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Impact of the sexual abstinence period on the production of seminal reactive oxygen species in patients undergoing intrauterine insemination: A randomized trial

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Abstract

Aim: To evaluate whether the duration of sexual abstinence has impact on oxidative stress in semen samples.

Methods: Oxidative reaction was tested for different levels of reactive oxygen species (ROS) by nitro blue tetrazolium assay in 90 patients with the diagnosis of unexplained or male factor infertility that were grouped into 3 groups as 0–2 (group 1), 3–4 (group 2) and >4 days (group 3) of duration of sexual abstinence. Subsequently, the remaining semen was prepared by gradient method for ovarian stimulation and intrauterine insemination (IUI) cycles to compare pregnancy rates in terms of different levels of ROS and different abstinence periods.

Results: Increased staining pigment intensity was related to higher level of ROS in >4 days' group as compared to groups 0–2 and 3–4 days (70% vs 43.3% and 50%, $P = 0.013$ and $P = 0.014$, respectively). Pregnancy rates significantly decrease with prolonged abstinence period (26.7%, 16.7% and 6.7% in groups 1, 2 and 3, respectively, $P = 0.039$). Progressive motile sperm count after gradient method of sperm preparation for IUI was highest in 3–4 days of abstinence period than shorter and longer abstinence groups.

Conclusion: Longer duration of sexual abstinence causes higher oxidative stress and decreases pregnancy rates in IUI cycles.

Key words: intrauterine insemination, semen analysis, oxidative stress, oxisperm, sexuel abstinence.

Introduction

Ejaculatory abstinence period is one of the factors that may affect semen parameters and pregnancy rates with fertility treatment. The standard duration of sexual abstinence before semen analysis is 2–7 days as recommended by the World Health Organization (WHO).[1] However, there is no definitive evidence about the recommended period of sexual abstinence. It is suggested that semen volume and concentration increase[2,3] and motility decrease[3] with prolonged abstinence. Previous studies have reported conflicting results about the effect of abstinence duration on pregnancy success. In a study, ≤2 days of abstinence has been reported to increase pregnancy rates before intrauterine insemination (IUI) despite the decrease in total motile sperm.[4] On the other hand, higher pregnancy rates have been achieved in IUI by <7 days of abstinence.[5] Also, regardless of the semen parameters, it was shown that ejaculatory abstinence period has an impact on pregnancy rates and it should not be longer than 3 days for IUI success.[6]

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Increased abstinence period can lead to increased semen reactive oxygen species (ROS) in semen. This relation was emphasized in a study in which oxidative stress was evaluated indirectly by total antioxidant capacity (TAC), which was able to eliminate oxygen radicals, was higher in 1-day ejaculatory abstinence than 4 days.[7] It was also found that increased oxidative stress was associated with ART failure.[8] However, no any study previously evaluated the relations between abstinence duration with oxidative stress and oxidative stress with cycle outcome at once.

Thus, in this prospective randomized study, we aimed to investigate the relation between the sexual abstinence period with the formation of ROS by nitro blue tetrazolium assay and also its impact on the outcomes of IUI cycles in unexplained or male factor infertility.

Methods

A total number of 90 male partners of infertile couples who applied to the ART Unit of Gazi University Faculty of Medicine between June 2014 and June 2015, were included to the study. Patients were allocated into three groups as 0–2, 3–4 and >4 days of duration of sexual abstinence from June 2014 to June 2015 (Fig. 1**)**. The sample size was calculated as 90 for statistical analysis. Computer-assisted randomization was used to generate random allocation. The parallel assignment was performed to equalize the number of cases in each group as an interventional model of the study. All authors were blinded except YD who generated the random allocation and assigned participants for the interventions.

This study was approved by Local Ethics Committee of Gazi University Faculty of Medicine with a number of 145/2013. An informed consent was obtained from all participants. This study was also registered to ClinicalTrials.gov Protocol Registration and Results System with an ID and title of NCT02401997. The effect of the sexual abstinence period on the production of ROS in patients who planned to take part in intrauterine insemination, respectively.

