

Short Report

## Nitric Oxide Level in Seminal Plasma and Its Relation with Sperm DNA Damages

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Received 14 October 2006; revised 8 April 2007; accepted 9 May 2007

### ABSTRACT

**Background:** Evidence supports the involvement of nitric oxide (NO) in a variety of male reproductive processes such as spermatogenesis, spermiogenesis, sperm motion, sperm metabolism and sperm capacitation. However, low concentration of NO is essential in biology and physiology of spermatozoa, but high amounts of NO is toxic and has negative effects on sperm functions. On the other hand, it is established that high amounts of NO have detrimental effects on DNA. The integrity of sperm DNA is an important factor in successful fertility and embryo development. It is hypothesized that supra physiological concentrations of NO in seminal plasma cause sperm DNA damage. The aim of this study was to determine sperm DNA damage by comet assay and its correlation with NO level in seminal plasma of fertile and infertile men. **Methods:** Semen samples were collected from 45 patients and 70 healthy donors. The stable metabolites of NO (nitrite and nitrate) in seminal plasma were measured by Griess assay and DNA damage was determined using single cell gel electrophoresis (comet) assay method. **Results:** The NO concentration in the seminal plasma of infertile males was significantly higher than fertile males ( $5.74 \pm 1.01 \mu\text{M/L}$  vs.  $3.88 \pm 0.53 \mu\text{M/L}$ ). There was a significant positive correlation between the NO concentration and sperm DNA comet value in infertile males ( $P < 0.01$ ,  $R = 0.598$ ). **Conclusion:** These results indicate that the overproduction of NO in genital tract of infertile males has a potential pathogenetic role in the reduction of sperm DNA integrity. *Iran. Biomed. J. 11 (4): 259-264, 2007*

**Keywords:** Nitric oxide (NO), Infertility, Sperm, DNA integrity

### INTRODUCTION

Nitric oxide (NO) is a biological messenger molecule produced by one of the essential amino acids, L-arginine, by the catalytic action of the enzyme NO synthase (NOS). NO is known to be involved in diverse physiological and pathophysiological processes in various organ systems, including the human male and female reproductive tracts. The dual role of NO as a protective or toxic molecule is due to several factors such as the isoform of NOS involved, concentration of NO and the type of cells where it is synthesized [1-4].

The physiological role of NO in male reproductive process has been previously identified and

expression of all isoforms of NOS in male genital tract cells suggested a definite role for NO in contractile, haemodynamic, hormonal aspects of testicular and epididymal function as well as in spermatogenesis and germ cell degeneration [5-7]. NO is detectable in seminal plasma and it has been reported that NO concentration in seminal plasma of some infertile males is significantly higher than the healthy males [8]. However, the sources of overproduction of NO in seminal plasma of infertile males are unknown but some studies suggest that it may be produced by induced genital tract cells such as Leydig cells, epididymal or vas deferens epithelial cells or spermatozoa itself and finally in some conditions such as subinfectious or inflammatory disease of male genital tract, induced leukocytes are

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the source of high concentration of NO in seminal plasma [9].

The effects of NO on sperm are dose-dependent. At physiological concentrations, NO has positive effects on sperm motility while at supra-physiological concentrations, NO has negative effects on sperm functions and decreases sperm motility [10-16].

On the other hand, it is established that high concentration of NO induces DNA damage [17]. NO reacts rapidly with superoxide to form highly toxic peroxy nitrite (ONOO<sup>-</sup>). Both NO and ONOO<sup>-</sup> have demonstrated ability to directly damage DNA [18]. Therefore, high amounts of NO in seminal plasma can cause sperm DNA damages and as reported previously, fragmentation of sperm DNA is a main factor in male infertility [19-21]. Intact human sperm DNA is an essential prerequisite for successful fertilization and embryo development. Thus, sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique [20].

Although the extent of DNA damage is closely related to sperm function and male infertility, the origin of such damage is still largely controversial. It is believed that despite of improper packaging and ligation during sperm maturation and germ cell apoptosis, oxidative stress is an important factor in sperm DNA damage [22]. The role of oxygen reactive species (ROS) in sperm DNA fragmentation and relation between concentration of ROS in seminal plasma and sperm DNA fragmentation have been previously reported [22-25]. To date, however, the overproduction of NO and its negative effects on sperm functions, such as motility, viability and metabolism have been reported in infertile males [11-16], but there is no report on the relation between seminal plasma NO concentration and sperm DNA damages.

