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Physiological IMSI (pIMSI) Improves Results Obtained with IMSI in Patients with Idiopathic Infertility

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Abstract

Study Background: Intracytoplasmic morphology-selected sperm injection (IMSI) is a procedure in which spermatozoa are digitally analysed prior to injection into the oocyte. This increases the chance of selecting a good sperm, but physiological selection is not carried out. Here, we combine physiological pre-selection of spermatozoa with IMSI for patients with idiopathic infertility.

Methods: 290 patients, attending for assisted reproduction, were prepared for oocyte retrieval using standard controlled ovarian hyperstimulation protocols. Spermatozoa were preselected for ICSI with either digitalised morphological selection (IMSI) or IMSI combined with hyaluronic acid (HA) pre-selection techniques (pIMSI). Biological and clinical parameters were recorded and the clinical pregnancy rate noted.

Results: HA- pre-selection of mature spermatozoa led to a significant increase in pregnancy and implantation rates in patients treated with IMSI.

Conclusions: These data suggest that the combination of HA pre-determination of spermatozoa and advanced morphological analysis enables sperm selection on both a physiological and morphological basis and increases the probability of selecting a high quality spermatozoa.

Keywords: ICSI; IMSI; Idiopathic infertility; Assisted reproduction; Hyaluronic acid

Introduction

Idiopathic infertility is described as the failure to conceive after at least a year of unprotected intercourse in the absence of known pathologies. The proportion of patients attending for assisted reproduction that are diagnosed with idiopathic infertility is currently about 25-30%. As research and clinical techniques advance, this number tends to decline as causes are discovered. However, these groups of patients remain difficult to treat with assisted reproductive technologies.

A recent probable cause of male-factor infertility has been attributed to the presence of high levels of fragmented DNA in spermatozoa [1-4]. These data indicate that apparently normal spermatozoa often fertilise the oocyte creating an embryo in which the male genome is damaged to the extent that implantation and formation of a healthy foetus cannot occur. Although fragmented DNA in spermatozoa can be diagnosed, techniques enabling the selection of normal spermatozoa from the population contained within an ejaculate are still experimental.

Two techniques that enable to some extent the selection of physiologically normal spermatozoa have recently been developed. One of these is termed intracytoplasmic morphology-selected sperm injection (IMSI). Here, spermatozoa are selected for ICSI and analysed digitally prior to the microinjection procedure in order to deselect morphologically abnormal spermatozoa. With this technique, abnormalities not visible in standard ICSI procedures have been observed [5-11]. IMSI increases the pregnancy rate during ICSI cycles, and some data suggests that the level of pregnancy termination is also decreased [7,11-15]. Abnormalities in spermatozoa observed with MSOME have been correlated with the presence of fragmented DNA by TUNEL assay [16,17]. A second technique recently introduced to assisted reproduction is that of sperm selection with hyaluronic acid (HA). In this technique, mature sperm with HA receptors are distinguished from immature and abnormal sperm since these do not express such receptors. This technique is otherwise referred to as

'physiological ICSI' (pICSI) since the HA selection mimics the binding of spermatozoa to the zona pellucida of the oocyte – a physiological sperm selector [18-23]. It has been shown that HA-bound sperm have lower levels of persistent histones, fragmented DNA, apoptotic markers and aneuploidies than unbound spermatozoa [24-28].

The combination of HA sperm selection and IMSI are perfectly compatible in the assisted reproduction routine, and have been applied to the selection of spermatozoa for IMSI [29]. We applied this technique to couples affected by idiopathic infertility during IMSI procedures. We have termed this technique physiological IMSI (pIMSI). In this work, we compare pIMSI to the standard IMSI technique.

