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RESEARCH ARTICLE

COMPARISON OF ZETA POTENTIAL AND HA-BINDING (PICSI) METHOD IN THE SELECTION OF MATURE, GOOD QUALITY SPERM

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ABSTRACT

This study was aimed to compare two sperm preparation techniques zeta potential vs. HA based PICSI in terms of motility, morphology and DNA fragmentation. Study was performed in 42 semen samples from men with suspected infertility. Both techniques were used in each sample and evaluated % of motility, % of morphology and % of DNA fragmentation. Mean % of motility, morphology and DNA fragmentation were 73.31, 78.44, and 75.91 with standard deviation of 6.35, 7.31, and 9.21 respectively with Zeta potential. The PICSI yielded the values of 78.88, 78.77 and 80.2 with standard deviation of 5.19, 6.74, and 8.42 respectively. Wilcoxon signed ranked test showed significant difference in the results of motility, morphology and DNA fragmentation with p-values less than 0.05. The Zeta potential was found to be better in terms of DNA fragmentation but PICSI yielded better results in motility and morphology. This study results showed that Zeta potential is an effective method to select mature sperms with acceptable motility, morphology and less DNA fragmentation. So Zeta potential is comparable to PICSI and it is less time consuming, economic and can be used for all normal semen samples especially with suspected high oxidative stress.

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INTRODUCTION

Male infertility refers to a male's inability to cause pregnancy in a fertile female. In human it accounts for 40-50 % of infertility (Brugh and Lipshultz, 2004; Hirsh, 2003; Farhi *et al*., 2008). Current routine sperm selection procedures or techniques are not adequate in the selection of sperms on the basis of necessary quality parameters which includes motility, morphology, viability, DNA integrity, apoptosis and maturity. Since the sperm carrying all the genetic information for the future developments, more care must be taken in the selection of sperms with high DNA integrity. Several advanced sperm selection methods like, Magnetic activated cell sorting (MACS), electrophoresis- surface charge method, Zeta potential method, and Hyaluronic acid (HA) binding method were introduced to improve the quality of embryo and ICSI outcome (Ralf, 2012; Nasr *et al*., 2012; Morrell and Rodriguez, 2011; Ebner *et al*., 2012). These methods aim at isolating mature, structurally intact, non apoptotic spermatozoa with high DNA integrity. Among these methods, Zeta potential and HA binding methods shows significant improvement in ICSI outcome (Tamer and Said, 2011; Nasr-Esfahani, 2011; Kheirollahi-Kouhestani, 2009; Parmegiani, 2010; Worrilow *et al*., 2013). By using these two methods in the selection of mature sperms with low DNA fragmentation, controversial study reports were published (Zahedi *et al*, 2013; Razavi *et al*., 2010). This study was aimed to compare Zeta potential method and HA bounded method (PICSI- Preselected intra cytoplasmic sperm injection) method in the selection of mature sperms with motility, normal morphology and low DNA fragmentation.

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MATERIALS AND METHODS

A. Design of study and sampling method

This study was conducted at Al-Baraka fertility hospital, Bahrain during the period of Jan 2014 to May 2014. This study was carried out in 42 infertile patients with a history of recurrent abortions and unknown infertility. Ethical committee approval was taken before starting the sample collection for the study. Semen samples were collected after taking the informed

consent of the patients. Initially samples were analyzed for routine parameters and divided into two- One for Zeta potential method and second for PICSI method.

B. Sperm selection methods and procedure

The sperm selection methods and procedure were as follows:

(i) Zeta potential method

The Zeta potential method of sperm selection is based on the negative electro kinetic potential of the sperm. For this study, the Zeta potential method was carried out as per the procedure described by Chan *et al*. (2006). Immediately after semen liquefaction, the sample was washed in a sterile centrifuged tube with media containing serum. The supernatant was then discarded so that the tube was only with the minimum amount of serum containing medium. After this the pellet was mixed with 1 ml of serum free media. The medium was then exposed to a positive surface charge as follows: the tube was placed inside a latex glove up to the cap. The tube was then grasped by the cap, rotated two or three turns and rapidly removed from the glove. Each tube was kept at room temperature for 1 min to allow adherence of the charged sperm to the tube wall and then centrifuged at 200g for 5 min. The medium and pellet were then discarded in order to eliminate any non-adhering sperm and other cells. Centrifugation does not alter the net charge on the tube. The tube surface was washed with 200 micro liters of media containing serum in order to neutralize the charge on the tube wall and to detach adhering sperm. The collected medium at the bottom of each tube was used to rinse the wall of the same tube several times in order to increase sperm recovery. Sperm parameters were then examined under microscope.

