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Magnetic-activated sperm enrichment (MASE) versus density gradient centrifugation (DGC) impact on ICSI outcome

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Abstract
Background: Male factor infertility contributes to around 40% of the infertility clinical scenarios around the globe (Kumar N. & Singh A. K. 2015). Male infertility is usually reflected in semen analysis parameters. However, some core problems within the spermatozoa itself which does not reflect with just a simple semen analysis. One of the major debilitating core factors for such infertility is DNA fragmentation which can be described as DNA breakage reflecting nuclear damage that impacts embryonic molecular and cellular development course (Seli, E., Gardner, D.K., Schoolcraft, W.B., Moffatt, O., & Sakkas, D. 2004. Density gradient centrifugation (DGC) as a routine sperm preparation technique implemented as a diagnostic tool for overall reduction of bad quality spermatozoa but does not have a single selection per spermatozoon prior to ART (Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G., & Bianchi, U. 2000). Magnetic Activated Sperm Enrichment (MASE), a new spermatozoa preparation for ART technique uses both Annexin V & lectins namely LCA and PNA to bind to the apoptotic, necrotic and malformed or damaged sperm acrosomal and glycocalyx regions (Sutovsky & Kennedy 2013). Such offers a single selection to spermatozoa for fertilization. Study aim is to compare between two processing techniques for spermatozoa in ICSI management cycles (MASE) vs (DGC). Factors for comparison included fertilization, cleavage rate, Blastocysts formed and confirmed pregnancies. Statistics performed using SPSS.
Methodology: A prospective research study implemented at el nada IVF centre between February 2019 and September 2019 were 132 couples undergoing fertility treatments were included in the study. All cases showed only male factor infertility as all females were reported normal, their age ≤ 36 years, Normal basal FSH level, BMI ≤ 30, No uterine abnormalities, Not PCO syndrome, endometrial thickness ≥ 9 mm. sperm sample provided per case was split into 2 groups (group 1 processed with MASE and group 2 processed with DGC) for sperm selection prior to ICSI. Embryos per group were cultured under the same conditions. ET was randomized and split into 2 groups (66 per technology).
Results: A total of 3066 oocytes were injected for the two groups, Magnetic Activated Sperm Enrichment (MASE) and Density gradient centrifugation (DGC) (mean +/- SD =11.88 ± 0.3 MASE group vs 11.05 ± 0.29 DGC group), overall enhancement to the quality of the embryos formed from spermatozoa sorted with magnetic activated sperm enrichment (MASE) in comparison to common density gradient column centrifugation (DGC) as regards fertilization rate (mean +/- SD =10.02 ± 0.24 MASE vs 9.08 ± 0.25 DGC) (p value =0.008), cleavage rate (mean +/- SD = 8.08 ± 0.2 MASE vs 6.9 ± 0.24 DGC)(p value =0.0001), Blastocysts formed (6.13 ± 0.19 MASE vs 4.86 ± 0.2 DGC) (p=0.00001), 5AA grade (4.07 ± 0.15 MASE vs 3.74 ± 0.15) were significant (p=0.0001) and in favour of MASE while fair grade embryos (5BA) for the MASE group (2.75 ± 0.24) versus DGC (2.81 ± 0.20) (p=0.0008). 53 confirmed pregnancies out of 66 (MASE) vs 35 out of 66 (DGC). Chi-square test was performed (0.05 significance), fisher's exact significance (0.002).
Conclusion: MASE as a laboratory tool is a highly effective protocol for sperm selection in ICSI procedures in a statistically significant manner in comparison to DGC, however future research efforts are recommended to be conducted in a multicentric manner with larger sample sizes taking in consideration racial and ethnic differences of cases as response to the outcome.