All participants had a diagnosis of unexplained or male infertility and planned to receive IUI treatment. Inclusion criteria were at least 1 year of regular unprotected sexual intercourse, existence of tubal patency and normal uterine cavity in hysterosalpingography (HSG) and normal or subnormal sperm parameters in semen analysis according to the WHO criteria.[9] All groups also contain a number of cases with low sperm morphology but with normal or subnormal concentration and/or motility to evaluate the relation of oxidative stress with morphology and the policy of requirement of having at least two COH IUI cycles before IVF in these cases in our country. Exclusion criteria were impotence and ejaculatory dysfunction, the presence of systemic diseases (cardiac diseases, unregulated diabetes, kidney diseases, etc.), tubal and/or uterine pathologies in HSG and decrease in ovarian reserve.

Ovarian stimulation for IUI cycle was initiated on the 3rd day of the menstruation by using recombinant gonadotropins (Gonal-F,Merck-Serono). Serial transvaginal ultrasonography (TVU) and measurement of plasma estradiol level were used to assess the ovarian response. hCG (Ovitrelle, Merck-Serono) was administered to trigger ovulation when the diameter of dominant follicles (one or two) reached 18 mm on TVU. IUI was performed after 36 h of triggering ovulation. For determining the level of oxidative stress, all semen samples were stained by using the following method of testing the amount of reactive oxygen species. Then, the remaining semen was prepared by 'gradient' wash method to be used wfor IUI. Vaginal progesterone (Crinone gel, Merck-Serono) was utilized to support of the luteal phase. Two weeks after the IUI, a pregnancy test was applied. A clinical pregnancy was determined by presence of gestational sac on TVU, accordingly.

Testing the amount of ROS

For liquefaction of reactive gel containing nitroblue tetrazolium (NBT), which is the content of the OxiSperm kit (Halotech DNA SL), the Eppendorf tube was heated in the microwave for 1 min or in a 90°C water bath for 5 min. The tube was kept in a water bath or in the oven for 5 min at 37°C to reduce heat. The reactive gel in the Eppendorf tube was combined with the sample of the semen, taking care not to form bubbles during mixing. The mixture in the Eppendorf tube was kept in a refrigerator at 4°C for 5 min. The mixture was incubated at 37°C for 45 min. The color after incubation was assessed by the naked eye or light microscope and oxidative stress level was reported by comparing with the baseline color. To the principle of this approach, when NBT reacts with superoxide anions, it reveals a blue pigment known as formazan described from Baehner *et al.*[10] According to oxidative stress radicals, color intensity increases in the semen sample. Four levels of oxidative stress are determined

