



## An analysis of the outcome of Intra-cytoplasmic sperm injection (ICSI) using Fresh or Frozen sperm

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Complete List of Authors:	Kalsi, Jas; UCL Hospitals NHS Trust, Department of Andrology Thum, M.Y.; The Lister Hospital Muneer, Asif; UCL Hospitals NHS Trust, Department of Andrology; The Lister Hospital Pryor, John; The Lister Hospital Abdullah, Hossam; The Lister Hospital Minhas, Suks; UCL Hospitals NHS Trust, Department of Andrology; The Lister Hospital
keywords:	ICSI, Fresh, Frozen, Sperm, Aetiology
Abstract:	<p>Introduction</p> <p>Male patients with azoospermia or severely impaired semen parameters can be offered ICSI in order to successfully father children. However, the outcome from ICSI using fresh or frozen sperm remains controversial.</p> <p>The aim of this study was to compare the outcome of first-attempt ICSI-ET cycles using frozen thawed testicular sperm (FTTS), fresh testicular sperm (FTS), frozen thawed epididymal sperm (FTES) and fresh epididymal sperm (FES) respectively in order to determine which of these has the most successful ICSI outcome with respect to fertilisation, pregnancy and birth rate. The outcomes were also assessed according to the underlying aetiology of azoospermia.</p> <p>Method</p> <p>The records of 493 patients undergoing first-attempt ICSI between 1993 and 2008 were retrospectively reviewed. FTS was used in 112 cycles, FTTS was used in 43 cycles, FES was used in 279 cycles, and FTES was used in 59 cycles. Within each group, the aetiology of the azoospermia was recorded according to history, clinical examination and histological analysis (n= 316). The fertilisation, clinical pregnancy, and delivery rates were calculated for each group with respect to the type of sperm retrieval used.</p>

	<p>Results</p> <p>Analysis of the data showed no significant differences between any of the 4 groups in the fertilisation, pregnancy or delivery rates (<math>p &gt; 0.05</math>). There were no significant differences seen between fresh sperm (testicular + epididymal) versus frozen sperm (testicular + epididymal) or between epididymal sperm (fresh + frozen) versus testicular sperm (fresh + frozen) in any of the outcomes measured (<math>p &gt; 0.05</math>). However, sub-set analysis demonstrated a statistically higher fertilization and pregnancy rate for FTTS over fresh sperm. When comparing the aetiology, we found no significant difference in the fertilization rate, clinical pregnancy and delivery rates between obstructive (OA) and non-obstructive (NOA) groups. However, sub-set analysis demonstrated a higher pregnancy and birth rate for FTTS over fresh in both OA and NOA groups.</p> <p>Conclusion</p> <p>The results from this study suggest using frozen sperm in ICSI cycles is a reliable and favourable method that has the same outcome compared to fresh sperm. Furthermore using testicular and epididymal sperm have similar ICSI outcomes whether fresh or frozen samples are used. However, results suggest a tendency for higher pregnancy and birth rates for frozen vs. fresh testicular sperm in both OA and NOA aetiologies. The aetiology of azoospermia does not significantly affect the outcome of first attempt ICSI. The higher rates in the frozen groups suggest that these patients may have had better quality semen when they were initially harvested and frozen.</p>

## **An analysis of the outcome of Intra-cytoplasmic sperm injection (ICSI) using Fresh or Frozen sperm**

**Jas Kalsi<sup>1</sup>, M.Y. Thum<sup>2</sup>, Asif Muneer<sup>1,2</sup>, John Pryor<sup>2</sup>, Hossam  
Abdullah<sup>2</sup>, Suks Minhas<sup>1,2</sup>**

**Department of Andrology, UCL Hospitals NHS Trust<sup>1</sup> and The Lister  
Hospital, Chelsea<sup>2</sup>**

### **Abstract**

### **Introduction**

Male patients with azoospermia or severely impaired semen parameters can be offered ICSI in order to successfully father children. However, the outcome from ICSI using fresh or frozen sperm remains controversial.

The aim of this study was to compare the outcome of first-attempt ICSI-ET cycles using frozen thawed testicular sperm (FTTS), fresh testicular sperm (FTS), frozen thawed epididymal sperm (FTES) and fresh epididymal sperm (FES) respectively in order to determine which of these has the most successful ICSI outcome with respect to fertilisation, pregnancy and birth rate. The outcomes were also assessed according to the underlying aetiology of azoospermia.

