SPERM DNA FRAGMENTATION AND MALE AGE: RESULTS OF *IN VITRO* FERTILIZATION TREATMENTS

Running title: IVF RESULTS ACCORDING TO MALE AGE

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ABSTRACT

The aim of the present study was to assess the effects of sperm DNA fragmentation according to different paternal age groups in couples comprised of normozoospermic men and infertile women undergoing conventional IVF.

The results obtained in 163 conventional IVF cycles were analysed retrospectively. Couples where the woman's age was between 30 and 37 years old were included. Sperm DNA fragmentation was studied using the TUNEL assay. Four groups were determined according to male age and sperm DNA fragmentation: #1: ≤39 years and TUNEL assay ≤20%; #2: ≤39 years and TUNEL assay >20%; #3: ≥40 years and TUNEL assay ≤20%; and #4: ≥40 years and TUNEL assay >20%.

No significant differences were found in semen parameters or fertilization rate between groups. Groups with <20% sperm DNA fragmentation showed significant differences in other parameters, including higher blastocyst formation rate (#1: 63% and #3: 60% vs #2: 43% and #4: 41%, $p<0.05$) and higher expanded blastocyst formation rate (#1: 42% and #3: 40% vs #2: 21% and #4: 18%, $p<0.05$). Abortion rate was significantly higher in #4 (42% and 46% vs 5%, 25% and 5% in #1, #2 and #3, respectively, $p<0.05$).

Our results showed a lower blastocyst formation rate in IVF treatments when males had a high sperm DNA fragmentation. Furthermore, if those males were older than 40 years, we found a higher abortion rate. These results show the need to inform about potential risks to couples looking for fertility treatments whose male is over 40 years of age.

Keywords: Assisted reproduction - Sperm DNA fragmentation - Male age - Abortion rate

INTRODUCTION

Advanced maternal age (>35 years) is known to be associated with a progressive decrease in fertility and the occurrence of embryonic chromosomal alterations (Leader et al., 2018). However, the influence of male age on reproductive outcomes has been largely ignored. Nonetheless, in the last few years, the influence of the paternal component on embryo quality and success in achieving pregnancy both in natural pregnancies and in IVF treatments has gained better understanding (Kumar et al., 2013). Furthermore, it has been suggested that several parameters of embryo quality are strongly influenced by sperm quality, and sperm DNA fragmentation often being the main factor (Colaco and Sakkas, 2018), and this DNA fragmentation is strongly linked to paternal age (Wyrobek et al., 2006). Some studies showed that DNA sperm repairing mechanisms are altered by advanced male age, which induces an increase in the number of sperm with altered DNA (Muratori et al., 2019).

Studies showing a decrease in male fertility potential after the age of 40 have recently reported that this age group accounts for more than 25% of males who start highly complex treatments with their partners worldwide (Evenson et al., 2020; Kaarouch et al., 2018; Stone et al., 2013). In addition, some studies have suggested that sperm DNA fragmentation levels are linked to poor embryonic quality, low blastocyst development rate, higher global aneuploidy rate, low implantation rate, and recurrent abortions (Borges et al., 2019; Colaco and Sakkas, 2018).

Different studies recommend considering an altered semen sample when it shows >20% sperm DNA fragmentation (Agarwal et al., 2016; Van Montfoor et al., 2004; Zini et al., 2008), while others propose >30% of affected sperm (Colaco and Sakkas, 2018). These cut-off values depended on the sperm DNA fragmentation detection technique applied (Sakkas and Alvarez, 2010).

Based on the above, the aim of this study was to investigate the effects of sperm DNA fragmentation according to different paternal age groups in couples comprised of normozoospermic men and infertile women undergoing conventional IVF.

MATERIALS AND METHODS

A retrospective study was conducted in order to analyse the results obtained by 163 couples who underwent IVF treatments at Fertilis Reproductive Medicine, Buenos Aires, Argentina, between January 2019 and April 2020, which were retrospectively evaluated. The Institutional Ethics Committee approved the study protocol.

