Effect of cold atmospheric pressure plasma on DNA integrity in patients with asthenospermia

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Abstract: In this paper, we focused on the possible biomedical applications of the atmospheric pressure plasma, where we used plasma ignited in Argon (Ar) gas flow to study the effects of cold plasma on DNA integrity. Atmospheric pressure plasma, by needle, contains almost of equal amounts of positive and negative charges and has much potential for medical applications. A prospective study carried on 60 samples of human semen; each sample divided into 2 parts, before and after exposure to plasma, and then study the DNA integrity in both parts. Sperm DNA integrity was determined using a modified alkaline single cell gel electrophoresis (comet assay) and acridine orange test (AOT). The results showed that atmospheric pressure plasma give a significant (P< 0.05) decreasing in DNA damage for prepared sperm. Plasma for sperm preparation, decreases DNA damage.

Keywords: Cold plasma, comet assay, DNA damage, Sperm

1. Introduction

The need for assisted reproduction technologies (ART) for the establishment of pregnancies has steadily increased worldwide. Therefore, it is of vital importance that an efficient sperm preparation technique used for retrieval of high-quality spermatozoa contributes to the creations of high-quality embryos, with high implantation potential. The World Health Organization (WHO) defines the infertility as an inability of a couple to achieve conception or bring a pregnancy to term after one year or more of regular unprotected sexual intercourse. Conception is normally achieved within 12 months in 80%–85% of couples using no contraceptive measures [1]. Male infertility is due to the complete absence of sperm in the ejaculate and is relatively uncommon. Male subfertility can be due to low numbers of sperm, a low percentage of sperm with effective progressive movement or abnormalities in the sperm’s ability to fertilize an egg [2,3]. Male infertile patients are often classified as oligozoospermic, asthenozoospermic, or teratozoospermic on the basis of concentration, motility, morphology or any of these combinations [4].

Asthenozoospermia is therefore one of the major causes of infertility or reduced fertility in men [5]. Therefore, the cause of infertility in infertile men with normal semen parameters could be related to abnormal sperm DNA [6].

Non-thermal atmospheric pressure plasma is composed of UV light, radicals, positive and negative charges traveling in a flow of gas in a plasma needle. One reason for the plasma needle advantageous is because even though the electrons and other species which are generated might be hot due to their high kinetic energy, the overall gas is at room temperature[7,8]. This property of the plasma needle can be used to treat living tissue. Non-thermal atmospheric pressure plasmas can be used for biomedical applications such as in
dentistry in which it is used for teeth bleaching [9,10]. Plasma can generate a wide array of species and therefore the manner by which plasma attacks DNA can occur through various different reaction pathways. Plasma can generate “excited atoms and molecules, charged particles, electrons, and UV light,” all of which can have different effects on the DNA molecule [11]. The aim of this research is to study the effect of cold plasma atmospheric pressure at room temperature, on DNA integrity, by the comet assay technique.

2. Experimental work:

Material and methods:
The prospective study was carried on 60 semen samples in the Biotechnology Research Center laboratories, Al-Nahrain University, and Kamal Al Samuray Hospital laboratories (Test tube baby and infertility laboratories) from November 2017 to June 2018. Twenty samples were selected for comet assay test and forty samples for Acridine orange test. Their mean age was 20–49 years, smoker and non-smoker, alcoholic and not alcoholic, no history of chronic drug intake, no chronic disease like hypertension, diabetes mellitus and varicocele. The samples were obtained from men after a minimum 3 days of abstinence and ejaculated into a clean, wide-mouthed container. After liquefaction for 30 minutes in 37°C incubator, routine semen analysis was performed manually according to World Health Organization (WHO) to assess semen volume, pH, viscosity, liquefaction, sperm count, sperm motility, sperm agglutination, strict sperm morphology, and cell contamination (WHO 2010). After that we take the sperms with Low spermatozoal motility (Asthenozoospermia). Samples is prepared by swim-up technique. Add 5ml flushing medium to the native semen sample and mix. Centrifuge for 5 minutes at 3500 RPM. Remove supernatant and add 3ml Flushing medium to the pellet and perform swim-up method. Then each semen samples were divided into 2 parts, one part is before exposure to plasma needle and the other part after plasma system treatment at room temperature for constant time (120sec). And then study DNA integrity in both parts. Sperm DNA integrity was determined using a modified alkaline single cell gel electrophoresis (comet) assay and acridine orange test (AOT).

