoa both positively and negatively due to the direct influence of laboratory interventions on cytoskeleton assemblies (24). The markedly reduction in sperm concentration and sperm agglutination was observed following both sperm processing techniques. These results may be due to beneficial effect of preparation technique by removal of dead, immotile spermatozoa, and semen debris in such away only superior quality motile spermatozoa were harvested and unfortunate quality spermatozoa (25). The results indicate that sperm agglutination is not specifically immune reaction by generation of AS-Abs in varicocelic infertile compared with non varicocelic men; or it may be due to cytotoxic materials which secreted from the inflammatory cells which causes clumping and agglutination due to presence of antisperm antibodies. In addition, sperm agglutination either specific or non specific causes sperm clustering which prevent the sperm motility and activity (26). The percentages of sperm motility, progressive sperm motility, and normal sperm morphology, sperm agglutination, sperm HOST, and sperm Eosin test were significantly increased after sperm processing. Really, the enhanced sperm functions were a normal response for sperm biology after removal of seminal plasma and sperm agglutination by sperm preparation techniques (27). However, spermatozoa are particularly susceptible to the damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (28). There are numerous detailed reports on human antisperm antibodies and interference of some of them with reproductive processes. It is supposed that binding of antisperm antibodies to sperm surface inhibits sperm function and fertilization and the presence of circulating antisperm antibodies in serum of women has been implicated as a contributing factor to infertility. In these studies, the incidence of subsequent

pregnancy in infertile couples was reduced significantly if one or both partners had antisperm antibodies in serum or in genital tract secretions (29). According to other reports, the prevalence of ASA positive cases in men and women with unexplained infertility was significantly more than cases with explained infertility (30). The concentrations of IgA and IgG in semen were not correlated but there was a strong tendency for them to be directed against the same region of the spermatozoa encouraging the belief that they may be directed against similar antigens. This would account for their similar effects on IVF rates. Although it is too early to employ determination of antibody specificity as a diagnostic tool, in the long term it may be the only way to offer an accurate prognosis to patients with antisperm antibodies (31). The direct immunobead test may offer a more accurate picture of distribution of antibody on the sperm surface and might supply a more accurate prognosis than the indirect test applied here. However, it can only be used for samples which contain sufficient motile spermatozoa which restricts its application in clinical practice (32). The prostate and vesicle infection and subclinical reproductive tract infection may lead to dysfunction of sperm and changes in semen parameters, and the latter may consequently lead to infertility. Some possible pathophysiological mechanisms of the development of infertility are linked either to inhibition of the spermatogenesis resulting from testicular damage or the autoimmune process (33). The prevalence of ASAs among infertile males with varicocele was found to be lower than males without varicocele. These results postulated that the presence of ASA is of a little relevance in varicocele associated infertility. However, data about influence of varicocele on ASAs formation are contradictory. However, the surgical correction of varicocele did show significant differences in semen parameters in men with or without ASA (34). Theoretically, in patients with varicocele, the testes suffers deleterious tissue effect which sometimes leads to a complete atrophy that attributed to the prolonged venous stasis and hyperthermia and might induce antibody formation. In addition, reduced Ecadherin and α-catenin expression at the junctions between adjacent Sertoli cells in varicocele cases might also lead to a disruption of blood-testis barrier and the production of antibodies (35). Furthermore, local ASAs stimulate interferon γ production, which plays, a role in enhancing directly phagocytic cells to produce hydrolyase, lipase, and esterase and indirectly by proteins phosphorelation through activation of certain enzymes such as protein kinase (36). Varicocele may play a role in pathogenesis of male infertility patients. However, tolerance to self-antigens is developed during embryonic and early fetal life. The seminal antigens are not present in organism during that time and therefore these antigens will be handled by the organism as foreign (37). In general, 20%- 50 of patients do not appear to improve significantly after the operation, in order to avoid antibody production against these substances, organism develops two mechanisms, one of which is blood-testis barrier and other involves an immunosuppressive action of semen (38). The poor semen parameters, elevated level of ASAs, and infertility in men are linked with history of cryptorchism, orchitis, varicocoele, epididymitis and accidental or surgical trauma of male genital tract (39). However, only a few patients have no clear etiologic

factor for ASAs and infertility, although ASA may form as a result of exposure of sperm antigens to the rectal mucosa, and they have been detected in the sera of a high percentage of homosexual men (40).

