Correlation between *in vitro* preparation techniques and asthenozoospermic semen with bacteriospermia on intra-uterine insemination outcomes

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Abstract

Background: The sperm preparation techniques used for intrauterine insemination (IUI) and other reproductive techniques are heavily based on the sperm parameters and their survival. Furthermore, sperm preparation methods allow for the removal of immotile sperm, leukocytes, and immature germ cells from the inseminated volume.
Objectives: This study has been aimed to evaluate the effectiveness of in vitro preparation technique(s) for the elimination of bacteria from semen samples that will be used for IUI.
Subjects, Materials and Methods: Fifty one patients were enrolled for intra –uterine insemination who attended Infertility Clinics at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University during the period from November, 2021 till the end of May, 2022.The semen samples from husbands were divided into normozoospermia,and asthenozoospermia following semen fluid analysis according to (WHO ,2021 &2010). The semen samples were cultured under specific conditions using different type of media including (blood, MacConkey, chocolate and mannitol agar), to identify bacterial growth before and after in vitro preparation and activation using simple washing and direct swim up techniques for IUI .
Results: There was a highly significant different in progressive motile sperm (p=0.001) after in vitro sperm activation compared to before activation in infected normozoospermic and asthenozoospermic infertile men growth of pathogenic bacteria. The main bacterial growth recoded in this study was Streptococcus feacalis , Klebsiella, Staphylococcus aureus ,E coli. Two out of 51 couples were pregnant following IUI one of them from normozoospermic man (10.0%) and the other from asthenozoospermic man (5.5%) with total 3.9% pregnancy rate.
Conclusions: From practical point of view, Both preparation techniques prior to IUI leads to significant increases in certain sperm function parameters with significant bacterial reduction in most semen samples but not all semen bacterial clearance was obtained. The study recommended to culturing all semen samples not responded to the treatment.
Key words: Asthenozoospermia, IUI, in vitro preparation techniques , Bacterial growth.

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Introduction

Male infertility is a common and challenging condition that has a variety of etiologies, one of which is bacteriospermia. It is estimated that approximately 15% of all infertility cases are caused by uropathogen infections, and in most cases, bacteria are involved in infection and inflammation, leading to the development of bacteriospermia (Sharma et al.,2022). Excess leukocyte infiltration in the urogenital tract occurs in response to bacterial load, causing oxidative stress (OS). They may also cause sperm death by inducing apoptosis. Acute bacteriospermia is related to another clinical condition known as leukocytospermia, and both impair male fertility potential through OS-mediated sperm damage, resulting in male infertility (Sandipan et al.,2022).Some studies found that bacterial infection has a negative impact on the male urogenital tract and is clearly associated with poor semen quality (Yanic et al.,2022), whereas others found that the presence of bacteria has no effect on their parameters, indicating that no definite relationship between semen parameters and bacteriospermia has been established (Basima ,2019).On the other hand, intrauterine insemination (IUI) is the first-line fertility treatment modality in couples with unexplained or mild male subfertility, and sperm parameters have a significant effect on IUI success when different sperm preparation techniques have been described over time, and these techniques may eventually vary from laboratory to laboratory (Kim et al.,2017).The purpose of sperm preparation techniques is to maximize motile sperm concentration whilst also removing seminal plasma, debris, prostaglandins, and other potentially harmful substances that may cause uterine contractions and bacterial contamination (Nazli et al.,2021).Multiple washing and centrifugation steps are used in traditional sperm preparation methods. Direct swim-up (DSW), conventional swim-up (CSW), and density gradient method (DGC) are common methods used in laboratories. DGC is generally preferred when the sperm count is low. Swim-up techniques involve moving sperm from a pre-washed pellet into a different medium (Rappa et al.,2016)There are rare studies search on the semen bacterial growth before and after semen preparation and activation for IUI in Iraq ,therefore the study will determine the prevalence and type of bacteria present in semen of infertile men who are subjected for IUI procedure.Evaluate the effectiveness of in vitro preparation technique(s) for the elimination of bacteria from semen samples that will be used for IUI.

Patients, Materials and Methods

The current Cross-sectional study includes fiftyone infertile men who attended the Infertility Unit, High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University in Baghdad, during the period from 22 th November 2021 till 17 th May 2022. Twenty eight out of 51 couples were fit for Intra Uterine Insemination (IUI) enrolled in the study. The men were subjected to clinical examination by the andrologist in charge the male infertility Unit.