Plasma needle system

Plasma needle system includes four main parts:

- High A.C voltage power supply.
- Plasma needle.
- Argon gas.
- Flow meter.

DNA Integrity assessment:

Acridine orange test (AOT) [12] Sperm smear is prepared and let it dry. Immerse the sperm smear in methanol-glacial acetic acid (at a ratio of 3:1) overnight at room temperature for fixation, slide was stained after drying. There was daily preparation of the staining solution from the stock AOT solution in a ratio of 1/1000 mL of distilled water and stored at 4 °C and in dark place. Staining solution was prepared by adding 10 mL of stock solution to 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M Na$_2$HPO$_4$, 7 H$_2$O and the solution was maintained at room temperature. The slides after staining with Acridine orange (0.19 mg/mL, pH 2.5) left at room temperature for 5 minutes, rinsed gently by distilled water, coverslip was put and viewed by fluorescence microscope. Sperm cell heads with good DNA integrity had green fluorescence, and those with diminished DNA integrity had orange - red staining. Samples were scored within 1 hour after staining. Counting fifty sperm were count, and scoring the percentage of green, yellow –red stain sperm.

Comet Assay
It is a single cell gel electrophoresis assay (SCGE) for simple assessment of cellular DNA destruction in live cell. Molten agarose were mixed with sperm cells, applied to the comet slides. Lysis buffer and alkaline solution was added to these embedded cells and this treatment would lead to DNA relaxation and denaturation. To separate intact DNA from fragmented DNA, the samples were electrophoresed a horizontal chamber, dried, stained with a DNA dye and examined by fluorescent microscope. In electrophoresis fields, damage cellular DNA would travel faster than intact DNA, making a classic comet tail shape under the microscope. Degree of DNA fragmentation is generally visually assessed by comet tail measurement [13]. Fifty randomly selected sperm were counted per sample to quantify the comet cell. The score was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scores ranging from 1.2 to 2 were considered of low DNA damage (LD), from 2.1 to 3 of medium DNA damage (MD), and up to 3 of high DNA damage (HD) [14].

Statistical analysis

The SPSS program version 20 (Statistical package for social science) was employed for both data analysis and entry. Continuous variable presented as mean ± SD. Annova and Independent sample t test was used to test the significance of association of variables. P-value of < 0.05 was considered significant.

3. Results and Discussion

3.1. DNA Fragmentation

Acridine orange test (AOT):

Table 1. showed the percentage of DNA fragmentation of the prepared sperm (PS) before and after exposure to plasma needle at room temperature. Figure 2. illustrated the image of AOT. The percentage of DNA fragmentation before exposed to plasma needle for age 20-25 was 24.16% ± 4.14 SD and after treated with cold plasma, the DNA fragmentation decreases to 12.33% ± 3.18 SD. The percentage for age 26-30 (before treated with plasma needle) was 23.33% ± 5.165 SD, then after treated it became 14.27% ± 2.16 SD. For the age from 31-49 at control environment (before plasma treatment) the percentage was 25.83% ± 4.70 SD, when after exposed to cold plasma the DNA fragmentation decreases to 15.27% ± 3.38 SD at constant time 120 sec, Therefore, all DNA fragmentation results were significant (P<0.05).

