J.Natn.Sci.Foundation Sri Lanka 2014 42 (2): 149-154
DOI: http://dx.doi.org/10.4038/jnsfsr.v42i2.6999

RESEARCH ARTICLE

Macrophage migration inhibitory factor (MIF) in seminal fluid as a marker of male factor infertility: a pilot study in Sri Lankan men

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Revised: 9 December 2013; Accepted: 30 January 2014

Abstract: Recent clinical and epidemiological studies worldwide suggest an increasing incidence of male factor infertility (MFI). Paucity of information on the biochemical analysis of seminal fluid in Sri Lanka prompted undertaking a pilot study to establish a clinical marker for the male fertility status in Sri Lanka based on the level of the cytokine, macrophage migration inhibitory factor (MIF) in seminal fluid, an area hitherto unexplored locally. The analysis was carried out on the semen samples of infertile males (N = 61) where age matched individuals (N = 30) with proven past paternity served as controls. D-dopachrome tautomerase assay was performed to assess the MIF level in semen while other seminal fluid parameters were assessed according to the standard WHO criteria. The present study revealed an abnormal biphasic profile of MIF in the seminal fluid of individuals with impaired sperm parameters, which was either significantly below or above the range of MIF tautomarase activity typical of normal fertile men ($p < 0.000$). This is the first report in a Sri Lankan population. The receiver operating characteristic (ROC) plot analysis established a cutoff point of 3.375 µg MIF/mL of semen (at 90 % sensitivity: 81.2 % specificity; 0.923 accuracy) to differentiate fertile from infertile males (excluding azoospermics and severe oligozoospermics). The MIF concentrations significantly correlated with the semen pH in the azoospermic and severe oligozoospermic group. As MIF was clearly indicative of the male fertility status by estimates of sensitivity and specificity of the D-dopachrome tautomerase assay, MIF may be developed as a potential marker of male infertility in Sri Lanka.

Keywords: Azoospermia, biphasic MIF profile, D-dopachrome tautomerase assay, macrophage migration inhibitory factor (MIF), male factor infertility (MFI), severe oligozoospermia.

INTRODUCTION

Male infertility is an extremely common problem affecting 1 in 25 men globally. Almost 10 − 15 % couples have been reported to be childless worldwide, where in 50 % of these cases, different male related factors are the causative element (Seshagiri, 2001; Sharlip *et al*., 2002). Despite active research, the underlying causal factors remain unknown in a large percentage (40 %) of men. The high incidence of these unexplained infertility cases is unfortunate since empirical treatment of these males can be both emotionally and financially draining for the individual.

Since its rediscovery as a proinflammatory cytokine, macrophage migration inhibitory factor (MIF) has demonstrated a widespread distribution and depending on its location, to play different roles (Frenette *et al.*, 2005). Recent studies have indicated a broader scope for MIF activities including a role in reproduction, as it affects the sperm maturation and spermatozoa motility (Aljabari *et al*., 2007). In the male reproductive tract, MIF has been found in both the testes and the epididymis. In the testes, it is expressed by the Leydig cells and is involved in the paracrine regulation of testicular function (Meinhardt *et al*., 1996). MIF has also been found to be produced in a regional specific manner by the epithelial cells of the epididymis with maximal expression in the caput (Eickhoff *et al*., 2001; Frenette *et al*., 2002).

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In addition MIF was localized in the membranous vesicles secreted by the epithelial cells of the epididymis, which are in close contact with the epididymal sperms (Eickhoff *et al*., 2004). It is one of the proteins transferred to spermatozoa during the epididymal transit (Frenette *et al*., 2005). MIF is also present in large quantities in human semen, probably originating from the prostatic secretion containing membranous vesicles similar to epididymosomes and are described as prostasomes. Therefore, MIF may be a "moonlighting protein" inhibiting sperm motility in the epididymis and playing another function in seminal plasma, possibly modulation of the female immune response (Frenette *et al*., 2005).

In contrast to all other known cytokines, MIF has several unusual intrinsic enzymatic activities such as its ability to catalyze keto-enol isomerization (i.e. tautomerization) reactions. MIF tautomerizes D-dopachrome or L-dopachrome methyl esters to their corresponding indole derivatives (Rosengren *et al*., 1996).