Keywords: gradient, ICSI, Spermatozoa, fragmentation

Introduction
Infertility affects almost 8-12% around the globe (Skakkebak *et al*; 2006). Male affects amost 40 – 50% of them (Kumar, N., & Singh, A. K. 2015) [6]. DNA fragmentation (Seli, E., Gardner, D.K., Schoolcraft, W.B., Moffatt, O., & Sakkas, D. 2004) [11] is considered an important factor other than the conventional parameters as morphology, count and motility (WHO 2010). Radiation, in vivo Reactive Oxygen Species (ROS), environmental pollutants exposure lead to DNA fragmentation. A threshold index for such nuclear damage is established within each cell after which the cell is unable to fix its DNA breakage and initiate apoptosis (Seli, E. *et al*., 2004) [14] (Alvarez *et al.* 2017) [3]. Intra Cytoplasmic Sperm Injection (ICSI) has a major role in males with severe male factor infertility (Palermo *et al.* 1992). However, we cannot determine DNA fragmentation percentage in the selected spermatozoa for oocyte injection that might affect fertilization failures and poor embryo quality due to their poor nuclear integrity (Bounartzi *et al*. 2016) (Pacey *et al.* 2015) (Simon *et al*. 2016). Various technologies as comet assay, tunnel test and SCD aimed to estimate the DNA fragmentation

index (DFI) for the entire sample (Ribas-Maynou J1. *et al*. 2013) [10] (Vandekerckhove *et al*., 2016) [16]. However, they cannot label the DNA integrity for the selected spermatozoon prior to ICSI.

Density gradient centrifugation (DGC) as a routine sperm preparation technique aimes at reduction of the overall DNA fragmentation (Kheirollahi-Kouhestani *et al*. 2009) (Rappa *et al*. 2016). However, is aimed at an overall reduction and not specific per spermatozoon for the ICSI process. On the contrary, Magnetic Activated Cell Sorting (MACS) eliminates apoptotic spermatozoa using Annexin V particles that targets the external phosphatidylserine region which is a marker of cellular apoptosis (MACS) (Said *et al*. 2005), leading to selection of better spermatozoa with higher fertility potential which improves the ART outcomes (Tsung-Hsien Lee *et al*. 2009) [15] (Ziarati *et al*. 2018) [20].

Acrosome damage Altered sperm surface (Sutovsky *et al*. 2015) [7] (Ozmen B *et al*. 2007) [8] are correlated with DNA fragmentation, DNA damage and lately sperms fertility potential. The sperm glycocalyx is the thick coat of the sperm that takes part in sperm maturation, protection, acrosome reaction, oocyte recognition and fertilization (Schroter S. *et al*. 1999) [12] (Diekman AB 2003) [5]. Glycans, Lectins are natural found glycoproteins on the surface of the sperm have been used in the animal industry to label and sort sperm multiple defects prior to insemination (Sutovsky *et al*. 2015) [7]. An interesting finding was a higher conception rates found in nano purified spermatozoa for cattle that underwent insemination (Odhiambo *et al*. 2014) [7].

Lectin PNA (Arachis hypogaea/peanut agglutinin) has an affinity toward disaccharides with terminal galactoses, including those present in the sperm acrosomal matrix. Consequently, lectin PNA binds to the malformed or damaged acrosomal membranes/matrix.

In this study, we compare the effect of Magnetic Activated Sperm Enrichment to traditional Density Gradient Centrifugation in terms of fertilization, Embryo development & pregnancy.

Methodology

This prospective study was approved by the ethical committee of ElNada fertility center Located in Cairo Egypt. One-hundred and thirty-two infertile couple undergoing ART treatment between February 2019 and September 2019 were included in the study. All cases showed only male factor infertility as all females were reported normal, their age ≤ 36 years, Normal basal FSH level, BMI ≤ 30, No uterine abnormalities, Not PCO syndrome, endometrial thickness ≥ 9 mm.

All patients had undergone controlled ovarian stimulation by the same gynaecologist. The dose of gonadotropins was individualized based on the patient's age, history, and response to medication. Cycles were monitored using serial transvaginal ultra-sounds to asses' follicular growth. Administration of hCG occurred when follicular size was appropriate. Transvaginal oocyte retrieval was performed approximately 36 hours later. Patients received transvaginal progesterone beginning the night of the retrieval until 8 weeks 'of gestation.