Inclusion criteria: Twenty eight men were divided into normozoospermia and , asthenozoospermia, following semen analysis according to WHO 2021 and 2010

Exclusion criteria: Patient with azoospermia and patient under treatment with antibiotics.

-Semen sample collection

According to WHO 2021: the sample was collected insterile wide-mouthed container that is made of glass or plastic from infertile men. The sample container was kept at surrounding temperature, between 25 °C and 37 °C, it was labelled with the patient's name then it was placed on the bench or in an incubator (37 °C) while the semen liquefies.After complete liquefaction, the semen analysis was performed by macroscopic and microscopic assessment according to standard criteria of WHO 2021 and 2010.If the sample was fit for Intra

Uterine Insemination (IUI) it was inoculated on different culture media for microinfection test such as (Blood agar, chocolate agar, MaConkey's agar and Mannitol agar) before and after preparation techniques.

-Semen culture

Semen samples were cultured before and after preparation technique respectively on different type of media including blood agar, chocolate agar, MacConkey agar and manitol agar (figure 3-3), then incubated under 37℃. Whereas cultivated chocolate agar, were incubated at 5% CO2 candle jar (McGowan et al.2008).Different methods were used to isolate and identify aerobic and anaerobic bacteria. Isolated colonies were identified by colony morphology, gram staining, and biochemical tests (Domes et al, 2012). The bacterial concentration of greater than 10^3 CFU/ml for certain pathogens and greater than 10^4 for occasional pathogens were considered as significant.

-Female investigation and ovulation induction

Stimulation with gonadotropins was began on cycle day 3 under the guidance of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies' gynecologist. Serial ultrasounds and blood estradiol levels were used to monitor treatment response. Ovulation was induced by recombinant human chorionic gonadotropin (Ovidrel®250 mg; Serono Laboratories, Inc., Norwell, MA) when at least one follicle measuring 16 mm developed. Intrauterine insemination was conducted 36 hours following the delivery of human chorionic gonadotropin, with a cleaned male partner. A pregnancy test was taken about 16 days following the IUI treatment, and a serum b-human chorionic gonadotropin level of more than 6 mIU/mL was declared positive.

- **In vitro sperm preparation and activation for IUI**

After the semen were collected and analysis they were prepared using two spermpreparation techniques namely; simple wash technique and direct swim-up technique and performed according to WHO 2021.Certain sperm function parameters were account and the prepared samples were used for IUI.

-Intra-uterine insemination procedure

In the first step of the procedure ,the cervix was visualized by using a sterile non lubricated Cusco's speculum. The next step, the catheter was introduced to the uterine cavity till it reach the fundus. After that the syringe containing 0.3-<1ml of a prepared activated sperm sample was pushed slowly to uterine cavity. Following 15 -30 mins the female was allowed to leave the theater. The next day of insemination ,the patient was instructed to use progesterone tablets (Duffaston® 10 mg/ day) for two weeks as a luteal phase support.

Results

Comparison the effect of microorganism growth on certain sperm function parameters of normozoospermia patients was demonstrated in table 1 and table 2, in infected normozooospermic infertile men growth of pathogenic bacteria ,there was a significant difference in all sperm parameters after *in vitro* activation that include sperm concentration (p=0.001), progressive motile sperm (p=0.001),non-progressive motile sperm (p=0.007),immotile sperm (p=0.002)and round cell mean is (p=0.001).However there morphologically normal sperm percentage was not shown a significant (p=0.525)difference after activation.

while whereas table 2 with no significant bacterial growth in uninfected normozoospermia infertile men there was significant difference in sperm concentration mean*(p=0.043)*, progressively motile sperm mean *(p=0.001)*, immotile sperm mean *(p=0.001)*as well as round cells mean *(p=0.002)* while no significant differences in sperm non- progressive motile sperm mean *(p=0.146)* andmorphologically normal sperm mean *(p=0.074)* .