### **Method**

The records of 493 patients undergoing first-attempt ICSI between 1993 and 2008 were retrospectively reviewed. FTS was used in 112 cycles, FTTS was used in 43 cycles, FES was used in 279 cycles, and FTES was used in 59 cycles. Within each group, the aetiology of the azoospermia was recorded according to history, clinical examination and histological analysis (n= 316). The fertilisation, clinical pregnancy, and delivery rates were calculated for each group with respect to the type of sperm retrieval used.

## Results

Analysis of the data showed no significant differences between any of the 4 groups in the fertilisation, pregnancy or delivery rates ( $p>0.05$ ). There were no significant differences seen between fresh sperm (testicular + epididymal) versus frozen sperm (testicular + epididymal) or between epididymal sperm (fresh + frozen) versus testicular sperm (fresh + frozen) in any of the outcomes measured ( $p>0.05$ ). However, sub-set analysis demonstrated a statistically higher fertilization and pregnancy rate for FTTS over fresh sperm.

When comparing the aetiology, we found no significant difference in the fertilization rate, clinical pregnancy and delivery rates between obstructive (OA) and non-obstructive (NOA) groups. However, sub-set analysis demonstrated a higher pregnancy and birth rate for FTTS over fresh in both OA and NOA groups.

## Conclusion

The results from this study suggest using frozen sperm in ICSI cycles is a reliable and favourable method that has the same outcome compared to fresh sperm. Furthermore using testicular and epididymal sperm have similar ICSI outcomes whether fresh or frozen samples are used. However, results suggest a tendency for higher pregnancy and birth rates for frozen vs. fresh testicular sperm in both OA and NOA aetiologies.

The aetiology of azoospermia does not significantly affect the outcome of first attempt ICSI. The higher rates in the frozen groups suggest that these patients may have had better quality semen when they were initially harvested and frozen.

## Introduction

The technique of intracytoplasmic sperm injection (ICSI) has been used since 1994 and has revolutionised the management of male factor infertility. Using this technique azoospermic patients have the ability to father children after either epididymal or testicular sperm extraction. Previous studies have demonstrated that the success rates of ICSI with fresh testicular or epididymal spermatozoa are equivalent to those achieved by in vitro fertilization (IVF) using ejaculated spermatozoa (Aboulghar et al., 1997; Ghazzawi et al., 1998; Van et al., 1998). However, the ability to cryopreserve sperm before undergoing ICSI allows more flexibility and increases the range and number of therapeutic options available.

Frozen sperm avoids the need for repeated surgical sperm retrieval procedures. Moreover, having frozen sperm on stand-by results in more effective treatment planning such that concurrent sperm and oocyte retrievals may not be required (Oates et al., 1996). Previous studies report that using cryopreserved epididymal and testicular spermatozoa for ICSI yields an acceptable rate of both fertilization and pregnancy (Kupker et al., 2000; Palermo et al., 1999).

However, it is unknown whether using either fresh or frozen sperm with ICSI is associated with better outcomes (Tournaye et al., 1999; Wood et al., 2002), as previous reports suggest that cryopreservation reduces the fertilizing capacity of sperm (Holden et al., 1997; Thompson-Cree et al., 2003). Furthermore, the retrieval site (testicular or epididymal) of the sperm which is subsequently frozen has been suggested to have an impact on the outcome when used in subsequent cycles (Wood et al., 2002).

To address whether there is a significant impact of cryopreservation and whether there is a difference between testicular and epididymal sperm on the outcomes using ICSI, we retrospectively compared the outcomes of first-attempt ICSI-ET (intra-cytoplasmic sperm injection and embryo transfer) cycles when using frozen-thawed testicular sperm (FTTS), fresh testicular sperm (FTS), frozen-thawed epididymal sperm (FTES) and fresh epididymal sperm (FES). We also determined if outcomes were affected according to the underlying aetiology of the azoospermia.

## Material and Methods

The hospital records of patients undergoing first-attempt ICSI between 1993 and 2008 were retrospectively reviewed. In total 493 couples were included in the study and all had first attempt ICSI cycles. Fresh testicular sperm (FTS) was used in 112 cycles, frozen thawed testicular sperm (FTTS) was used in 43 cycles, fresh epididymal sperm (FES) was used in 279 cycles, and frozen thawed epididymal sperm (FTES) was used in 59 cycles. The fertilisation, clinical pregnancy, and delivery rates were calculated for each group.