Table 2. showed the results of Comet assay [(ND), (LD), (MD), (HD)] before treated with cold plasma (needle) and after exposure at room temperature for the prepared (PS) at constant time (120 sec). The percentage for No DNA damage before exposure to plasma needle was 14.19 % ± 2.16 SD, it increases to 22.47% ± 3.71SD after exposure to plasma needle. For low DNA damage the percentage at control (before plasma jet treatment) was 21.23% ± 3.07SD and it increases to 31.27% ± 4.24 SD after treated with cold plasma. For Medium DNA damage before treatment the percentage was 28.84% ± 3.36 SD and it decreases to 22.57% ± 4.05 SD after exposure to plasma needle. The percentage for High DNA damage at control (before plasma needle treatment) was 35.44% ± 4.15SD it decreases to 23.81% ±4.19 SD. The differences in percentages of no, medium and high DNA damage was significant where the (P<0.05), while there was no significant difference in percentage of low DNA damage. Figure 3. show the percentage of DNA damage.

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The differences in percentages of no, medium and high DNA damage was significant where the ($P<0.05$), while there was no significant difference in percentage of low DNA damage. Figure (3) show the percentage of DNA damage.

3.2. Discussion

The results of DNA fragmentation either by acridine orange test (AOT) or Comet assay provide evidence that plasma needle exposure results in less DNA fragmentation and improves DNA integrity of prepared spermatozoa. These results are similar to those in the literature, which showed that irradiation with a He-Ne laser of thawed ram sperm leads to an increase of the motility, viability and functional integrity of the sperm membrane [15]. The results of the both tests AOT and comet assay gave relatively similar predictive values for DNA fragmentation, although rapid fading of fluorescence, and heterogeneous staining of slides, and as some reports show that AOT gives higher DFI value makes AOT a test of questionable value in clinical practice. However, the AOT, is simple, less expensive, quick procedure of DNA integrity evaluation test for diagnostic and prognostic purpose in basic andrology laboratories and can provide valuable information about overall sperm chromatin status. While the major limitation of comet assay, labor intensive, has observer subjectivity and requires experience to evaluate it [16,17].

4. Conclusion

From these results it was concluded that using the atmospheric plasma needle as a method of treatment may exert beneficial effects in the improvement of DNA integrity and repair the damage in DNA. More studies are recommended to find out fertilization point of the sperm.

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Figure 1. The plasma needle system
Table 1. Acridine Orange test for DNA fragmentation% of the prepared sperm by using atmospheric pressure plasma jet (needle) for age (20 – 49). Capital Letters A and B for comparison between before and after treated with plasma. $P < 0.05$ significant.

| Age (Year) | DNA fragmentation (%) (mean±SD) |  |
|------------|---------------------------------|--|
|            | Before treated                  | After treated with plasma |  |
| 20-25      | A 24.16±4.14                    | B 12.33±3.18               |  |
| P-value    | 0.41                            |  |  |
| LSD        | 4.71                            |  |  |
| 26-30      | Before treated                  | After treated with plasma |  |
|            | A 23.33±5.165                   | B 14.27±2.16               |  |
| P-value    | 0.023                           |  |  |
| LSD        | 5.55                            |  |  |
| 31-49      | Before treated                  | After treated with plasma |  |
|            | A 25.83±4.70                    | B 15.27±3.38               |  |
| P-value    | 0.015                           |  |  |
| LSD        | 5.41                            |  |  |

Figure 2. A normal green AOT, B abnormal red AOT
Table 2. The Comet percentage of prepared semen before and after treated with plasma. Capital Letters A and B for comparison between before and after treated with plasma.

|                      | No damage % (mean+SD) | Low damage % (mean+SD) | Medium damage % (mean+SD) | High damage % (mean+SD) |
|----------------------|-----------------------|------------------------|---------------------------|------------------------|
| **Before treated**   |                       |                        |                           |                        |
| A 14.19+2.16         | A 21.23+3.07          | A 28.84+3.36           | A 35.44+4.15             |
| **After treated with plasma** |               |                        |                           |                        |
| B 22.47+3.71         | B 31.27+4.24          | B 22.57+4.05           | B 23.81+4.19             |
| **P-value**          | 0.017                 | 0.028                  | 0.025                     | 0.033                  |
| **LSD**              | 2.06                  | 4.13                   | 3.09                      | 5.27                   |

Figure 3. Scoring categories for comet assay (A: Normal, B: Low DNA Damage (LD); C: Medium DNA Damage (MD); D: High DNA Damage (HD).