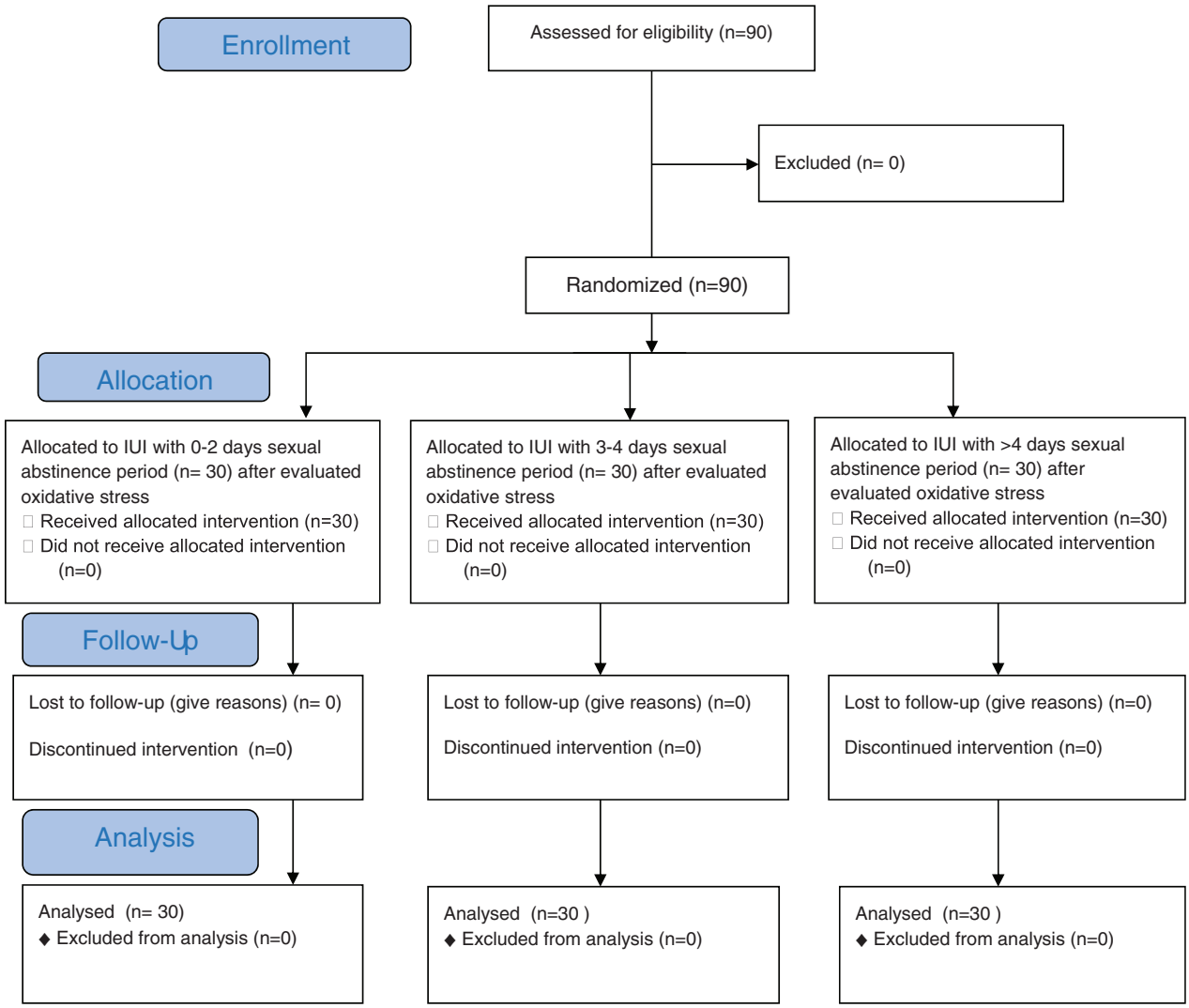

Figure 1 Consolidated Standards of Reporting Trials (CONSORT) Flow Diagram.

according to the burden of reactive oxygen radicals as described on the website of the manufacturer http://www.halotechdna.com/wp-content/uploads/2018/04/iu_eng%20oxisperm2018.pdf.

Gradient method of sperm preparation

Colloid silica solution (Sprem Grad, Vitrolife) with covered silane and tamponed with bicarbonate and HEPES was used for gradient solution. The solution was prepared as 40% and 90% in concentration density gradient solution by using G-IVF PLUS (Vitrolife). 90% and 40% medium was placed into a falcon conical tube, respectively. The tube was incubated for 15 min at 37°C. Subsequently, semen was added on to the tube slowly with a pipette and centrifuged at 1400*g* for 10 min. After supernatant removed, 3 mL sperm wash solution G-IVF PLUS (Vitrolife) was added on and centrifugation was repeated at 1400*g* for 10 min. Last, the supernatant discarded and remained 0.3–0.5 mL sperm solution was used for IUI.

Primary outcome measure was the relationship between the level of oxidative stress and the period of sexual abstinence. Clinical pregnancy rate was the secondary outcome measure. Since this is a pilot study, each group consists of 30 cases, because of estimated sample size of at least 79 cases to have 80%

Table 1 Comparison of demographical and clinical characteristics between abstinence groups

Variables	0–2 days (n = 30)	3–4 days (n = 30)	>4 days (n = 30)	P-value
Age, male (y)	31.8 ± 4.1	30.5 ± 3.5	33.1 ± 5.4	0.073†
Age, female (y)	28.4 ± 4.0	27.8 ± 3.3	28.4 ± 5.0	0.795†
Marriage duration (y)	4 ± 2.7	4.1 ± 2.4	4.1 ± 3.4	0.991†
Infertility period (y)	2.7 ± 2.1	3.1 ± 1.7	3 ± 2.2	0.781†
Infertlity type				
Primary, n (%)	21 (70)	21 (70)	21 (70)	
Secondary, n (%)	9 (30)	9 (30)	9 (30)	
Male BMI, (kg/m^2)	26.4 ± 4.0	25.3 ± 3.1	25.5 ± 3.1	0.430†
Female BMI, (kg/m^2)	24.2 ± 4.6	25.7 ± 4.2	23.3 ± 3.5	0.718†
Smoking history, n (%)	15 (50)	11 (36.7)	9 (30)	0.270‡
Alcohol history, n (%)	3 (10)	4 (13.3)	–	0.133‡
Leucocyte existence, n (%)	3 (10)	5 (16.7)	3 (10)	0.661‡

Data were presented as mean ± standard deviation. †One-way analysis of variance (ANOVA),; ‡Pearson's χ^2 test. and BMI, body mass index; y, year.