Within each group the aetiology of the azoospermia was recorded (n= 316) according to history, clinical examination or biopsy result. The fertilisation, clinical pregnancy, and delivery rates were calculated for each group with respect to the type of sperm retrieval used.

All males with a history of azoospermia were evaluated at our andrology clinic to confirm their diagnosis prior to commencement of ICSI. If no sperm were seen, these patients were offered sperm retrieval after a full assessment and counselling with a consultant urologist.

## Treatment protocol

Stimulation protocol: Ovarian stimulation was carried out with either recombinant FSH, human menopausal gonadotrophin or urinary FSH. A trans-vaginal scan was performed prior to ovarian stimulation to ensure the ovaries were quiescent. For long protocol, patients were down regulated with either Nafarelin or Buserelin at mid luteal phase. For Cetrotide protocol, gonadotrophin releasing hormone antagonist was commenced when the leading follicle reached 12mm. When follicles reached pre-ovulatory size (18 to 22mm), 10,000 IU of hCG was administered. Oocytes were aspirated using trans-vaginal ultrasound guidance 34 to 36 hours after hCG administration. Embryo transfer was performed on day 2 or day 3 using a soft catheter with trans-abdominal ultrasound guidance. All patients received progesterone 400mg pessaries as a supplement throughout the luteal phase. A pregnancy test was performed two weeks after the embryo transfer.

### **Retrieval of testicular spermatozoa**

The technique of micro-dissection testicular sperm extraction as previously described was used (Schlegel, 1999) was used to retrieve sperm after 2005. Prior to this patients underwent multiple biopsy TESE. Using high powered ICSI microscopes testicular tubules were dissected immediately by an embryologist and any sperm obtained was either frozen or used for injection for a concurrent cycle of ICSI.

Percutaneous epididymal sperm aspiration (PESA) or Microsurgical epididymal sperm aspiration (MESA) were performed as previously described (Schroeder-Printzen et al., 2000) under a local or general anaesthesia. For MESA, spermatozoa were obtained from the microsurgically opened epididymal tubule with a micropipette or a 24-gauge cannula using a binocular microscope (magnification x25). Each specimen was examined for the presence of motile spermatozoa using a phase contrast microscope (magnification x400), starting at the cauda epididymis and continuing to a tubule 0.5 cm above the first until motile spermatozoa were aspirated. For PESA, a needle is placed into the epididymis through the skin blindly to aspirate sperm. The aspirated sample is then assessed for the presence of sperm using a phase contrast microscope (magnification x 400).

### **Freezing and thawing of PESA/MESA or TESE sperm**

For freezing, an equal volume of sperm freezing medium was added slowly and drop wise to the semen, agitating the sample gently throughout. Samples of 1-1.5ml were aliquotted into pre-labelled cryotubes. The aliquots were placed in the vapour phase of a liquid N<sub>2</sub> (-147°C) bank for 15 minutes and then transferred into the liquid phase for storage (-190°C). For thawing, a single cryotube was removed from the sperm storage vessel into a holding flask containing liquid N<sub>2</sub> (-190°C). The sample was then removed from holding flask and kept at room temperature for 15 minutes or until sample had thawed.

### **Statistical analysis**

Data was collected from Medical System for IVF (MedicalSys, London, UK) and analysed with Statistics Package for Social Sciences (SPSS, Surrey, UK). Descriptive statistical analysis was performed initially to examine the normality of distribution of all

continuous variances for parametric statistical tests. Associations between fresh or cryo-preserved sperm groups with pregnancy rates, miscarriage rates and live birth rates were examined with Chi-square Cross Tabulation test. Analysis of variance was then conducted to assess the relations between fresh or cryo-preserved sperm groups with women mean age, MII / total oocytes collected ratio, number of oocytes injected, average number of normal fertilized embryos and average no of embryos transferred. Statistical significant was set at  $P < 0.05$ .

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## Results

The mean maternal age was 33.7 years (range 21-47). There was no difference in maternal age between the 4 groups.

### **Comparison of fresh epididymal spermatozoa and FTES for ICSI**

There were no differences between the fresh epididymal spermatozoa (FES) and FTEPS groups in terms of maternal age, number of oocytes retrieved, oocyte maturity and number of embryos transferred (Table 1). A statistically higher number of oocytes were injected in the frozen group ( $p=0.018$ ) but the two groups did not differ in either fertilization rate (FR), pregnancy rate (PR), live birth rate or miscarriage rate ( $p>0.05$ ).

