Biochemical Assays in Spermatology

9

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Abstract

Declaration of a male as fertile involves complete evaluation of sperm cell quality as well as that of seminal plasma. Qualitative and quantitative measurements of seminal contents yield valuable insight to arrive at a conclusive finding about cellular damages and functional status of accessory sex glands. This chapter dwells about measurements of acrosin, hyaluronidase and transaminases as a marker of sperm cell damages. Moreover, protocols related to determination of various enzymes and other seminal contents, viz. neutral α-glucosidase (for epididymis function) and acid phosphatase, ascorbic acids and zinc (for prostatic function), are described as a means to assess functional status of accessory sex glands. Estimation of seminal fructose as a means to evaluate functional status of seminal vesicle has been provided elsewhere.

Keywords

Acrosin • Citric acid • Zinc • GOT • Transaminase • neutral α-glucosidase

Contents

9.1 Introduction

During ejaculation, semen is produced from a highly concentrated suspension of sperm cells, stored in the paired epididymides and mixed with, and diluted by, secretions from the accessory sex glands. It is emitted in several boluses, and about 90% of semen volume is made up of secretions from the accessory organs [1], mainly the prostate and seminal vesicles, with minor

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contributions from the bulbourethral (Cowper's) glands and epididymides.

The total fluid volume contributed by the various accessory glands reflects the secretory activity of the glands. Normal functioning of accessory sex glands is a prerequisite for optimum fertility in males. Though clinical examination of accessory sex glands by rectal examination is possible to a certain extent, it seldom produces confirmatory diagnosis of normal functioning of examined organs. Under such circumstances, biochemical examination of markers of semen, for example, catalase, neutral α-glucosidase, transaminases and others produced by accessory sex glands and testis itself, yields results that are of much value in assessing sexual health of males.

Exposure of spermatozoa to oxygen during semen processing for long-term preservation causes excessive production of detrimental free radicals. Generation of free radicals due to lipid peroxidation plays an important role in sperm ageing. The lipid peroxidation cascade is initiated when spermatozoa are attacked by free radicals, resulting in a loss of unsaturated fatty acids from the plasma membrane and a corresponding decline in the survival and fertilizing ability of affected spermatozoa [2]. Under such circumstances, endogenous enzymatic antioxidant system, i.e. superoxide dismutases (SOD), catalases (CAT) and glutathione peroxidase (GSHPx), may offer protection to sperm cells [3]. However, the equilibrium between the amount of free radical production and endogenous enzymatic antioxidant system decides the amount of damages a sperm cell would undergo [4].

The chapter 'Biochemical Assays in Spermatology' covers twin aspects of estimation of markers to assess functional health of accessory sex glands and determination of sperm cell damages by evaluating cellular enzyme, for example, acrosin and hyaluronidase concentration in seminal plasma. Since the chapter covers protocols involving different parameters of seminal plasma, relative merits of each assay have been provided in that particular section itself.

9.1.1 Acrosin Estimation

Principle

Acrosin is located in the deeper parts of acrosome. This protein hormone can be extracted at low pH in its inactive form (as zymogen) which is finally converted into acrosin. Hydrolysis of acrosin with a synthetic arginine releases a chromophoric product [5].

Materials

Semen sample, sonicator, spectrophotometer, centrifuge. Refrigerated centrifuge, water bath, Tris-HCl buffer, 2.5 mM benzoyl-DL-arginine p-nitroanilide (BAPNA)), 20 mM calcium chloride

Detergent Mixture

0.3 m NaCl.
0.1% Hyamine.
0.1% Triton.
Dilute to 100 mL DW.

Procedure

Processing of Semen Sample

(a) Mix an aliquot of semen containing 10^6 spermatozoa in 2.0 mL Tris-HCl buffer (0.05 m, pH 7.4).
(b) Centrifuge at 3000 rpm for 10 min.
(c) Discard the supernatant and resuspend the pellet in Tris-HCl and centrifuge.
(d) Collect the sperm pellet, and add 1.5 mL of detergent solution in 1 mM HCl.
(e) Vortex for 5 min.
(f) Sonicate the suspension for about 90 s (with 30 s break) at 40–67 μ amplitude.
(g) Centrifuge the sonicated sample at 10,000 rpm at 4 °C for 15 min.
(h) Collect the supernatant and store at – 20 °C.
(i) This is further used for acrosin and hyaluronidase estimation.