Table (1): In vitro sperm processing undergo sperm preparation technique in varicocelic patients with antisperm antibodies (AS-ABs) positive and (HOST-Eosin) Negative

Parameters	Conventional layering technique		Centrifugation swim-up technique	
	Pre-processed	Post-processed	Pre-processed	Post-processed
Sperm Concentration ($\times 10^6$ sperm/ml)	40.35±6.31	22.75±3.65 [a]	41.90±3.24	27.55±3.12 [a]
Sperm Motility (%)	51.00±2.55	76.60±2.07 [a]	54.00±2.42	79.25±2.15 [a]
Progressive sperm Motility (%)	31.90±1.66	54.85±1.43 [a]	37.00±1.93	58.35±2.20 [a]
Sperm Agglutination (%)	12.50±2.40	0.00±0.00 [a]	14.00±2.13	0.00±0.00 [a]
Normal Sperm Morphology (%)	44.50±3.20	80.25±2.09 [a]	49.50±2.53	83.25±2.06 [a]
Sperm HOST (%)	44.23 ± 8.2	68.20 ± 6.3 [a]	42.20 ± 7.1	60.12 ± 7.3 [a]
Sperm Eosin (%)	45.20 ± 6.3	52.33 ± 5.1 [a]	42.22 ± 5.3	54.10 ± 5.2 [a]
AS-Abs assay by IBT	54.21± 5.2(+)	41.10± 5.3(+)	52.13± 5.1(+)	40.20± 4.2(+)

Values are Mean ± S.E.M

a: means a highly significance (P<0.001) different from pre-treatment with glutathione

No. of infertile patients=30 for both layering and centrifugation technique

Mean of age ± S.E.M for infertile subjects prepare with simple layering technique (30.05 ± 4.87 years)

Mean of age ± S.E.M for infertile subjects prepare with centrifugation technique (31.75 ± 6.10 years)

Table (2): In vitro sperm processing undergo sperm preparation technique in non varicocelic patients with antisperm antibodies (AS-ABs) Negative and (HOST-Eosin) positive

Parameters	Conventional layering technique		Centrifugation swim-up technique	
	Pre-processed	Post-processed	Pre-processed	Post-processed
Sperm Concentration ($\times 10^6$ sperm/ml)	40.35±6.31	22.75±3.65 [a]	41.90±3.24	27.55±3.12 [a]
Sperm Motility (%)	51.00±2.55	76.60±2.07 [a]	54.00±2.42	79.25±2.15 [a]
Progressive sperm Motility (%)	31.90±1.66	54.85±1.43 [a]	37.00±1.93	58.35±2.20 [a]
Sperm Agglutination (%)	12.50±2.40	0.00±0.00 [a]	14.00±2.13	0.00±0.00 [a]
Normal Sperm Morphology (%)	44.50±3.20	80.25±2.09 [a]	49.50±2.53	83.25±2.06 [a]
Sperm HOST (%)	57.23 ± 7.1	72.23 ± 5.3 [a]	51.20 ± 6.2	66.10 ± 6.2 [a]
Sperm Eosin (%)	52.20 ± 4.3	62.31 ± 5.1 [a]	50.20 ± 5.3	52.10 ± 5.1 [a]
AS-Abs assay by IBT	34.20± 3.2(-)	22.12± 2.1(-)	32.10± 3.2(-)	21.00± 2.3(-)

Values are Mean ± S.E.M

a: means a highly significance (P<0.001) different from pre-treatment with glutathione

No. of infertile patients=30 for both layering and centrifugation technique

Mean of age ± S.E.M for infertile subjects prepare with simple layering technique (30.05 ± 4.87 years)

Mean of age ± S.E.M for infertile subjects prepare with centrifugation technique (31.75 ± 6.10 years)

Figure (1): In vitro sperm processing by (CLT) in varicocelic patients with antisperm antibodies (AS-ABs) Negative and (HOST-Eosin) positive.