Table 2 Comparison of semen parameters between abstinence groups

Variables	0–2 days (n = 30)	3–4 days (n = 30)	>4 days (n = 30)	P-value
Total sperm count (millions)	123.9 ± 78.9	175 ± 99.3	134.2 ± 73.3	0.052†
Concentration (million/mL)	43.7 ± 20	54.7 ± 23.4	53 ± 33	0.233‡
Total motility (%)	60.8 ± 14.4	69 ± 12.4	60.9 ± 16.6	0.051†
Progressive motile sperm counts (millions)	64.2 ± 48.8	92.8 ± 53.8	71.0 ± 49.5	0.080†
Morphology (%)	2.9 ± 2.1	2.5 ± 1.8	2 ± 1.8	0.210†
Post-gradient concentration (million/mL)	57.1 ± 37.7$^{(3–4\ days)}$	86.1 ± 45.5$^{(0\text{-}2\ days)}$	60.4 ± 42.7	**0.017**†
Post-gradient motility (%)	80.2 ± 12.7	86 ± 9.2$^{(>4\ days)}$	77 ± 14.4$^{(3\text{-}4\ days)}$	**0.045**‡
Post-gradient progressive motile sperm counts (millions)	22.9 ± 16.3$^{(3\text{-}4\ days)}$	35.9 ± 20.9$^{(0–2,\ >4\ days)}$	23.7 ± 19.9$^{(3\text{-}4\ days)}$	**0.016**†

Data were presented as median.; †One-way analysis of variance (ANOVA). and ‡Kruskal–Wallis test, superscripts indicate a significant difference between that group, bold values indicate $P < 0.05$.

power to detect a difference of 20% in the levels of ROS in pairwise comparisons of 3 groups. Statistical Package for Social Sciences (SPSS, version 11.5, Statistics, 2013) was used for statistical analyses. The homogeneity of the variances was investigated by the Kolmogorov–Smirnov test. Normally distributed parametric data were compared with one-way analysis of variance test. Kruskal–Wallis test or Mann–Whitney U test was used to compare nonparametric data, when appropriate. Categorical variables were compared by "χ^2 test." The parameters which affect the oxidative stress level were investigated with Spearman correlation. $P < 0.05$ was considered as statistically significant.

Results

Demographical and clinical characteristics, including male and female age, marriage duration, infertility period, infertility type, male and female body mass index (BMI), history of smoking and alcohol consumption were similar between groups (Table 1). WHO parameters of semen analysis were also similar among three groups (Table 2). However, post-gradient progressive motile sperm counts significantly better in 3–4 days of sexual abstinence group than the others (Table 2).

Table 3 shows the relationship between duration of abstinence and level of oxidative stress, in which Group 3 (>4 days) had significantly higher level of oxidative stress than groups 0–2 and 3–4 days of abstinence period (P = 0.013 and P = 0.039, respectively). The difference of the level of oxidative stress was not significant between 0–2 and 3–4 days of abstinence period.

There was no significant correlation between oxidative stress and sperm morphology (r = −0.067 and P = 0.532).

Pregnancy rate significantly decreased with the prolonged sexual abstinence period, (P = 0.039) (Table 4).

Table 3 Frequency of oxidative stress in groups of sexual abstinence

	0–2 days (n = 30)	3–4 days (n = 30)	>4 days (n = 30)	P-value
Oxidative stress				**0.014***
Level 1	9 (30.0%)	8 (26.7%)	3 (10.0%)	
Level 2	8 (26.7%)	7 (23.3%)	5 (16.7%)	
Level 3	13 (43.3%)$^{(>4\ days)}$	15 (50.0%)$^{(>4\ days)}$	21 (70.0%) $^{(0–2,\ 3\text{-}4\ days)}$	
Level 4	–	–	1 (3.3%)	

*χ^2 test, superscripts indicate a significant difference between that group ($P < 0.05$). The difference between 0–2 days and > 4 days (P = 0,013) and between 2–4 days and > 4 days was statistically significant (P = 0.039).

