A new technique for the precise location of lactate and malate dehydrogenases in goat, boar and water buffalo spermatozoa using gel incubation film

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Summary. Gel incubation film, which contained gelatin to prevent the diffusion of enzyme during chemical reaction and phenazine methosulfate to operate as a hydrogen acceptor between NADH and tetrazolium, was used and light microscopy revealed that lactate dehydrogenase was located in the head and tail of the spermatozoa as well as in the midpiece, whereas malate dehydrogenase was confined to the midpiece in spermatozoa of the animals examined. In goat spermatozoa, lactate dehydrogenase was associated mainly with the inner acrosomal membrane in the head, the mitochondrial matrix in the midpiece and with flagellar fibrils in the tail, whereas malate dehydrogenase was present only in the mitochondrial matrix.

Keywords: gel film histochemical technique; spermatozoa; goat; boar; water buffalo; lactate dehydrogenase; malate dehydrogenase

Introduction

In mammals, energy for sperm motility depends on glycolysis and respiration. Biochemical analyses in spermatozoa of rabbit (Storey & Kayne, 1975), buffalo (Sidhu & Guraya, 1979), bull, boar and ram (Harrison & White, 1972) have shown that the enzymes of the glycolytic pathway and Krebs' cycle are mainly bound to the midpiece, but are partly distributed in other regions; lactate dehydrogenase (LDH, EC 1.1.1.27) and malate dehydrogenase (MDH, EC 1.1.1.37) are found in the head and tail fractions in spermatozoa of bulls (Mohri et al., 1965) and humans (Clausen, 1969). However, histochemical studies have indicated that LDH (Balogh & Cohen, 1964; Dokov & Kirov, 1971; Mathur, 1971; Sidhu & Guraya, 1979), MDH (Balogh & Cohen, 1964; Mathur, 1971; Sidhu & Guraya, 1979) and some other enzymes of the glycolytic pathway and Krebs' cycle (Edwards & Valentine, 1963; Balogh & Cohen, 1964; Ayyagari & Mukherjee, 1970; Dokov & Kirov, 1971; Mathur, 1971; Sidhu & Guraya, 1979) occur only in the midpiece in spermatozoa of various species. Histochemical study with electron microscopy verified the location of LDH in the mitochondrial matrix of bull spermatozoa (Baccetti et al., 1975).

In spermatozoa, close compacting of organelles presents problems in their separation. Thus, any biochemical approach for location of enzymes is complicated. Histochemical demonstration of LDH and MDH in spermatozoa has been performed in aqueous media using tetrazolium salt, which produces an insoluble coloured compound known as formazan at the site of enzyme activity. Under these circumstances, however, estimation of the location of these enzymes is unreliable, because of diffusion of enzymes from tissue to the incubation medium (Friede et al., 1963; Novikoff, 1963; Fahimi & Amarasingham, 1964; Kalina & Gahan, 1965) and interference by diaphorase (Farber et al., 1956).

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The present study assesses the precise location of LDH and MDH in spermatozoa of goat, boar and water buffalo, by a histochemical technique using gel incubation film (Lojda et al., 1979).

Materials and Methods

Spermatozoa

Goat spermatozoa were collected from cauda epididymides and from ejaculates of Japanese Native bucks (Capra hircus) during the breeding season. Boar spermatozoa were collected from cauda epididymides of Landrace boars (Sus scrofa). Water buffalo (Syncerus caffer) spermatozoa were obtained from ejaculates collected as a result of a conditioned reflex.

Preparation

The sperm samples of each species were washed twice with Ca-free Ringer solution (283 mosmol kg⁻¹ H₂O) by centrifugation at 355 g for 10 min. Smears of washed spermatozoa were prepared on glass slides, dried at room temperature for 5 min and subjected to histochemical evaluation.

Reagents

Nitro-blue tetrazolium (nitro-BT), dl-lactate sodium salt and phenazine methosulfate were purchased from Nakarai Chem. Ltd (Kyoto, Japan), L-malate sodium salt from Wako Chem. Ltd (Osaka, Japan), gelatin from Difco Lab. (Michigan, USA) and nicotinamide adenine dinucleotide (NAD) from Sigma Chem. Co. (Michigan, USA).

