Materials and Methods –

Institutional Review Board approval as well as informed consent was obtained and semen samples were collected from 6 healthy, medically fit and solvent subjects after 6 defined ejaculatory abstinence (EA) periods (1, 2, 5, 7, 9 and 11 days). Abstinence periods were: short = 1 day, optimal = 2 days and long = 5, 7, 9 and 11 days. Each donor served as his own control. Measurement of volume, pH, sperm concentration, motility, viability and round cell count was carried out manually as described in the World Health Organization (WHO, 2010). We also examined the effect of short and long abstinence on the advanced parameters such as levels of reactive oxygen, DNA damage.

Standard Semen Analysis
After semen collection, semen samples were evaluated for macroscopic and microscopic characteristics. A 5 µL aliquot of well-mixed semen sample was loaded on a Microcell Counting chamber (Vitros, Rochester, NY) for assessment of sperm motility and motility. Sperm morphology was measured by the noting the filling time of the Microcell counting chamber.

Sperm Morphology and Vitality
Sperm vitality was assessed using one-step Eosin-Nigrosin staining procedure. At least 200 spermatozoa were evaluated per sample by 400X magnification. The percentage of dead (colored pink) and live (unstained) cells were evaluated. Smears were stained using a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology. Percentage normal sperm were examined according to Kruger’s strict criteria (WHO, 2010).

Measurement of Hyposmotic Swelling (HOS) Test
Sperm membrane functional integrity was measured by the hyposmotic swelling (HOS) test. 100 µL of semen sample was mixed with 0.9 mL of hypotonic solution and incubated for 60 minutes at 37°C. After incubation, 1 drop of semen mix was evaluated using Microcell counting chambers (Vitros, Râncago, CA). The percentage of spermatozoa showing tail swelling was counted in duplicate under 40X objective.

Measurement of Reactive Oxygen Species
The chemiluminescence assay using luminol (5-aminoo-2, 3-dihydoxy-1-phthalazidene; Sigma Chemical Co., St Louis, MO) was used to measure the ROS levels. Luminol (10 µL, 5 mM) dissolved in dimethylsulfoxide (DMSO) was added to 400 µL of sample. Negative controls were prepared by adding 100 µM of N,N-dimethylformamide (DMF) to 400 µL of PBS. The chemiluminescent signal was measured for 15 min using a luminometer (Alokaum plus 953; Oakville, TN) and the results were expressed as RLU/s/X106 sperm.

Measurement of Sperm DNA Damage
Sperm DNA strand breaks were evaluated using a flow cytometric terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct, BD Biosciences). Terminal deoxynucleotidyltransferase (TdT) catalyzes a template-independent addition of deoxyuridine triphosphate to the 3'-hydroxyl (OH) termini of double and single stranded DNA. Sperm (2-5 X 106 cells/mL) were washed twice in PBS, re-suspended in 50 µL of 1 X TdT reaction buffer, and fixed on ice for 15 to 30 minutes. After fixing in ethanol (70%), assay kit controls, internal negative (no TdT) and positive controls were added, and the reaction was allowed to proceed for 1 hour at room temperature. Samples were washed twice in PBS and fixed with 70% ethanol in PBS for 30 minutes. A 500 µL solution of TUNEL assay kit was prepared and 50 µL of each sample was added to 485 µL of TUNEL assay solution. A total of 10,000 cells were evaluated. The FITC-green fluorescence was measured on a flow cytometer equipped with a 515-nm argon laser as a light source (BD Accuri C6 Flow Cytometer; Beckton Dickenson, San Jose, CA).

Table 1: Basic and advanced semen parameters after different abstinence periods (Table 2; Figures 1, 2, 3, 4).

Conclusions
1. Our longitudinal study establishes the impact of both short and long abstinence time on sperm parameters. Although abstinence of 1 day had a significant impact on sperm parameters, none declined below WHO (5th ed) reference values.

2. Short abstinence periods may be recommended to reduce viscosity and DNA fragmentation.

3. Collecting samples for fertility evaluation, sperm banking and ART may be recommended to observe shorter abstinence to reduce the deleterious effects of longer abstinence.

Table 2: Linear mixed model analysis to demonstrate the incremental effect of abstinence periods on various semen parameters.

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