Evidence suggests that MIF levels in seminal fluid may be indicative of the spermatozoa quality, and therefore male fertilizing ability. An increased level of MIF in seminal plasma was related with poor sperm motility in a study conducted in the USA (Al-Abed *et al.*, 2005). They identified an abnormal biphasic profile of MIF in the seminal fluid of oligozoospermic and azoospermic individuals as compared to normozoospermic individuals. This association may differ in the Sri Lankan male population mainly due to disparate environmental conditions and genetic factors.

MIF concentration in semen and the fertility status of Sri Lankan males has remained unexplored. The present study was undertaken with the main objective of demonstrating the possibility of introducing a quantitative biochemical test, which is highly reliable in distinguishing fertile from infertile semen samples based on the MIF levels in seminal fluid. The specific objectives were to establish i) a correlation between the MIF levels in human seminal fluid and the fertility status, and ii) associations between the MIF levels in seminal fluid and sperm parameters (sperm count, motility, morphology, volume and pH of seminal fluid).

METHODS AND MATERIALS

Ethical approval

The Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka approved this study (ERC/09/006; 26/03/2009) and written informed consent of all the participants was obtained for their voluntary participation.

Subjects

Infertile males (N = 61) recruited from the Reproductive Biology Laboratory, Faculty of Medicine, University of Colombo, and the *Vindana* Reproductive Health Centre, Colombo, Sri Lanka served as the test subjects. An age-matched group of men (N = 30) with proven paternity within the past two years with normal semen parameters according to the WHO (1999) guidelines was selected and recruited as the control group.

Semen collection and seminal fluid analysis (SFA)

Fresh semen samples were collected from the participants following three to five days of abstinence from sexual activity. SFA was undertaken as initial macroscopic and microscopic investigations as per the procedures described in the WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction (WHO, 1999).

In the first 5 mins of semen analysis, the specimen container was placed on the bench and examined for liquefaction. Between 30 – 60 mins of ejaculation, SFA involved assessing for liquefaction and the appearance of semen, measuring the semen volume and pH, preparation of wet mounts for assessing microscopic appearance, sperm motility, preparation of semen smears for assessing sperm morphology and assessing the sperm number and sperm vitality. Sperm morphology was assessed by fixing and staining of semen smears 4 h after ejaculation.

Determination of MIF level in seminal fluid

Seminal fluid was analyzed for MIF tautomerase activity using L-dopachrome methyl ester essentially according to Al-Abed *et al*. (2005). An orange-coloured fresh stock solution of 2.4 mM L-dopachrome methyl ester (1 mL) was prepared by oxidation of 4 mM L-3,4-dihydroxyphenylalanine methyl ester (0.4 mL) with 8 mM sodium periodate (0.6 mL).

To determine the MIF activity at room temperature, 300 μL of freshly prepared dopachrome solution and 675 μL of diluted semen (75 μL of semen in 600 μL of PBS) was mixed in a 1 mL sample cuvette. The time required for the orange-coloured L-dopachrome methyl ester to convert into a colourless solution was determined spectrophotometrically at 475 nm.

The D-dopachrome tautomerase assay was performed with a standard series of MIF solutions (Al-Abed *et al*.,

2005). MIF level in each test and control semen sample was calculated using the standard curve plotted.

The infertile group was further categorized into subgroups according to WHO (1999), for the analysis associated with the tautomerase activity.

Statistical analyses

Data management and statistical analyses were performed using the computer software package SPSS 15.0 for windows (SPSS Inc., USA).

Comparisons of normally distributed variables of the independent samples were performed using analysis of variance (ANOVA). Correlations between the parameters were sought by Pearson's correlation coefficient.

When the variable distribution was not normal, nonparametric statistical analysis was applied. Kruskal-Wallis and Mann-Whitney U tests were used to perform comparisons of median differences. Spearman's correlation coefficient was used to examine correlations.

Cutoff points in terms of sensitivity and specificity of MIF values were established by constructing a receiver operating characteristic (ROC) curve, where the area under the curve (AUC) provided the accuracy of the dopachrome assay (Obuchowsky, 2005). A statistically significant difference was accepted at $p < 0.05$.

RESULTS

The study group of 91 participants comprised the test group of infertile men (N = 61) and the fertile (N = 30) control group. To avoid any confounding effect, the age of the fertile group was matched with the infertile group (Kruskal-Wallis test; $p = 0.919$).

As the accrued data was not normally distributed (Kolmogorov-Smirnov one-sample test; 0.005) non-parametric tests were used in the analyses of data.