Histochemical procedure

Light microscopy. The incubation media for LDH and MDH were prepared according to Lojda et al. (1979). After incubation, the aqueous and gel film media were removed with distilled water, and slides were mounted in glycerol jelly.

Aqueous incubation media. The media were prepared to demonstrate the imprecise location due to NADH-diaphorase interference and enzyme diffusion. The final concentrations of the constituents in the assay were 25 mmol phosphate buffer 1⁻¹ (pH 7-4), 0·1% nitro-BT, 0·0065% potassium cyanide, 0·1% magnesium chloride, 100 mmol substrate 1⁻¹ (dl-lactate and L-malate sodium salt) and 1 mg NAD ml⁻¹. The media were adjusted to pH 7-2 and filtered. Spermatozoa were incubated in aqueous media at 37°C for 30 min.

Gel incubation film media. The media, prepared to demonstrate the precise location of the enzymes, contained 6% gelatin and 0·05 mg phenazine methosulfate ml⁻¹ in the aqueous incubation media. Gelatin was added to prevent the diffusion of enzymes during incubation and phenazine methosulfate was added to operate as a hydrogen acceptor. The media were adjusted to pH 7-2, filtered, poured into Petri dishes to give a layer about 3 mm thick and congealed at 4°C for 2 min. The gel films were cut into pieces about 2 × 4 cm, placed upon the slides and incubated at room temperature for 1 h in dark.

Electron microscopy. Washed epididymal spermatozoa from goats were used for electron microscope studies of histochemistry. The media for LDH and MDH were prepared according to Wenzel & Behrisch (1971) and contained gelatin to prevent the diffusion of enzymes during incubation. The final concentrations of the constituents in the assay were 20 mmol Sörensen phosphate buffer 1⁻¹ (pH 7-6), 300 mmol sodium potassium tartrate 1⁻¹, 21 mmol cupric sulfate 1⁻¹, 10 mmol substrate 1⁻¹ (dl-lactate and L-malate sodium salt), 1·5 mmol potassium ferricyanide 1⁻¹, 2·5 mg NAD ml⁻¹, 0·01 mg phenazine methosulfate ml⁻¹ and 6% gelatin. The media were adjusted to pH 7.0 and filtered.

The sperm samples from goat were embedded in the gelatin medium and incubated at room temperature for 1 h in the dark. After incubation, the gels were removed by careful washing with warm phosphate-buffered saline (PBS, 10 mmol l⁻¹, pH 7-4) and the sperm samples were washed for 1 h in several changes of ice-cold PBS. The samples were then fixed for 1 h with ice-cold 2·5% glutaraldehyde in PBS and then fixed with ice-cold 1% osmium tetroxide in PBS for 1 h. After washing in ice-cold PBS, the samples were dehydrated in graded acetone and embedded in epoxy resin. Ultrathin sections were counterstained for 10 min with 4% uranyl acetate and examined in a JEM-100B electron microscope at 80 kv.

Control. To test specificity of the enzyme assay, medium lacking substrate or medium containing substrate, but lacking phenazine methosulfate, were used as controls.
Results

Light microscopy

Lactate dehydrogenase

**Incubation in aqueous media.** The reaction product formazan was observed only in the midpiece of goat (Fig. 1a), boar (Fig. 1b) and water buffalo spermatozoa (Fig. 1c).

Fig. 1. Location of lactate dehydrogenase in spermatozoa incubated in aqueous media, from (a) goat cauda epididymides; (b) boar cauda epididymides and (c) water buffalo ejaculate. Formazan deposits are present in the midpiece, but absent in the head and tail; × 1400.

**Incubation in gel film media.** In gel media, formazan deposits were located not only in the midpiece, but also in the head and tail of spermatozoa in the three species (Fig. 2a–d). Formazan deposits were also found in the equatorial segment of boar spermatozoa (Fig. 2c). Some spermatozoa had damaged acrosomes or damaged membranes (Fig. 2b–d), which might have been caused during preparation.

Malate dehydrogenase

**Incubation in aqueous media.** In goats, formazan occurred only in the midpiece of the spermatozoa (Fig. 3a). The same staining pattern was observed in the other species.

**Incubation in gel film media.** In spermatozoa of all the animals examined, the deposition of precipitate was comparable to that obtained with aqueous media. Deposits occurred only in the midpiece of spermatozoa of boars (Fig. 3b) and water buffaloes (Fig. 3c), showing a distribution similar to that in goats.