In the present study, we have correlated NO concentration in semen and DNA fragmentation of sperm cells from normal and infertile men in order to acquire a deeper insight about the role of NO in the pathophysiology of human spermatozoa.

## MATERIALS AND METHODS

All chemicals (Analytical grade) were obtained from Sigma (USA), unless stated otherwise.

**Collection of semen samples.** Sperm samples were obtained from 45 men attending Fatemieh Infertility

Clinic (Hamadan, Iran) who had a history of infertility at least for a year. Controls consisted of samples obtained from 70 donors of unproven fertility. Semen samples were collected by masturbation after 3-5 days of sexual abstinence and allowed to liquefy at 37°C for at least 30 minutes. After liquefaction, they were evaluated according to the WHO (1999) guidelines, patients who had  $<20 \times 10^6$ /ml sperm concentration,  $<50\%$  motility or  $<30\%$  normal forms, were considered to have abnormal semen parameters. The liquefied semens were centrifuged at 300 g for 10 minute and then the supernatant was withdrawn and stored at -70°C until Nitrite assay. Pellet was resuspended in 1 ml Hams F10 medium and washed using a PurSperm (Nidacon, Sweden) discontinuous gradient (40 and 80%). The washed sperms used to DNA damage assay using single cell gel electrophoresis method.

**Single cell gel electrophoresis (comet) assay.** The comet assay which was first described by Singh *et al.* [26] is a sensitive technique that detects the presence of DNA strand breaks and alkali labile damages in the individual cells. The DNA fragments so produced migrate towards the anode pole at the rate inversely proportional to the size of the fragment during electrophoresis. In this study, the comet assay was performed using modification of Angelis method [27] in order to detect both single and double stranded breaks.

