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Research Article

Presence of a Biochemical Marker in Human Semen that Differentiate Ejaculates into Two Distinct Groups

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Abstract

Background: Conventional semen analysis is limited in diagnosing unexplained male infertility. Novel biomarkers are needed to improve clinical assessment.

Objective: To investigate the presence of a potential biomarker (PB) in semen capable of oxidizing 3,3',5,5'-tetramethylbenzidine (TMB), and to determine its association with semen quality.

Methods: Semen samples (n=227) from men referred for analysis were tested for PB activity by incubating 20 µl semen with 100 µl TMB for 5 min. Samples producing a blue color were scored PB-positive. Stability of PB was evaluated by six freeze–thaw cycles. Semen parameters were compared between PB-positive and PB-negative groups using Pearson's correlation and Student's t-test.

Results: PB activity was detected in 63 (27.8%) ejaculates; 164 (72.2%) were PB-negative. No significant correlation or group differences were observed between PB status and semen parameters. PB activity remained stable after repeated freeze–thaw cycles.

Conclusion: A subset of ejaculates contains a stable biomarker capable of oxidizing TMB. Its independence from standard semen parameters suggests it represents a novel factor, potentially relevant to unexplained male infertility. Further biochemical characterization is warranted.

Keywords: Male infertility; Seminal plasma; Biomarker; Peroxidase; Oxidative stress

Introduction

There is a pressing need for innovative and reliable biomarkers to improve the diagnosis of male infertility, particularly in cases of unexplained infertility, as semen analysis alone is often insufficient [1].

Seminal plasma, containing 35–55 mg/ml of proteins and enriched with RNAs, lipids, and other metabolites, can influence sperm function and male fertility [2,3,4]. Mass spectrometric profiling of seminal plasma is being actively investigated for male infertility diagnosis [5].

Peroxidase-positive leukocytes in semen may generate excessive reactive oxygen species (ROS), leading to oxidative damage of sperm lipids, proteins, and DNA [6]. These leukocytes are routinely detected by histochemical staining for peroxidase activity. Given that peroxidase is both stable and potent, we hypothesized that an enzyme of this type—or a similar molecule—might serve as a potential biomarker (PB). Specifically, such a biomarker could oxidize 3,3',5,5'-tetramethylbenzidine (TMB) to produce a blue reaction product [7].

To evaluate this possibility, we analyzed 227 human semen samples.

Materials and Methods

Sample Collection

Semen samples were collected from 227 apparently healthy men referred for routine semen analysis. Specimens were obtained by masturbation following informed consent for the use of surplus material that would otherwise be discarded. All samples were first assessed for semen quality for clinical purposes, after which residual specimens were de-identified and used for research. Clinical and research analyses were conducted independently, with no overlap or access to identifiable patient information. Therefore, this study did not qualify as human subjects research and did not require institutional review board approval.

PB Activity Assay

PB activity was assessed by adding 20 µl of semen to 100 µl of TMB substrate (Southern Biotech, Cat. Nos. 0410-01 and 0412-01; Surmodics, Cat. Nos. TMBW-0100-01 and LSTP-0100-01) in a 2 ml polypropylene microcentrifuge tube. Samples were vortexed for 5–10 seconds and incubated at room temperature for 5 minutes. Those developing a blue color were scored as "PB present"; others were scored as "PB absent."

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Table 1: Mean ± SD of semen parameters by Potential Biomarker present (PB) status.

Parameter	PB Present (n=63)	PB Absent (n=164)
Days of Abstinence	3.4 ± 2.5	3.5 ± 2.1
Semen Volume (ml)	2.8 ± 1.8	3.2 ± 1.9
Sperm Concentration (×10⁶/ml)	70.7 ± 56.2	78.0 ± 49.0
Motility (%)	53.4 ± 15.5	54.4 ± 15.5
Progressive Motility (%)	31.8 ± 14.7	32.0 ± 14.2
Round Cells (HPF)	3.1 ± 3.1	2.5 ± 3.0
Morphology (%)	3.5 ± 2.7	3.1 ± 2.3

No significant differences (p<0.05) between groups.