Control

No reaction occurred in spermatozoa incubated in aqueous or gel media lacking the substrate (Fig. 3d). In spermatozoa incubated in the gel media containing the substrate, but lacking phenazine methosulfate, deposits occurred in the midpiece.

Electron microscopy

Subcellular location of lactate and malate dehydrogenases was examined in goat spermatozoa using gel media.
Fig. 2. Location of lactate dehydrogenase in spermatozoa incubated in gel media from (a) goat cauda epididymides; (b) goat ejaculate; (c) boar cauda epididymides and (d) water buffalo ejaculate. Formazan deposits are present not only in the midpiece, but also in the head and tail (arrows); and in the equatorial segment in (c); ×1400.

**Lactate dehydrogenase**

**Head.** The electron-dense reaction product copper ferrocyanide was mainly associated with the inner acrosomal membrane (Fig. 4a) and partly with the plasma membrane covering the acrosomal and post-acrosomal regions.

**Midpiece.** The electron-dense deposits were seen in the mitochondrial matrix (Fig. 4b), but not on the plasma membrane covering the mitochondrial sheath.

**Tail.** Deposits were associated with the fibrillar components (Fig. 4c).

**Malate dehydrogenase**

Reaction product was confined to the midpiece in goat spermatozoa, in the mitochondrial matrix (Fig. 4d).
Fig. 3. Localization of malate dehydrogenase in spermatozoa from (a) goat cauda epididymides incubated in aqueous media (formazan deposits are present in the midpiece, but not in the head or tail); (b) boar cauda epididymides and (c) water buffalo ejaculate incubated in gel media (deposits are present in the midpiece, but not in the head or tail); and (d) water buffalo ejaculate incubated in gel media, but lacking the substrate (formazan deposits are absent); × 1400.

Control

Incubation in media lacking the substrate gave negative results. In incubation in a medium containing substrate, but lacking phenazine methosulfate, deposits were seen in the mitochondrial matrix.

Discussion

Incubation in aqueous media showed that lactate and malate dehydrogenases were located only in the midpiece of spermatozoa in the three species studied. These findings correspond well with previous reports (Balogh & Cohen, 1964; Dokov & Kirov, 1971; Mathur, 1971; Baccetti et al., 1975; Sidhu & Guraya, 1979) in mouse, rat, cat, dog, guinea-pig, boar, bull, buffalo and human spermatozoa, all of which were performed in aqueous incubation media. In dehydrogenase histochemistry using aqueous media, however, there are technical problems: (i) coenzyme(NAD)-linked
Fig. 4. Ultrathin sections through spermatozoa from goat cauda epididymides incubated in gel media with lactate dehydrogenase: (a) sperm head (copper ferrocyanide deposits are mainly associated with the inner acrosomal membrane (arrows) and partly with the plasma membrane, \( \times 43\,000 \)); (b) midpiece (deposits are visible in the mitochondrial matrix, \( \times 38\,000 \)); and (c) tail (deposits are demonstrated in flagellar fibrils, \( \times 58\,000 \)). (d) Ultrathin section through the head and midpiece of spermatozoa from goat cauda epididymides incubated in gel media with malate dehydrogenase, deposits are present only in the midpiece; \( \times 27\,000 \).
dehydrogenases such as LDH and MDH may diffuse from tissue into incubation media (Friede et al., 1963; Novikoff, 1963; Fahimi & Amarasingham, 1964; Kalina & Gahan, 1965) and (ii) the tetrazolium salt is not directly reduced by the NAD-linked dehydrogenase, but by the diaphorase (NAD-specific reductase) and produces insoluble coloured formazan at the site of diaphorase (Farber et al., 1956). From the staining reaction obtained for LDH and MDH after incubation in aqueous media in this and other studies, it is therefore not clear whether the absence of staining in the sperm head and tail reflects the absence of LDH and MDH or is a result of the diffusion of these enzymes or the absence of the diaphorase rather than the absence of LDH and MDH. These facts seem to account for the false or imprecise evaluation of the location of LDH and MDH.

