Seminal xanthine oxidase: Appropriate fluorometric assay for the examination of spermatozoa disorders

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ABSTRACT

A fluorometric assay for semen xanthine oxidase activity that could be used as a back-up to current tests in the differential diagnosis of spermatozoa disorders is described. The assay is based on the H2O2-dependent oxidation of thiamine catalyzed by peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)). The method is sensitive, precise (CV below 7.9%), and linear up to 40 U/l. The analytical recovery of the present method is estimated. The comparison with the UV method gave good correlation (r = 0.994). The method is applied to the measurement of the XO activity in seminal plasma of fertile men and Asthenozoospermic patients. Reference values for seminal xanthine oxidase activities determined with the present method on 30 healthy persons are 70.37±23.1.

KEYWORDS

Xanthine oxidase; Fluorometric assay; Spermatozoa disorders; Hematin; Hemoglobin; Thiamine.

INTRODUCTION

The key role of reactive oxygen species in the pathophysiology of sperm function has been considered comprehensively[1]. Their production in semen has been related to loss of motility, decreased capacity for sperm-egg fusion, loss of fertility[2] and defective sperm role[3]. Human spermatozoa are recognized to be susceptible to lipid peroxidation because of its high content of unsaturated fatty acids[4] and attack of ROS such as hydrogen peroxide on the sperm membrane phospholipids may be a reason of idiopathic subfertility. XO (XO, Xanthine:oxidoreductase, E.C.1.2.3.22) is recognized to be a potent producer of superoxide and hydrogen peroxide, which are the most injurious ROS for spermatozoa[5]. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and of the latter to uric acid[6]. The enzyme is present in intestine, liver, milk[7] and in most mammalian tissues, including seminal fluids[2]. In view of the problem of the ROS source in seminal plasma, it is essential to examine the levels of xanthine oxidase in semen.

Previous assays for XO employ one of the following principles. In the first, XO activity is determined spectrophotometrically by measuring the formation of uric acid from xanthine[8]. This assay undergoes certain disadvantages. It is sensitive, but cannot
detect the low levels of enzyme found in human seminal fluids in normal as well as under pathological conditions.

In the second assay, XO activity is determined by using the \(^{14}\text{C}\)Xanthine as described by Dougherty\(^9\). The disadvantages of this assay involve the need for special technique, and it is reagents have a limited shelf life.

The third assay involves the high-performance liquid chromatographic techniques\(^{10}\). This type of assays has some disadvantages such as the unavailability of laboratory equipment to perform in a large number of laboratories and the need for advanced skills to complete.

Battelli et al.,\(^11\) described a competitive enzyme-linked immunosorbent assay (ELISA) to measure the xanthine oxidase level. This method measures both active and inactive XO protein, which makes this method unsuitable for clinical use.

Atlante et al.,\(^{12}\) and Beckman et al.,\(^{13}\) developed a method in which the conversion of pterine into isoxanthopterin by hydrogen peroxide is monitored fluorometrically to measure XO activity. This assay disregarded the interaction that results from the presence of the catalase enzyme.

In this paper, precise fluorimetric method is introduced. The present assay has used 3-aminothiazol to eliminate the interaction with catalase. The assay is uncomplicated and the reagents used are relatively stable.

**PRINCIPLE**

XO oxidizes xanthine to hydrogen peroxide (\(\text{H}_2\text{O}_2\)):

\[
\text{Xanthine} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{Xanthine Oxidase}} \text{Uric Acid} + \text{H}_2\text{O}_2
\]

Hydrogen peroxide (\(\text{H}_2\text{O}_2\)) reacts stoichiometrically with thiamine to generate fluorescence thiochrome (at \(\text{Ex/Em} = 370/425 \text{ nm}\) in the presence of a mixture of hematine and hemoglobin, which acts as peroxidase mimics. Aminotriazole (AT) is included to inhibit catalase enzyme and prevents the interference with measurements, as shown in Figure 1. Since the fluorescence intensity is proportional to XO level; the XO activity can be accurately measured.