Acrosin Estimation

(a) Incubate the sperm extract (pH 8.0) at 25 °C, 15 min for auto-activation of proacrosin.
(b) Incubate 0.1 mL of the above sample with 0.5 mL of the 0.05 M Tris buffer (pH 8.0) for 10 min at room temperature.
(c) Add 0.5 mL of 20 mM $CaCl_2$ solution.
(d) Add 0.5 mL BAPNA.
(e) Record the absorbance at 410 nm at 30 s interval.

Calculation
The formula used to measure enzyme activity with /10^6 spermatozoa is

$$\text{Enz.activity} = \left(\text{OD}^{\text{t}} - \text{OD}^{\text{c}} \times 10^6\right) \div \left(1485 \times \text{Sperm conc.in million}\right)$$

where:

Enz. activity = μIU acrosin/10^6 sperm/unit time
OD^t = mean OD test
OD^c = mean OD control

9.1.2 Hyaluronidase Estimation

Principle
This enzyme is present in the sperm acrosome. Since integrity of acrosome is directly involved in fertilizing capacity of semen, measure of hyaluronidase activity upon its leakage from damaged acrosome makes sense.

Hyaluronidase is a hyaluronic enzyme, i.e. 'endohexosaminidase', and catalyses the degradation of hyaluronic acid with the liberation of acetyl glucosamine terminal groups which can be measured in a spectrophotometer.

Materials
Semen sample, sonicator, spectrophotometer, centrifuge. Refrigerated centrifuge, water bath, Tris-HCl buffer

Detergent Mixture
0.3 m NaCl.
0.1% Hyamine.
0.1% Triton.
Dilute to 100 mL DW.

Hydrochloric Acid and Acetate Buffer
50 mM acetate (pH 4.0)
150 mM NaCl
5.78 mL acetic acid

Solution A (100 mmol)
5.78 mL acetic acid.
Dilute to 1000 mL DW.

Solution B (100 mmol)
13.6 g CH3 CooN4, 3H2O.
Dilute to 1000 mL DW.

Acetate Buffer
41 mL solution A.
9 mL solution B.
Adjust the pH to 4.0.
0.875 g NaCl.
Dilute to 1000 mL DW.

Hyaluronidase (1.25gm/L)
62.5 mg hyaluronic acid.
Dilute to 50 mL acetate buffer.

N-acetyl Glucosamine Standard Solution
10 mg N-acetyl glucosamine.
Dilute to 10 mL acetate buffer.

Tetraborate (0–8 mol/L, pH 9.1)
24.44 g K2 B4 O7 4H2O.
Dilute to 100 mL DW.
Adjust to pH 9.1 with KOH5 mol/L.

Dimethylaminobenzaldehyde (1% w/v)
10 g 4-dimethylaminobenzaldehyde.
Dilute to 100 mL acetic acid (containing 12.5% v/v HCl).
Just before use, dilute with 9 volume of acetic acid.

Procedure

(a) Carry out processing of semen sample as described for acrosin estimation.
(b) Pipette 0.8 mL of the hyaluronic acid solution and incubate for 15 min.

(c) Add 0.2 mL of the sample.
(d) Incubate the mixture for 10 min.
(e) To 0.5 mL of test sample, add immediately 0.1 mL of tetraborate solution.
(f) For standard, add 0.5 mL of tetraborate solution for colour reaction.
(g) Heat for 3 min in boiling water bath, followed by cooling in running tap water.
(h) Add dimethylaminobenzaldehyde reagent 3.0 mL to all (test and standard) and mix it well.
(i) Incubate for 50 min at 37 °C in a water bath followed by cooling in running tap water.
(j) If necessary, centrifuge the solution to make it clear.
(k) Pour into cuvettes and immediately measure the OD.

Calculations

The enzyme activity is expressed in mole N-acetyl glucosamine liberated per min. Refer to the standards to calculate the amount of acetyl glucosamine liberated in incubation time (in minutes). The reading of the 19 g standard (10 µmol) is taken for the calculation.

The formula used to measure enzyme activity with /10^6 spermatozoa is

$$\text{Enz.activity} = \left(3767 \times 5 \times \text{OD sample}\right) \div \left(665 \times \text{OD standard}\right)$$

9.1.3 Catalase Test

Principle

The catalase is one of the important enzymatic antioxidant defence systems in sperm cells. Catalase catalyses the dissociation of H_2O_2 into H_2O and O_2 [6], reduces the oxidative stress and finally enhances sperm motility [7, 8]. The addition of antioxidants such as catalase to bull semen has been shown to offer protective effect on spermatozoa [9]. Catalase is found in the cytoplasm of cells, but sperm cells, which are essentially devoid of cytoplasmic components, contain little if any catalase [10].