(ii) Hyaluronic acid sperm binding (PICSI) method

PICSI is a method based on the HA binding of sperms. The presence of HA binding sites on sperm outer membrane is regarded as a sign of sperm maturity, and constitutes the basic principle for a sperm binding assay (Attila *et al*., 2005). HA is a natural occurring compound present in cervical mucus, cumulus cells and follicular fluid, the binding method is considered to have minimal bio safety risks for both the embryo and the patient (Nicolás *et al*., 2012). For this study we used PICSI dishes from ORIGIO Mid Atlantic Devices Inc (Mt Laurel, NJ, USA). Each PICSI dish contains three microdots of hyaluronan in a polystyrene culture dish. Washed semen sample was added into the dish and the sample was allowed it to interact with the surface coating of HA for 15 minutes. The sperm attached to the dish was picked up with the ICSI pipette and this was subjected for analysis. Figure 1 shows the microscopic view of the PICSI dish.

(C) Outcome Measures

After processing, each sample was subjected for analysis to assess the motility, morphology and DNA fragmentation. The details of tests were as follows.

(i) Motility test

To determine the motility, Makler counting chamber (Sefi Medical Instruments, Israel) was used. After liquefaction 10 micro liter of the semen sample was loaded into the counting chamber. Then the sample was allowed to settle for 1 minute. Objective lens magnification was at 20x. Sperm count was then carried out in 10 squares. The entire grid was counted only if the sperm concentration was less than ten sperm per row. Semen concentration was expressed in millions/milli liter. Then the total count was calculated as per the following formula.

Total count = motile sperm + immotile sperm in 100squares of Makler counting chamber divided by 10.

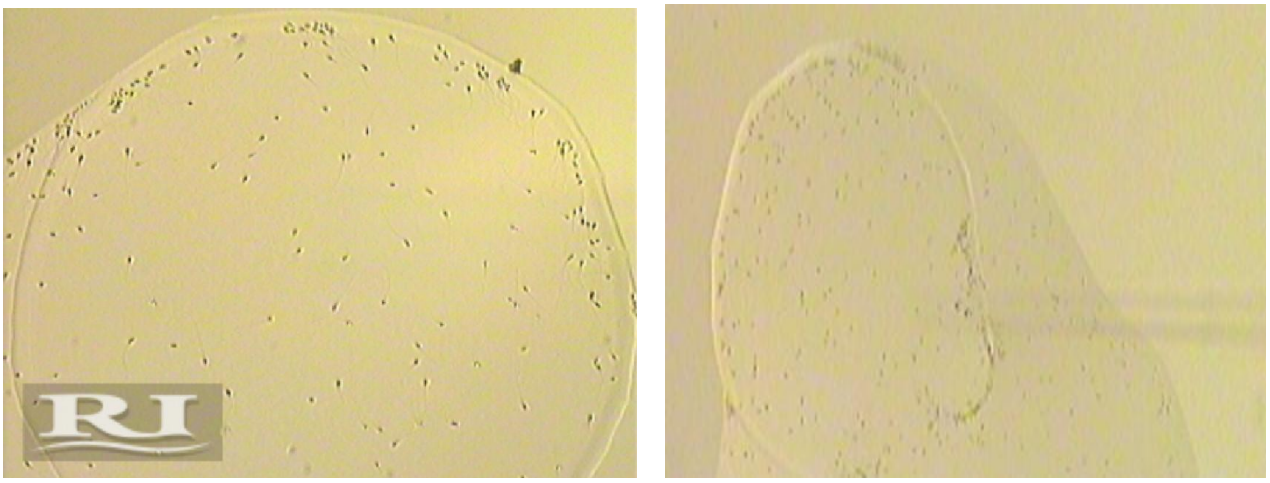

Figure 1. (a) Mature sperms bounded at the HA spot and immature sperms moves freely in the dish (b)picking of mature HA bounded sperm using injection needle

The sperm motility was assessed in at least 100 sperm and expressed as percent of motile sperm (sum of rapid progression plus slow progression sperm). The following formula was used.