The unknown concentration of MIF in semen samples tested were calculated using a reference standard curve ($r^2 = 0.96$) initially plotted with known concentrations of MIF.

There was a marked difference between the average time required for dopachrome decolourization between fertile and infertile individuals. It was found that 75 µL of normozoospermic seminal fluid tautomerizes 1 mM L-dopachrome methyl ester between 2 – 6 minutes, with an average of 4.82 minutes. However, based on the decolourization reaction it was apparent that the infertile group was clearly divided into two sub groups; individuals with azoospermia or with severe oligozoospermia (< 10^6 spermatozoa/mL) (N = 13) required between 30 s – 2.0 minutes for this conversion (average of 1.81 minutes) indicating a high level of MIF in their semen, while the other infertile group (N = 48) [other oligozoospermic individuals ($>10^6 < 20 \times 10^6$ spermatozoa/mL), asthenozoospermics, teratozoospermics, asthenoteratozoospermics, oligoasthenozoospermics, oligoteratozoospermics, oligoasthenoteratozoospermics], required on average 6.88 minutes (range 4.5 to 12 minutes) for tautomerization. Thus a clear cut biphasic profile of MIF tautomerase activity in males with abnormal sperm parameters was evident, which was either below or above the range typical of normozoospermic individuals. The MIF levels were significantly different (Mann-Whitney U test; $p < 0.000$) between any two of the above three groups *i.e.* fertile, azoospermic and severe oligozoospermic, and the other infertile groups (Figure 1).

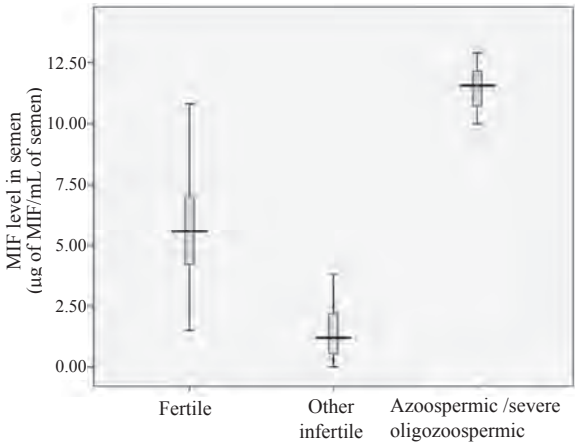

Figure 1: MIF levels in seminal fluid obtained from fertile and infertile individuals. The MIF levels significantly differed between any two groups of the above three *i.e.* fertile (N = 30), azoospermic and severe oligozoospermic (N = 13), and the other infertile (N = 48) groups (Mann Whitney U test; $p < 0.000$)

To describe the ability of the L-dopachrome assay to correctly diagnose the fertility status of an individual, a receiver operating characteristic (ROC) curve was plotted (Figure 2). The analysis established a cutoff point of 3.422 µg MIF/mL of semen to differentiate the fertile group from the other infertile group with 90 % sensitivity, 81.2 % specificity and 0.923 accuracy (AUC). A cutoff point for the azoospermic and severe oligozoospermic group from the fertile group could not be established due to the limited number of males in this group (N = 13).

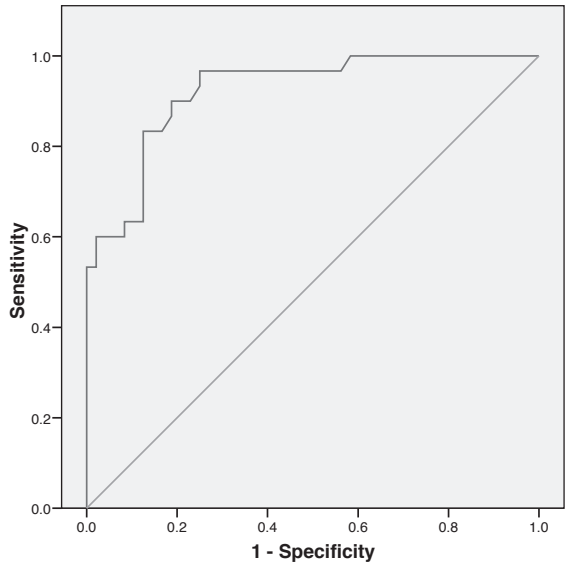

Figure 2: Receiver operater curve (ROC) to differentiate fertile from other infertile individuals. Area under the curve was 0.923 indicating that this assay is 92 % accurate at distinguishing fertile from the other infertile group.