Inclusion criteria included the following:

- fresh transfer of embryos on Day 5 of embryo culture
- women: aged between 30 and 37 years; mature oocytes (MII): ≥4;
- men: WHO sperm values (WHO, 2010):
  Volume: ≥1.5 mL
  Concentration: ≥15 x 10^6 sperm/mL
  Progressive motility: ≥32%
  Viability: ≥58%
  Morphology: ≥4%

Males included were between 28 and 55 years of age. Males with azoospermia, cryptozoospermia, retrograde ejaculation, leukocytospermia, or varicocele, those who had been exposed to chemotherapy, radiation therapy or pesticides and other toxics, and

those with a history of infection or fever in the three months prior to the treatment were excluded. Cases in which, for some reason, IVF had to be performed by means of intracytoplasmic sperm injection (ICSI) or using frozen semen sample were also excluded.

Women with uterine factor, reproductive tract infection diseases, anovulation, or premature ovarian failure were excluded.

Semen samples were collected by masturbation in a sterile bottle after 2 to 5 days of sexual abstinence. Semen volume was measured after 30-60 minutes and semen concentration and motility evaluated using a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Next, sperm morphology was evaluated according to Kruger's Strict Criteria (Kruger et al., 1986). To this end, a 5-10-µL semen aliquot was placed on a slide and a smear was performed and allowed to air dry. The slide was then submerged in 96% alcohol for 20 min for fixation, and finally immersed in Giemsa staining for 10 min. After this, the slide was washed with water and allowed to dry at room temperature.

Sperm DNA fragmentation levels were determined by means of the Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay (Lopes et al., 1998). To this end, Teflon Printed Slides for TUNEL (EMS, USA) were submerged for at least 2 h in Poly-L-Lysine 0.1% (Sigma, USA), and then rinsed with ultrapure water (Sigma, USA) and dried at room temperature. Semen samples were processed in a 15-ml centrifuge tube containing two layers of Pure Ception (SAGE, USA) at 90% and 50% (WHO, 2010). Samples were centrifuged at 300g for 20 min, washed at 300g for 10 min and re-suspended in 0.4 ml of heated human tubal fluid medium supplemented with 0.3% Human Albumin (SAGE, USA). Then, the samples selected for this study were fixed with 37% formaldehyde (Sigma, USA) and stored at 4-8°C until use.

To evaluate DNA fragmentation, 30-µL aliquots of the samples were placed on excavated slides in duplicate, and the slides then placed in a wet chamber for 24 h at 4-8°C. After that time, the samples were washed three times for 5 min with 10 µL of phosphate buffered saline (PBS) 1X (Sigma, USA). Methanol (Sigma, USA) was then added for 90 sec and the slides washed again three times with PBS 1X. Then, 10 µL of blocking solution (PBS + 0.5% Bovine Serum Albumin (BSA) (Sigma, USA) was added and allowed to act for 45 min inside the wet chamber at 4-8°C. Subsequently, another three washes with PBS 1X were performed.

A mixture of 30 µL of the fluorescent label and 5 µL of enzyme (In situ Death Cell Detection kit, Roche, USA) was added to each well of the excavated slides, protected from light and the slides then placed in a wet chamber for 1 h on a thermal plate at 37°C. Next, three 5 min washes were performed with 10 µL of PBS 1X and the slides then allowed to dry completely at room temperature, always avoiding exposure to light. Finally, 5 µL of Vecta-Shield mounting agent (Vector Lab, USA) were added to each well and a 24 x 50 mm slide cover slip was placed above the well. Finally, slides were observed under a fluorescence microscope (Nikon, Eclipse 200, Japan) at 1000x magnification with immersion oil. Sperm with fluorescence greater than 50% in the cytoplasm were considered positive, whereas the rest were considered negative (Figure 1). One extra well of the excavated slide was incubated with DNAse (1 U/mL; Sigma, USA) for 30 minutes at 37°C as a positive control, and in another well the TUNEL solution was omitted as a negative control. The number of sperm with fragmented DNA was recorded as the average of two counts of 100 sperm each (total 200) and the percentage of cells with positive TUNEL was calculated. Semen samples with TUNEL levels ≤20% were considered normal, whereas those with TUNEL levels >20% were considered altered.