### **Comparison of fresh testicular spermatozoa and FTTS for ICSI**

There were no differences between the fresh testicular spermatozoa (FTS) and FTTS groups in maternal age, number of oocytes retrieved and oocyte maturity (Table 1). However there was a difference for the frozen over the fresh group with respect to number of embryos transferred ( $p=0.001$ ), FR ( $p=0.021$ ) and PR ( $p=0.047$ ). Despite this there was no difference between the groups in the live birth rate and miscarriage rates.

### **Analysis of outcome with respect to aetiology**

When all groups were compared there were no significant differences seen between the groups with respect to FR, PR or live birth rates. Sub-set analysis was then performed to assess if there was any differences within the aetiological groups. In the obstructive group (OA), there was found to be a statistically higher PR and live birth rate for frozen testicular sperm (FTTS) over fresh sperm, but not for frozen over fresh epididymal sperm. In the non-obstructive group (NOA), there was found to be a difference for frozen over fresh testicular sperm with respect to PR, live birth rate and miscarriage rate.

## Discussion

Previous studies have suggested that using frozen-thawed testicular sperm (FTTS) may be associated with a reduced fertilizing potential compared to frozen-thawed epididymal sperm (Ulug et al., 2005a). There is also evidence suggesting that testicular and epididymal spermatozoa are more sensitive to cooling than ejaculated spermatozoa (Gilmore et al., 1998). One study evaluated the impact of cryopreservation on sperm obtained from patients with azoospermia and used for ICSI using frozen-thawed epididymal spermatozoa (FTES), frozen-thawed testicular spermatozoa (FTTS) and compared the results with fresh spermatozoa for ICSI in the same individuals (Ulug et al., 2005a). The results suggested no difference between the two groups with respect to fertilisation or clinical pregnancy rates. However, only 16% of the NOA and 39% of the OA patients had their sperm frozen. Moreover, the impact of cycle optimisation with second and subsequent cycles cannot be excluded from the results of this study.

The effects of cryopreservation on sperm have been previously reviewed. Extensive cryoinjury to spermatozoa can occur during several steps of the freeze-thaw process, including the cooling, thawing and the addition or removal of the cryoprotectant such as Glycerol (Agca and Critser, 2002). It has previously been shown that freezing of sperm can cause swelling and rupture of the inner and outer acrosomal and plasma membranes and therefore making them unusable (Nogueira et al., 1999). Moreover, the production of oxygen free radicals has been found to increase during both the cooling and freeze-thaw processes leading to free radical injury secondary to plasma membrane lipid peroxidation (Chatterjee and Gagnon, 2001). Some studies suggest that cryopreservation of ejaculated spermatozoa results in an increase in the proportion of sperm with broken necks after thawing (Verheyen et al., 1997), and this may in turn be associated with a lower fertilization capacity. Conversely, other studies have shown that the fertilisation rate (FR) in ICSI cycles using frozen-thawed, surgically retrieved spermatozoa did not differ significantly compared with FR using fresh, surgically retrieved spermatozoa (Friedler et al., 1998; Gil-Salom et al., 1996; Habermann et al., 2000; Hourvitz et al., 2008). Moreover, similar outcomes have been reported following FTES and FTTS (De, I et al., 1998).

Reports have indicated that the use of FTTS results in lower FR and pregnancy rate (PR) compared with fresh testicular spermatozoa (Holden et al., 1997; Thompson-Cree et al., 2003).

The effects of cryopreservation on clinical outcome can be best determined by comparing patients undergoing cycles of ICSI with both fresh and frozen–thawed spermatozoa in their first cycle. This is the first study to have specifically examined this. Previous studies have used data from either consecutive cycles or from unmatched control populations in order to compare the outcomes (Ulug et al., 2005b). Using data from consecutive cycles can be misleading as higher success rates observed in couples undergoing a second or subsequent ICSI cycle may result from optimizing the stimulation of the female partner and not just from male factors (Ulug et al., 2005a). Furthermore these studies have involved only a small number of patients, which can make statistical analysis difficult to interpret (n=19) (Cayan et al., 2001) (n=24) (Friedler et al., 1998). In the present study, we compared the clinical outcomes in a large series of couples undergoing a first cycle of ICSI using fresh and frozen–thawed surgically retrieved spermatozoa. Our results suggest that cryopreservation does not have a significant impact on outcome of first attempt ICSI regardless of the source of sperm. Indeed the results suggest that the frozen testicular sperm has a higher fertilisation and pregnancy rate compared to fresh samples. This is in contrast to other studies which have reported lower FR in FTTS compared to fresh testicular spermatozoa (De Croo et al., 1998; Park et al., 2003). Our results may be partly explained by an inherent bias to only freeze sperm with good characteristics that may survive the freeze-thaw cycle.