***% Motility** = (motile sperm/Total count) x 100*

(ii) Morphology test (% normal sperms)

Sperm morphology was assessed by Diff quick staining (Cryo lab international) method. In this 10 micro liter of semen

sample was taken on a slide and smear was prepared. This was kept for air drying and the slides were immersed in fixative for 15 seconds and drained the excess solution by placing slides vertically on absorbent paper. The slides were immersed sequentially in diff quick stain-1(Eosin) for 10 seconds and dipped in Diff quick stain-2 (Methylene Blue) for 5 seconds. Then the slides were rinsed briefly in tap water to remove excess stain and kept for air dry. Each slide was examined with oil immersion at 1000x magnification in bright field microscopy. Tygerberg strict criterion was used (Menkveld *et al*., 1990). The sperm morphology was categorized into normal and abnormal. Abnormal sperms were again sub divided based on the site of abnormality- head, mid piece, tail extra residual cytoplasm (ERC). As per the strict criteria, the sperm was classified as normal when the head was oval with the acrosome occupying 40–70% of the head, absence of mid piece and tail defects, and absent or small cytoplasmic droplets with the appropriate head dimensions. The percentage of sperm with strict normal morphology was calculated by the formula as follows.

Normal Forms (%) = normal sperms / the total number of sperms evaluated x 100.

A specimen was classified in the normal sperm morphology category when the percentage of strict normal morphology was over 4%. Figure 2 shows the sperm with normal and abnormal morphology.

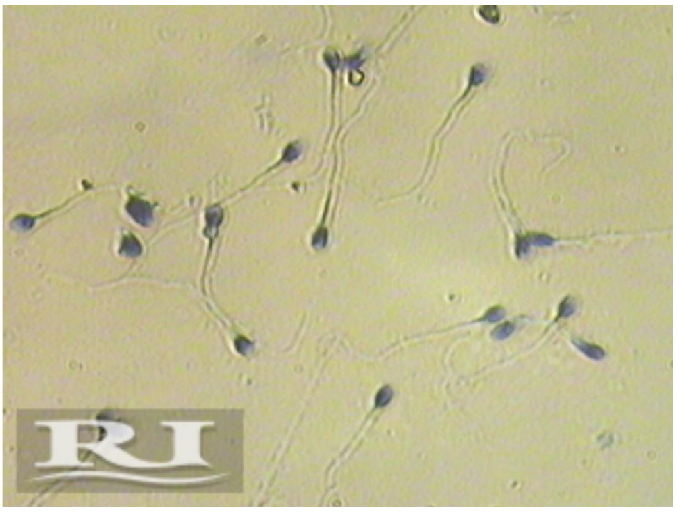

Figure 2. Slide with normal and abnormal sperm morphology

(iii) DNA fragmentation test

For DNA fragmentation study, the Halo sperm kit (Halotech Dna, SL) was used. The test procedure was based on the sperm chromatin dispersion (SCD) test (Fernandez *et al*., 2003, 2005). As an initial step, the lysis solution was kept at room temperature of 22^{o}C. The semen sample was diluted in culture medium to a concentration of 5-10 million per milliliter. The agarose gel was then dissolved by melted in a microwave or in water for 5minutes at 90-100^{o}C. The agarose eppendorf was transferred to a water bath where temperature was maintained at 37^{o}C and left it for 5minute until temperature became even. 25 micro liter of semen sample was added to agarose eppendorf and mixed well.14-20 micro liter of cell suspension was taken from agarose eppendorf on to the treated slides and covered with glass cover slip. Care was taken to avoid air bubbles. The slides were kept in horizontal position throughout the process. Then the slides were placed on the cold surface in refrigerator at 4^{o}C and left the sample 5minute to become gel form. Then acid denaturation (AD) solution was prepared. For this, 80microliter of AD was added to 10milli liter of distilled water and the resultant mixture was placed in the incubation tray. The slides cover was then removed by sliding it of gently with the help of lancet. After this, immediately the slides were immersed into AD solution and left to incubate for 7minutes. Afterwards, placed this in another incubation tray containing 10ml of lysis solution and kept it for incubation for 25minutes. Then slides were kept horizontally into a tray containing abundant distilled water in order to wash lysis solution. This was kept for incubation of 5minutes. The slides were then placed horizontally into a tray with 70% ethanol (2min) followed by 90% ethanol (2min) and finally 100% ethanol (2min). Kept the slides for air dry at room temperature followed by diff quick staining. After staining, slides were visualized to analyze the DNA fragmentation. Sperm with big halo and medium halo shows no DNA fragmentation (violet color). With small halo or without halo degenerated shows sperm with DNA fragmentation (blue color). Figure 3 represents a slide with halo and non halo sperms.