Male clinical examination by the same andrologist, conventional semen analysis according to the guide lines of the world health organization (WHO) Using Fresh ejaculate, samples were split into two groups. The first was processed using sperm wash (Sperm wash by global) followed by Magnetic Activated Sperm Enrichment (MASE) prior to ICSI. The second group samples were prepared using Density Gradient Centrifugation prior to ICSI.

ICSI was done as described by (VanSteirtegham *et al*. 1993 and Al-Hasani *et al*. 1995) [8]. Oocytes aspiration and pickup was performed in 120 mm petri dishes using HEPES buffered media followed by denudation using 170 micro meter cook strippers prior to injection. Oocytes were split per female into two groups; the first group was injected with the 1st group male spermatozoa treated with MASE, the second was injected with the 2nd group male spermatozoa treated with DGC. Injected oocytes per each group were group cultured using Global total non-sequential media. Embryo transfer protocol according to Schoolcraft *et al*. 2001 [14].

The patient was placed in the lithotomy position with full bladder. A sterile bivalve speculum was placed in the patient's vagina, and the cervix was exposed. Excess mucus and debris were cleared from the ectocervix using sterile cotton swabs dampened with phosphate-buffered saline. The embryos were loaded into the transfer Labotect-catheter (ref 13366, Germany) by the embryologist, and the catheter was passed to the transfer physician in less than 1 min. The embryos were then deposited approximately 1 cm from the uterine fundus under ultrasound guidance. After approximately 5 seconds the catheter was gently rotated 180° and removed, with care being taken to keep the plunger of the catheter depressed until it had been completely removed from the cervix. The embryologist immediately flushed the catheter with media to check for retained embryos, blood, or mucus. Patients remained supine for 20 minutes after the procedure.

Randomization of the study was built upon patients counselling. However, which embryo to be transferred from which group was only known by the corresponding embryologist. Chemical and clinical pregnancies were confirmed at 2 weeks and 6 weeks post embryo transfer.

Statistical methods

Statistics were done using SPSS for the following parameters regarding the two groups; number of fertilized oocytes, number of four cell stage formed, number of blastocysts formed and clinical pregnancy.

Results

Age of males undergoing treatment was split into 4 groups (1st group < 32 years, 2nd group 32-36 years, 3rd group 37-39 years and 4th group >39 years).

Females corresponding to the male partners were split into 4 groups according to age (1st group < 30 years, 2nd group 30-33 years, 3rd group 33-36 years).

A total of 3066 oocytes were injected for the two groups (11.88 ± 0.3 MASE group vs 11.05 ± 0.29 DGC group) injections were performed using the same exact conditions, same equipment, manipulator, embryologist and medium for each group. Fertilized embryos formed (10.02 ± 0.24 MASE vs 9.08 ± 0.25 DGC) were significantly different (p=0.008). Day 3 cleavage embryos retrieved (8.08 ± 0.2 MASE vs 6.9 ± 0.24 DGC) were significantly higher (p=0.0001). The more embryo culture proceeded; significance was higher and in favour to the MASE group regarding embryo survivability and quality obtained per

patient. Day 5 embryos retrieved (6.13 ± 0.19 MASE vs 4.86 ± 0.2 DGC) were highly significant (p=0.00001) indicating an overall enhancement to the quality of the embryos formed from spermatozoa sorted with magnetic activated sperm enrichment (MASE) compared to common density gradient column centrifugation (DGC). Blastocysts obtained for on day 5 which scored 5AA grade (4.07 ± 0.15 MASE vs 3.74 ± 0.15) were significant (p=0.0001) and in favour of MASE while fair grade embryos (5BA) for the MASE group (2.75 ± 0.24) versus DGC (2.81 ± 0.20) also came with significance (p=0.0008) and in favour or MASE than DGC. Chemical pregnancy was performed by the 14th day after transfer per patient. A total of 53 out of 66 came out as positive chemical pregnancies for the MASE group patients versus a total of 35 positive chemical pregnancies for the DGC group patients. Chi square test was performed with a significance level of 0.05, fisher test exact significance found (0.002). Selection for embryo transfer was randomized while female factor infertility was excluded from the study as no infertility or subfertility issues were reported during initial examination and follow up.