Cryopreservation has previously been shown to have a detrimental effect on the morphology of both testicular and ejaculated spermatozoa because of the formation of intracellular ice, which results in the plasma membrane rupturing (Mossad et al., 1994; O'Connell et al., 2002; Verheyen et al., 1997). This, in turn, may allow free radical oxygen species to access sperm nuclei, adversely affecting DNA integrity (Dalzell et al., 2004). The cytoplasm of testicular spermatozoa is not usually sufficient for antioxidant protection and may thus permit oxidative damage (Aitken et al., 1998). Compared with epididymal sperm, testicular sperm are more vulnerable because their chromatin packaging is not completed until the SH bonds are oxidized during transit through the epididymis. Moreover, prolonged incubation after thawing of cryopreserved testicular spermatozoa may damage nuclear DNA, thus reducing the quality of sperm used for ICSI (Dalzell et al., 2003).

In this study, when all aetiological groups were assessed there was found to be no significant difference on the outcome with respect to the underlying aetiology. This is in contrast with previous studies which have demonstrated a lower FR in the FTTS group (Vernaev et al., 2003; Wood et al., 2004). Moreover, sub-set analysis suggests a higher success rate for frozen testicular sperm in both the OA and NOA groups versus their respective fresh counterparts. However, these results must be interpreted with caution as they probably represent an inherent bias to cryopreserve good quality sperm only and also as the numbers in the frozen groups are small. Our interpretation of the results would suggest that frozen sperm is as effective as fresh sperm irrespective of the underlying aetiology.

In conclusion, we have shown that surgically retrieved spermatozoa can be efficiently used for ICSI after freezing and thawing, without significantly compromising outcome, independent of the site of retrieval or the underlying aetiology. Freezing of surgically retrieved spermatozoa allows ICSI cycles to be more appropriately planned and may therefore increase the probability of conception in couples with infertility secondary to azoospermia.

Using frozen sperm has the advantage of avoiding repeated surgical sperm retrieval with each cycle and ensures the availability of sperm before beginning the IVF cycle which may reduce the cost and avoid unnecessary cycles.

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Table II						
	Non-obstructive		Obstructive			
	Testicular		Epididymal		Testicular	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Number of patients	41	7	173	42	28	15
Women mean age $\pm$ SD	33.61 $\pm$ 5.1	34.14 $\pm$ 4.1	34.03 $\pm$ 4.8	33.77 $\pm$ 5.2	36.48 $\pm$ 5.7	35.75 $\pm$ 3.1
Average no. of oocytes collected $\pm$ SD	12.89 $\pm$ 7.8	11.86 $\pm$ 2.9	11.57 $\pm$ 6.4	13.48 $\pm$ 6.8	10.39 $\pm$ 6.3	12.69 $\pm$ 8.2
MII / total oocytes collected ratio (%)	80.8 $\pm$ 16.6	86.0 $\pm$ 5.1	82.5 $\pm$ 15.4	77.8 $\pm$ 17.2	84.6 $\pm$ 16.6	84.2 $\pm$ 14.8
Number of oocytes Injected $\pm$ SD	10.32 $\pm$ 6.8	10.14 $\pm$ 2.3	8.9 $\pm$ 5.4	11.3 $\pm$ 6.3	8.77 $\pm$ 5.5	10.94 $\pm$ 7.6
Average no of normal fertilized embryos $\pm$ SD	5.25 $\pm$ 3.8	6.43 $\pm$ 1.9	5.25 $\pm$ 3.5	6.07 $\pm$ 4.3	5.5 $\pm$ 3.5	6.3 $\pm$ 4.7
Average no of embryos transferred $\pm$ SD	2.36 $\pm$ 0.89	2.29 $\pm$ 0.76	2.24 $\pm$ 0.81	2.14 $\pm$ 0.79	2.10 $\pm$ 0.87	1.81 $\pm$ 0.65
Pregnancy rate (%)	<b>36.6%<sup>a</sup></b> (15/41)	<b>57.1%<sup>a</sup></b> (4/7)	<b>46.2%<sup>b</sup></b> (80/173)	<b>35.7%<sup>b</sup></b> (15/42)	<b>32.1%<sup>a</sup></b> (9/28)	<b>60.0%<sup>a</sup></b> (9/15)
Live birth rate (%)	<b>31.7%<sup>a</sup></b> (13/41)	<b>57.1%<sup>a</sup></b> (4/7)	<b>33.5%<sup>b</sup></b> (58/173)	<b>23.8%<sup>b</sup></b> (10/42)	<b>28.6%<sup>a</sup></b> (8/28)	<b>60.0%<sup>a</sup></b> (9/15)
Miscarriage rate (%)	<b>13.3%<sup>a</sup></b> (2/15)	<b>00.0%<sup>a</sup></b> (0/4)	<b>27.5%<sup>b</sup></b> (22/80)	<b>33.3%<sup>b</sup></b> (5/10)	<b>11.1%<sup>a</sup></b> (1/9)	<b>0.0%<sup>a</sup></b> (0/9)
<p><sup>a</sup> Significant statistical comparison using Chi-square Cross Tabulation test with <math>P &lt; 0.05</math> between fresh and frozen within one particular group.</p> <p><sup>b</sup> Not significant statistically between fresh and frozen within one particular group.</p>						