**REAGENTS**

1. Hemoglobin (\(5 \times 10^{-7} \text{ M}\)).
2. Hematin stock solution is prepared by dissolving 0.05 g hematin in 25 ml 0.2 N sodium hydroxide. This solution remains stable for 3 months when refrigerated.
3. Hematin working solution is prepared by diluting 0.5-ml of hematin stock solution to 50 ml of 25 mM sodium phosphate buffer (pH 7.4).
4. Peroxidase mimics solution is prepared by mixing equal volumes of Hemoglobin and Hematin working solution. (It is prepared immediately before using).
5. Xanthine Solution (0.15 mM) is prepared by dissolving 0.228 gm in a minimal volume of NaOH 100 ml. Add approximately 90 ml of phosphate buffer (pH 7.4). Adjust to pH 7.4 at 25°C with either 1 M NaOH or 1 M HCl. Dilute to a final volume of 100 ml. prepared fresh)
6. Thiamin stock solution (10 mM) is prepared by dissolving 337 mg of thiamine hydrochloride in 100 ml of water. Refrigerated at 4 °C, this solution stable for one month. A working solution (2x10^{-3} \text{ M}) is prepared daily by diluting one volume of stock solution to five volumes with distilled water.
7. Hydrogen peroxide (100 \(\mu\text{M}\)) is freshly diluted and standardized daily using a molar extinction coefficient of 43.6 M\(^{-1}\) cm\(^{-1}\) at 240 nm.
8. Sodium phosphate buffer pH 7.4 (50 mM) is prepared by dissolving 1.1 g of Na\(_2\)HPO\(_4\) and 0.27 g of KH\(_2\)PO\(_4\) in 100 ml distilled water.
9. 3-aminothiazole (50 mM): is prepared by dissolv-
ing 0.42 gm of 3-aminotriazole in 100 ml of phosphate buffer (pH 7.4).

10. An $K_2HPO_4$–NaOH buffer solution (pH-12) is prepared by adjusting 100 mM $K_2HPO_4$ (1.36 g of $KH_2PO_4$ dissolve in 100 ml D.W) to pH-12 with 2 M NaOH (8 g of NaOH in 100 ml dissolve in D.W).

**PROCEDURE**

Pipette the following reagents into suitable tubes:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Test Blank</th>
<th>STD1</th>
<th>STD2</th>
<th>STD3</th>
<th>STD4</th>
<th>STD5</th>
<th>STD Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>30 $\mu$L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$H_2O_2$ STD</td>
<td></td>
<td>30 $\mu$L</td>
<td>50 $\mu$L</td>
<td>100 $\mu$L</td>
<td>200 $\mu$L</td>
<td>300 $\mu$L</td>
<td>500 $\mu$L</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>500 $\mu$L</td>
<td>500 $\mu$L</td>
<td>500 $\mu$L</td>
<td>480 $\mu$L</td>
<td>430 $\mu$L</td>
<td>330 $\mu$L</td>
<td>230 $\mu$L</td>
<td>530 $\mu$L</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C for 5 min. Then add:

- xanthine solution 400 $\mu$L
- $H_2O_2$ 1500 $\mu$L
- Thiamin 250 $\mu$L
- Peroxidase- mimics 250 $\mu$L
- Sample 30 $\mu$L

Mix by inversion and incubate at 37°C for exactly 30 minutes. Then add:

- *Phosphate buffer (pH 12). 1500 $\mu$L
- Thiamin 250 $\mu$L
- Peroxidase- mimics 250 $\mu$L
- ** Sample 30 $\mu$L

* There is no need to use concentrated acids or high temperature to stop the reaction that catalyzed by a xanthine oxidase because this enzyme inhibited completely when the pH equal to 12. (ref. 14); ** Serum is added to test blank to insure the prevention of overlapping of peroxides in sample (semen plasma) with hydrogen peroxide that formed from the reaction, which catalyzed by xanthine oxidase.