Table 1: Comparison of certain sperm parameters between before and after activation in infected normozoospermic infertile men

Certain sperm function Parameters		Before activation (Mean ± SD)	After activation (Mean ± SD)	p value
Sperm concentration (x10^6/ml)		*44.29 ± 4.8*	*19.86 ± 4.3*	*< 0.001₸ S*
Sperm motility%	*Progressive motile %*	*48.57 ± 4.5*	*85.00 ± 4.1*	*< 0.001₸ S*
	Non progressively motile %	*18.57 ± 4.3*	*9.28 ± 3.2*	*0.007 ₸ S*
	Immotile sperm %	*32.86 ± 4.9*	*5.71 ± 1.3*	*0.002 ₸ S*
Morphologically Normal sperm %		*37.57 ± 4.3*	*42.29 ± 7.2*	*0.525 ₸ NS*
Round cells cell/HPF		*5.86 ± 1.1*	*1.43 ± 0.5*	*< 0.001 ₸ S*

SD: Standard deviation; NS: Not significant (p ≥ 0.05);S: Significant (p < 0.05) ;₸: Paired sample t test.

Table 2: Comparison of semen parameters between before and after activation in uninfected normozoospermic infertile men

Certain sperm function Parameters		Before activation (Mean ± SD)	After activation (Mean ± SD)	p value
Sperm concentration (x10^6/ml)		*45.57 ± 7.2*	*26.71 ± 4.7*	*0.043 ₸ S*
Sperm motility%	*Progressive motile %*	*39.29 ± 4.8*	*83.29 ± 9.6*	*< 0.001₸ S*
	Non progressively motile %	*22.57 ± 6.9*	*8.28 ± 1.3.8*	*0.146 ₸ NS*
	Immotile sperm %	*47.43 ± 6.1*	*9.01 ± 5.8*	*< 0.001₸ S*
Morphologically Normal sperm %		*35.14 ± 4.4*	*40.43 ± 6.8*	*0.074 ₸ NS*
Round cells cell/HPF		*4.29 ± 0.6*	*0.43 ± 0.3*	*0.002 ₸ S*

SD: Standard deviation; NS: Not significant (p ≥ 0.05) ;S: Significant (p < 0.05) ;₸: Paired sample t test.

The results in asthenozoospermic group were almost statistically similar to normozoospermic group as shown in table 3 .In infected asthenozoospermic infertile men growth of pathogenic bacteria, there was a significant difference in all certain sperm function parameters after *in vitro* activation which include sperm concentration *(*p=*0.001),*progressive motile (p=0.001),non-progressive motile sperm (p=0.003),immotile sperm (p=0.001)and round cells (p=0.001), except in morphologically normal sperm percentage that was no significantly difference(p=0.069).

While whereas in table -4 with non significant bacterial growth ,there was a significant difference in sperm concentration mean *(*p=*0.001)*, progressively motile sperm mean *(*p=*0.001)*, immotile sperm *(*p=*0.001)* as well as round cells mean *(*p=*0.005)* while no

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significant differences in ,non- progressive motile sperm mean *(*p*=0.315)* andmorphologically normal sperm *(*p= 0.443).

Table 3: Comparison of certain sperm function parameters between before and after activation in infected asthenozoospermic infertile men

Certain sperm function Parameters		**Before activation**	**After activation**	**p value**
Sperm concentration (x10^6/ml)		*26.79 ± 1.9*	*10.95 ± 1.2*	*< 0.001Ŧ S*
Sperm motility%	*Progressive motile %*	*24.42 ± 1.7*	*73.79 ± 5.7*	*< 0.001Ŧ S*
	Non progressively motile %	*20.11 ± 2.5*	*9.79 ± 1.9*	*0.003 Ŧ S*
	Immotile sperm %	*56.89 ± 2.4*	*16.31 ± 4.5*	*< 0.001Ŧ S*
Morphologically Normal sperm %		*33.42 ± 1.8*	*36.00 ± 2.3*	*0.069 Ŧ NS*
Round cells cell/HPF		*5.89 ± 0.9*	*2.32 ± 0.48*	*< 0.001Ŧ S*

SD: Standard deviation; NS: Not significant (p ≥ 0.05) ;S: Significant (p < 0.05) ;Ŧ: Paired sample t test.