Stability Testing

Two PB-positive semen samples were frozen at –20 °C. Each sample underwent six freeze–thaw cycles, during which 20 µl aliquots were removed at each thaw, incubated at 4 °C, and subsequently tested with 100 µl of TMB. PB status was recorded after 5 minutes of incubation.

Statistical Analysis

The mean ± standard deviation (SD) of semen parameters were calculated. Associations between PB status and semen quality parameters were analyzed using Pearson correlation coefficients, and group differences were assessed with unpaired Student's t-tests. A p-value < 0.05 was considered statistically significant.

Results

Among the 227 semen samples, 63 (27.8%) were PB-positive and 164 (72.2%) were PB-negative. Mean ± SD values for semen quality in both groups are presented in Table 1.

There was no significant correlation between PB status and semen parameters using Pearson correlation analysis, nor were there significant differences between PB-positive and PB-negative groups by unpaired Student's t-test ($p > 0.05$).

Freeze–thaw stability testing showed no change in PB status after six cycles, indicating PB stability under these conditions.

Discussion

Seminal plasma is known to contain antioxidant molecules and enzymes, which may be deficient in some individuals [8,9]. If PB activity were related to oxidative balance, differences in TMB reactivity might have been expected. However, no association was found between PB status and round cell counts, which often include peroxidase-positive leukocytes. This suggests that PB activity may not originate from leukocytes alone.

Other proteins in seminal plasma, including those capable of binding iron or containing reactive heme groups, could potentially account for the observed blue coloration with TMB. Notably, the absence of significant correlations between PB status and semen parameters strengthens the hypothesis that PB represents an independent and novel factor in semen [10,11].

Our findings suggest that PB may represent a previously unrecognized biochemical entity unrelated to standard semen quality measures, analogous to unexplained male infertility. Further studies are needed to identify the molecular basis of PB activity, which may involve an enzyme with a reactive heme center or another metal-containing protein.

References

1. Agarwal A, Baskaran S, Parekh N. et al. Male infertility. Lancet. 2021; 397: 319–333.
2. Camargo M, Intasqui P, Bertolla RP. Understanding the seminal plasma proteome and its role in male fertility. Basic Clin. Androl. 2018; 28: 1-6.
3. Samanta L, Parida R, Dias TR, et al. The enigmatic seminal plasma: A proteomics insight from ejaculation to fertilization. Reprod. Biol. Endocrinol. 2018; 16: 41.
4. Candenas L. Chianese, R. Exosome Composition and Seminal Plasma Proteome: A Promising Source of Biomarkers of Male Infertility. Int. J. Mol. Sci. 2020; 21: 1-27.
5. Preianò M, Correnti S, Butt TA, et al. Mass Spectrometry-Based Untargeted Approaches to Reveal Diagnostic Signatures of Male Infertility in Seminal Plasma: A New Laboratory Perspective for the Clinical Management of Infertility? Int J Mol Sci. 2023; 24: 4429.
6. Sharma R, Gupta S, Agarwal A, et al. Relevance of Leukocytospermia and Semen Culture and Its True Place in Diagnosing and Treating Male Infertility. World J Mens Health. 2022; 40: 191-207.
7. Zhang X, Yang Q, Lang Y, et al. Rationale of 3,3',5,5'-Tetramethylbenzidine as the Chromogenic Substrate in Colorimetric Analysis. Anal Chem. 2020; 92: 12400-12406.
8. Jones R et al. Peroxidative breakdown of phospholipids by human spermatozoa, spermicidal properties of fatty acid peroxides and protective action of seminal plasma. Fertility and Sterility. 1979; 31: 531-537.
9. Smith R et al. Total antioxidant capacity of human seminal plasma. Human Reproduction. 1996; 11: 1655-1660.
10. Jeyendran RS, Zaneveld LJD. Controversies in the developments and validation of new Sperm Assays. Fertility Sterility. 1993; 59: 726-728.
11. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen Sixth Edition. Vol Edition, V. 2021.