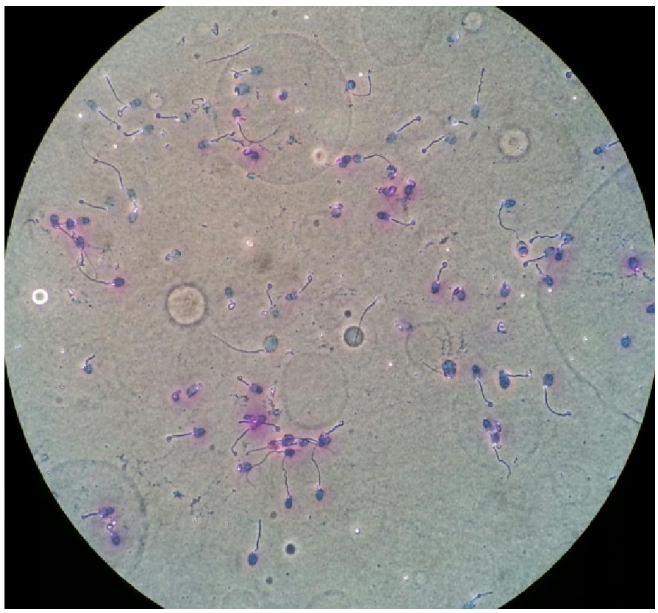

Figure 3. Slide shows sperms with no DNA fragmentation (Violet color) and with DNA fragmentation (Blue color)

(D) Statistical analysis

For the data analysis, statistical tests were performed. Basic descriptive statistics (mean ± standard deviation) were calculated for different parameters such as total motility, normal sperm morphology and DNA fragmentation. Wilcoxon signed rank test was performed to find out the significance in above parameters while comparing Zeta potential vs. HA binding (PICSI) method. A P-value < 0.05 was considered statistically significant.

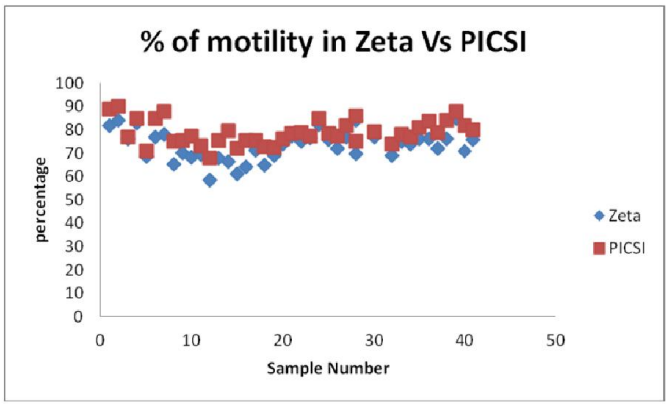

Figure 4. Scatter diagram shows percentage of motility vs. sample Number in Zeta and PICSI method

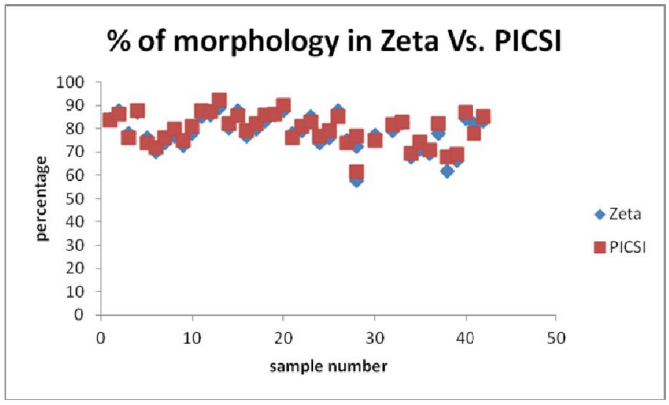

Figure 5. Scatter diagram shows percentage of morphology vs. sample Number in Zeta and PICSI method