Male patients were divided into four groups according to their age and TUNEL staining pattern in their semen samples:
Group 1: patients aged ≤39 years old with normal TUNEL assay (≤20%)
Group 2: patients aged ≤39 years old with altered TUNEL assay (>20%)
Group 3: patients aged ≥40 years with normal TUNEL assay (≤20%)
Group 4: patients aged ≥40 years old with altered TUNEL assay (>20%)

All the female patients were stimulated with recombinant follicle stimulating hormone (FSH) (Gonal-F, Merck-Serono, Germany) combined with human menopausal gonadotropin hormone (hMG) (Menopur, Ferring, Sweden). An initial dose of 150 to 300 international units (IU) of gonadotropins was administered for 5 days, adjusting it according to ovarian response. Upon reaching an average follicular diameter of 14 mm or oestrogen levels of 300 pg/mL, a daily dose of gonadotropin-releasing hormone (GnRh) antagonist (Cetrorrelix, Cetrotide NR, Merck-Serono, Germany) was administered until discharge of ovulation, for which a simple dose of 10,000 IU of human chorionic gonadotropin (hCG) (Gonacor 5000, Ferring Pharmaceuticals, Switzerland) was administered 34-36 h prior to follicular aspiration.
After follicular aspiration, oocytes were place in Quinn's Advantage protein plus Fertilization medium (SAGE, USA). The embryos produced after conventional IVF were grown until Day 3 in Quinn's Advantage protein plus Cleavage medium (SAGE, USA), and then placed in Quinn's Advantage protein plus Blastocyst medium (SAGE, USA) until culture Day 5. The culture was carried out at 37°C in ESCO mini-Miri incubators in 5% oxygen and 6% carbon dioxide. Embryos from Day 5 were evaluated according to the Istanbul criteria (Balaban et al., 2011). All the transfers were performed on culture Day 5, transferring only one embryo per patient using a Rocket-Echo Cath (Rocket Medical, England) catheter and cryopreserving the remaining embryos. Clinical pregnancy was confirmed by ultrasound 6 weeks after embryo transfer.
The following parameters were determined: i) fertilization rate: MII oocytes fertilized with respect to total inseminated MII; ii) blastocyst formation rate: embryos that reached blastocyst stage with respect to the number of fertilized oocytes; iii) expanded blastocyst formation rate: embryos that reached expanded blastocyst stage with respect to the number of fertilized oocytes; iv) clinical pregnancy rate: clinical pregnancies with foetal cardiac activity with respect to total treatments; v) multiple pregnancy rate: pregnancies with more than one gestational sac relative to total pregnancies; vi) abortion rate: patients with pregnancies interrupted before 20 weeks of gestation with respect to the total of patients with a gestational sac; and vii) ongoing pregnancy rate: pregnancies ongoing after 20 weeks' gestation with respect to total treatments. All parameters were defined according to the ICMART Glossary (Zegers-Hochschild et al., 2017).

Statistical analysis

Statistical analysis was performed using GraphPad InStat 7 software (Graphpad Software Inc., San Diego, CA). The Kruskal Wallis test was used in the analysis of non-parametric data. Qualitative variables were analysed with the Chi-square test. Statistical significance was set at <0.05.

RESULTS

Groups 1 and 2 comprised male patients with an average age of 34.9±4.2 years, whereas Groups 3 and 4 involved male patients with an average age of 45.1±3.7 years. DNA fragmentation levels measured using the TUNEL assay were 12.1±4.8% in groups with "normal fragmentation" (Groups 1 and 3) and 27.7±6.3% in groups with "altered fragmentation" (Groups 2 and 4).

Comparison of the study groups showed that semen characteristics such as viscosity, volume, concentration and vitality showed no significant differences (Table 1). In addition, female patients showed no significant differences in age, infertility factor distribution, progesterone level at the time of ovulation discharge, and endometrial thickness at the time of follicular aspiration (Table 2). Finally, analysis of the results of IVF treatments in the four groups showed no significant differences in number of total oocytes recovered, number of mature oocytes (MII) recovered, fertility rate or pregnancy and multiple pregnancy rates. In contrast, results showed significant differences in blastocyst formation rate and expanded blastocyst formation rate, both of which were lower in the two groups with DNA fragmentation levels >20% (Groups 2 and 4). Results also showed that the abortion rate of the group with male age ≥40 years and altered DNA fragmentation levels (Group 4) was significantly higher; nevertheless, ongoing pregnancy rate was not different among groups (Table 3).

DISCUSSION

Although some researchers have previously found a correlation between advanced paternal age and alteration in conventional semen parameters (Aitken et al., 2009; Alshahrani et al., 2014), in the present study we found no significant differences in these semen parameters between the group of males aged ≤39 years and that of males aged ≥40 years. However, based on the fact that other types of alterations should not be underestimated, we evaluated the effect of sperm DNA fragmentation, and found that the increased level of sperm DNA fragmentation (>20%) led to a significant decrease in the results of IVF treatments in terms of the percentage of embryos that reached blastocyst stage and those that reached expanded blastocyst stage. This benefits groups with low sperm DNA fragmentation, as they will have a higher cumulative pregnancy rate.