Catalase test is carried out to detect concentration of catalase enzyme in the semen samples. It is an indicator of presence of pus and blood and the bacterial contamination in the semen. This is not a routine test in semen evaluating laboratories. Estimation of catalase in semen sample [11] using spectrophotometer as well as using ELISA kit [12] is described below.

Materials

mPBS, phosphate buffer, Percoll, Tris-HCl

PBS Modified (mPBS, pH 7.4)

2.7 mM KCl.
1.5 mM KH2PO4.
8.1 mM Na2HPO4.
137 mM NaCl.
5.55 mM glucose.
1.0 mM pyruvate.
Dilute to 1000 mL DW.

Percoll (40 and 80%, v/v)

40 or 80 mL Percoll.
Dilute to 100 mPBS.

Procedures

Spectrophotometer Method

Sperm Washing [13]

(a) Wash fresh and frozen-thawed semen samples using Percoll density gradient.
(b) Take a 15 mL centrifuge tube and pour 1 mL layer of 40% Percoll.
(c) Gently pipette 1 mL layer of 80% Percoll over it.
(d) Now carefully layer 1 mL of semen sample on top of two-step Percoll column.
(e) Centrifuge the prepared column at 10,000 × g, 5 °C for 10 min.
(f) Discard the supernatant.
(g) Wash the sperm pellet twice using Tris-HCl buffer (pH 7.4), at 600 × g for 10 min.
(h) Adjust the suspension at a concentration of 10^9 sperm cells.
(i) Sonicate the above suspension and collect supernatant for estimation of catalase.

Catalase Activity

(a) Take 5 mL of the above lysate in a cuvette.
(b) Add 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2.
(c) Record OD at 240 nm for 1 min.

Observations
The molar extinction coefficient of H_2O_2 (43.6 M cm^{-1}) is used to determine the catalase activity. One unit of activity is equal to 1 mM of H_2O_2 degraded per mL.

ELISA Kit

(a) Take 5 mL of fresh or frozen-thawed semen samples in a cryo-centrifuge tube.
(b) Centrifuge the sample at 1600 × g for 5 min to remove seminal plasma.
(c) Discard the supernatant carefully.
(d) Add 15 mL of 1% Triton X-100 solution into the precipitate.
(e) Incubate the suspension for 20 min.
(f) Centrifuge again at 4000 × g, 25 °C for 30 min.
(g) Suspend the precipitate in DW, and shake well.
(h) Collect the supernatant containing crude extract of enzymes.
(i) Analyse using ELISA kit.

Observations
Higher concentration of catalase in the sample indicates increased antioxidant activity [14].

Points to Ponder
Avoid turbidity of the solution while carrying out assay involving spectrophotometry.

9.1.4 Transaminase/Phosphatase (GOT/GPT) Activity

Principle
Enzymes are protein in nature and are all relatively unstable requiring immediate estimation after collection of blood/seminal plasma sample. Transaminases are intracellular enzymes located in the midpiece of sperm cell. During stress or damages of spermatozoa, these enzymes leak to the seminal plasma. Thus, estimation of its quantity gives an idea of extent of spermatozoa cell damage. Some examples of transaminases are glutamic-oxaloacetic acid transaminase (GOT) now referred to as aspartate aminotransferase (AST), stable for 3 days at room temperature or 28 days at –20 °C. Another transaminase enzyme is glutamic-pyruvic transaminase (GPT) now referred to as alanine aminotransferase (ALT). The assay is based on the principle that the oxaloacetic acid is produced by transamination, when GOT reacts with substrate (alpha-ketoglutamic acid and aspartic acid) dicarboxide spontaneously to pyruvate. This in turn reacts with 2,4-dinitrophenylhydrazine to produce a brown-coloured hydrazine. This hydrazine is measured in spectrophotometer at 510 nm wavelength. The results are expressed in mole/min/litre.

For estimation of enzymes, commercial test kits designed for human use are available. They can be used for animal samples as well, but a cross-check is always required. In certain cases, substrate concentration, pH or some activators have been found to be below par.

