

Effect of Sperm Chromatin Integrity on Parameters of In Vitro Fertilization

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Abstract

Infertility affects 15% of couples seeking children worldwide and male factor is evident in about 30-50% of the cases. To assess the effect of sperm chromatin integrity on the outcomes of intracytoplasmic injection, sperm DNA compactness was evaluated by acridine orange staining in 40 fertile and 44 infertile men attending one infertility clinic. The results showed no significant difference in the percentage of sperm with integrated DNA between the fertile and the infertile groups ($p > 0.05$). Also, no significant difference ($p > 0.05$) between fertile and infertile men was found in terms of fertilization rate, and pregnancy rate. However, a significant difference was found in developmental rate ($p < 0.05$). When the percentage of sperm with DNA fragmentation was $\geq 38\%$, there was a decline in embryos quality. The current work concludes that conventional semen analysis is moderately predictive of an individual's infertility. Thus, a need arises for new assays for the evaluation of sperm quality.

المخلص

عالمياً، تبلغ نسبة الأزواج الراغبين بالإنتاج والعاجزين عن تحقيق ذلك بسبب إحدى مشاكل العقم 15%، حيث يكون الرجل مسؤولاً عن 30-50% من مثل هذه الحالات. تهدف هذه الدراسة إلى قياس معدل تكسر المادة الوراثية للحيوانات المنوية الموجودة في عينات السائل المنوي لأربع واربعة عشر رجلاً عقيمًا ولأربعين رجلاً خصيباً (بناءً على التحليل التقليدي للسائل المنوي)، وذلك باستخدام طريقة صبغة صباغة الحيوانات المنوية بمادة الأكردين البرتقالية. كما أنها تهدف إلى تقييم تأثير التكسر في المادة الوراثية للحيوان المنوي على مخرجات عملية الحقن المجهرية للحيوان المنوي داخل الغشاء البلازمي للبيضة، ومقارنة معدل هذه المخرجات بين الأشخاص العقيمين والخصيبين. توصلت الدراسة إلى أنه لا يوجد فرق معنوي بين الرجال العقيمين والخصيبين من حيث معدل تكسر المادة الوراثية للحيوانات المنوية أو معدل الإخصاب والحمل، ولكن يوجد فرق معنوي بين المجموعتين في معدل التطور الجنيني. كما وجدت الدراسة أيضاً أنه في حالة كون نسبة الحيوانات المنوية ذات المادة الوراثية المتكسرة $\leq 38\%$ فإن ذلك يؤثر على نوعية الأجنة الناتجة من الحقن المجهرية وذلك بزيادة أعداد الأجنة متوسطة الجودة والردية. إن تحليل السائل المنوي بالطريقة التقليدية قد لا يكون ذا قيمة لإعتماده كمرجع لاختيار الحيوان المنوي المناسب من أجل عملية الحقن المجهرية داخل الغشاء البلازمي للبيضة. لذلك فإن تحليل معدل تكسر المادة الوراثية في الحيوان المنوي يعتبر قيمة أخرى فعالة.

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1. Introduction

Infertility is defined as the inability to conceive after one year of regular, unprotected intercourse (WHO, 1999). It affects approximately 15% of all couples seeking to have children (Sharma *et al.*, 2004). Male factor is evident in about 30%-50% of clinical infertility cases worldwide (Larson-Cook *et al.*, 2003) and in 35% of infertility cases in Jordan (Tahtamouni *et al.*, 2002). Poor sperm quality is represented by abnormal semen parameters, including low sperm concentration, poor sperm motility, and abnormal sperm morphology (Chen *et al.*, 2006).

Over the last decade, the use of assisted reproductive techniques (ART) to overcome the problem of couple infertility has increased, and the use of intracytoplasmic sperm injection (ICSI), initially suggested for cases of severe male factor infertility, has been expanded (Gandini *et al.*, 2004). ICSI bypasses all processes of natural selection presented by sperm-oocyte physical interaction, which is still present in conventional in-vitro fertilization

(IVF), and relies on the direct injection of sperm into the oocyte cytoplasm. And this increased the concern about sperm genome integrity and the safety of ICSI-born progeny (Gandini *et al.*, 2004).

The prominent risks of ICSI come about from forcing fertilization with sperm that might contain hidden DNA damage (Larson-Cook *et al.*, 2003). The exact mechanism(s) by which chromatin abnormalities/DNA damage arise in human spermatozoa is not precisely understood, but three main theories have been proposed: namely, defective sperm chromatin packaging, apoptosis and oxidative stress (Lopes *et al.*, 1998; Agarwal *et al.*, 2003; Sharma *et al.*, 2004). Infertile men display higher levels of loosely packaged chromatin and damaged DNA (Virant-Klun *et al.*, 2002).

