Comparing Seminal Plasma Biomarkers between Normospermic and Azoospermic Men

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Abstract

Introduction: Azoospermia affects more than 10% - 15% of infertile male subjects attending infertilty clinics. At present, testicular biopsy is the golden standard procedure for evaluating spermatogenesis status in men with azoospermia . Semen collection and analysis is a non-invasive method and has proven to be valuable in the evaluation of spermatogenesis. Identification of seminal plasma markers with testicular or extra-testicular origins have a great value in predicting the prescence of sperm in testicular tissue and presumptive cause of azoospermia. The aim of this study was to find such markers by comparing the content of seminal plasma using different methods in normospermic and azoospermic men.

Matherials and Methods: Semen samples were collected from 200 men attending Avicenna Infertility Clinic (AIC) in Tehran, Iran. Semen samples were analysed according to WHO guidlines. The subjects were divided into two groups: normospermic (n = 100; group one) and azoospermic men (n = 100; group two) according to semen analysis results. Seminal plasma was separated by high speed centrifuagation and stored in -20° *C*. Four markers including fructose, neutral alpha glucosidase (NαG), inhibin B and anti-Müllerian hormone (AMH) were measured in seminal plasma. Fructose and NαG were evaluated by spectrophotometry, while inhibin B and AMH were assessed by ELISA method. The spermatogenesis status in the azoospermic group was evaluated by histopathological method following testicular biopsy.

Results: Fructose concentration showed no difference between the two groups. However, it was significantly correlated with sperm count ($p < 0.01$, r = -0.408). Seminal plasma inhibin B (OR: 1.01; 95%: CI: 1.005 - 1.016), AMH (OR: 1.63; 95% CI: 1.17 - 2.28) and NαG, (OR: 1.07; 95% CI: 1.04 - 1.1) levels were higher in normospermic subjects compared to azoo-spermic men. There were significant differences in inhibin B and AMH concentrations between the two groups based on the presence or absence of mature sperm in testicular biopsies ($p < 0.01$). Inhibin B concentration was positively correlated with sperm count in the normospermic group, however, NαG concentration correlated with sperm count of normospermic men ($p < 0.01$, r = 0.345) and the subjects'age in both groups.

Conclusion: Inhibin B and AMH were correlated with the presence of sperm in testicular tissue samples. According to non-specific changes in inhibin B and AMH concentrations, identification of more specific molecular markers in seminal plasma to definitely evaluate the status of spermatogenesis is recommended.

Keywords: Anti-Mullerian Hormone, Azoospermia, Fructose, Inhibin B, Male infertility, Neutral alpha glucosidase, Seminal plasma, Spermatogenesis.

Introduction

Infertility is a problem that affects more than 10 - 15% of couples during reproductive years and male factor infertility is the cause of up to half of the cases. (1, 2). Most male factor infer-

tilities are due to testicular failure which results in a decline in semen quality (3). Most male factor infertilities are represented as a wide range of semen quality from normal spermogram to

absence of any sperm in semen. The absence of sperm in semen results from sever failure in testicular function or obstruction of extra-testicular ducts (4). Azoospermia, defined as complete absence of spermatozoa in ejaculate, is present in less than 1% of men in the normal population, and 10 - 15% of infertile men (5). Azoospermia results from obstruction of extra-testicular ducts (obstructive) or testicular dysfunction (non-obstructive), (6, 7). In obstructive azoospermia (OA) sperm can be retrieved by micro-surgical sperm aspiration (MESA), percutaneous sperm aspiration (PESA), testicular sperm aspiration (TESA) or testicular sperm extraction (TESE) in more than 90% of cycles. Non-obstructive azoospermia (NOA) is caused by severe impairment of spermatogenesis and it is considered the most critical cause of male infertility (7). In men with NOA, only a few foci of spermatogenesis may be present in the testis. In about 30% of NOA cases, sperm can be retrieved after multiple testicular biopsies, however in more than 70% of men with NOA sperm is not found in the testis following multiple biopsies and testis may also be atrophied following these multiple procedures (8). Therefore, prognosis of sperm presence in NOA cases and evidence of its absence in testicular tissue in negative cases have remarkable values in the treatment of male infertility.