Materials and Methods

Patients

All patients in the present work were attending the Centro Fecondazione Assistita, Clinica Villa del Sole, Naples for assisted reproduction technology. Couples were characterised with idiopathic infertility when the female partner had no apparent cause of infertility after normal hysterosalpingography, hysteroscopy and ultrasound monitoring of the menstrual cycle; where hormonal values were within the normal range i.e. basal FSH <10IU/l, body mass index (BMI=weight (kg)/height (m)^2) < 29, menstrual cycle range 24-35 days

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(intra-individual variability ± 3 days); if the karyotype of both subjects of the couple was normal and if the male partner was normospermic. Female patients were prepared using standard controlled ovarian hyperstimulation protocols including downregulation of the pituitary gland with a GnRH agonist (Decapeptyl, Ipsen, Italy) followed by ovarian stimulation with exogenous FSH (Gonal-F, Serono, Italy). A single member of the medical staff co-ordinated all stimulation protocols ensuring standardisation. Oocyte retrieval was performed 36 hours after the administration of 10,000 IU hCG when 2-3 follicles of 18-20 mm diameter were observed by ultrasound examination, and blood 17 β-oestradiol levels reached 150-200 pg/ml/follicle over 18 mm. Luteal phase supplementation was achieved with intramuscular injections of prontogest 50 mg/day. Sperm samples were collected by masturbation and examined after liquefaction. All samples were washed using a silicon-based gradient of 40% overlaid over an 80% silicon solution (COOK Sperm Gradient, Ireland). The sample was centrifuged for 20 minutes at 1000 G, followed by a wash in Hams F-10. The final precipitate was resuspended to a final concentration of 1 x 10^6 sperm/ml and conserved in an atmosphere of 37°C and 6% CO_2 until required.

Intracytoplasmic sperm injection was performed on all patients diagnosed with idiopathic infertility to avoid the risk of failed fertilization. All oocytes in the present project were treated with ICSI 3 hours after oocyte retrieval (60 minutes after removal of the cumulus complex). A single team of biologists co-ordinated all biological work, ensuring that both culture protocols and embryo assessment were standardised. Oocytes were processed for ICSI using commercial IVF medium (COOK, Limerick, Ireland), pre-equilibrated to 37°C and 6% CO_2. In standard procedures, spermatozoa previously selected by the IMSI or pIMSI technique were placed in a drop of PVP in a Falcon 1006 ICSI dish (Beckton Dickinson, New Jersey, USA), and microinjected using an ICSI setup consisting of a Nikon Eclipse Ti-U microscope, stage heated to 37°C and Hoffman contrast objectives. Micromanipulation was performed with Narishige micromanipulators and COOK microtools (ICSI and holding pipette, COOK, Ireland). Zygote quality was scored 16-17 hours after ICSI. Embryo quality on day 3 was assessed 64-65 hours after insemination. Zygote and embryo evaluation was performed according to previous data [30]. Two or three embryos were transferred in all cases on the third day after oocyte retrieval. The establishment of a pregnancy was considered as a positive β-hCG test of over 60 IU/l 14 days after embryo transfer. The implantation rate was calculated by the observation of foetal heart beats after ultrasound analysis, 8 weeks after the establishment of pregnancy.

Intracytoplasmic morphology-selected sperm injection (IMSI) was offered to patients after at least two cycles of ICSI in which pregnancy was not obtained. In the standard IMSI procedure, motile spermatozoa were preselected on the basis of morphology using a 40x Nomarski objective. Purified sperm samples were placed in a solution of 10% PVP in a Willco GWSt-5030 glass-bottomed petri dish (WillCo Wells BV, Amsterdam, Netherlands) and individual spermatozoa immobilised, captured and placed in groups of 10 in a separate drop of diluted PVP solution (5% final concentration) in the same dish and analysed under differential interference contrast optics using a 100x oil-immersion objective. The resolution (r) obtained with this system is defined as $r=\lambda/2NA$ or 0.2 μm with λ defined as 560 nm. Computer-enhanced analysis and measurement of individual spermatozoa was achieved through digital recording of individual cells with a Nikon Digital Sight DS-2MBW camera followed by image analysis and measurement using the Nikon NIS-Elements image enhancement package (Nikon NIS-Elements, Florence, Italy). Spermatozoa were considered morphologi-

cally suitable for ICSI procedures where the head of the sperm was a regular oval shape, 4.5-4.9 μm in length and 3.1-3.5 μm in width, and was characterised by a maximum of a single vacuole not more than 4% the total area of the sperm head (5). The midpiece was also required to be a regular, rectangular shape of between 4.0 and 5.0 μm in length (16). Upon selection of a sufficient number of spermatozoa to complete the technique, selected spermatozoa are replaced into the PVP drop of a standard prepared Falcon 1006 ICSI dish and subsequently injected into oocytes through standard ICSI procedures.