**RESULTS AND DISCUSSION**

Oxidation of non-fluorescent thiamine to fluorescent thiochrome with peroxidase or peroxidase mimics as a catalyst by hydrogen peroxide is often used to estimate thymine\cite{15,16} or to estimate hydrogen peroxide\cite{17}. Previous methods, which focused on measuring hydrogen peroxide, have used hemin, hematint\cite{18}, metal-porphyrin complex \cite{19} or hemoglobin \cite{17} as peroxidase mimics. In the present assay, a new fluorometry for assay xanthine oxidase via hydrogen peroxide determination is presented, in which a mixture of hemat (HT) and hemoglobin (HB) is used as mimetic enzyme of peroxidase for fluorogenic reaction between thiamin and hydrogen peroxide. The obtained results demonstrated that the mixture of HB and HT is a promising peroxidase mimic. The emission spectra of thiamine is shown
in Figure 2.

The concentration of 3-aminotriazole required to prevent interference with catalase that is founded in the sample, is investigated by assaying catalase activity in the presence of different concentrations of this inhibitor. Figure 3 indicates that the degree of catalase inactivation depends on the concentration and duration of pre-incubation with aminotriazole.

Aminotriazole (50mM) is included in all subsequent assays of XO; this concentration is likely to be in excess of that necessary to inactivate the catalase present in seminal fluids. The increment of xanthine oxidase activity after adding 50mM-aminotriazole reaches up to 40% and stays constant with higher concentration of this inhibitor. However, five min incubation with 50mM-aminotriazole allowed adding 100 k-unit of catalase (obtained from Himedia (Product Code: TC037)) to reaction mixture without any effect on the measured oxidase activity.

A crude xanthine oxidase has been purified from bovine milk according to method described by Shivraj et al.,[20]. It is used to compare present method with others that are used for assessment of xanthine oxidase. Results obtained by the present method are compared with those of UV-method[8]. Identical sample, buffer, and substrate were used in both methods. Results have been shown in TABLE 1. The method is applied to the measurement of the XO activity in seminal plasma of 30 fertile men (age 31.6±3.3 year) and 30 Asthenozoospermic patients (age 32.5±3.23 year). Results have been shown in TABLE 2.

It is evident from the above that the results of this method can be used to assess oxidative stress of seminal fluids. The obtained results are consistent with results of previous studies[21].

Accuracy of the entire assay protocol is measured by recovery of hydrogen peroxide added to sample detailed in TABLE 3.
TABLE 1: Statistical Analysis of The Values Obtained for Xanthine Oxidase by UV- Method and Present Method.

<table>
<thead>
<tr>
<th>No. of Samples</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of UV- method (U/L)</td>
<td>9.84</td>
</tr>
<tr>
<td>Mean of test method (U/L)</td>
<td>9.94</td>
</tr>
<tr>
<td>Mean of both methods (U/L)</td>
<td>9.89</td>
</tr>
<tr>
<td>Regression coefficient B</td>
<td>0.9863</td>
</tr>
<tr>
<td>Regression coefficient A</td>
<td>0.0137</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9942</td>
</tr>
</tbody>
</table>

TABLE 2: Levels of Xanthine Oxidase in seminal plasma of fertile and subfertile men.

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Asthenozoospermic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase (mU/L)</td>
<td>70.37±23.1</td>
<td>132.37±30.15</td>
</tr>
</tbody>
</table>

The precision of the assay is measured in-run on a single sample specimen and between run over a period of three weeks with aliquots from the same sample pool but different reagent’s preparations. The results are shown in TABLE 4.

The assay presents a number of advantages more than the existing methodologies. These advantages include; less quantity of sample is required (0.03 ml); the reagents are relatively stable; instrumentals, and apparatus are not complicated and available in most research laboratories, and the assay is free from interference.

REFERENCES