We also observed a marked effect of the combination of advanced male age and an altered level of sperm DNA fragmentation on the abortion rate, since this group showed an abortion rate of 46% as compared with the other three male groups, which showed a rate ranging between 5 and 25%. These findings are consistent with numerous studies that have reported a positive correlation between increased male age and sperm DNA damage (Agarwal et al., 2008, Johnson et al., 2011, Nijs et al., 2011, Ramasamy et al., 2015, Sharma et al., 2015). Despite this, ongoing pregnancy rate was not significant among groups. We believe that this might be due to the limited number of cases analysed. At present, we are recording pregnancy data from patients who have failed in the first transfer attempt and are undergoing the second transfer. Our hypothesis is that those groups with lower sperm DNA fragmentation will reach a higher cumulative pregnancy rate. We would also like to explore in further studies the take-home baby rate, and it may be interesting to analyse the possible effect of fragmentation on the offspring.

There are several techniques for sperm DNA fragmentation analyses including Comet assay, SCD (Sperm Chromatin Dispersion), SCSA (Sperm Chromatin Structure Assay) and TUNEL. We applied TUNEL in our study because previous reports have shown a

high correlation between test results and pregnancy rates, yielding a high predictive value (Alvarez & Lewis 2008; Borini et al., 2006; Greco et al, 2005). In addition, we used a cut point of 20% based on previous reports where this threshold value for TUNEL assay distinguished between fertile controls and infertile men, with a high specificity and sensitivity (Sergerie et. al, 2005).

As known, sperm provides 50% of the embryo's genome, thus making it vitally important for embryo development. The new embryo's genome begins to express on Day 3 and, until that moment, development is almost exclusively dependent on the oocyte while the sperm acts only as a trigger of the process (Ortega et al., 2018). Until recently, the influence of male age on reproductive outcomes has been largely ignored. However, in the last few years, the influence of the paternal component on embryo quality and on the success in achieving pregnancy both in natural pregnancies and in IVF treatments has gained greater concern (Kumar et al., 2013).
In order to have a better understanding of the correlation between sperm DNA fragmentation and IVF results, it is important to address the mechanisms that we consider are leading to this damage. As some authors show, there are three main mechanisms that cause DNA fragmentation: apoptosis induction, an increasing production of reactive oxygen species (ROS), and impairment of sperm chromatin maturation. These mechanisms can be induced by a variety of factors such as lifestyle, drugs, diseases, aging, exposure to pollutants and infections (Muratori et al., 2019; Sakkas and Alvarez, 2010). Although we attempt to exclude all these factors by means of our patient selection, we have to consider other hidden variables impossible to study, such as epigenetics and metabolic effects that sperm DNA fragmentation could cause (Colaco and Sakkas, 2018).
Some researchers have described a possible mechanism of sperm DNA damage repair by the oocyte during fertilization stage or at a later embryonic stage, which would lead to the development of mosaic embryos (Jaroudi et al., 2009, Kaarouch et al., 2015). A possible explanation of this phenomenon in male patients older than 40 years is that this mechanism does not seem to be sufficient to repair the sperm damage, and we may think that the damage is either very extensive or undetectable by the tests carried out in our study. Thus, this may cause the significant increase in the abortion rate in this group of patients. Several studies found that sperm DNA fragmentation test values did not always correlate with pregnancy rate. Some authors believe that this is related to the type of DNA fragmentation mechanism in the sample. In some cases the damage can be repaired by the oocyte and in others, it cannot. Therefore, two patients could have the same DNA fragmentation value by the same test but prognosis could be different (Sakkas and Alvarez, 2010). Other researchers believe that repair failures in sperm DNA damage may lead to *de novo* mutations and structural chromosomal alterations in the germline of male patients of advanced age, and that these mutations will be transmitted to the embryos (Beal et al., 2017). This can explain the increased abortion rate in this group of patients (Priskorn et al., 2014).
It is important to consider DNA fragmentation in the sperm sample due to the fact that ICSI, a widely used technique, may facilitate the entry of damaged sperm into the oocyte, since its normal morphology does not necessarily mean that there is no DNA damage. It is relevant to identify the male factors that affect embryo quality, considering that when they are severe, there is an increased risk of transmitting genetic disorders to the offspring (Colaco and Sakkas, 2018). Furthermore, recent studies have also assessed the correlation between paternal age and an increased risk of autism onset in born children (Sandin et al., 2016). These studies have reported that, for the general