HA-binding of sperm samples

HA-binding of sperm samples was performed using the Sperm Slow kit (Origio, Denmark). A 1 μl drop of the prepared sperm samples was placed in a 10 μl drop of Gamete buffer (COOK, Ireland) on a Willco GWSt-5030 glass-bottomed petri dish (WillCo Wells BV, Netherlands). This was then gently fused with a 10 μl drop of Sperm Slow (Origio, Denmark) in order to maintain the integrity of the HA solution. Spermatozoa binding to the HA were characterised by vigorous tail beating with slow forward progression, whereas those that did not bind maintained rapid progression despite the presence of HA. The proportion of spermatozoa exhibiting HA binding was calculated by random selection of a pool of spermatozoa. Spermatozoa preselected by HA treatment were then placed in a drop of 5% PVP solution on the same dish and analysed for morphology as above.

Prospective, randomised trial

Two hundred and ninety couples diagnosed with idiopathic infertility agreed to be inserted into the trial during a cycle of assisted reproduction. These couples were assigned a number, and patients were selected for standard IMSI or pIMSI by picking numbers with random number tables. Couples were included in the trial after at least 1 year of unprotected intercourse, two cycles of assisted reproduction without pregnancy and a failed cycle of IMSI in our centre within the past year.

Statistical analysis

All data were plotted as mean ± standard deviation, or as percentages, unless stated. All plots and statistical analysis was calculated using Excel (Microsoft, USA) or web-based statistical analysis sites. Significance of data was tested with either Student's t-test, ANOVA or the chi-squared test. The z-test with Yates correction was used to test the significance of proportions where necessary. Power of the test was calculated to be 98.6% in the present data.

Results

Statistical analysis of ICSI cycles in CFA Naples during the period 2007-2010 revealed a pregnancy rate of 29.8% for patients affected by idiopathic infertility (194/652 patients, maternal age 34.6 ± 5.5 years, Table 1). Since this group of patients, although apparently fertile, are difficult to treat successfully, we tested new techniques for the selection of spermatozoa. IMSI cycles in our laboratory have been previously shown to increase the pregnancy rate for a wide range of infertility diagnoses in patients where the male partner was affected by sub optimal semen characteristics [16]. However, in patients affected with idiopathic infertility, little benefit was observed. In a pool of 86 patients affected with idiopathic infertility treated with IMSI between 2009 & 2010, a pregnancy rate of 32.5% (28 couples), not significantly greater than that of ICSI in the same class of patients (Table 1) was achieved. We noted that in this patient population, the proportion of spermatozoa selectable for ICSI was higher than previous data (77.9%, [16], see Table 1), suggesting that the population of spermatozoa in idiopathic

	ICSI	IMSI	pICSI	*p*
Patients	652	86	12	
Mean age years ± sd (range)	34.6 ± 5.5 (27- 37)	33.8 ± 3.5 (30-37)	33.8 ± 1.9 (31-37)	0.40
Number of oocyte retrievals	652	86	12	
Number of mature oocytes retrieved (mean ± sd/patient)	7008 (12.4 ±3.0)	1118 (13.2 ± 4.0)	161 (13.4 ± 2.0)	0.05
Proportion of sperm selected for ICSI	7008/7008 (100%)	1118/1434 (77.9%)	161/453 (35.5%)	
Number of oocytes fertilised (mean ± sd/patient %)	5677 (81.0 ± 6.7%)	917 (81.0 ± 11.2%)	113 (71.2 ± 6.0%)	
Number of grade A embryos (% of total)	4088 (72.0%)	624 (68.0%)	77 (68.1%)	
Number of transfers	652	86	12	
Number of embryos transferred (mean ± sd/transfer)	1941 (2.8 ± 1.0)	258 (3.0 ± 1.0)	36 (3.0 ± 0.0)	0.18
Number of grade I embryos transferred	1941	258	36	
Number of clinical pregnancies (% pregnancies/transfer)	194 (29.8%)	28 (32.5%)	3 (25.0%)	
Number of foetal heart beats (Implantation rate %)	255 (13.1%)	42 (16.3%)	4 (11.1%)	
Pregnancies to term	186 (95.9%)	25 (89.3%)	3 (100%)	
Live births	221 (86.6%)	31 (73.8%)	3 (75.0%)	

Notes: Data is presented as mean ± sd with range where necessary. p<0.05 is considered significant.