Units

(a) The international unit =1 IU = 1 μ mol of substrate utilised or product formed per min at the stated temperature.
(b) 1 unit = 10^3 milli- units (m units)
(c) 1 m unit = 1 n mol substrate utilised or product formed per min, and results are usually reported as m units/mL or units/I serum at °C.
(d) Enzyme concentration may now be expressed in forms of nkat or pkat where 1 unit = 16.67 nkat.

Materials

Phosphate Buffer (pH 7.4)
11.3 g dry anhydrous disodium hydrogen phosphate.
2.7 g dry anhydrous potassium dihydrogen phosphate.
Dilute to 1000 mL DW.
Store at 4 °C.

GOT Substrate (pH 7.4)
13.3 g of DL-aspartic acid.
0.146 g of alpha-ketoglutaric acid.
90 mL N NaOH solution.
Adjust pH to 7.4.
Dilute to 500 mL with phosphate buffer.
This solution is divided into 20 mL portions and stored at −15 °C.

GPT Substrate
9 g of alanine.
90 mL DW.
2.5 mL N NaOH to adjust the pH to 7.4.
0.146 g of alpha-ketoglutaric acid and dissolve.
Dilute to 500 mL with phosphate buffer.
This solution is divided into 20 mL portions and stored at −15 °C.

Stock Pyruvate Standard
220 mg of sodium pyruvate.
Dilute to 100 mL of phosphate buffer.
This solution is divided into 10 mL portions and stored at −15 °C.

Working Pyruvate Standard (DNPH)
10 mL standard pyruvate solution.
50 mL phosphate buffer.
Store at −15 °C.
Prepare fresh every week.

24-Dinitrophenyl Hydrazine
19.8 mg of dinitrophenyl hydrazine.
10 mL of conc. HCl.
Dilute to 100 mL.
Keep in brown bottle in room temperature.

0.4 N Sodium Hydroxide
16 g of NaOH.
Dilute to 1000 mL DW.

Procedure

Test (T)
(a) Warm the 0.5 mL test substrate in water bath at 37 °C for 3 min.
(b) Add 0.1 mL of seminal plasma, and mix gently.
(c) Incubate for 60 min exactly.
(d) Remove the tubes from the bath.
(e) Add 1.5 mL of DNPH immediately and mix well.

Control (C)
(a) Mix 0.5 mL substrate with 0.5 mL of DNPH solution and add 0.5 mL of plasma into it.

Standard (S)
(a) Mix 0.5 mL of DNPH with 0.4 mL of substrate and 0.5 mL DW.

Blanks (B)
(a) Mix 0.5 mL of substrate, 0.1 mL of DW and 0.5 mL of DNPH in a test tube.
(b) Allow DNPH to react in all tubes for 20 min in room temperature.
(c) Add 5 mL of NaOH (0.4 N), wait for after 10 min, mix well and compare the colour at 510 nm.

Calculations
Calculate the pyruvate formed (min/litre):

$$\text{Pyruvate formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 0.4 \times \left(1/60\right) \times 1000/0.1$$

or

$$\text{Pyruvate formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 67\,\mu\text{mol}$$

Procedure (GPT Estimation)
Follow the same procedure as the above except use of GPT substrate and incubation time of 30 min.

Calculation

$$\text{GPT formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 0.4 \times \left(1/30\right) \times 100/0.1$$

or

$$\text{GPT formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 133\,\mu\text{mol}$$

Points to Ponder

(a) As enzymes are temperature sensitive, assay results should always be mentioned along with temperature at which it was carried out.
(b) Addition of glycerol increases the release of GOT in extracellular fluid [15].

9.1.5 Neutral α-Glucosidase

Principle

Seminal plasma contains both a neutral α-glucosidase isoenzyme, which originates in the epididymis, and an acid isoenzyme contributed by the prostate. The prostatic isoenzyme can be selectively inhibited by sodium dodecyl sulphate (SDS) [16]. This permits measurement of the neutral α-glucosidase reflecting epididymal function. Glucosidase converts the synthetic glucopyranoside substrate to p-nitrophenol, which turns yellow on addition of sodium carbonate. The complex thus formed absorbs light at 405 nm.

$$\text{p-nitrophenol-}\alpha\text{-glucopyranoside} \xrightarrow{\alpha\text{-glucosidase}} \text{p-nitrophenol} \xrightarrow{Na2CO3} \text{Complex}$$

This assay is made more sensitive by accounting for non-glucosidase-related substrate breakdown by using the inhibitor castanospermine. The method described below is for use with a 96-well plate reader with sensitivity 1.9 mU/ mL [17].