Table 1: Illustrating Means, Standard Error Mean & Standard deviation

	N	Mean	S.E.	Std. Deviation
injected (MASE)	132	11,88	0.30	3,50
injected (DGC)	132	11,05	0.29	3,31
fertilized (MASE)	132	10,02	0.24	2,70
fertilized (DGC)	132	9,08	0.25	2,86
Cleavage (MASE)	132	8,08	0.20	2,35
Cleavage (DGC)	132	6,90	0.24	2,74
Blastocyst (MASE)	132	6,13	0.19	2,14
Blastocyst (DGC)	132	4,86	0.2	2,31
Good Quality (5AA) MASE	132	4.07	1.231	0.151
Good Quality (5AA) DGC	132	3.74	1.231	0.151
Fair Quality MASE (5BA)	132	2.75	2.023	0.249
Fair Quality DGC (5BA)	132	2.81	1.653	0.203

Table 2: Illustrating T- test results for the study investigated parameters

Confidence Interval CI=(0.95)	Paired Differences					t	Df	Sig. 2 tailed
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			p-value
Injected MASE vs DGC	0.83	4.38	0.38	0.07	1.58	2.17	131	0.032
Fertilized MASE vs DGC	0.94	4.00	0.35	0.25	1.63	2.70	131	0.008
Cleavage MASE vs DGC	1.18	3.55	0.31	0.57	1.79	3.83	131	0.000
Blastocyst MASE vs DGC	1.27	3.12	0.27	0.73	1.81	4.68	131	0.00

Table 3: Illustrating pregnancies performed statistics via Chi-Square test.

Statistic	Value	df	Asymp. Sig. (2-tailed)	Exact Sig. (2-tailed)	Exact Sig. (1-tailed)
Pearson	11.05	1	0.001		
Chi-Square Likelihood Ratio	11.29	1	0.001		
Fisher's Exact Test					
Continuity Correction	9.85	1	0.002	0.002	0.001
Linear-by-Linear-Association	10.96	1	0.001		
N of Valid Cases	132				

Table 4: Illustrating pregnancies performed statistics via Chi-Square test.

macs or gradient	clinical pregnancy		Total
macs or gradient	+ ve FHS	- ve FHS	Total
MASE	53.00 80.30%	13.00 19.70%	66.00 100.00%
Gradient	35.00 53.03%	31.00 46.97%	66.00, 100.00%
Total	88.00 66.67%	44.00 33.33%	132.00 100.00%