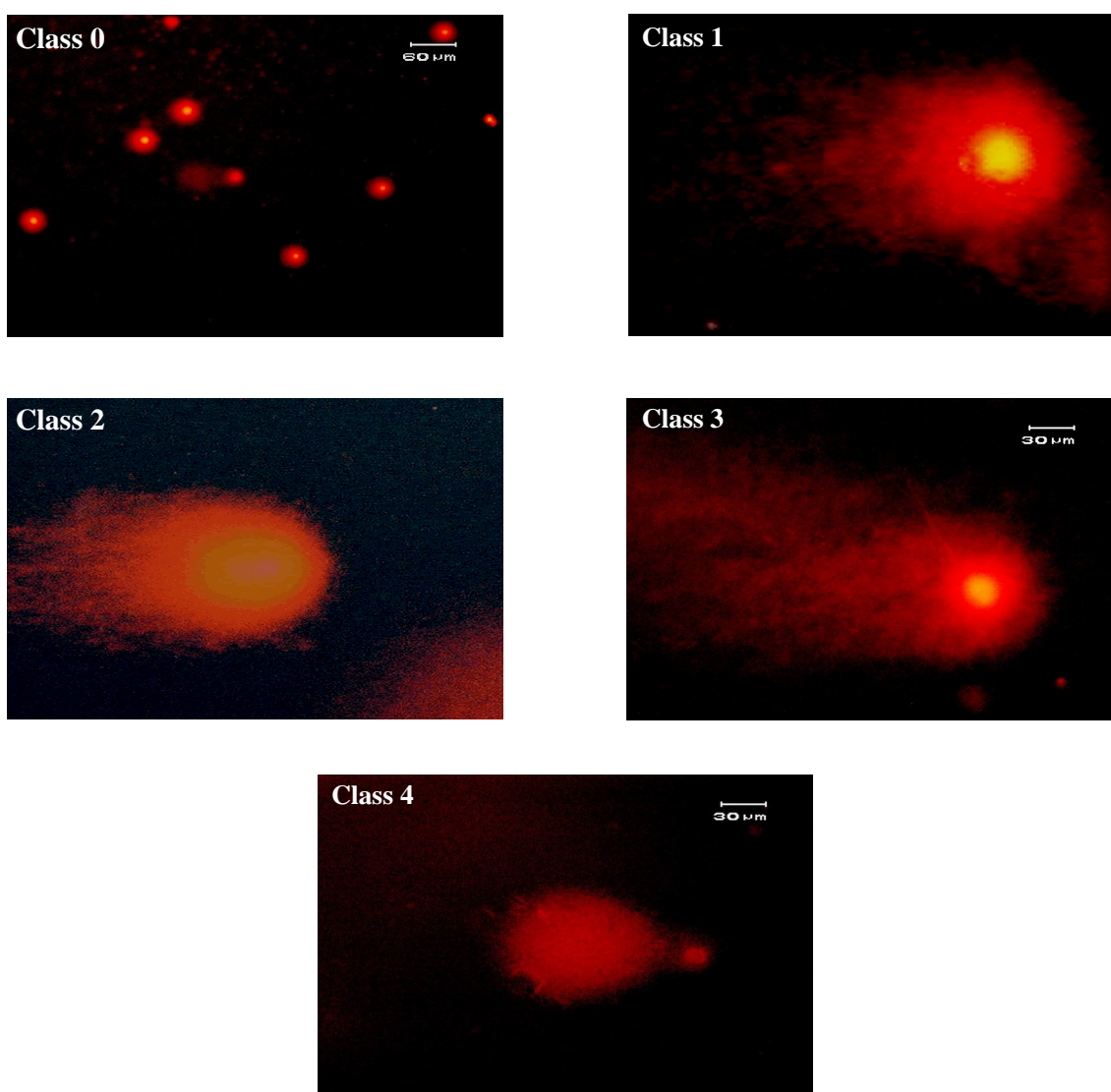
Pre cleaned slide (ROTH, Germany) were dipped in a solution of 1.5% (w/v) normal malting point agarose in PBS, a cover slip was then placed on top, and allowed solidifying at room temperature over night. The next day cover slip was removed and 100 microliters suspensions of spermatozoa in 1% (w/v) low melting point agarose (LMPA), at a concentration of  $1 \times 10^4$  cell/ mL, was pipetted on the slides and covered with a cover slip. The slides were allowed to solidify then 1% LMPA was used to form a third layer. After solidification, the slides were placed in cold lyses buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 1% Dimethylsulfoxide, and 10 mM Dithiothreitol at pH 10 at 4°C for 30 min) and protected from light. The slides were then incubated at 37°C in 10 µg/ml of Proteinase K in lyses buffer for 2.5 h.

Following cell lyses, all slides were placed in a horizontal electrophoresis tank filled with electrophoresis buffer (10 mM Tris containing 0.08 M boric acid and 0.5 M EDTA) and were kept for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 25V adjusted to 300

mA. When electrophoresis was completed, the slides were drained and flooded with neutralization buffer (0.4 mol/l Tris; pH 7.5). After neutralization step, the slides were stained with ethidium bromide (20 µg/ml dissolved in distilled water) and mounted with a cover slip. Cells were visualized at 200× using a fluorescent microscope (Nikon, Japan).

Each cell with fragmented DNA had the appearance of a comet with a brightly fluorescent head and a tail to one side formed by DNA, which contained strand breaks that were drawn away during electrophoresis.

**Measuring comet by visual scoring.** Analysis of comet cells was performed using the scoring method of Collins [28]. In this method, the cells were divided into 5 classes from 0 (no tail) to 4 (almost all DNA in tail) according to comet appearance and each cell was assigned a value of 0 to 4 (Fig. 1). At least 100 cells were randomly selected from each slide and observed under the microscope, the comet value for each slide was calculated between 0 and 400. Because two slides were prepared for each sample, the mean of comet value of two slides was calculated and considered for each case.



**Fig. 1.** Sperm cells processed using single cell gel electrophoresis (Comet) assay showing 5 classes of comet from Class 0, cells with intact and undamaged DNA, to Class 4 cells with high degree of DNA damage.