population, the rate of autism among children born to male parents over the age of 50 is 66% higher than that of those born to 20-year-old fathers. In addition, the rate of autism in children born to male parents aged between 40 and 49 years was 28% higher than that of those born to 20-year-old fathers. These results, together with those reported in the present study; clearly show the need to inform these potential risks to couples looking for fertility treatments whose male component is over 40 years of age.
One way to reduce sperm DNA damage is to perform lifestyle changes such as avoiding smoking, pollutants, contaminants, and factors that increase oxidative stress (Cui et al., 2016; Henkel and Franken 2011; Rybar et al., 2011; Wright et al., 2014). These could help increase the reproductive success rates in this group of patients. However, a reliable method to treat semen samples with high levels of DNA fragmentation should be found, as the ones used so far have not clearly demonstrated to be as reliable (Cakar et al., 2016; Nadalini et al., 2014; Romany et al., 2014; Tavalaee et al., 2012). In this regard, a microfluidic system that has been recently developed is showing encouraging results, and so far seems to be the most suitable alternative for the treatment of this type of sperm condition (Nosrati et al., 2017; Parrella et al., 2019, Quinn et al., 2018; Shirota et al., 2016). This system uses a chip made of polymethylmethacrylate and has a microfilter that only allows sperm with low DNA fragmentation levels and with the highest progressive motility to pass. This allows collecting sperm samples with probable lower percentage of DNA damage and increasing the chances to obtain good quality embryos (Samuel et al., 2018; Yetkinel et al., 2019). Given the results in this study, we have decided to introduce this technique in order to remove affected sperm in those patients with high DNA fragmentation values.

Our results show a significant effect of advanced paternal age on IVF results, making it necessary to study sperm DNA fragmentation levels in male patients who will undergo this type of treatment.

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TABLES

Table 1.- Comparison of the semen parameters of the four study groups

	Group 1 ≤39 years old and normal DNA fragmentation	**Group 2 ≤39 years old and altered DNA fragmentation**	**Group 3 ≥40 years old and normal DNA fragmentation**	**Group 4 ≥40 years old and altered DNA fragmentation**
N	52	32	49	30
Mean male age	35.9 ± 2.8 a	35.7 ± 2.6 a	45.6 ± 3.9 b	46.4 ± 3.4 b
Normal viscosity	30/52 (58%)	16/32 (50%)	27/49 (55%)	16/30 (53%)
Volume (ml)	3.1 ± 1.8	3.4 ± 1.8	2.9 ± 1.9	2.8 ± 1.9
Concentration (10^6spz/mL)*	62.3 ± 55.5	73.6 ± 52.1	69.7 ± 52.0	63.7 ± 56.2
Total count (10^6 spz)*	192.5 ± 143.7	232.4 ± 161.1	220.9 ± 196.3	195.6 ± 163.8
Progressive spermatozoa (%)	44.9 ± 19.9	44.6 ± 16.5	45.2 ± 21.9	45.9 ± 23.2
Vitality (%)	74.1 ± 11.0	73.8 ± 10.1	74.5 ± 13.2	71.9 ± 15.1
Normal sperm morphology (%)	7.5 ± 4.9	6.6 ± 4.7	6.8 ± 4.7	7.0 ± 4.5
Concentration after processing (10^6spz/mL)*	65.7 ± 30.3	67.2 ± 32.6	65.5 ± 38.2	67.9 ± 31.8
Motility after processing (%)	92.9 ± 5.3	93.1 ± 4.1	93.9 ± 5.2	92.9 ± 6.1
Total count after processing (10^6spz)*	25.1 ± 11.9	24.9 ± 12.2	24.8 ± 11.6	24.7 ± 11.3

* spz = spermatozoa. a,b Values with different letters inside the line differ significantly ($p<0.05$)

Table 2.- Comparison of the female patients' characteristics in the four study groups