Not long ago, prognosis of sperm retrieval in NOA men was mostly limited to physical examinations (testis volume and consistency), hormonal profile and genetic workup, but the final documentation for the presence or absence of sperm retrieval was given via an invasive diagnostic procedure called TESE. Therefore, application of non-invasive methods with a high sensitivity, specificity and predictive value has a great clinical value and it prevents up to more than 70% of testicular biopsies that result in inadequate sperm for ICSI (9).

Seminal plasma results from a mixture of various secretions from different parts of the male reproductive organ (10). Less than 10% of the seminal plasma results from secretion from seminiferous tubules and epididymis (11), therefore, evaluation of semen components and its biochemical characteristics provide valuable non-invasive biomarkers for evaluating the function of different accessory sex glands and seminiferous tubules involved in semen production.

Seminal plasma contains several components with different origins such as inhibin B, Anti-Müllerian hormone (AMH), Neutral alpha glucosidase (NAG) and fructose. Inhibin B could be used as a marker for the identification of spermatogenesis and as an indicator for the level of interruption in spermatogenesis in men with non-obstructive azoospermia (12). Inhibin B in men with normal fertility is higher than men with impaired spermatogenesis. The fact that semen does not contain inhibin B after vasectomy, further confirms the theory that the testis is the main source of this hormone (13). Anti-Müllerian hormone (AMH) is of testicular origin and it is secreted by Sertoli cells in seminiferous tubules (14, 15). NAG has epididymal origin and its measurement in semen is a simple and sensitive method for determining the type of azoospermia (16, 17). Fructose concentration in seminal plasma could indicate ejaculatory duct obstruction and seminal vesicle function or its hypoplasia (18). Therefore, the four aforementioned markers can evaluate different parts of male reproduvtive ducts.

The aim of this study was to compare the classical hormones used to evaluate male infertility (FSH, LH, and testosterone) by the aforementioned new markers of spermatogenesis in men with normospermia and NOA and also to evaluate their correlation with histopathological results in men with NOA.

Materials and Methods

Two-hundred semen samples were collected from infertile men aged 29 - 43 years, attending Avicenna Infertility Clinic (AIC) affiliated to Avicenna Research Institute (ARI) in Tehran, Iran. Semen samples were collected by masturbation in a sterile wide-mouthed cup. Semen analysis was immediately performed according to WHO guidelines.

Semen samples were centrifuged for 5 minutes at 3,000 *rpm*. The supernatant seminal plasma was quickly and carefully recovered and put to freeze at -20° *C* for upcoming measurements.

The specimens were categorized into two groups according to the results of spermiograms: normospermic (group 1, n = 100) and azoospermic men

(group 2, n = 100). Concentrations of fructose, NAG, inhibin B and AMH were determined in the stored seminal plasma. Fructose concentration and NAG activity were measured according to WHO guidelines. For measuring inhibin B and AMH in semen, an antiprotease cocktail in tablet form (complete antiprotease) was added to seminal plasma immediately after thawing. Levels of inhibin B and AMH were determined using the ELISA sandwich method (DSL, USA). Testosterone, follicle stimulating hormone (FSH) and leutinizing hormone (LH) were measured by chemiluminescent immunoassay (Diasorin, Italy).

In the normozoospermic group, measurement of inhibin B and AMH in undiluted seminal plasma revealed values in excess of the maximum amount for the standard curve. Therefore, the samples were diluted to 1:2.

Testis biopsy was performed on 78 out of 100 azoospermic men. Testicular biopsy had been performed earlier for the other patients in elsewhere, therefore mostly had documented histopathological results and were reluctant to undergo the procedure. Sperm extraction was also performed in the azoospermic group. Subsequently, cytological evaluation for the presence of mature sperm in the testis was performed on wet tissue and histopathological evaluation was done for the presence of different lineage of spermatogenic cells on paraffin-embedded tissue blocks by two skilled clinicians including an embryologist and a pathologist, respectively.

All of the statistical analyses were performed by SPSS software, version 13.0. Correlation and intergroup comparison of the values were carried out by linear regression and student t-test. Spearman's rank correlation test was used to assess correlations and non-parametric Mann-Whitney test was used to assess differences between the groups. P-values less than 0.05 were regarded as significant.