Table 1: ICSI, IMSI and pICSI cycles for idiopathic infertility.

infertility is not highly affected by morphological abnormalities visible with high power optics.

Physiological intracytoplasmic sperm injection (pICSI) is a technique in which physiologically mature spermatozoa are selected through their capacity to bind hyaluronic acid [18-20]. We compared the hyaluronic acid binding capacity parameters of spermatozoa from idiopathic and tubal factor infertility patients. In 15 control samples (no selection criteria), 53.5 ± 4.6% of spermatozoa analysed appeared bound to the HA (Figure 1). In a pool of 10 tubal factor infertility patients (in which there is no presumable male factor), 55.3 ± 4.8% of spermatozoa were bound to the HA, not significantly different from that of controls (Figure 1). Interestingly, a pool of 12 patients affected with idiopathic infertility were characterised by a significantly lower proportion of spermatozoa bound to HA (161/453 sperm examined, 35.5%, Figure 1). Individual patients were characterised by a wide range of HA binding capacities in idiopathic infertility patients (Figure 2). These data suggest that patients affected by idiopathic infertility are often characterised by low ratios of sperm binding to HA. We tested whether the pICSI technique could assist in sperm selection in patients affected by idiopathic infertility. In a group of 12 patients, 3 pregnancies were obtained (25%), not significantly higher than that of the control population (Table 1).

Recent work has suggested that HA-binding of spermatozoa does not select for normal morphology forms (28). We tested whether physiological selection of spermatozoa also selected sperm of good morphology by examining spermatozoa selected by HA binding in high power optics. A total of 1765 spermatozoa from 18 patient semen samples were analysed. Interestingly, no morphological selection appeared to have occurred. 16.28 ± 5.95% spermatozoa, examined from the control population (i.e. not bound to HA) were found to have good morphology after Kruger morphology analysis. After HA pre-selection, a total of 17.22 ± 6.42% (6.92 ± 23.27 % improvement) were found to have normal morphology. Again, a large variation in individual patients was noted (Figure 3). These data suggest that HA selection of spermatozoa does not enrich for normal morphology forms.

Since the IMSI technique selects spermatozoa on the basis of morphology alone, and pICSI selects spermatozoa on the basis of physiology without advanced morphological selection, we tested whether a combination of the techniques i.e. physiological selection of spermatozoa followed by morphological analysis, could improve the pregnancy rate in this group of patients. We therefore initiated a prospective, randomised trial to test this hypothesis. In total, 290 patients were selected for the trial after informed consent. Patients were divided into two groups of 145 patients (Table 2). After controlled ovarian hyperstimulation, 5 couples selected for IMSI, and 2 couples
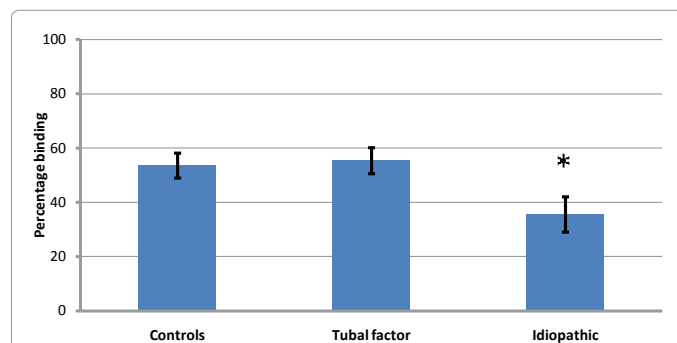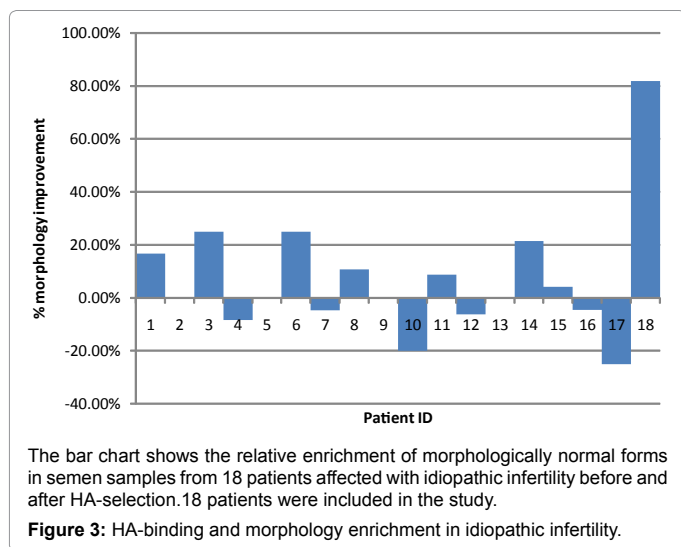