Materials

Solution 1
4.56 g dipotassium hydrogen phosphate ($K_2HPO_4.3H_2O$).
Dilute to 100 mL DW.

Solution 2
2.72 g potassium dihydrogen phosphate (KH2PO4).
Dilute to 100 mL DW.

Buffer A (0.2 M Phosphate, pH 6.8)
Mix equal volume of solutions 1 and 2 until the pH is 6.8

Buffer B
1 g SDS.
Dilute to 100 mL DW.

Colour Reagent A (0.1 M Sodium Carbonate, for Stopping the Reaction)
6.2 g sodium carbonate (Na2CO3.H2O)
Dissolve to 500 mL DW

Colour Reagent B
0.1 g SDS
Dissolve in 100 mL of colour reagent A

Substrate p-Nitrophenol Glucopyranoside (PNPG) (5 mg/mL)
0.1 g PNPG.
Dissolve in 20 mL buffer B.
Warm the solution on a hot plate at 50 °C for 10 min with continuous stirring.
Keep solution at 37 °C during use.

Glucosidase Inhibitor for Semen Blanks (10 mM, Castanospermine, Stock Solution)
18.9 mg castanospermine.
Dilute to 10 mL DW.

Glucosidase Inhibitor for Semen Blanks (1 mM, Working Solution)
1 mL glucosidase inhibitor stock solution.
Dilute to 10 mL DW.
Freeze 1 mL aliquots at −20 °C.

p-Nitrophenol (5 mM, PNP, for Standard Curve, Stock Solution 1)
69.5 mg PNP.
Dilute to 100 mL DW.

PNP for Standard Curve (Stock Solution 2)
400 µL of PNP stock solution in a 10 mL test tube.
Dilute to 10 mL with colour reagent B.

PNP for Standard Curve (Working Solution)

50/100/150/200/250 μL PNP stock solution 2 in five separate test tubes.

Dilute to 10 mL with colour reagent B.

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take two vials for test (T) and add 15 μL of seminal plasma in each.
(f) Take another vial for blank (B) and add 15 μL of DW.
(g) Add 100 μL of PNPG substrate solution at 37 °C to each tube.
(h) Vortex each tube and incubate at 37 °C for 2 h.
(i) Stop incubation after 2 h by adding 1 mL of colour reagent A, and mix.
(j) Transfer 250 μL of samples and standards to the 96-well plate.
(k) Use DW as blank to set the reading to zero.
(l) Read the plate in a 96-well plate reader at 405 nm wavelength within 60 min.

Calculation

(a) Read the concentration of PNP produced by the sample from the standard curve (μM) by comparing OD values.
(b) Discard values that lie above the top standard and reassay after dilution using buffer A.
(c) Multiply by the correction factor (0.6194*) to obtain the activity of neutral α-glucosidase in undiluted seminal plasma (IU/L).
(d) Subtract the activity (IU/L) of the castanospermine seminal plasma blank from each sample to obtain the corrected (glucosidase-related) activity.
(e) Replicates should agree within 10% (difference between estimates/mean of estimates) x 100 ≤ 10%.
(f) If the above value is greater than 10%, then repeat the assay on two new aliquots of seminal plasma.
(g) Multiply the corrected glucosidase activity by the whole volume of semen (mL) to obtain the glucosidase activity (million U) of the ejaculate.

Points to Ponder

(a) SDS will precipitate on storage at 4 °C, but redissolves on gentle warming.
(b) While making PNPG, a few crystals may remain undissolved.
(c) Make a fresh solution of PNPG for each assay.
(d) Warming of PNP solution is necessary to dissolve it completely. Store at 4 °C in the dark in aluminium foil-covered or brown glass bottle.
(e) Make up a fresh PNP standard solution every 3 months.
(f) Maintenance of exact temperature and timings are crucial while incubating the samples during neutral α-glucosidase estimation.
(g) One international unit (IU) of glucosidase activity is defined as the production of 1 μM of product (PNP) per minute at 37 °C.
(h) In this procedure, the activity is derived from 15 μL of seminal plasma in a total volume of 1.115 μL over 120 min; therefore, the correction factor is (1115/15)/120 = 0.6194.
(i) Before interpreting the result, always check the value from the lower and upper reference limit reported by others.