Results

The mean ages of the normozoospermic and azoospermic participants were 35.3 ± 6.2 (ranging from 29 to 42 years) and 36.64 ± 6.12 years (ranging from 30 - 42 years), respectively. There were no significant differences concerning the seminal plasma volume, pH and age between the two groups. Sperm parameters in normospermic men have been summarized in Table 1.

Table 1. Sperm parameters in 100 normospermic men attending Avicenna Infertility Clinic

Parameters	Values
Sperm concentration ($\times 10^6$/*ml*)	115.0 ± 69.9
Total sperm count ($\times 10^6$/*ml*)	422.2 ± 288.1
Normal morphology *(%)*	19.8 ± 5.9
Motility *(%)*	80.2 ± 5.9
grade a	20.1 ± 9.2
grade b	27.2 ± 6.7
grade c	10.7 ± 4.4
WBC ($\times 10^6$/*ml*)	0.09 ± 0.02

- The reported parameters have been presented as M ± SD

The levels of the aforesaid biomarkers in the two groups have been summarized in Table 2. The range of inhibin B and AMH concentration in the seminal plasma were widely dispersed in the two groups of patients. Seminal concentration of NAG, inhibin B and AMH were significantly ($p < 0.05$) different between the two groups (Table 2).

As shown in Table 2, the normospermic controls presented a significant increase in NAG activity, (OR: 1.07; 95% CI: 1.04 - 1.1) and concentrations of inhibin B (OR: 1.01; 95% CI: 1.005 - 1.016) and AMH (OR: 1.63; 95% CI: 1.17 - 2.28) in comparison with those of the azoospermic group.

There was a significant correlation between fructose (p = 0.009; r = -0.263), neutral α-glucosidase (p = 0.013; r = 0.261) and inhibin B (p < 0.05; r = 0.381) with sperm count. In addition, NAG, negatively correlated with the concentration of fructose.

A positive correlation was found between concentrations of inhibin B and AMH (p < 0.001, r = 0.795). In addition, NAG had a positive correlation with inhibin B (p < 0.001, r = 0.412) and

Table 2. Comparing biochemical parameters between normospermic and azoospermic men attending Avicenna Infertility Clinic

Variables	Azoospermic	Normospermic
Neutral α-glucosidase (*mU/ml*)	14.1 ± 10.7	25.6 ± 16.3
Fructose (*mg/dl*)	263.1 ± 89.8	283.4 ± 98.1
Inhibin B (*pg/ml*)	88.6 ± 129.9	629 ± 965.2
AMH (*ng/ml*)	0.7 ± 3.1	5.5 ± 7.5
FSH (*mIU/ml*)	24.4 ± 18.0	-
LH (*mIU/ml*)	9.0 ± 5.6	-
Testosterone (*ng/ml*)	7.2 ± 2.5	-

- The reported parameters have been presented as M ± SD
- Mann-Whitney

Table 3. Histopathological results of testicular biopsies in azoospermic men in comparison with their serum FSH and inhibin B concentrations

Histopathological results	Patients No.)	Inhibin B (ng/ml)	FSH (mIU/ml)
Germ cell aplasia	47	57.137 ± 50.34	26.87 ± 17.85
Arrest in spermatogenesis	23	236.94 ± 242.138	14.02 ± 17.83
Hypospermatogenesis	8	65.83 ± 63.62	31.08 ± 17.49

- Mann-Whitney

AMH ($p < 0.001$, $r = 0.489$). There was a negative correlation between levels of inhibin B and AMH with serum concentrations of FSH and LH in the azoospermic group.

Mature spermatozoa was retrieved only in eight men following testicular biopsy and despite multiple biopsies on both testes in 70 patient, there were no mature sperm that could be used for ICSI. The seminal inhibin B concentration was significantly different ($p < 0.05$) between positive and negative outcomes of TESE due to the presence of mature sperm (Table 4). Table 3 shows the histopathological results of testicular biopsies in comparison with the concentrations of FSH and inhibin B. Mature sperm was retrieved only in about 10% of men undergoing testicular biopsy.