Sperm DNA instability may have possible negative impact on the outcomes of ART including fertilization, blastocyst development, and pregnancy rates (Seli *et al.*, 2004; Huang *et al.*, 2005). It has also been reported that sperm concentration, motility and morphology might be affected by sperm DNA fragmentation (Saleh *et al.*, 2003).

Rapid advances in reproductive molecular biology in the last two decades have resulted in techniques to assess sperm DNA fragmentation. Acridine orange (AO) staining

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is a simplified microscopic and cytochemical method for determining sperm DNA integrity; allowing the differentiation between normal, double-stranded and abnormal, single-stranded sperm DNA, using the metachromatic properties of the dye (Virant-Klun *et al.*, 2002). The fluorochrome AO intercalates into double-stranded sperm DNA as a monomer and binds to a single stranded sperm DNA as an aggregate. The monomeric AO, bound to normal double-stranded DNA, fluoresces green, whereas the aggregated AO on single-stranded DNA fluoresces yellow to red (Virant-Klun *et al.*, 2002). Increased red fluorescence indicates increased sensitivity to denaturation. Sperm cells having DNA that is sensitive to denaturation also have DNA strand breaks (Gopalkrishnan *et al.*, 1999). AO staining is a well-established method to study nucleic acid changes since the early sixties (Bertalanffy, 1963).

Some studies show that sperm single-stranded DNA, detected by AO staining, affects the fertilization process in a classical IVF program negatively (Virant-Klun *et al.*, 2002). In addition, when the level of spermatozoa with single-stranded DNA was increased, there was a significantly lower fertilization rate and a higher percentage of fragmented embryos after ICSI. On the other hand, no correlation was found between the level of spermatozoa with single-stranded NA, pregnancy rate, and live-birth rate achieved by ICSI (Virant-Klun *et al.*, 2002).

The current work aims at assessing the degree of sperm DNA fragmentation, measured by AO staining, in fertile vs. infertile men. Also, it aims at evaluating the effect of sperm single-stranded DNA on ART outcomes.

2. Materials and Methods

2.1. Sample Selection

The study population consisted of 84 chosen couples presented for ICSI at the Infertility Unit of Farah Hospital, Amman-Jordan. All men were informed of the research and all gave their consent for participation.

2.2. Seminal Fluid Collection and Analysis

Semen samples were collected by masturbation into sterile jars after 48 to 72 hours of sexual abstinence. Semen analysis for the 84 men, attending the infertility center, was performed according to the method described in the World Health Organization (WHO) laboratory manual (1992). Analyzed semen parameters were: pH of the semen, concentration of sperm/ml, % motility, and % normal morphology. According to semen sample analyses, 44 couples were diagnosed with male factor infertility, while the remaining 40 men were fertile and were used as the control group.

2.3. Data Collection

Data about the chosen 84 couples were obtained by referring to medical records. The data included type, duration and cause of infertility, patients' age, and ICSI information. The latter also included number of picked up, injected and fertilized oocytes, number and type(s) of developed and transferred embryos, and pregnancy test result confirmed by the determination of serum beta-HCG 15 days after embryo transfer (ET) to the uterus.

In all couples, fertilization rate [no. of fertilized oocytes (presence of two pronuclei after 18-20 hr of injection) per all oocytes] was calculated. Forty eight hours after the ICSI procedure (Day 2), the no. of the fertilized oocytes which developed into embryos (at least two cells and preferably three or four cells, cleaving stage) (Larson-Cook *et al.*, 2003) was determined as the developmental rate (no. of embryos per fertilized oocyte). According to Farah infertility unit, embryos were classified into four types (I-IV). Type I or excellent embryos had regular, spherical blastomeres of an equal shape and size, with no fragmentation. Type II or good embryos had regular, spherical blastomeres of an equal shape and size, with some fragmentation (<30%). Type III or fair embryos had blastomeres slightly irregular in size and shape with considerable fragmentation (30-50%). Type IV or bad embryos had embryos with barely defined blastomeres with considerable fragmentation (>50%) (Xia, 1997).

2.4. Evaluation of Sperm Chromatin Integrity by Acridine Orange Staining

Acridine orange staining was performed according to the method of (Tejada *et al.*, 1984; Virant-Klun *et al.*, 2002). After washing and drying, the slides were immediately observed under a fluorescent microscope (Olympus, Japan; excitation of 450–490 nm). Averages of 200 sperm cell were counted on each slide by the same examiner. Sperm displaying green fluorescence were considered having normal DNA content, whereas sperm displaying a spectrum of yellow–orange to red fluorescence were considered having damaged DNA. The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of sperm with red, orange, or yellow fluorescence to all sperm counted per sample.

