Correspondence

Unexplained early pregnancy loss: Role of paternal DNA

Sir,

Recurrent miscarriage (RM, recurrent spontaneous abortion - RSA) is defined as the loss of three or more consecutive pregnancies. Classic factors associated with RM include parental chromosome translocations, uterine malformations, endocrine and autoimmune factors. Even after extensive investigation approximately 40 per cent of RM cases remain unexplained and are classified as unexplained RM (URM). Female factor is believed to play a major role in recurrent miscarriages, but the role of male factor has only recently been realised. Sperm DNA integrity plays a vital role in embryogenesis and foetal well being and sperm DNA damage may lead to pre- and post-implantation losses, early pregnancy loss and congenital malformations. This pilot study was planned to analyse sperm factor in unexplained RM and determine cut-off value of DNA damage in cases of URM.

After approval of the study protocol from the ethics committee of the institute [All India Institute of Medical Sciences (AIIMS), New Delhi, India], a total of 25 idiopathic RM cases and 20 control men were enrolled for the study from February 2010 to May 2011. These cases were referred from department of Obstetrics and Gynaecology, AIIMS, a tertiary research and referral hospital after detailed work up for female factor by the clinician. Selection criteria included a history of at least three prior pregnancy losses at <20 wk of gestation, with all pregnancies fathered by the same partner. All known causes for RM were ruled out and idiopathic cases were recruited. In brief, female partners of the couples had normal haemogram (Hb, TLC, DLC, ESR, platelets, PT, CT) with normal ovarian function (normal ultrasonography of ovary for PCOS), a normal uterus confirmed by vaginal ultrasound and/or hysterosalpingography/hysteroscopy, and had normal endocrinological parameters (level of T₃, T₄, TSH, FSH, LH, PRL), with normal perspeculum and pervaginal examination. Other causes for RM like thrombophilia both inherited (Proglob C) and acquired (anticardiolipin-IgG and IgM) were also screened and male partners having normal semen parameters only were included in the study. Also the karyotype of both partners were analysed to rule out any chromosomal abnormality. Male partners of couples who have recently fathered a child (not older than 2 yr) were enrolled as fertile controls. Informed consent was obtained from both the cases and controls.

Semen samples were analysed as per the WHO guidelines. An aliquot of 100 µl of the raw semen was stored at -80°C till sperm chromatin structure assay (SCSA) analysis was done. The SCSA was performed as per the protocol described by Evenson & Jost. Frozen aliquots of semen were placed in 37°C water bath until just thawed, after which samples were diluted with TNE buffer (pH 7.4, working solution, 0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA) to 1-2×10⁶ sperm cells per ml; 0.20 ml aliquots of diluted samples were mixed with 0.40 ml of acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). After 30 seconds, the cells were stained by adding 1.2 ml acridine orange (AO) solution containing 6 μg AO (chromatographically purified; Polysciences Inc, USA) per ml buffer (0.037 M citric acid, 0.126 M Na₂HPO₄, 0.0011 M EDTA disodium, 0.15 M NaCl, pH 6.0). After complete analysis of the sample, X-mean (red fluorescence) and Y-mean (green fluorescence) values were recorded manually after selecting gate for sperm cells using FlowJo software (Oregon, USA). Extent of DNA denaturation (damage) was expressed in terms of the DNA fragmentation index (DFI), which is the ratio of red to total (red plus green) fluorescence, i.e. the level of denatured DNA over the total DNA. The
percentage of high DNA stainability cells (HDS) was also recorded in each sample manually from the graph plot. HDS represents another distinct population in semen that characterizes immature spermatozoa with incomplete chromatin condensation. The DFI values of the same patient and controls were analysed in duplicate by a single operator and mean value was recorded.

Statistical analyses were performed using MedCalc trial version for Windows (MedCalc Software, Mariakerke, Belgium). Data are represented as mean ± standard deviation. The data was analysed by using Student’s t-test to compare DFI of cases and controls (29.35±4.93 vs 21.0±4.1, P<0.001). Receiver operating characteristic (ROC) curve analysis was applied to find the cut-off value of DFI to discriminate patient from control. The mean DFI of cases was 29.35 ± 4.93 with a range of 19.45 to 35.75. The median DFI of cases was significantly (P<0.05) higher when compared to controls. However, in controls, the lowest DFI was 18.34 and the highest was 28.67. Using ROC curve analysis, a threshold value of 23.54 was obtained to discriminate from control group. The area under the curve was 0.975 (P<0.001; 95% CI, 0.845-1.000), with 97 per cent sensitivity (95% CI, 84.7 - 99.9) and 100 per cent specificity (95% CI, 79.4 - 100.0).

Sperm DNA is highly compact, organized crystalline structure, the size of which is 1/6 to 1/20 that of somatic cells. This genome is organized into a central protamine bound fraction (toroid) while the peripheral histone bound fraction retains the nucleosomal structure. It is this peripheral loosely bound chromatin which has genes of critical developmental importance and maintains genomic imprints. This 15 per cent peripherally located histone bound chromatin is highly susceptible to environmental insult especially oxidative stress. We have shown in a previous study that sperm reactive oxygen species (ROS) levels are elevated in RM cases. We have also reported that DFI in infertile cases is approximately 30 per cent. Thus it is possible that cases with 30 per cent DFI and above are infertile where as cases with lower DFI (24%) are able to initiate pregnancy but have sufficient DNA damage which adversely affects embryogenesis and results in recurrent early pregnancy loss. Oxidative stress results in generation of ethenonucleoside, an oxidative product which inhibits oocyte nucleotide excision repair. Thus sperm DNA damage results in impaired embryonic development and recurrent miscarriages.

The role of sperm factor in recurrent assisted and spontaneous conception loss is now being realised. Earlier studies have suggested that pregnancy is unlikely to occur when sperm nuclear DNA fragmentation index values are above certain threshold value. However, a cut-off value for DNA damage in unexplained recurrent miscarriages cases is still not documented. In our earlier study, we have established a cut-off value in infertile men unable to conceive even after 6 years of marriage. This preliminary study on URM following spontaneous conception has established cut-off value of sperm DFI (24%) in URM cases. This will help to understand aetiology in a large numbers of couples experiencing URM. High level of DNA damage in cases of RM in our study is in accordance with previous studies. In the study by Liu et al, DFI of 37.5 per cent of the subjects was over 30 per cent as compared to the control group, where only 25.8 per cent of the subjects had DFI over 30 per cent. The chief cause of loss of DNA integrity was oxidative stress. Smoking has been shown to increase seminal leukocyte concentration by 48 per cent and cause a 107 per cent increase in seminal ROS levels. Peake and his associates documented that extremes of exercise (too little and rigorous exercise) are linked with oxidative stress. Another group reported that obesity produces oxidative stress as adipose tissue releases proinflammatory cytokines that increases leukocyte production of ROS. Psychological stress results in decline in semen quality. Psychological stress has also been reported to result in poor sperm quality which was mediated by supraphysiological ROS levels and low antioxidant levels. Thus minimizing lifestyle triggers of oxidative stress can prevent oxidative stress induced DNA damage.

The results of this study show that sperm DNA damage may be a cause of URM, and paternal factors may play a critical role in embryonic development.

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