9.1.6 Citric Acid

Principle

Seminal vesicle is the principle source of citric acid. Its production is controlled by testosterone. Season affects its secretion greatly. Colorimetric method based on pentabromoacetone [10] is described below.

Materials
Sulphuric acid, potassium permanganate, potassium bromide, sodium nitrite, urea, sodium sulphide, ethylene glycol (pure), light petroleum (20–40 BP), spectrophotometer with blue filter

Reagent A
mL sulphuric acid (15 N, H_2SO_4)
0.3 mL potassium permanganate (0.3 M, $KMnO_4$)
0.2 mL potassium bromide (1 M, KBr_2)

Reagent B
10.35 g sodium nitrite (1.5 M, NaNO2).
Dilute to 100 mL DW.

Reagent C
12.012 g urea (2 M, NH2CONH2).
Dilute to 100 mL DW.

Reagent D
1 g sodium sulphide.
8 mL ethylene glycol (pure).
Dilute to 12 mL DW.

Procedure

(a) Take 0.1 mL of fresh semen in a centrifuge tube.
(b) To this, add 1.9 mL of 10% TCA solution.
(c) Centrifuge at 2000 rpm for 15 min.
(d) Transfer the entire supernatant to a 100 mL separating funnel.
(e) Add reagent A to the above funnel, mix well and leave for 15 min.
(f) To the above tube, add 0.5 mL of reagent B to remove excess of permanganate.
(g) Add 0.5 mL of reagent C to decompose the excess nitrite.
(h) Shake well to allow escape of gas formed due to reaction.
(i) Add 10 mL of light petroleum (20–40 BP) and shake vigorously for 60 s.
(j) Carefully remove the aqueous layer so as not to disturb petroleum layer.
(k) Wash the petroleum twice with 3 mL of DW.
(l) Transfer measured quantity of petroleum layer to stoppered test tube (2 × 20 cm).
(m) Add 6 mL of reagent D, and shake the content thoroughly for 15 min for the yellow colour to develop.
(n) Set the reading of spectrophotometer at '0' (445 nm, blue filter) using a sodium sulphide solution.
(o) Measure the OD of the test samples and plot against standard curve.
(p) Draw a standard curve using the readings of known concentration of citric acid standard.

9.1.7 Estimation of Acid Phosphatase

Principle
Acid phosphatase is a hydrolase enzyme that catalyses the hydrolysis of various phosphate esters at optimum acidic pH. Although it is found in high concentrations in the prostate, bones, blood cells, the spleen and other organs [18], one of the molecular variants of the enzyme (isoenzymes), i.e. prostate isoenzyme, has the highest significance in assessing the prostate's function [19]. In animals, this enzymatic biomarker detected for the first time in a bull [20] is primarily related to the metabolic function of spermatozoa in ruminants.

In essence, the colorimetric procedure of Seligman and co-workers [21] consists of incubation of the enzyme source with a solution of sodium/3-naphthyl phosphate buffered to an appropriate pH (4.8 for acid phosphatase) at 37.5 °C for 2 h. Two molecules of p-naphthol released during the reaction are coupled with tetrazotized diorthoanisidine to yield an insoluble, purple azo dye. This dye is then extracted with ethyl acetate for measurement of the colour density in a photoelectric calorimeter.

Materials
Sodium carbonate (1.0 M), trichloroacetic acid (40%, TCA), anhydrous ethyl acetate

Substrate
2 mg sodium p-naphthyl phosphate (0.0008 M).
Dilute to 10 mL DW.

Verona1 Buffer (pH 9.1, 0.1 M)
950 mL sodium diethyl barbiturate (0.1 M)
50 mL hydrochloric acid (0.1 M)

Acetate Buffer (pH 4.8, 0.2 M)
120 mL sodium acetate (0.2 M)
80 mL acetic acid (0.2 M)

Tetrazotized Diorthoanisidine
20 mg powder.
Dilute to 5 mL cool DW.
Prepare fresh.

Working solution (Buffered Substrate)
10 mL substrate solution.
10 mL of either Veronal or acetate buffer.
Add this solution to the enzyme preparation as described below.