Table 4 Comparison of pregnancy rates between abstinence groups

	0–2 days (n = 30)	3–4 days (n = 30)	>4 days (n = 30)	P-value
Pregnancy				**0.039***
Absent	22 (%73.3)	25 (%83.3)	28 (%93.3)	
Present	8 (%26.7) **(>4 day)**	5 (%16.7)	2 (%6.7) **(0-2day)**	

*χ^2 test, superscripts indicate a significant difference between that group ($P < 0,05$). The difference between 0–2 days and > 4 days was statistically significant (P = 0.040).

Also, increased level of oxidative stress related a tendency to decrease in pregnancy rates, but the difference was not statistically significant (20%, 20%, 14.3% and 0%, in levels 1, 2, 3 and 4 of oxidative stress, respectively, P = 0.464).

Discussion

Oxidative stress has some impact on sperm function and there is a correlation between male sexual abstinence and oxidative stress.[7,11-14] So, we purposed to assess the effect of the abstinence duration on oxidative stress and success of IUI and found a statistically significant increase in oxidative stress levels in group >4 days of abstinence period as compared to shorter abstinence groups.

There are few studies in the literature evaluating the effect of abstinence duration on ROS. In a previous study, ROS were unrelated to abstinence period and did not affect the sperm parameters based on semen samples obtained from 1 to 10 days abstinence periods of a single fertile male.[15] However, when oxidative stress was quantified by indirect methods including, TAC and sperm membrane lipid peroxidation in semen samples after the 4-day abstinence period, it was found that increasing period of abstinence reduced TAC but not lipid peroxidation.[7] Consistently, we found significant differences in level of oxidative stress among different periods of the abstinence groups that was highest in group >4 days of abstinence with using a direct method of determination of oxidative stress.

The relation between sperm ROS and treatment success has mostly been evaluated in IVF studies and there is little about oxidative stress and pregnancy success after IUI in the literature. Pregnancy outcome after ART was affected adversely by the increase in seminal oxidative stress and sperm DNA fragmentation index.[8] In another study evaluating the effect of the ROS on intracytoplasmic sperm injection (ICSI) and IUI treatment by 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a marker of oxidative stress, 8-OHdG was found to be significantly higher in the non-pregnant group after IUI, but this was not related to ICSI outcome.[16] Although different sperm abstinence periods with regard to IUI success were investigated in several studies, our study is yet to be the first study comparing sperm ROS and IUI success alone in different sexual abstinence groups. Although we observed a significant decrease in pregnancy rates with prolonged abstinence period, the increased levels oxidative stress level was tended to be associated with lower pregnancy rates that may be due to the small population in the groups, as a limitation of this study.

For the relation of oxidative stress and sperm morphology, a cytoplasmic droplet has been correlated with ROS levels and it was suggested that abnormal sperm could be a source of abnormal ROS production.[17] Also, a study showed that patients with teratozoospermia have significantly higher seminal ROS than healthy donors and it was stated that sperm morphology may be directly correlated with oxidative stress.[18] Nevertheless, there was no significant correlation between oxidative stress level and sperm morphology in our study.

Many published studies have shown a relation between sperm parameters and sexual abstinence.[19-22] When sperm parameters were compared between 1, 3, 5 and 8 daily abstinence duration, sperm volume and sperm concentration were increased with duration of abstinence.[19] However, total and progressive motility and morphology were not changed with the duration of abstinence. In another study, evaluating the effect of abstinence on the sperm parameters of 2–3, 4–5 and 6–7 days, sperm concentration and total count was reported to be significantly higher in the group of 4–5 days as compared to 2–3 days, and also it was stated that the progressive motility was increased in the group of 4–5 days as compared to other groups.[20] When the effect of sexual abstinence period on sperm morphology was evaluated, the best morphologic results were obtained by 1 day abstinence period and the increasing periods of abstinence resulted in morphologic deterioration.[21] In contrast, another study reported that abstinence duration was not related to motility and morphology.[22] Unlike previous studies, basal total number, concentration, total and progressive motile sperm count and morphology were similar among different abstinence periods. However, post-gradient motility and progressive motile sperm counts were better in 3–4 days of abstinence in this study.

In conclusion, our study revealed that the duration of sexual abstinence is associated with increased seminal oxidative stress and also with decreased pregnancy rates. Although there is a tendency to decrease pregnancy rates with higher oxidative stress the difference did not reach statistically significance that indicates a need to confirm these results in larger population.

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Disclosure

None declared.

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