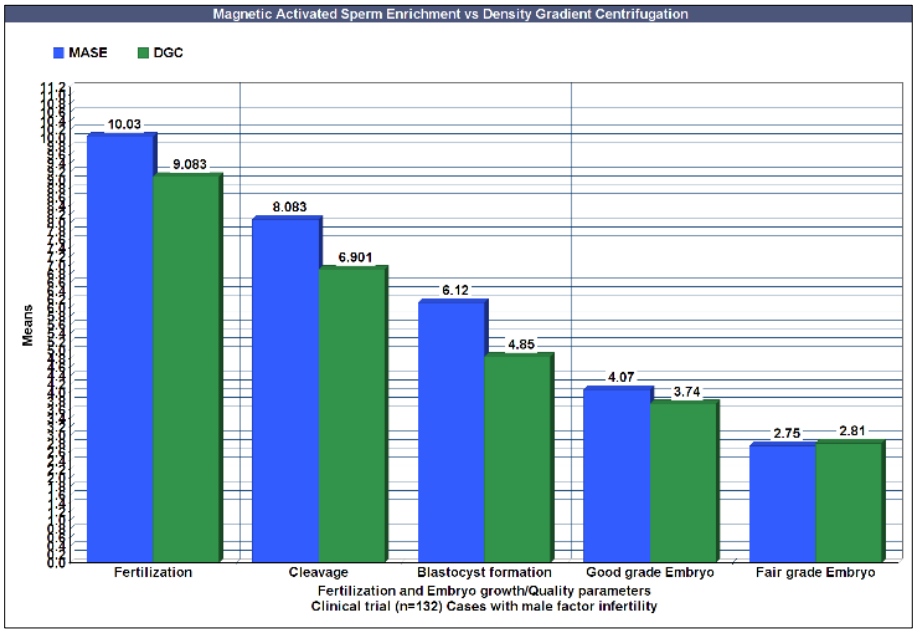

Fig 1: Illustrating the results for the post ICSI 5 days Embryo culture.

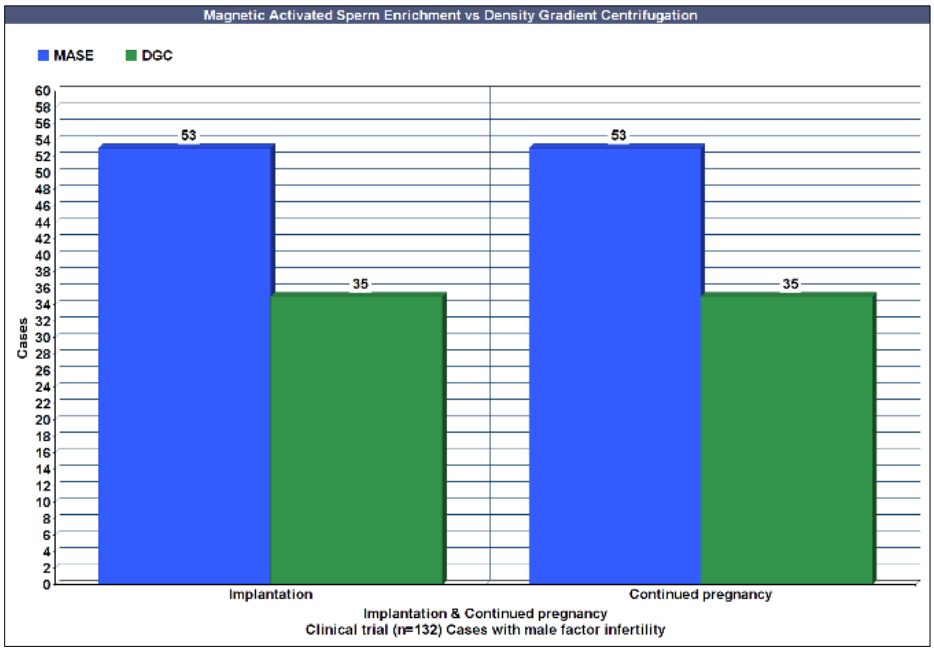

Fig 2: Illustrating the results for Implantation and pregnancy rates

Discussion

Sperm DNA fragmentation is an uprising issue in every day practise in infertility clinics. Sperm selection in cases of high DNA fragmentation is considered a state of art in IVF lab practise. Sperm selectivity of normal spermatozoa would enhance the ICSI management cycles in regards to live birth rates and pregnancy rates.

As regards to human semen processing, DGC is considered a highly useful protocol used in IVF labs for selection of the motile portion with a good morphology from the ejaculate. Even though frequently applied it lacks the capability for spermatozoa selection having the best DNA structural integrity that is a cornerstone factor for ART cycle success as regards the outcome (Avenda~no *et al*., 2009) [2]. Numerous reproductive research studies revealed and displayed a statistically significant fertilization failure rate, impaired embryonic development and pregnancy loss after ART management in which males had a high sperm DNA fragmentation rate in comparison to low DNA fragmentation cases (Seli, Gardner, Schoolcraft, Moffatt, & Sakkas, 2004; Velez de la Calle *et al*., 2008; Zini, Boman, Belzile, & Ciampi, 2008) [14].