The chart is a comparison of the proportion of HA-bound spermatozoa in three groups of patients. Controls are an unselected population (n=15). *Tubal factor* are normospermic males whose partner has a diagnosed tubal factor infertility (n=10). *Idiopathic* are patients whose infertility diagnosis has not been determined (n=12). Data are means with standard deviation. The asterisk delineates significant differences (p<0.05)

Figure 1: Proportions of hyaluronic acid bound semen in specific patient groups.
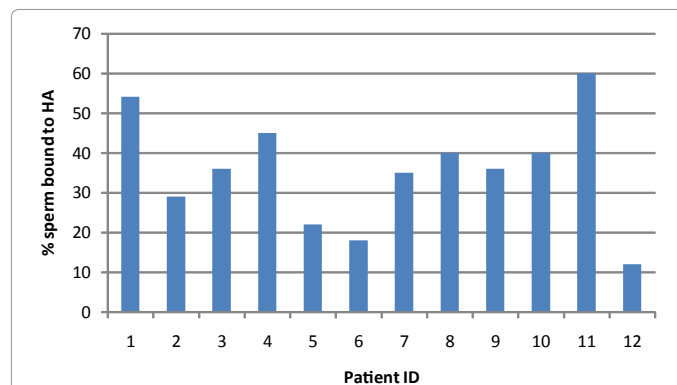
The bar chart shows proportions of sperm bound to HA in individual samples of patients characterised with idiopathic infertility. Data is presented as proportion of sperm HA-bound.12 patients were included in the trial

Figure 2: Variation of HA binding in individual patients affected by idiopathic infertility.

The bar chart shows the relative enrichment of morphologically normal forms in semen samples from 18 patients affected with idiopathic infertility before and after HA-selection.18 patients were included in the study.

Figure 3: HA-binding and morphology enrichment in idiopathic infertility.

	IMSI	pIMSI	p
Patients	145	145	
Mean age years ± sd (range)	34.6 ± 2.1 (29- 37)	34.5 ± 1.6 (30-37)	0.65
Number of oocyte retrievals	140	143	
Number of mature oocytes retrieved (mean ± sd/patient)	1596 (11.8 ±2.8)	1866 (12.1 ± 3.0)	0.39
Number of oocytes fertilised (mean ± sd/patient %)	1325 (83.3 ± 9.1%)	1562 (84.0 ± 11.1%)	0.62
Number of grade A embryos (% of total)	994 (75.0%)	1156 (74.0%)	0.57
Number of transfers	140	142	
Number of embryos transferred (mean ± sd/transfer)	364 (2.6 ± 0.8)	355 (2.5 ± 1.1)	0.52
Number of grade I embryos transferred	364	355	
Number of clinical pregnancies (% pregnancies/transfer)	50 (35.6%)	85 (59.6%)	0.00
Number of foetal heart beats (Implantation rate %)	55 (15.1%)	107 (30.1%)	0.00
Pregnancies to term	48 (96.0%)	84 (98.8%)	0.50
Live births	50 (90.9%)	101 (94.4%)	0.50

Notes: Data is presented as mean ± sd with range where necessary. p<0.05 is considered significant

Table 2: Clinical data for trial.

in the pIMSI group had their cycles cancelled due to poor response to the therapy (Table 2). Table 2 summarises the clinical data obtained during the trial. Interestingly, the percentage of fertilisation was not statistically significant between the two groups, 83.3 ± 9.1% of injected oocytes fertilised after IMSI, whereas 84 ± 11.1% of oocytes fertilised after pIMSI (Table 2). Furthermore, sufficient numbers of grade A embryos were produced to permit transfer of grade A embryos in all cases (Table 2). In the present trial, the group of patients undergoing IMSI achieved a total of 50 pregnancies out of the 140 transfers completed (35.6%). Cycles of pIMSI however resulted in 85 pregnancies