**Table 1.** Characteristic of the study population, semen analysis parameters and the mean of NO concentration and Comet Value. There were no significant differences in the mean of age of participants and morphology of sperms in two groups but the means of sperm concentration, motility, NO concentration and Comet Value in fertile males are significantly higher than infertile males ( $P < 0.001$ ).

	Fertile (Mean $\pm$ SD)	Infertile (Mean $\pm$ SD)	P value (Mean $\pm$ SD)
Age (y)	31.40 $\pm$ 4.60	33.20 $\pm$ 5.50	NS
Sperm concentration (* 10 <sup>6</sup> )	63.80 $\pm$ 16.70	39.70 $\pm$ 26.30	<0.001
Sperm motility (%)	53.00 $\pm$ 5.00	29.80 $\pm$ 13.70	<0.001
Sperm Morphology(%)	25.00 $\pm$ 6.00	13.48 $\pm$ 5.60	NS
NO concentration( $\mu$ mol/L)	3.88 $\pm$ 0.53	5.47 $\pm$ 1.01	<0.001
Comet Value	12.90 $\pm$ 7.59	48.77 $\pm$ 24.42	<0.001

**Measurement of NO in seminal plasma.** NO concentration was assessed by monitoring seminal plasma concentration of stable oxidation products of NO metabolites (NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>). Nitrite was determined using Griess reaction by adding 1% sulfanilamide and 0.1% naphthylethlenediamine dihydrochloride in 2% phosphoric acid and by recording absorbance at 540 nm with a spectrophotometer after 10-minute incubation at 37°C in the dark. Nitrate was detected after reduction to nitrite using nitrate reductase, as described elsewhere [29].

**Statistical analysis.** Data are reported as mean  $\pm$  SD. The comparisons between two groups were tested by student's *t*-test using SPSS11. Correlation between two continuous outcomes was evaluated using Pearson correlation coefficients.  $P < 0.05$  are considered as statistically significant.

## RESULTS AND DISCUSSION

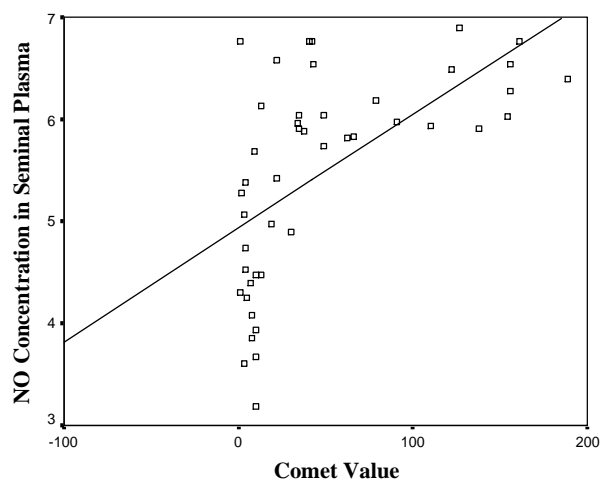
The mean of participants' age, semen analysis parameters, NO concentration and comet value in two groups are summarized in Table 1. As shown in the Table, there are no significant differences in the mean of participants' age in two groups but the mean of sperm concentration and sperm motility of fertile males were significantly higher than that of infertile males ( $P < 0.001$ ).

The mean of NO concentration in the seminal plasma of infertile males was significantly higher than that of fertile males ( $P < 0.001$ ). Also, the mean of comet value in the sperms of infertile males was significantly higher than that of fertile males (12.9  $\pm$  7.59 vs. 48.77  $\pm$  24.42,  $P < 0.001$ ).

There was significant positive correlations between NO concentration and comet score ( $R = 0.598$ ;  $P < 0.001$ ) in the infertile group (Fig. 2). This correlation was not found in the fertile group.

Our results contrary to Huang *et al.* [9] and in agreement with Rewelli *et al.* [10] provide evidence that NO concentration is significantly lower in the healthy males than the infertile males. Also our data are confirmed the results of others [19-20] about the higher level of sperm DNA fragmentation in infertile males compare to fertile male.

Furthermore, for the first time, our findings suggest that high concentrations of NO play a deleterious effect on spermatozoa DNA integrity and significant positive linear correlations between seminal plasma NO concentration and sperm DNA fragmentation in the infertile males has been identified in this study.