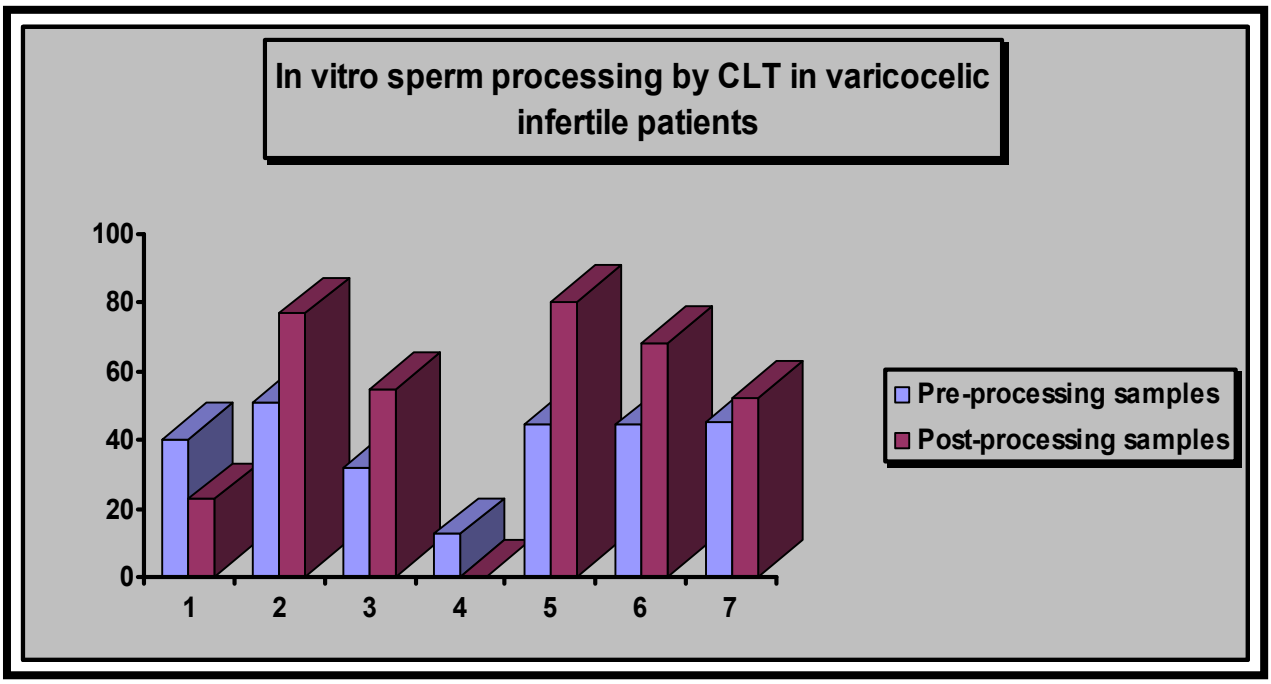


Figure (2): In vitro sperm processing by (CSUT) in varicocelic patients with antisperm antibodies (AS-ABs) Negative and (HOST-Eosin) positive.