A prior research group of investigators have implemented a similar research effort to the current study in which they used nanotechnology molecular-based cell targeting using biocompatible magnetic nanoparticles (MNP for detectability and removal of damaged sperms in a magnetic field. Fresh semen

has been mixed with various amounts (0, 87.5, and 175. g) of MNP-conjugate (Annexin V-MNP and Lectin-MNP) and incubated (10 to 15 min) for 37 °C in Exp. 1. In Exp. 2, extended semen was mixed with optimal concentrations of MNP-conjugates and incubated (0, 30, 90, or 120min). In Exp.3, the synergistic effects of both MNP-conjugates (87.5. g – 30 min) on spermatozoa was evaluated, followed by sperm fertility evaluation. Sperm motion, viability, and morphology characteristics were evaluated in all study subjects recruited.

The researchers have revealed among their study results that the motility of nano selected spermatozoa was improved in a statically significant fashion (P value <0.05). The sperm viability, have been evaluated via assay of reactive oxygen species and acrosomal integrity, plasma membrane, and mitochondrial membrane was not different between nano selected and control spermatozoa.

In conclusion similar to the current research results it was shown that the benefit of magnetic nano selection for high-throughput targeting of damaged sperm, for removal and rapid and effortless enrichment of semen doses with highly motile, viable, and fertile spermatozoa.

The current and prior research study findings could be justified by the fact that at molecular and cellular levels that the usage of magnetic fields represent a cost effective diagnostic as well as therapeutic method for better sperm quality selection based on the protein-coated-beads used for targeting apoptotic cell membrane of low quality sperm, enhancing a better proportion of sperms used in ICSI, in the current research we justify the higher study results due to their ICSI into a good quality M2 oocytes retrieved from the female study subjects.

Conclusion

MASE as a laboratory tool is a highly effective protocol for sperm selection in ICSI procedures in a statistically significant manner in comparison to DGC, however future research efforts are recommended to be conducted in a multicentric manner with larger sample sizes taking in consideration racial and ethnic differences of cases as response to the outcome.

Compliance with Ethical Standards

Funding: This study was privately funded by El Nada Fertility Center

Conflict of Interest and Authorship Conformation

We hereby affirm the following:

1. All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
2. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
3. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

Human Subjects were involved and signatures are available on their applications where the technology was provided free of charge and with full consent of each and every couple subject to the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

"The trial for magnetic activated sperm enrichment versus density gradient centrifugation impact on ICSI outcome was conducted in the period between February 2019 to august 2019 at El Nada Fertility Center on 132 couples with male factor infertility. Informed consent was obtained from all individual participants included in the study.

El Nada fertility Center registration number: 80 in 7/2/2019 Ministry of Health and Population – Arab Republic of Egypt" – El Nada Ethics committee main declaration attached with the manuscript.

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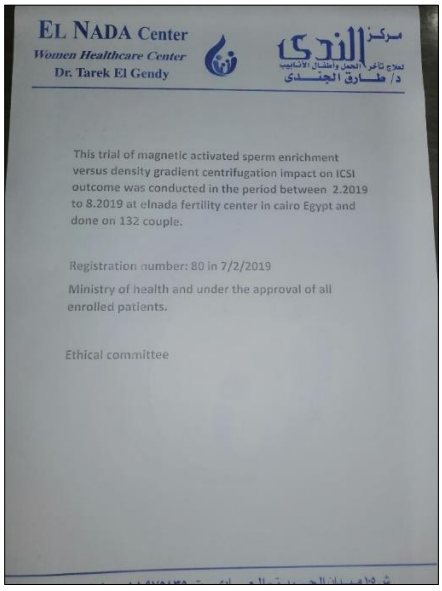
EL NADA Center
Women Healthcare Center
Dr. Tarek El Gendy

مركز الندى
د/ طارق الجندى

This trial of magnetic activated sperm enrichment versus density gradient centrifugation impact on ICSI outcome was conducted in the period between 2.2019 to 8.2019 at elnada fertility center in cairo Egypt and done on 132 couple.

Registration number: 80 in 7/2/2019

Ministry of health and under the approval of all enrolled patients.

Ethical committee

Fig 3

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