	Group 1 ≤39 years old and normal DNA fragmentation	**Group 2 ≤39 years old and altered DNA fragmentation**	**Group 3 ≥40 years old and normal DNA fragmentation**	**Group 4 ≥40 years old and altered DNA fragmentation**
N	52	32	49	30
Female age	33.6 ± 2.7	34.7 ± 2.4	34.5 ± 4.3	33.5 ± 3.0
Tubal disconnection	23/52 (44%)	17/32 (53%)	25/49 (51%)	18/30 (60%)
Salpingectomy	23/52 (44%)	13/32 (41%)	21/49 (43%)	12/30 (40%)
Tubal ligation	5/52 (10%)	1/32 (3%)	2/49 (4%)	0/30 (0%)
Cervical factor	1/52 (2%)	1/32 (3%)	1/49 (2%)	0/30 (0%)
Progesterone (picograms/mL)	0.5 ± 0.3	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.2
Endometrium (mm)	8.0 ± 2.4	8.0 ± 2.2	7.9 ± 2.7	7.9 ± 2.5

Table 3.- Comparison of IVF results obtained by the four study groups

	Group 1 ≤39 years old and normal DNA fragmentation	**Group 2 ≤39 years old and altered DNA fragmentation**	**Group 3 ≥40 years old and normal DNA fragmentation**	**Group 4 ≥40 years old and altered DNA fragmentation**
N	52	32	49	30
Number of oocytes recovered	8.3 ± 3.8	8.2 ± 4.3	7.8 ± 3.5	8.0 ± 3.9
Number of mature oocytes recovered (MII)	6.2 ± 2.4	6.0 ± 3.2	5.9 ± 4.0	6.0 ± 3.3
Fertilization rate	283/321 (88%)	165/194 (85%)	24/287 (86%)	150/182 (82%)
Blastocyst formation rate	178/283 (63%) a	83/194 (43%) b	172/287 (60%) a	75/182 (41%) b
Expanded blastocyst formation rate	119/283 (42%) a	41/194 (21%) b	115/287 (40%) a	33/182 (18%) b
Clinical pregnancy rate	22/52 (42%)	12/32 (38%)	19/49 (39%)	11/30 (37%)
Multiple pregnancy rate	1/52 (1%)	0/32 (0%)	0/49 (0%)	0/30 (0%)
Abortion rate	1/22 (5%) a	3/12 (25%) b	1/19 (5%) a	5/11 (46%) b
Ongoing pregnancy rate	21/52 (40%)	9/32 (28%)	18/49 (37%)	6/30 (20%)

(a,b) Values with different letters inside the line differ significantly ($p<0.05$)

FIGURES

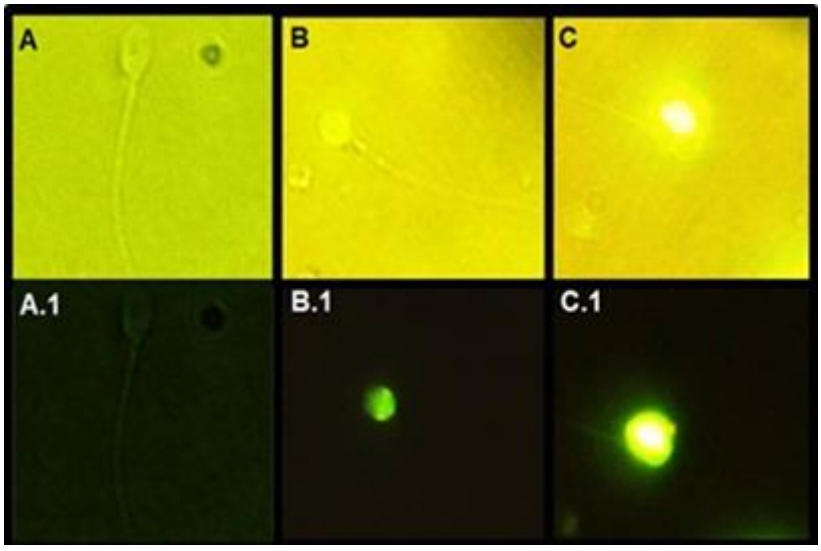

Figure 1: Spermatozoa with different TUNEL staining patterns (1000x). A: Negative sperm in white light; A.1: negative sperm with 0% fluorescence under UV light. B: Negative sperm with <50% fluorescence in white light; B.1: Negative sperm with <50% fluorescence in UV light. C: Positive sperm with >50% fluorescence in white light; C.1: Positive sperm with >50% fluorescence in UV light (from Riva et al., 2018)