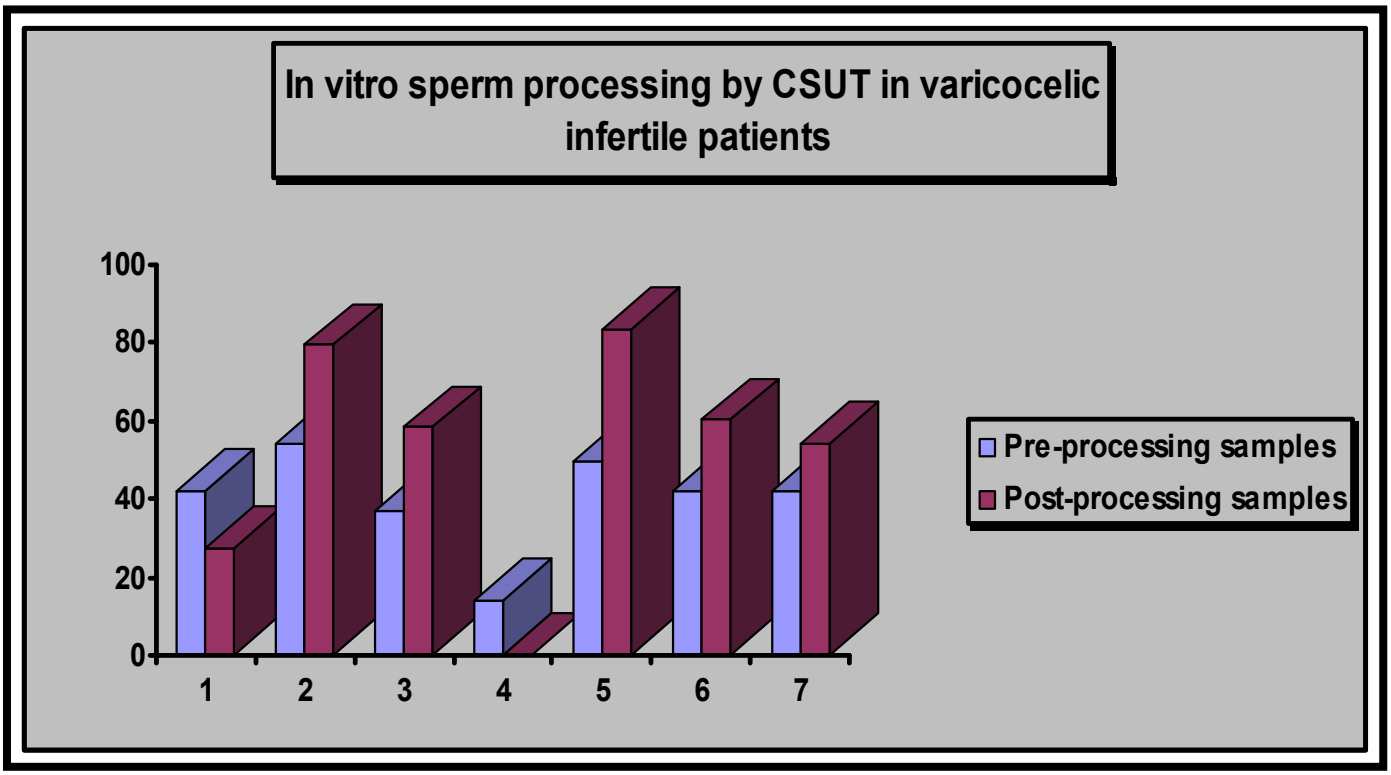


Figure (3): In vitro sperm processing by (CLT) in non varicocelic patients with antisperm antibodies (AS-ABs) Negative and (HOST-Eosin) positive.