(59.6%). The pregnancy rate was highly statistically significant between groups (p=0, z-test). The implantation rate of pIMSI patients was also significantly greater than that of IMSI patients. For the IMSI group, 55 gestational sacs were observed out of the 364 embryos transferred (15.1%). In the group of patients undergoing pIMSI, 107 gestational sacs were observed after the transfer of 355 embryos (30.1%). No significant differences were observed in patients undergoing IMSI and pIMSI treatments with respect to the number of pregnancies to term, or the percentage of gestational sacs that resulted in live births (Table 2).

Discussion

Idiopathic infertility remains a 'pathology' which is difficult to treat with assisted reproduction. This is in large part due to the fact that the underlying cause of failure to conceive has not been diagnosed, leaving the couple untreated. In recent years, improvements in diagnostic techniques and the advancement of genetic testing has led to the reduction in the percentage of patients left undiagnosed. Tests on the physiology of spermatozoa such as the DNA fragmentation assay have been introduced to diagnose male factor infertility in cases where no apparent pathology is present [16]. One of the problems with males diagnosed with high levels of fragmented DNA is how to positively select for unfragmented DNA in a population of spermatozoa such as that obtained in an ejaculate. Gradients, glass wool filtration and other tests select for live spermatozoa, but no or little physiological selection is achieved.

IMSI is a technique whereby high resolution optics and digital analysis have revealed morphological abnormalities in spermatozoa [5-11]. This has aided in the selection of high quality spermatozoa for ICSI, and leads to an increase in pregnancy rates over classical ICSI techniques [7,11-15]. However, the technique does not detect physiological abnormalities or sperm maturity. Hyaluronic acid binding of spermatozoa detects physiological maturity of spermatozoa, but gives a low level of selection for morphology. However, this technique has been shown to increase pregnancy rates in assisted reproduction cycles.

In the present data, we found little increase in pregnancy rates after either IMSI or pICSI alone in patients affected by idiopathic infertility. However, our data suggests that the two techniques are based on mutually exclusive selection criteria i.e. IMSI selects for good morphology, but not necessarily mature spermatozoa and pICSI selects physiologically mature spermatozoa, but without enrichment for morphologically normal forms. Since the application of hyaluronic acid sperm selection techniques and morphological selection of spermatozoa with IMSI are not mutually exclusive, we combined them into a new technique which we termed 'physiological intracytoplasmic morphology-selected sperm injection (pIMSI). This technique will enable the selection of morphologically normal, physiologically mature spermatozoa. We compared the clinical results obtained with this technique with IMSI alone in a group of patients diagnosed with idiopathic infertility. Couples in the two groups had previously attempted 2 cycles of IVF and a third cycle with IMSI without success prior to the pIMSI cycle. Couples were characterised by similar characteristics such as age, BMI and response to gonadotrophins. Couples were randomly selected for IMSI or pIMSI to exclude bias in the patient characteristics.

In this trial, patients treated by IMSI or pIMSI had similar clinical results with respect to fertilisation rate, percentage of grade I embryos, number of grade I embryos transferred, pregnancies and birth rates. However, the percentage of pregnancies achieved after pIMSI was significantly higher than the results achieved in standard IMSI cycles.

Furthermore, the implantation rate of embryos produced with pIMSI was also significantly higher than that of IMSI.

The data suggest that the combination of physiological selection of spermatozoa with hyaluronic acid prior to the morphological examination of these spermatozoa for intracytoplasmic sperm injection improves results over either physiological or morphological selection alone in patients with no known infertility pathology. The data suggest that a population of spermatozoa contains both physiologically mature and morphologically normal subpopulations, but these do not necessarily correlate. The proportion of physiologically and morphologically normal spermatozoa in an ejaculate may therefore be a determinant of fertility in an otherwise normospermic male. Interestingly, the selection of physiologically and morphologically mature spermatozoa does not lead to an increase in either fertilisation rate of an improvement in embryo quality, suggesting that no quality classification criterion currently available to the major part of assisted reproduction laboratories can assist in the selection of embryos in these patients. We suggest that pIMSI could develop into a powerful tool for the treatment of patients affected by idiopathic infertility.

Acknowledgements

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