Table 2. The outcome of first attempt ICSI-ET with respect to aetiology.

Table I-a: IVF-ICSI treatment outcome with fresh and frozen epididymal retrieved sperm.			
	Fresh Epididymal	Frozen Epididymal	P-value
Number of patients	279	59	NA
Women mean age $\pm$ SD	33.61 $\pm$ 5.1	33.92 $\pm$ 5.4	NS 0.673
Average no. of oocytes collected $\pm$ SD	11.86 $\pm$ 6.6	13.39 $\pm$ 6.3	NS 0.098
MII / total oocytes collected ratio (%)	78.7 $\pm$ 17.6	83.2 $\pm$ 14.2	NS 0.058
Number of oocytes Injected $\pm$ SD	9.3 $\pm$ 5.7	11.2 $\pm$ 5.6	0.018
Average no of normal fertilized embryos $\pm$ SD	5.07 $\pm$ 3.5	6.07 $\pm$ 3.8	NS 0.051
Average no of embryos transferred $\pm$ SD	2.15 $\pm$ 0.78	2.26 $\pm$ 0.86	NS 0.329
Pregnancy rate (%)	<b>44.4%</b> (124/279)	<b>40.7%</b> (24/59)	NS 0.351
Live birth rate (%)	<b>35.4%</b> (99/279)	<b>30.5%</b> (18/59)	NS 0.295
Miscarriage rate (%)	<b>20.3%</b> (25/124)	<b>25.0%</b> (6/24)	NS 0.392
Table I-b: IVF-ICSI treatment outcome with fresh and frozen testicular retrieved sperm.			
	Fresh Testicular	Frozen Testicular	P-value
Number of patients	112	43	NA
Women mean age $\pm$ SD	33.96 $\pm$ 5.4	33.57 $\pm$ 4.6	NS 0.184
Average no. of oocytes collected $\pm$ SD	11.82 $\pm$ 6.7	12.59 $\pm$ 7.6	NS 0.528
MII / total oocytes collected ratio (%)	81.3 $\pm$ 17.4	75.5 $\pm$ 19.7	NS 0.731
Number of oocytes Injected $\pm$ SD	9.42 $\pm$ 5.1	9.78 $\pm$ 6.6	NS 0.709
Average no of normal fertilized embryos $\pm$ SD	4.62 $\pm$ 3.1	6.04 $\pm$ 4.4	0.021
Average no of embryos transferred $\pm$ SD	2.18 $\pm$ 0.75	1.74 $\pm$ 0.71	0.001
Pregnancy rate (%)	<b>39.3%</b> (44/112)	<b>55.8%</b> (24/43)	0.047
Live birth rate (%)	<b>29.5%</b> (33/112)	<b>39.5%</b> (17/43)	NS 0.320
Miscarriage rate (%)	<b>25.0%</b> (11/44)	<b>29.2%</b> (7/24)	NS 0.461
NS = difference not statistically significant ( $P > 0.05$ )			
NA = not applicable.			

Table 1. Outcome of first attempt ICSI-ET with respect to source of sperm