Possible correlations were sought between the MIF concentration and the sperm parameters such as motility, concentration, morphology and pH. A strong and highly significant correlation between the MIF concentration with semen pH was evident in the azoospermic and severe oligozoospermic group (Figure 3; Spearman's correlation coefficient, $r = 0.728$, $p < 0.01$). Conversely no other sperm parameter signified a correlation with the MIF concentration in either of the infertile groups.

DISCUSSION

Biochemical analysis of seminal fluid at present is limited to the macroscopic and microscopic parameters that determine the male fertility status in Sri Lanka. Consequently this study was undertaken to investigate the association between the concentrations of MIF of seminal fluid of fertile and infertile individuals to seek the possibility of establishing a clinical marker based on MIF to ascertain the male fertility status in Sri Lanka.

The current study revealed a biphasic profile of MIF tautomerase activity in individuals with abnormal sperm parameters, which was either below or above the range typical of normal fertile individuals. The group of high MIF values included all the infertile individuals with either azoospermia or severe oligozoospermia, whereas all the other infertile individuals reported a low MIF concentration well below the MIF level of normal individuals. MIF levels, either higher or lower than that of normozoospermics is indicative of impaired fertilizing ability, which corresponds with the finding that a MIF threshold concentration is required for sperm maturation and movement (Aljabari *et al*., 2007).

This biphasic property of MIF cytokine in seminal fluid was also reported in a previous study carried out by Al-Abed *et al.* (2005). Variations in the average time required to decolourize the dopachrome ester among the three groups of Sri Lankan men compared with those of their American counterparts may probably be due to the disparate demographic and environmental factors of the two countries. Previous studies corroborated that semen parameters may differ due to demographic and environmental, socio economic factors (Li *et al*., 2009). However, this concept seems to be a matter for controversy (Agarwal, 2006).

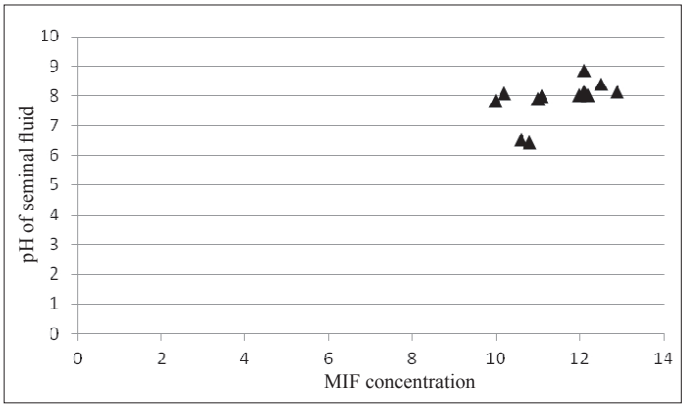

Figure 3: Correlation between MIF concentration and pH of seminal fluid of azoospermic or severe oligozoospermic individuals

In the study, Al-Abed and coworkers (2005) had observed the biphasic profile between the severe oligozoospermic ($< 10^6 \times 10^6$ spermatozoa/mL) and azoospermics group and the other infertile group consisting of only asthernozoospermics and other oligozoospermics. Conversely, in the present study the other infertile group consisted of other oligozoospermic individuals ($> 10^6 < 20 \times 10^6$ spermatozoa/mL), asthenozoospermics, teratozoospermics, asthenoteratozoospermics, oligoasthenozoospermics, oligoteratozoospermic and oligoasthenoteratozoospermics. Therefore, it would be of interest to further investigate how these profiles differ among different socio demographic groups, locally and globally.

MIF levels of the three groups were found to be significantly different. This gave a directive to check the accuracy of the assay as it is indicative of the ability of using this assay as a marker for male fertility status in Sri Lanka. The receiver operating characteristic (ROC) curve analysis, revealed a cutoff point of 3.422 µg MIF/mL of semen with 90 % sensitivity and 81.2 % specificity describing the ability of the test to enable correct diagnosis of infertility when conditions are actually present and to correctly rule out when it is truly absent or fertile. The area under the curve (AUC = 0.923) indicates that this test is 92 % accurate in distinguishing fertile from infertile individuals excluding azoospermics and severe oligozoospermics.