Table 4 **Comparison of semen parameters between before and after activation in uninfected asthenozoospermic infertile men**

Certain sperm function Parameters		**Before activation (Mean ± SD)**	**After activation (Mean ± SD)**	**p value**
Sperm concentration (x10^6/ml)		*30.50 ± 4.3*	*12.58 ± 2.8*	*< 0.001Ŧ S*
Sperm motility%	*Progressive motile %*	*21.00 ± 2.4*	*66.17 ± 7.7*	*< 0.001Ŧ S*
	Non progressively motile %	*24.33 ± 3.4*	*18.91 ± 4.6*	*0.315 Ŧ NS*
	Immotile sperm %	*55.67 ± 2.9*	*18.41 ± 5.6*	*< 0.001Ŧ S*
Morphologically Normal sperm %		*37.50 ± 14.9*	*45.67 ± 28*	*0.443 Ŧ NS*
Round cells cell/HPF		*5.00 ± 1.5*	*1.17 ± 0.5*	*0.005 Ŧ S*

SD: Standard deviation; NS: Not significant (p ≥ 0.05) ;S: Significant (p < 0.05) ;Ŧ: Paired sample t test

Table 5 showed there was two ofnormozoospermic had a significant growth of Streptococcus feacalis and one of them had significant growth of E.coli whereas only one of infected normozoospermia infertile men with significant growth of Streptococcus feacalis after activation.

Table 5: Significant growth of pathogenic bacteria in semen of normozoospermic group before and after activation.

Type of bacteria	**Normozoospermic group**	
	Before activation	**After activation**

E.coli	*1 (10.0%)*	*0*
Streptococcus faecalis	*2 (20.0%)*	*1 (10.0%)*
klebsiella	*0*	*0*
Staphylococcus aureus	*0*	*0*

The type of significant growth of pathogenic bacteria in semen of asthenozoospermia group was presented in table 6 , the semen of two asthenozoospermic infertile men were found with significant growth of Streptococcus feacalis, E.coli , Klebsiella and one with staphylococcus aureus before activation , each one of the other three semen sample of asthenozoospermic infertile men was infected with significant growth of different pathogenic bacteria as the following (one with E.coli ,one with streptococcus faecalis, one with klebsiella and one with Staphylococcus aureus) after activation.

Table6: Significant growth of pathogenic bacteria in semen of asthenozoospermic group before and after activation.

Type of bacteria	**Asthenozoospermia group**	
	Before activation	**After activation**
E.coli	*2 (11.1%)*	*1 (5.5%)*
Streptococcus faecalis	*2 (11.1%)*	*1 (5.5%)*
klebsiella	*2 (11.1%)*	*1 (5.5%)*
Staphylococcus aureus	*1(5.5%)*	*1(5.5%)*

Out of 51 infertile men the partner of only one of each normozoospermia 1 (10.0%) and asthenozoospermia 1 (5.5%) infertile men were getting pregnancy with pregnancy rate$\left(\cdot\frac{2}{51}\times 100\right)$*=3.94%*

Table7: Pregnancy rate of the studied groups

Male infertility factors Groups	**Positive pregnancy N. (%)**	**Negative pregnancy N. (%)**	**p value**
Normozoospermic group	*1 (10.0%)*	*9 (90.0%)*	*0.563 Č NS*
Asthenozoospermic group	*1 (5.5%)*	*17 (94.4%)*	

NS: Not significant (p > 0.05) ;Č:Chi square test.