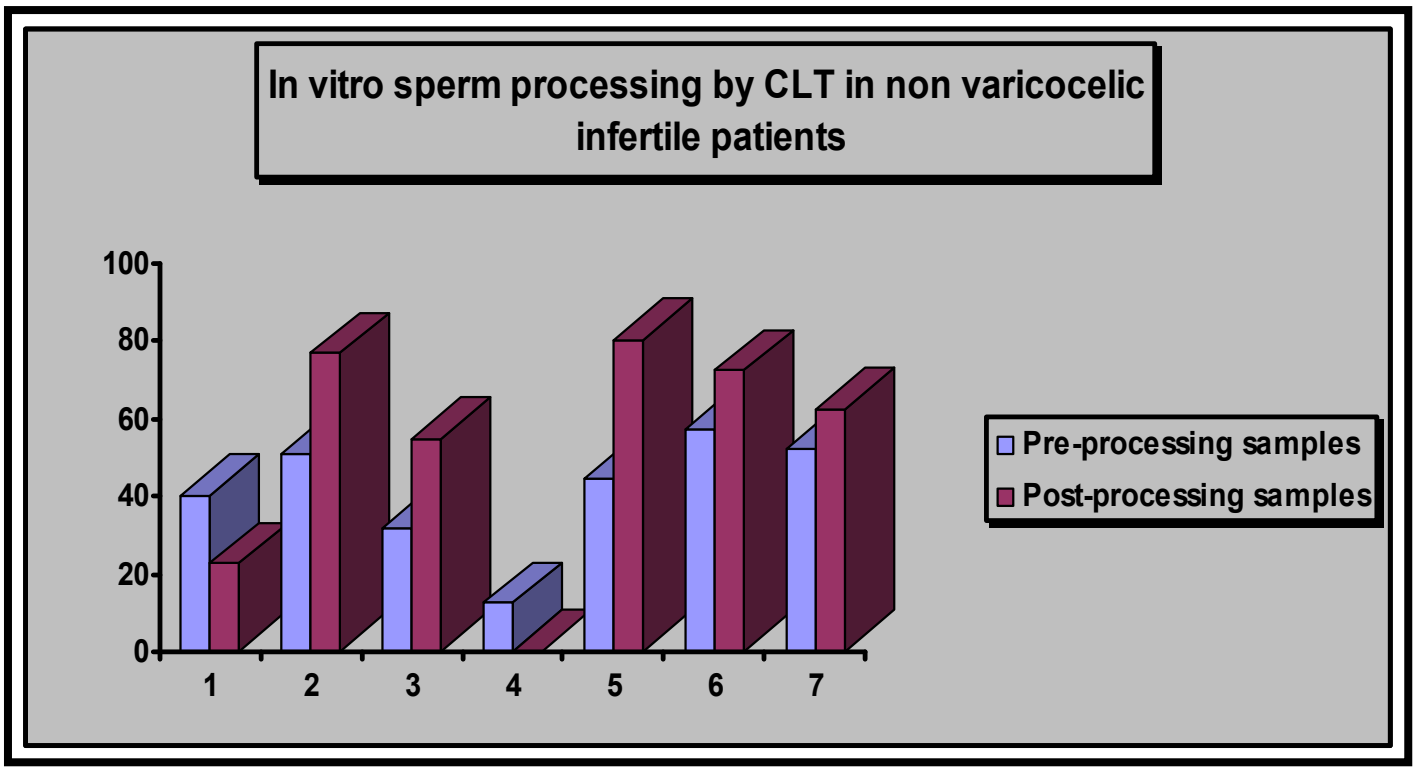
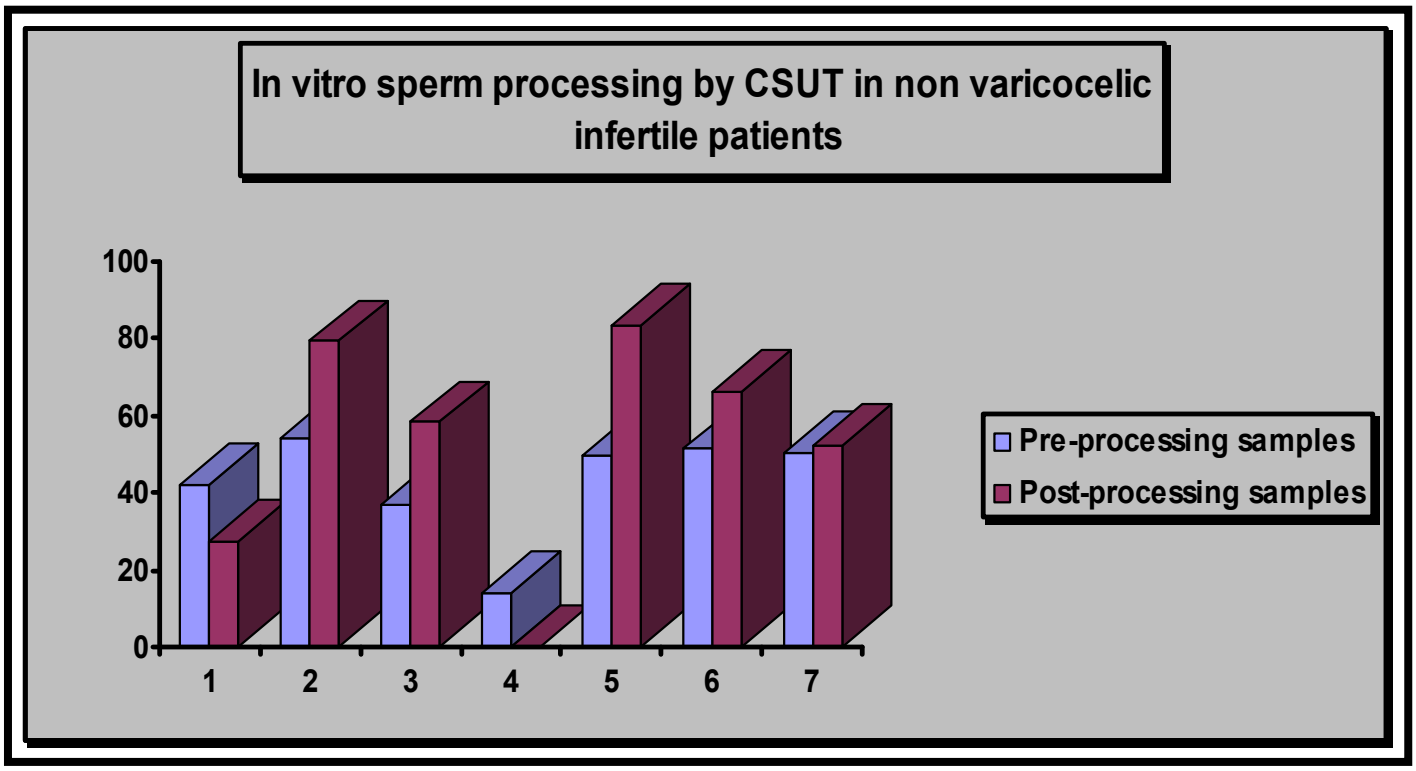