2.5. Statistical Analysis.

Statistical analysis was performed with STATISTICA for Windows software version 6.0 (Stat Soft Inc., USA). Student's t-test and ANOVA (One-way ANOVA) were used for the comparison of quantitative parameters. Correlation matrices were used for calculation of correlation coefficient. Data are stated as means \pm Standard Error of the Mean (S.E.M). Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. Seminal Fluid Analysis

Seminal fluid analysis (SFA) for eighty four men attending the Infertility Unit at Farah Hospital (Amman, Jordan) was performed. According to WHO criteria, 1992, forty men were considered fertile as they had normal semen, and the other 44 men were considered infertile based on their SFA (Table 1). The mean age for fertile and infertile men was 39.5 ± 1.2 and 38.3 ± 1.5 years, respectively. The cause of infertility was due to male factor in all of the 44 infertile couples. Nineteen of the infertile couples had primary infertility while 25 couples had secondary infertility with a duration of 6.3 ± 1.2 years.

The fertile and infertile groups differed significantly in the volume of the semen ($p < 0.01$), sperm concentration ($p < 0.001$) and in the percentage of motile sperm ($p < 0.001$), and normal sperm forms ($p < 0.0001$) (Table 1).

Table 1. Seminal Fluid Analysis. Values are expressed as mean \pm SEM.

Parameter	Fertile men (n=40)	Infertile men (n=44)	p-value
Volume (ml)	3.2 \pm 0.2	4.6 \pm 0.4	<0.01
pH	8	8	-
Sperm count (106/ml)	55.9 \pm 4.4	34.8 \pm 4.1	P<0.001
Progressive motile sperm (%)	60.5 \pm 1.2	50.9 \pm 2.2	P<0.001
Normal sperm forms (%)	19.2 \pm 0.8	10.1 \pm 2.0	P<0.0001

4. Evaluation of Chromatin Integrity of Sperm DNA by Acridine Orange Staining.

Sperm DNA fragmentation was detected with the acridine orange staining method, in which sperm with single-strand DNA was identified by the presence of evident yellow to red fluorescence. No significant difference in the percentage of sperm with fragmented DNA was found between the fertile (21.9 \pm 4.2%, 0.0-93.0%) and the infertile groups (26.7 \pm 5.0%, 0.0-91.5%) ($p>0.05$).

4.1. ICSI Outcomes

No significant difference in the mean of the fertilization rate was found between the fertile (78.8 \pm 2.6%) and infertile groups (69.4 \pm 4.7%) ($p>0.05$). On the other hand, a significant difference was found in the developmental rate between the fertile (99.1 \pm 0.6%) and the infertile groups (89.4 \pm 4.8%) ($p<0.05$). Biochemical pregnancy was confirmed by the serum beta-HCG determination 15 days after ET. In both the fertile and infertile groups, the positive pregnancy rate was almost similar (39.4% and 38.1%, respectively, $p>0.05$) (Table 2).

Table 2. Comparison between ICSI outcomes in fertile and infertile groups. Values are expressed as mean \pm SEM.

Parameter	Fertile group n=40	Infertile group n=44	P-value
DNA fragmentation rate (%)	21.9 \pm 4.2	26.7 \pm 5.0	P>0.05
Fertilization rate (%)	78.8 \pm 2.6	69.4 \pm 4.7	P>0.05
Development rate (%)	99.1 \pm 0.6	89.4 \pm 4.8	P<0.05
Pregnancy rate (%)	39.4 \pm 1.9	38.1 \pm 2.2	P>0.05

4.2. The effect of sperm DNA fragmentation on fertilization rate

No correlation was observed between DNA fragmentation and fertilization rate in the fertile group ($r=-0.02$, $p<0.00001$) and the infertile group ($r=-0.02$, $p<0.00001$).

4.3. The effect of sperm DNA fragmentation on developmental rate

A small significant negative correlation was observed between DNA fragmentation and developmental rate in the fertile group ($r=-0.20$, $p<0.00001$). A moderate significant

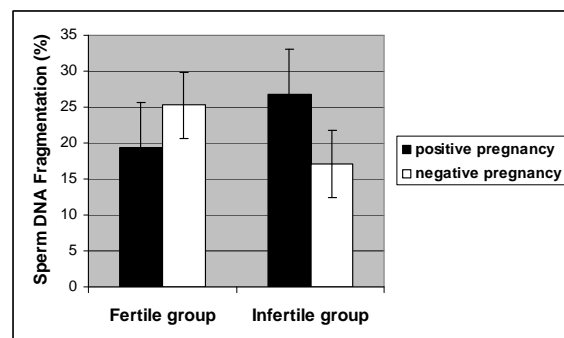
negative correlation was observed in the infertile group ($r=-0.51$, $p<0.00001$).