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take 1 mL of seminal plasma and dilute with 19 mL of DW.
(f) Take 1 mL of diluted seminal plasma and add 5 mL of buffered substrate (T).
(g) In another test tube, take 5 mL of buffered substrate (control, for non-enzymatic hydrolysis).
(h) Incubate both tubes for 2 h at 37.5 °C.
(i) After incubation, add four drops of sodium carbonate (1 M) solution to raise the pH to optimum level of coupling.
(j) Add 1 mL of tetrazotized diorthoanisidine solution, and mix thoroughly.
(k) Allow 3 min for coupling to take place.
(l) To each tube, add 1 mL of 40% TCA to precipitate protein and favour release of dye from protein complex.
(m) Add 10 mL of ethyl acetate, and mix thoroughly until an even emulsion is formed.
(n) Centrifuge tubes at 3000 × g for 10 min.
(o) Transfer 5 mL of supernatant to another Klett tube.
(p) Avoid evaporation of ethyl acetate by capping the tube.
(q) Determine OD in spectrophotometer through a green filter (540 nm).
(r) For preparation of standard curve, add ß-naphthol in the presence of plasma following the above procedure.
(s) Plot the test value to get the phosphatase activity.

Calculation
The number of units of acid phosphatase per 100 mL of seminal plasma was obtained by multiplying by 100 the number of mg of p-naphthol released in 2 h when 0.05 mL of sample was used.

Points to Ponder

(a) The enzyme concentration in bull was 24.7 ± 11.8 IU, depending on the method of quantitation [22].
(b) The substrate solution may be stored at 4 °C for a month with no significant spontaneous hydrolysis.
(c) The solution for tetrazotized diorthoanisidine compound is always prepared fresh, for it decomposes extensively on standing at room temperature for 20 to 30 min.
(d) However, colour does not fade on standing, but evaporation of ethyl acetate in over 1 h concentrates the solution affecting result.
(e) A calibration curve may be obtained with 0.01 to 0.08 mg of p-naphthol.
(f) Definition of one unit of phosphatase activity is defined as that amount of enzyme which liberates the colour equivalent of 10 mg of ß-naphthol per hour at 37.5 °C.

9.1.8 Estimation of Zinc

9.1.8.1 96-Well Plate Reader/kit Method

Principle
The compound 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol (5-Br-PAPS) binds with zinc, producing a change in colour, which can be measured at 560 nm.

5 - Br - PAPS + Zn^{2+} → 5 - Br - PAPS - Zn complex

The procedure is based on a commercial kit for measurement of serum zinc by spectrophotometric assay as described in 'WHO Laboratory Manual for the Examination and Processing of Human Semen' [23]. The procedure was described by Johnsen and Eliasson [24], modified for the use of a 96-well plate reader with sensitivity 4 μM/L [25].

Materials

Colour reagents A (2 × 60 mL vial) and B (1 × 30 mL vial) from the commercial kit and zinc standard (100 μM/L)

Zinc standard (100 μM/L, stock solution; store at − 20 °C)

- 0.144 g of zinc sulphate ($ZnSO_4.7H_2O$).
- Dilute to 50 mL DW.

Zinc Solution for Standard Curve

- Take 2, 4, 6, 8 and 10 mL of zinc stock solution in a clean test tube and add 8, 6, 4, 2 and 0 mL of DW, respectively, to each. This will produce 20, 40, 60, 80 and 100 μM/L concentration solution for drawing a standard curve.

Colour Reagent (25 mL)

20 mL of colour reagent A

- 5 mL of colour reagent B

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take 1.5 mL tube and add 5 μL of seminal plasma and 300 μL of DW.
(f) Mix by vortexing for 5 s.
(g) Add replicate 40 μL of sample from the above step to 96-well plate reader, and include replicate blanks (40 μL of DW) and 40 μL of each of the standards.
(h) Add 200 μL of colour reagent to each well, and mix for 5 min on a 96-well plate shaker.
(i) Read the plate at 560 nm using the DW blank to set the zero.

Calculation

(a) Concentration of the zinc in the sample is obtained from the standard curve.
(b) Reject results that are above the top standard, and reassay such samples at greater dilutions.
(c) Multiply the result by the dilution factor of 61 (5 μL of seminal plasma diluted with 300 μL of DW) to obtain concentration of the zinc (mM) in undiluted seminal plasma.
(d) Replicates should agree within 10% (difference between estimates/mean of estimates) x 100 ≤ 10%.
(e) If the above value is greater than 10%, then repeat the assay on two new aliquots of seminal plasma.
(f) Finally, multiply the zinc concentration by the whole volume of semen (mL) to obtain the total zinc content (μM) of the ejaculate.