A highly significant correlation between the MIF concentration with pH in the azoospermic and severe oligozoospermic group (N = 13) was evident in the current study. The relationship between the pH and post testicular (obstructive) azoopermia is well established; "the semen pH is characteristically low as a consequence of dysplasia or the absence of the seminal vesicles. When the fructose-rich alkaline secretion of the seminal vesicles is lost, the seminal plasma is formed mainly from the relative scanty and acidic prostatic secretion" (Aziz, 2013). As the pH of the medium becomes acidic, the human MIF molecule that consists of a trimer with each monomer containing two antiparellel α helices and six β strands, four of which forms a mixed β sheet (Sun *et al*., 1993), must be unfolded (Swope *et al*., 1998). Therefore it may be surmised that this pH induced unfolding of the protein may probably be the basis for the low MIF levels in low pH. Therefore, pH and MIF levels should be considered in conjunction with clinical data such as pre testicular, testicular or post testicular causes for azoospermia or severe oligozoospermia related to male infertility. As we did not have access to such information on the subjects, this analysis was not possible. Furthermore, due to paucity of available literature on the correlation of MIF concentration with the pH of seminal fluid, this cannot be explained further.

Currently human enzyme linked immunosorbent assay (ELISA) kits are commercially available to determine the MIF concentration in semen in which the MIF in the sample is captured by immobilized anti-MIF antibody, which is detected with peroxidase-labeled anti-MIF antibody and peroxidase substrate TMB. Although ELISA is a highly sensitive diagnostic tool and the ready-to-use kit has its advantages in that all the conditions have already been predetermined and the reagents prepacked and ready-to-use, the prohibitively high cost deters the use of these kits in local diagnostic laboratories. The in-house optimized methodology adopted during this study to assay the MIF concentration (with 81.2 % specificity, 1.1 µg/ mL sensitivity) is much more cost-effective than the commercial ELISA kits (with 100 % specificity, and sensitivity of < 6 pg/mL) currently available in the market and offers a more affordable system for routine infertility tests in a developing country such as Sri Lanka. The cost of testing a semen sample using this methodology is approximately US$ 0.46 while the MIF ELISA kit costs 50 fold more.

At present, the most important test for assessing male fertility is the semen fluid analysis (SFA) based on the WHO guidelines (1999). It is simple, inexpensive and easy to perform. However, its apparent simplicity and the subjective nature of this qualitative test can be very misleading (Keel, 2006), because in reality it requires much skill to perform a semen analysis accurately.

Therefore it is crucial that the SFA is carried out at a reliable Andrology laboratory that specializes in sperm testing, as the reporting is very subjective and depends upon the skill and the experience of the technician performing the test. When differentiating azoospermia from severe oligozoospermia, the laboratory technicians have to be more cautious as many severe oligozoospermic samples can be misinterpreted and reported as azoospermic. On the other hand, even in men with no semen abnormalities, performance anxiety (Patterson & O'Gorman, 1989; Saleh *et al*., 2003) may produce either azoospermic or severe oligozoospermic samples and it is important to accurately identify these men for appropriate management of the condition. Thus, introducing a quantitative test based on a biochemical marker is much more reliable, as the laboratory data lead to very crucial decision making in the management of infertile couples.

It is concluded that the simple D-dopachrome tautomerase assay as described by Al-Abed *et al.*

(2005) seems to be an accurate, sensitive, and specific quantitative test that is easy to use and locally affordable to differentiate between fertile and infertile males in Sri Lanka based on the MIF levels in seminal fluid. This method warrants further clinical validation by using larger sample sizes to compare different infertile groups with fertile controls.

Acknowledgement

The authors acknowledge: Prof. Yousef Al Abed, The Feinstein Institute for Medical Research, Manhasset, New York, USA for providing human MIF cytokine; Ms Madawi Warnakulasooriya and all the staff members of the Reproductive Biology Laboratory, Department of Obstetrics & Gynecology, Faculty of Medicine, University of Colombo; staff members of *Vindana*; Ms Anoma de Silva and Mr J.L.R.A. Jayakody of the Department of Zoology, Faculty of Science, University of Colombo for their help and technical assistance in this study; Professor W.D. Ratnasooriya, Emeritus Professor of the Department of Zoology, University of Colombo for his constructive comments and suggestions in improving the manuscript; and the University of Colombo, Sri Lanka for financial assistance.

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