**Fig. 2.** Correlation analysis between NO concentration in seminal and sperm comet value. There is positive correlation between NO concentration and comet value ( $R = 0.598$ ,  $P < 0.001$ ).

As established previously, NO is a double-edged sword, serving as a key-signal molecule in both physiological and pathological process [1-3]. A moderate amount of NO in seminal plasma is essential for sperm motility and capacitation [12-16]

but excessive amounts of NO might inhibit sperm motility and DNA damages and thus lead to male infertility. The harmful effects of NO are mediated by biologically activated molecules produced by the reaction of NO with the superoxide anion yielding ONOO<sup>-</sup> and peroxyntrous acid (ONOOH). The resulting molecules are strong oxidant that can cause molecular damage to a variety of tissues]. The acid ONOOH reacts with the cysteine residues of proteins or glutathione, forming S-nitrosothiols. S-nitrosylation causes deregulation of cellular signal transduction processes and also has harmful effects on cellular energetic through the inhibition of complex I in mitochondrial respiration promotion of DNA damage and/or apoptosis [23-25]. Previously, we demonstrated the role of NO in induction of apoptosis in embryonic cells [30]. In this study, a positive correlation between seminal plasma NO concentration and sperm DNA fragmentation in the infertile males has been identified. This correlation may be has an important role in infertility in some cases.

However, this study established negative effect of NO in high concentration on sperm DNA integrity but it is not the main factor of sperm DNA fragmentation in all of the infertile males. It should be considered that in the case of severely impaired sperm DNA integrity, factors other than the NO such as other free radicals, improper packaging and ligation of DNA during sperm maturation and germ cell apoptosis might result in sperm DNA damages.

## REFERENCES

- Thippeswamy, T., McKay, J., Quinn, J.P. and Morris, R. (2006) Nitric oxide, a biological double-faced janus- is this good or bad? *Histol. Histopathol.* 21 (4): 445-458.
- Fostermann, U., Class, E.I., Pollock, J.S., Nakane, M., Schwartz, P., Gath, I. and Kleinert, H. (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning and functions. *Hypertension* 23: 1121-1131.
- Moncada S. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharm.Rev.* 43 (2): 109-142.
- Rosselli, M. and Paul, J. (1998) Role of nitric oxide in the biology, physiology and pathology of reproduction. *Hum. Rep. Update* 4913: 3-24.
- Yanhe, L., Amiya, P., Sinha, H., Christina, W., Andrew, L. and Swerdloff, R. (2003) Functional role of inducible nitric oxide synthase in the induction of male germ cell apoptosis, regulation of sperm number, and determination of testes size: evidence from null mutant mice. *Endocrinology* 144 (7): 3092-3100.
- Zuni, A., Bryon, M.K., Magid M.S. and Schlegel, P.N. (1996) Immunohistochemical localization of endothelial nitric oxide synthase in human testis, epididymis, and vasdeferan suggest a possible role for nitric oxide in spermatogenesis, sperm maturation and programmed cell death. *Biol. Reprod.* 55: 235-291.
- O'Bryan, M.K., Schlatt, S., Gerdprasert, O., Phillips, D.J., Kretser, D.M. and Hedger, M.P. (2000) Inducible nitric oxide synthase in the rat testis: evidence from potential roles of both normal function and inflammation-mediated infertility. *Biol. Reprod.* 63: 1285-1293.
- Nobunago, T., Tokugawa, Y., Hashimoto, K., Kubota, Y. and Sawai, K. (1996) Elevated nitric oxide concentration in the seminal plasma of infertile males: nitric oxide inhibits sperm motility. *Am. J. Reprod. Immunol.* 36: 193-197.
- Huang, I., Jones, J. and Khorram, O. (2006) Human seminal plasma nitric oxide: correlation with sperm morphology and testosterone. *Med. Sci. Monit.* 12 (3): CR103-106.
- Revelli, A., Bergandi, L., Massobrio, M., Lindblom, B., Bosila, A. and Ghiyo, D. (2001) the concentration of nitrite in seminal plasma does not correlate with sperm concentration, sperm motility, leucocytospermia, or sperm culture. *Fertil. Steril.* 76 (3): 469-500.
- Aksoy, Y., Ozbey, I., Aksoy, H., Polat, O. and Akcay, F. (2004) seminal plasma nitric oxide concentration in oligo and/or asthenozoospermia subjects with/without varicocele. *Arch. Androl.* 48 (3): 181-185.
- Rosselli, M., Dubey, R.K., Imtharn, B., Macas, E. and Keller, P.J. (1995) Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decrease sperm motility and induces sperm toxicity. *Hum. Reprod.* 10: 1786-1790.
- Donnelly, E., Lewis, S.H., Tompson, W. and Chakaravarty, U. (1997) Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. *Mol. Hum. Reprod.* 3 (9): 755-762.
- Zhang, H. and Zheny, R.L. (1996) Possible role of nitric oxide of fertile and asthenozoospermic infertile human sperm functions. *Free Radic. Res.* 25: 347-354.
- Balercia, G., Moretti, S., Viynini, A., Mayaynini, M., Mantero, F. and Boscaro, M. (2004) Role of nitric oxide concentrations on human sperm motility. *J. Androl.* 25 (2): 245-249.
- Zhang, H., Zhou, Q.M., Li, X.D., Xie, Y., Duan, X., Min, F.L., Liu, B. and Yuan, Z.G. (2006) Ginsenoside R(e) increases fertile and asthenozoospermic infertile human sperm motility by induction of nitric oxide synthase. *Arch. Pharm. Res.*