Discussion

The data of present study revealed that there was a high significant decrease in sperm concentration, non-progressive motile sperm, immotile sperm and round cells after in vitro activation using (Flashing media) as compared to pre- activation. The study results are in agreement with the results reached by other studies (Al-Dujaily et al., 2019;Muhammed , 2015). This finding may beexplained logically due to inability of dead sperm and round cells to swim up and migrate into the upper layer of the culture media used in this study (Willem et al., 2014).Furthermore, the data of the present study found a high significant increase in the percentage of active motile sperm (progressive motility) and morphology, concentration in normozoospermic group and asthenozoospermic group(Tables 1 4). Such result is regarded as a normal response for sperm activity after the removal of seminal plasmasince it contains dead sperm, leukocytes, epithelial cells, particular debris and microbial contamination.At the same time, the medium utilized included beneficial ions and amino acids that increased sperm motility (Al-Dujaily et al., 2019).However, it has been suggested that microbial contamination produces a large number of oxygen radicals, which might have a detrimental impact on sperm functions such as active sperm motility. Prolonged interaction of spermatozoa with seminal plasma may impair sperm activity and fertilization capacity (Reza et al, 2021).Interestingly, the results of this study supported prior findings from other studies that found that there was no influence of any type of microbe present in human sperm on sperm characteristics (Kenny, 2018). Other study claim that there is no differences in seminal fluid parameters in the presence of bacterial growth or not (Huang et al., 2015).Thus, the variability in results might be attributed to early seminal fluid infection in infertile men, which did not reveal the negative effects of bacterial growth and/or did not reach the bacterial growth colony the titer of toxic production (Berjis et al .2018).Naturally, a low percentage of sperm motility may be attributed to an increase in reactive oxygen species produced by white blood cells, as well as infected microorganisms that damage sperm (Berjis et al., 2018; Agarwal et al., 2018).In the present study, it was found that out of 28 infertile men only 10 (35.7%) had a positive culture for different species of pathogenic bacteria in semen samples and this is in agreement with the results reported by (Fatemeh et al., 2021). Numerous factors can contribute to male infertility, although the detrimental effects of bacterial infection on male infertility remain controversial. (Fatemeh et al.,2021), In the present study, the semen quality in subfertile men has been evaluated with the chance of presence of at least one abnormality in sperm parameters to detect the possible bacterial infection that lead to male infertility. As the results showed, it is clear that Streptococcus is the most common isolated bacteria in studied samples matching 40% other detected bacteria include E.coli (30%) , Klebsiella (20%) and Staphylococcus aureus (10%) .Bacteriospermia affects semen parameters, sperm DNA integrity, and ROS production in the semen of subfertile and infertile men controversially. According to several studies, bacterial infection has a detrimental effect on the male urogenital tract (Rusz et al.,2012, ;Zhang et al.,2011). Many studies have found that bacteriospermia is significantly correlated to sperm parameters such as sperm count, motility, and morphology (Isaiah et al.,2011; Fatemeh et al.,2021) which have been demonstrated in the current study. Many studies have found that E. coli attachment to various sperm function parameters such as sperm count, motility (both in vitro and in vivo), sperm morphological abnormalities, and sperm agglutination (Fraczek et al., 2014; Isaiah et al.,2011;Kaur and Prabha,2014). This negative association between sperm quality and bacterial infection might be attributed to increased ROS production associated with inflammatory processes in semen infections as the study of (JE et al.,2005) demonstrated that enhanced ROS production led to impairment of sperm DNA and male infertility.

In the current study, the results of pregnancy rates after intra-uterine insemination in normozoospermic group and patients with asthenozoospermic revealed highly no significant differences. Out of 28 infertile men the partner of only one of each normozoospermia and asthenozoospermia with not significantinfection get pregnant with 3.9% pregnancy rate. In fact, It has been found that in vitro activation techniques were widely used in the treatment of infertility patients (Al-Dujaily and Abo-Regheef 2013).The success rate of IUI is determined by many factors such as the ovulation induction medicine used for stimulation, the triggering time, the number of cycles and the total number of motile sperms after washing various in sperm quality and quantity, activation methods, patient selection, various infertility factors, and others (Fatemeh et al.,2021; Al-Dujaily and Abo-Regheef 2013). Moreover, sperm parameters of male partners used in IUI are generally predictive factors that increase the likelihood of conception.A previous study revealed that when the amount of washed motile sperm is inadequate, the number of sperms inserted into the uterus decreases. The number of motile sperm, progressive motility, and sperm count were significantly higher in both pre- and post-activation IUI outcomes (Mohsin, 2019).From these results, it is clear that the procedure that had been chosen, direct swim up, produced a lower yield of spermatozoa than other procedures. As a result, it should be noticed that this process selects sperm for motility and is useful when the percentage of motile spermatozoa in sperm is low, which is suitable for IVF and ICSI procedures. (WHO, 2021).Consequently, it has been postulated that bacteria in semen samples have negative effects on sperm parameters and negatively influencing fertility status and worsening reproductive potential and both preparation technique prior to IUI leads to significant increases in certain sperm function parameters with significant bacterial reduction in most semen samples but not all clearance was achieved.

Conclusions: From practical point of view, Both preparation techniques prior to IUI leads to significant increases in certain sperm function parameters with significant bacterial reduction in most semen samples but not all semen bacterial clearance was obtained. The study recommended to culturing all semen samples not responded to the treatment.

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