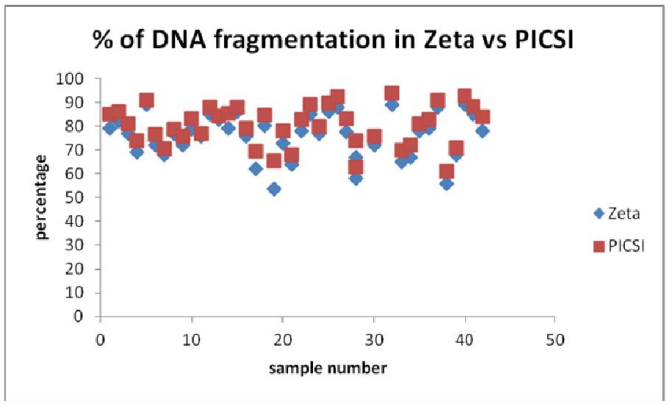

Figure 6. Scatter diagram shows percentage of DNA fragmentation vs. sample number in Zeta and PICSI method

RESULTS

In the comparison, the mean % of total motility, morphology and DNA fragmentation were 73.31, 78.44, and 75.91 with standard deviation of 6.35, 7.31, and 9.21 respectively with Zeta potential method. At the same time HA bounded PICSI yielded the values of 78.88, 78.77, and 80.2 with standard deviation of 5.19, 6.74, and 8.42 respectively. Figure4, 5 and 6 shows the test results in scatter plot form for motility, morphology and DNA fragmentation respectively. Wilcoxon signed ranked test showed significant difference in the results of motility, morphology and DNA fragmentation with p-values of less than 0.05 as shown in Table 1.

Table 1. Comparison Zeta potential method vs. HA binding (PICSI) method in terms of total motility, normal sperm morphology and DNA fragmentation

Parameter	Mean +/- SD		P-Value (using Wilcoxon signed rank test)
	Zeta potential method	HA binding (PICSI) method	
Motility %	73.31±6.35	78.88±5.19	0.00
Morphology %	78.44±7.31	78.77±6.74	0.00
DNA fragmentation %	75.91±9.21	80.2±8.42	0.00

DISCUSSION

Recent studies indicate that, basic sperm selection parameters are not adequate in the selection of good quality sperms since they are not able to identify a mature sperm with low chromosomal aneuploidies and DNA fragmentation (Said *et al*., 2011). Due to this reason many studies were focused to identify a better sperm selection technique to choose sperm with less DNA fragmentation, high motility and morphology. Among the sperm selection methods, the Zeta potential and HA binding methods became more popular due to their easiness to perform, less time consumption and to select sperm with less DNA fragmentation. Moreover, both techniques are highly useful in patients with reduced motility and morphology, in patients with history of recurrent abortion or miscarriage, poor fertilization, poor development of embryos, sperms collected by PESA and Frozen or cryo-preserved samples.

Mohammad *et al*. (2009) published his study results that Zeta method may be more efficient to select sperm with low DNA fragmentation than HA binding method. Naser *et al*. (2010) compared HA binding and zeta method and found that HA method can more efficiently select sperm with normal morphology. Presently the comparison studies are limited to trace out a conclusion in order to choose a suitable technique to enhance the clinical outcome. Our study results showed that DNA fragmentation is less in zeta method but the motility and morphology are better is PICSI. Mild higher results of motility and morphology in PICSI can be due to high efficiency of PICI dish with hyluronan media to capture mature sperms.

PICSI dishes are costlier compared to the expense of zeta potential method. Zeta potential can be performed in a normal andrology lab with less expensive consumables. So it can be utilize for all patients with normal count, especially in suspected cases of high oxidative stress. Disadvantage of zeta potential method is that, it needs experience in order to avoid the discharge or loss of electric potential which is imparting to the mature, less DNA fragmented sperms during the time of sperm processing. Although the Zeta potential method yielded many advantages, some studies indicated that the zeta method gives less number of sperms (Ishijima *et al*., 1991). This is the limitation of Zeta potential method to use for low count patients. Another study indicates that, in Zeta potential method, there can be a chance of selecting X- bearing sperm

due to their higher net negative charge compared to Y- bearing sperm (Kaneko *et al*., 1984, Cartwright *et al*., 1993). PICSI also has disadvantages. One study reported that there can be chance of injecting non-bounded sperm in ICSI and it can cause aneuploidies and DNA fragmentation in conceptus (Parmegiani *et al*., 2010).

Conclusion

This study results showed that Zeta potential is an effective method to select mature sperms with acceptable motility, morphology and less DNA fragmentation. So Zeta potential is comparable to HA binding PICSI and it is less time consuming, economic and can be used for all the normal semen samples especially with suspected high oxidative stress.

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