4.4. The effect of sperm DNA fragmentation on embryo quality

To elucidate the effect of DNA fragmentation on embryo quality, we divided the infertile group (data regarding the types of embryos were not available for the fertile group) according to DNA fragmentation rate into two groups, group I (sperm DNA fragmentation < 38%; n=32) and group II (sperm DNA fragmentation \geq 38%; n=12) (Table 2). No significant difference was found between the two groups in terms of no. of embryos of type I and II ($p>0.05$, and $p>0.05$, respectively). However, there was a significant difference between the two groups in the no. of type III and IV embryos ($p<0.05$ and $p<0.001$, respectively) (Table 3).

4.5. The effect of sperm DNA fragmentation on pregnancy rate

DNA fragmentation had no effect on whether a pregnancy occurred or not. For the fertile group, DNA fragmentation rate was 19.4% \pm 6.3 for those couples who achieved pregnancy, and 25.3% \pm 7.5 for those who did not, $p>0.05$. In the infertile group, DNA fragmentation rate was 26.8% \pm 7.5 and 17.1% \pm 4.6 %, respectively, $p>0.05$ (Figure 1).

Figure 1. The relation between sperm DNA fragmentation and pregnancy result. Values are expressed as mean \pm SEM. P > 0.05.

4.6. Primary and secondary infertility and sperm DNA fragmentation and ICSI outcomes

To compare primary with secondary infertility according to DNA fragmentation, fertilization rate and developmental rate, the infertile couples were divided into two subgroups: primary infertility (n=19) and secondary infertility (n=25). No significant difference in DNA fragmentation rate ($p>0.05$), fertilization rate ($p>0.05$) and the developmental rate ($p>0.05$) was found between infertile couples suffering from primary or secondary infertility (Table 4). Also, no significant difference was found in the pregnancy rate between primary and secondary infertile males (32% vs. 43%, $p>0.05$) (Table 4).

5. Discussion

Acridine orange (AO) staining is an established cytochemical method for determining sperm DNA integrity, allowing the differentiation between normal, double-stranded and abnormal, single-stranded sperm

DNA, using the metachromatic properties of the dye (Virant-Klun *et al.*, 2002; Martins *et al.*, 2007). The AO staining can be used to evaluate the integrity of the sperm nucleus, disorders of which can cause unexplained infertility or lower fertilization potential that may go undetected by routine analysis (Gopalkrishnan *et al.*, 1999).

There has been a tremendous amount of controversy in literature in terms of the degree of sperm DNA fragmentation in fertile versus infertile men. This could be attributed mainly to two reasons; the method of testing for chromatin integrity, and/or the way researchers define “fertile” and “infertile” male. Many techniques have been developed to test chromatin fragmentation. Three of these

Table 3. Quality of embryos in the fertile group according to the percentage of sperm with DNA fragmentation

Infertile group	Excellent embryos Type I	Good embryos Type II	Fair embryos Type III	Bad embryos Type IV
Group I (n=32) (Fragmentation rate ≤ 38%) No. of embryos= 79	46.2 ± 1.1%	27.9 ± 0.8%	24.6 ± 0.5%	1.3 ± 0.1%
Group II (n=12) (Fragmentation rate ≥ 38%) No. of embryos= 112	36.9 ± 0.9%	18.7 ± 0.3%	38.2 ± 1.2%	6.2 ± 0.3%
P-value	P > 0.05	P > 0.05	P < 0.05	P < 0.001

Table 4. Comparison between DNA fragmentation and ICSI outcomes in primary and secondary infertile men subgroups. Values are expressed as mean ± SEM.

Parameter	Primary infertility n=19	Secondary infertility n= 25	P-value
DNA fragmentation rate (%)	16.9 ± 5.5	25.8 ± 6.6	P > 0.05
Fertilization rate (%)	76.4 ± 4.7	80.7 ± 3.1	P > 0.05
Development rate (%)	89.3 ± 0.7	89.0 ± 1.1	P > 0.05
Pregnancy rate (%)	32.6 ± 2.3	43.9 ± 1.4	P > 0.05

are Sperm Chromatin Structure Assay (SCSA) (Evenson and Wixon, 2006), In Situ DNA Nick End Labeling Assay (TUNEL) (Seli *et al.*, 2004; Huang *et al.*, 2005; Sergerie *et al.*, 2005), and Acridine Orange Staining (AO) (Tejada *et al.*, 1984; Virant-Klun *et al.*, 2002). Many researchers consider AO technique not to be sufficiently reliable for clinical use (Evenson and Wixon, 2005). The primary reason is that the metachromatic property of acridine orange requires a very strict equilibrium concentration of the dye and perfectly flat glass slides and cover slips (Kapusinski *et al.*, 1983).