Points to Ponder

(a) The volumes of semen and reagents can be proportionally adjusted for spectrophotometers using 3 ml or 1 ml cuvettes. The appropriate corrections must be made in calculating the results.
(b) The colour reagent is stable for 2 days at room temperature or 1 week at 4 °C.
(c) Before interpreting the result, always check the value from the lower and upper reference limit reported by others.

9.1.8.2 Spectrophotometer Method

Principle

Even though atomic absorption spectrophotometry is an excellent method to measure concentration of zinc, another simple, reliable, highly sensitive method for the determination of zinc in biological fluids including seminal plasma [26] is described below. In the procedure, interfering trace metals are removed as insoluble

iodides or hydroxides prior to complex formation of zinc with dithizone in a Tris-buffered trichloroacetate centrifugate. Thereafter, absorbance of the chelate is read at 555 nm. The sensitivity of the new method is reported to be comparable to that of atomic absorption spectrophotometry.

Materials

Dithizone, sodium hydroxide, trichloroacetic acid (TCA), zinc standard (as described above), potassium iodide (KI), hydrochloric acid (6 M, HCl), Tris buffer (saturated)

Dithizone Reagent

10 mg of dithizone.
Dilute to 10 mL of Tris (1 M).

Procedure

(a) Separate seminal plasma from sperm cells as described above.
(b) Take 3 mL of seminal plasma in a test tube, add 30 mg of KI and mix well.
(c) Add 0.15 mL of TCA solution to the above tube.
(d) Shake the mixture well and allow to stand at room temperature for 10 min.
(e) Centrifuge the solution at 3600 × g for 30 min.
(f) This will yield about 2.5 mL of clear supernatant.
(g) Adjust the pH to 13.5 by adding 0.1 mL NaOH (10 M)/mL of the supernatant.
(h) Allow the mixture to stand for 15 min and then centrifuge at 3600 × g for 30 min.
(i) Discard the precipitate.
(j) Take 0.1 mL of the supernatant in another tube, add 0.1 mL HCl and mix well.
(k) Add 0.1 mL of saturated Tris buffer, and check pH (should be between 1 and 8.5).
(l) Add 0.1 mL dithizone reagent in 1 M Tris buffer to the above mixture.
(m) Absorbance is determined at 555 nm.
(n) Prepare blank (B) by substituting DW for seminal plasma.
(o) Draw standard curve as described before.

Calculations

(a) Subtract reading of B from test sample (T).
(b) Plot this value in the standard curve to determine the concentration of zinc.

Points to Ponder

(a) Vigorous shaking of dithizone solution is avoided as it results in loss of sensitivity in the assay.
(b) Remove traces of undissolved dithizone by filtration.

9.2 Background Information

Broadly, poor-quality semen may result from production of abnormal spermatozoa from the testis or from post-testicular damage to spermatozoa in the male reproductive tract, or the ejaculate may contain abnormal accessory gland secretions. During clinical examination of a male, secretions from accessory glands can be measured to assess glandular functions. For example, citric acid, zinc, glutamyl transpeptidase and acid phosphatase (prostate), fructose and prostaglandins (seminal vesicles) and free L-carnitine, glycerophosphocholine (GPC) and neutral _-glucosidase (epididymis) are measured to arrive at diagnostic findings. Neutral _-glucosidase has been shown to be more specific and sensitive for epididymal disorders than other two markers [17]. There are two isoforms of _-glucosidase in the seminal plasma: the major, neutral form (solely from the epididymis) and the minor, acidic form (mainly from the prostate). The amount of zinc, citric acid [27] or acid phosphatase [28] in semen gives a reliable measure of prostate gland secretion [29].

While offering a diagnosis, one must remember that although an infection can sometimes cause a decrease in the secretion of these markers, the total amount of markers present may still be within the normal range. Moreover, an infection can also cause irreversible damage to the secretory epithelium resulting in secretions remaining low even after treatment.

Since the total content of any accessory gland secretion in the ejaculate reflects the overall secretory function of that gland [30], this value is obtained by multiplying the accessory gland marker concentration by the total volume of the ejaculate.

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Key References

31. WHO laboratory manual 2010**.** See above [23] In-depth discussion and excellent background information related to various biochemical assays for semen quality assessment.
32. Kumar and Singh LP 2003**.** See above [5] Provides procedures for several assay for estimation of enzymes.