When the azoospermic men were categorized according to the presence or absence of mature sperm in testicular biopsies, there were significant differences in inhibin B and AMH concentrations between the two groups. There were no differences regarding the levels of NAG and fructose between the two groups (Table 4).

Discussion

In about 50% of cases with none-obstructive azoospermia, sufficient number of viable sperm can be retrieved to perform ICSI following testicular biopsy (19). Until recently, prediction of spermatogenesis in testis was performed by looking for several parameters including spermogram, testis volume and consistency and the endocrine profile (LH, FSH and testosterone) but in numerous cases sperm could not be found following multiple biopsy in the face of signs for sperm presence. On the contrary, despite conditions suggestive of a negative result for sperm presence, proper sperm has been found following multiple biopsies (19, 20). These disagreements reveal limitation of these techniques in predicting spermatogenesis status in the testis of azoospermic men.

Lately, some studies suggested a number of new biomarkers to predict the chances of finding proper spermatozoa following multiple testis biopsies, but only the presence of viable spermatozoa at the time of a preliminary diagnostic biopsy appeared to have a strong predictive value for future treatment via ICSI (19, 20). This explains the need to develop a series of non-invasive biomarkers with high predictive values to estimate the presence of mature sperm in the testis.

Several studies with questionable results have focused on the value of serum FSH and inhibin B to predict the status of spermatogenesis in the testis of NOA men, but the number of papers concerning the potential significance of seminal biomarkers of spermatogenesis are very small (21).

The present study compares some spermatogenesis biomarkers in seminal plasma by comparing the classical and newly introduced seminal plasma biomarkers in normospermic and azoospermic men. The results showed a significant difference in alpha-glucosidase, AMH and inhibin B concentrations in the seminal plasma of azoo-

Table 4. Comparing seminal plasma biomarkers with histopathological results from testicular biopsies in azoospermic men

Seminal biomarkers TESE outcome	Inhibin B (pg/ml)	NAG (mU/ml)	AMH (ng/ml)	Fructose (mg/dl)
Positive	133.69 ± 60.5	17.68 ± 10.78	0.022 ± 0.038	255.67 ± 161.12
Negative	81.32 ± 139.76	15.08 ± 10.76	0.9 ± 3.31	263.86 ± 84.71

- The reported parameters have been presented as Mean ± SD
- Mann-Whitney

spermic men in comparison with those of normo-spermic men. Furthermore, seminal inhibin B concentration was significantly different between positive and negative TESE outcomes due to the presence of mature sperm.

The neutral form of α-glucosidase originates from epididymis and it is totally absent in the accessory glands and in the testis; but an acidic isoenzyme can be detected in these organs (22). In this study, selective inhibitors were not used to separate neutral and acidic isoenzymes, but the assays were run at neutral pH, which should have inhibited the acidic isozyme, since it is highly sensitive to environmental pH (22).

Guerin *et al.* showed that patients with azoo-spermia presented significantly decreased NAG activity. In the present study, NAG activity decreased significantly in azoospermic men compared to normospermic men. Being in agreement with the findings of Henkel *et al.*, a significant correlation was noted between neutral α-glucosidase and sperm concentration in our study (23). In addition, Sandoval showed spermatogenesis arrest in NOA men to be associated with a decrease in α-glucosidase which was lower than the concentration seen in obstructive azoospermia (24). Duan's results on α-glucosidase in seminal plasma were similar to the findings of the present study and they were closely related to non-obstructive azoospermia. (25).

The present results on fructose confirm previous reports that fructose concentration is negatively correlated with sperm count. The results are in agreement with those of Gonzales *et al.*, who showed that seminal fructose concentration was inversely correlated with sperm count (26). These relationships can be related to the uptake of fructose by spermatozoa. Indeed, fructose present in the seminal plasma is considered as the main source of energy for sperm metabolism and motility in vitro (27). The first report on the increased seminal plasma fructose in samples from men with non-obstructive azoospermia was published by Buckett *et al.*, who postulated that the condition might be due to a decreased fructose metabolism in the absence of spermatozoa or, more likely, an increased fructose secretion by seminal vesicles (28).