The second reason for the controversy arises from the fact that some researchers choose their “fertile, control” group as men with normal SFA (Seli *et al.*, 2004; Huang *et al.*, 2005), while others choose them as men of proven fertility (fathering a child or achieving pregnancy) (Sergerie *et al.*, 2005). In the current work, our control, fertile men were chosen based on their normal seminal fluid analyses (SFA) (Table 1).

Our data show that sperm DNA fragmentation rate was not significantly different between fertile (21.9%) and infertile groups (26.7%) ($p > 0.05$), which agrees with the results reported by Chohan and colleagues, 2004, and it might support the hypothesis that conventional SFA is not of a paramount value in choosing a sperm for ICSI. Indeed, some investigators have used sperm DNA fragmentation rate as a marker to differentiate fertile from infertile men (Lopes *et al.*, 1998).

In the current work, there was no significant difference between fertile and infertile men in terms of fertilization rate (78.8% and 69.4%, respectively) ($p > 0.05$), and pregnancy rate (39.4% and 38.1%, respectively) ($p = 0.05$). A significant difference was found in the developmental rate (99.1% and 89.5%, respectively) ($p < 0.5$) (Table 2). It is possible that if the sperm DNA fragmentation rate is low, the oocyte might be able to repair the condition and the fertilization is successful (Huang *et al.*, 2005). On the other hand, the first steps of the development are subjected to maternal control, and the expression of paternal genes begins at the 4–8 cell stage. It is therefore at this stage that the consequences of paternal DNA integrity may become evident and thus impairing embryo development (Borini *et al.*, 2006). Most studies found that when the level of spermatozoa with single-stranded DNA was increased, there was a significantly lower fertilization rate after ICSI (Lopes *et al.*, 1998; Virant-Klun *et al.*, 2002; Huang *et al.*, 2005).

A significant negative correlation was observed between DNA fragmentation and developmental rate in the fertile ($r = -0.20$) and in the infertile groups ($r = -0.51$). This is in agreement with the extensive literature which showed that sperm DNA fragmentation might affect the outcome of assisted reproduction through its effect on embryo development (Virant-Klun *et al.*, 2002; Saleh *et al.*, 2003). Sperm DNA fragmentation probably prevents the formation of blastocysts and embryo development (Borini *et al.*, 2006).

In terms of embryo quality, infertile men had more embryos of type III (fair embryos) and type IV (bad embryos). The number of type III and type IV embryos increased when DNA fragmentation was $\geq 38\%$ (Table 3). This is in agreement with other reports. For example, a significant negative association between the percentage of sperm with DNA fragmentation and embryo quality was reported by Virant-Klun *et al.* (2002) and Huang *et al.*, (2005). However, few studies indicated that pregnancy rate might be affected by sperm DNA fragmentation (Benchaib *et al.*, 2003), while others showed that no correlation was found between DNA fragmentation and pregnancy rate (Virant-Klun *et al.*, 2002; Gandini *et al.*, 2004). Our data showed that DNA fragmentation had no effect on whether a pregnancy occurred or not (Figure 1). Usually, embryos of bad quality (type III and IV) are not transferred to the uterus, and if it happens it might then affect the pregnancy rate. Also, embryonic quality is not the only factor affecting implantation rate; implantation is a complex process involving several maternal factors (Virant-Klun *et al.*, 2002).

Data presented in Table 4 show a comparison between DNA fragmentation and ICSI outcomes in primary and secondary infertile males' subgroups. Primary infertility is the term used to describe a couple that has never been able to conceive a pregnancy, after at least 1 year of unprotected intercourse. On the other hand, secondary infertility describes couples who have previously been pregnant at least once, but have not been able to achieve another pregnancy (WHO, 1999). No significant difference was found in sperm DNA fragmentation, fertilization rate, developmental rate and pregnancy rate if the patient suffered from primary or secondary infertility (Table 4).

In summary, many factors affect the outcomes of ICSI. One of these factors is the integrity of sperm chromatin. Therefore, it becomes a priority to look for an assay which tests the chromatin integrity of the sperm used in ICSI technique. Such an assay must not be invasive or fatal for that sperm.

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