Figure (4): In vitro sperm processing by (CLT) in varicocelic patients with antisperm antibodies (AS-ABs) Negative and (HOST-Eosin) positive.



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تقييم متغيرات السائل المنوي بعد اجراء تقنيات تحضير النطف البشرية لمرضى العقم المصابين وغير المصابين بدوالي الخصية بعد اجراء التداخل الجراحي واستئصال الدوالي

باسم خميس كوتي*، ضياء عبد عودة جازع**

الخلاصة:

صممت الدراسة الحالية إلى تقييم ومقارنة نتائج متغيرات السائل المنوي وفحص كفاءة وحيوية النطف لمرضى العقم غير المصابين والمصابين بدوالي الخصية بعد اجراء التداخل الجراحي (استئصال الدوالي). تضمنت الدراسة (٣٠ عينة سائل منوي من مرضى مصابين بدوالي الخصية و٢٠ عينة سائل منوي من مرضى غير مصابين بدوالي الخصية تم اختيارهم كمجموعة سيطرة) وعينات السائل المنوي تم تحليلها قبل وبعد تحضير النطف بالطريقة التقليدية البسيطة وتم جمع العينات بواسطة الاستمناء في غرفة خاصة لجمع السائل المنوي.

أن فحوصات كفاءة النطف والتي تتضمن تركيز النطف، حركة النطف، الحركة التقدمية للنطف، تلازن النطف، النسبة المئوية للنطف السوية، فحص كفاءة الغشاء البلازمي للنطف،وفحص حيوية النطف تم تقييمها وفقاً الى مقررات منظمة الصحة العالمية(WHO1999) مع اجراء فحص الاجسام المناعية المضادة للنطف لغرض الكشف عن وجود تلك الاجسام على سطح النطفة حيث تم حضن النطف لفترة ٣٠ دقيقة في ٥% ثنائي أوكسيد الكاربون وبدرجة ٣٧ مْ. اظهرت النتائج فرقاً ($P<0.001$) معنوياً عالياً في معايير السائل المنوي لمرضى العقم غير المصابين بدوالي الخصية نتيجة عدم وجود الاجسام المضادة لديهم وكانت نتيجة الفحص سلبية (فحص كفاءة الغشاء البلازمي وحيوية النطف ايجابياً) مقارنة بمرضى دوالي الخصية حيث كانت نتيجة الفحص المناعي ايجابية (فحص كفاءة الغشاء البلازمي وحيوية النطف سلبياً). نستنتج من خلال الدراسة الحالية ان هناك علاقة معنوية عالية وقوية بين دوالي الخصية وعملية توليد الاجسام المناعية المضادة للنطف وبين الاجسام المناعية وفحص الغشاء البلازمي وحيوية النطف. نوصي في الدراسات المستقبلية بتقييم التاثير الضار للاجسام المضادة للنطف على الـ DNA ونوعية التطورات الجنينية بعد اجراء تقنيات الاخصاب الخارجي ونقل الاجنة.

مفتاح الكلمات: العقم الذكري، دوالي الخصية، الاجسام المناعية المضادة للنطف، استئصال الدوالي جراحياً

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