- 29 (2): 145-151.
17. Dimmler, S. and Zeiher, A.M. (1997) Nitric oxide and Apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide* 1 (4): 275-281.
  18. Ishikawa, T., Kondo, Y., Goad, K. and Fujisawa, M. (2005) Overexpression of endothelial nitric oxide synthase in transgenic mice accelerates testicular germ cell apoptosis induced by experimental cryptorchidism. *J. Androl.* 26 (2): 281-288.
  19. Saleh, R.A., Agarwal, A., Nelson, D.R., Nada, E.A., El-Tonsy, M.H., Alvarez, J.G., Thomas, A.J. and Sharma, R. (2002) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil. Steril.* 78: 313-318.
  20. Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, S., Lejeune, H. and Guérin, J.F. (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum. Reprod.* 18: 1023-1028.
  21. Sakkas, D., Moffat, O., Manicardi, G.C, Mariethoz E., Tarozzi, N. and Bizzaro, D. (2002) Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol. Reprod.* 66: 1061-1067.
  22. Moustafa, M.H., Sharma, R.K., Thornton, J., Mascha, E., Abdel-Hafez M., Thomas A., Agrawal A. (2004) Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum. Reprod.* 19 (1) 129-138.
  23. Saleh, R.A., Agarwal, A., Nada, E.A., Tonsy, M.H., Sharma, R.K., Meyer, A., Nelson, D.R. and Thomas, A.J. (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil. Steril.* 79: 1597-1605.
  24. Agarwal, A. and Saleh, R.A. (2002) Role of oxidants in male infertility: rationale, significance, and treatment. *Urol. Clin. N. Am.* 29: 817-827.
  25. Agarwal, A., Saleh, R.A. and Bedaiwy, M.A. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil. Steril.* 79: 829-843.
  26. Singh, N., McCoy, M., Tice, R. and Schneider, E. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184-191.
  27. Angelis, K.J. Dusinska, M. and Collins, A.R. (1999) Single cell gel electrophoresis: detection of DNA damage at different levels of sensitivity. *Electrophoresis* 20 (10): 2133-2138.
  28. Collins, A.R. (2004) The Comet Assay for DNA damage and repair, principles, application and limitation. *Mol. Biotechnol.* 26: 249-260.
  29. Harad, H. and Schmiot, H. (1995) Determination of nitric oxide via measurement of nitrite and nitrate in culture media. *Biochemica* 2: 22-23.
  30. Amiri, I., Sobhani, A., Abolhasani, F., Akbari, M. and Farimani, M. (2003) the effect of nitric oxide on mouse pre-implantation embryo development and apoptosis. *Iran. Biomed. J.* 7 (3): 107-111.