Changes in seminal plasma concentrations of both AMH and inhibin B are inter-correlated. Reaffirming previous findings, a significant positive correlation was observed between inhibin B concentration in the seminal plasma and sperm count, but such correlation was not the case for AMH (29). Moreover, inhibin B and AMH levels negatively correlated with serum FSH and LH concentrations which is in lines with previous studies (14, 30).

The higher concentration of seminal AMH in normospermic men compared with azoospermic subjects is also in agreement with previous studies (29, 31). AMH is preferentially secreted by Sertoli cells into the seminiferous lumen. However, the presence of developmentally more advanced spermatogenic cells may increase AMH secretion that is related to specific stages of the seminiferous epithelium cycle (32). According to the present study, a significant decrease in seminal plasma AMH is associated with the absence of spermatozoa in the semen of azoospermic men, revealing a close correlation between AMH concentration and progress in spermatogenesis. Undetectable seminal AMH in some normo-spermic men may be due to the presence of seminal proteases and their none-specific digestion of seminal protein content prior or during deep freezing of semen before biochemical analysis (29, 31).

In 11.5% of azoospermic men mature sperm was found following testicular sperm extraction (TESE), but it was much lower than other studies (31). This difference might be due to the more solid techniques used in those studies, such as testicular fine needle aspiration, multiple biopsies from different regions of testis, enzymatic digestion of biopsied seminiferous tubules and micro-dissection TESE (33). The present results support insufficiency of TESE in excluding the absence of focal spermatogenesis in testis of NOA men. Tsujimura *et al.*, compared multiple TESE and microdissection TESE in 37 and 56 azoospermic men, and the rates of sperm retrieval were 35.1% and 42.9%, respectively (34).

No correlations were found between seminal plasma AMH, NAG, fructose and sperm retrieval, confirming the results of previous studies (29, 31, 35). It was also found that seminal plasma inhibin B in normozoospermic men was significantly

higher than that of azoospermic subjects, sustaining the relation between inhibin B secretion and sperm concentration. Harmonious to previous studies, it also demonstrates a positive correlation between the concentrations of inhibin B and AMH with the levels of α-glucosidase in seminal plasma (30). In contrast to Yehia, Tunc and Duvilla results, a significant difference was noted in seminal plasma inhibin B concentration ($p < 0.01$) between positive and negative TESE outcomes (29, 30, 36). But the present study confirms Ballesca's findings in this regard (37).

Similar to Yehia's study, the present findings showed higher levels of inhibin B in azoospermic samples with maturation arrest at advanced stages of spermatogenesis compared to samples without any germ cells (Sertoli cell only syndrome or germ cell aplasia), (30). Sertoli cells are the predominant site for the production of inhibin B in the testis (38, 39). By using specific monoclonal antibodies against its α- and βB-subunits, Andersson *et al*. revealed that germ cells (but not Sertoli cells), could be immunostained for βB-subunit. Sertoli cells only contain α-subunits whereas the βB-subunits are localized in pachytene spermatocytes and in round spermatids (38). Therefore, these data discard the hypothesis that Sertoli cells are the only source of inhibin B in testis. It seems that biologically inactive α-subunits can only be found in the absence of germ cells in mature testis (Sertoli cell-only syndrome or germ cell aplasia). Seemingly, inhibin B is produced by Sertoli cells but its biological activity is dependent on the presence of specific germ cells. Pachytene spermatocytes and round spermatids may act as major modulators for inhibin synthesis in the early stages of sperm development (38, 40).

Conclusion

The present study revealed a significant relationship between levels of inhibin B and AMH in seminal plasma and presence of mature sperm in the testis of NOA men. This finding suggests that inhibin B and AMH are sensitive biomarkers in semen and could reflect the functional state of the seminiferous epithelium. Since inhibin B and AMH determinations are of a low cost and a valuable non-invasive predictor of spermatogenesis, their application is suggested in all NOA men. Serum and seminal plasma levels of inhibin B and AMH, in conjunction with serum FSH will

be measured prior to performing TESE. In addition, more attention should be paid to the function of Sertoli and spermatogenic cells in NOA men via application of high throughput techniques to find new specific biomarkers with high sensitivity and specificity.

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