Enzyme diffusion is overcome by mixing up the aqueous media with a tissue stabilizer, such as gelatin (Fahimi & Amarasingham, 1964; Khan et al., 1971), agarosa (Sigel & Pette, 1969) or polyvinyl alcohol (Kalina & Gahan, 1965; Altman, 1971). The effect of diaphorase can be minimized by using an electron carrier such as phenazine methosulphate (Wijhe et al., 1963), which transfers electrons from reduced NAD to the tetrazolium salt, independently of diaphorase. Hence, we believe that the precise location of LDH and MDH in spermatozoa will become certain when the viscosity of the incubation medium is increased and phenazine methosulphate used.

By using gel incubation film that contained gelatin to prevent the diffusion of enzymes during incubation and phenazine methosulphate to operate as a hydrogen acceptor, as reported by Lojda et al. (1979), we found that the staining reaction of MDH took place only in the midpiece in goat, boar and water buffalo spermatozoa, but that staining of LDH occurred in the head and tail as well as the midpiece. When compared with the staining in aqueous media, these results showed a marked difference in distribution of LDH. These findings provide a clear explanation for observation from the aqueous incubation media: the absence of staining in sperm head and tail obtained for LDH after incubation in aqueous media was not due to the absence of LDH, but occurred as a result of the absence of the diaphorase and/or of diffusion of LDH, while the result for MDH was due to the absence of MDH. Thus, we conclude that MDH is confined to the midpiece, whereas LDH is located not only in the midpiece, but also in the head and tail in goat, boar and water buffalo spermatozoa. This conclusion is emphasized by subcellular electron microscopy in goat spermatozoa: LDH was associated with the inner acrosomal membrane in the head, the mitochondrial matrix in the midpiece, and the flagellar fibrils in the tail; and MDH was associated with the mitochondrial matrix in the midpiece.

From the biochemical viewpoint, LDH and MDH in mammalian spermatozoa seem to be distributed in the midpiece. Storey & Kayne (1975) reported that LDH in rabbit spermatozoa is bound to the cell structure and resists removal by washing after hypotonic treatment, which does not affect the mitochondrial membrane, but ruptures the plasma and acrosomal membranes. Sidhu & Guraya (1979) concluded that LDH and MDH in buffalo spermatozoa are bound to the midpiece, because these enzymes were not readily extracted by sonication and were present histochimically in the midpiece. Harrison & White (1972) also described the association of LDH with mitochondria in bull, boar and ram spermatozoa. Unfortunately, such biochemical analyses were not done to distinguish between the midpiece and other parts of spermatozoa. Masaki & Hartree (1962) pointed out that metabolic activity is found throughout the cell in bull spermatozoa. In contrast, the results obtained by fractionation of bull (Mohri et al., 1965) and human (Clausen, 1969) spermatozoa have shown a similar finding to the present study: LDH is distributed not only in the midpiece fraction, but also in the head and tail fractions. However, our results for MDH do not agree with those of Mohri et al. (1965) for fractionated bull spermatozoa, showing that MDH is enriched in the midpiece and tail fractions. The location of MDH in goat, boar and water buffalo spermatozoa probably differs from that in bull spermatozoa.

It is not clear why LDH is located in the head and tail in goat, boar and water buffalo spermatozoa. LDH and MDH play a role in cellular glycolysis and respiration, respectively. The location of LDH, MDH and the cytochrome system (Mann & Lutwak-Mann, 1981) in the midpiece can be understood in relation to metabolic events. Clausen (1969) showed that LDH in human
spermatozoa consisted mainly of LDH-X (LDH isoenzyme) and that LDH-X was detected throughout the cell. Our findings for LDH seem to be in line with these studies on LDH-X. Ultrastructural observations by Talbot & Chacon (1982) on the entry of spermatozoa into mammalian oocytes suggest that the sperm inner acrosomal membrane exposed as a result of acrosome reaction serves in the initial binding of the sperm head to the oocyte membrane before gamete membrane fusion. A series of experimental studies on LDH-X has indicated that immunization of female rabbits (Goldberg, 1973) and mice (Lerum & Goldberg, 1974) with LDH-X results in a highly significant reduction in incidence of pregnancy. We have provided direct evidence that LDH in the head of goat spermatozoa was associated with the inner acrosomal membrane. LDH located in the sperm head of the three species studied may therefore relate to the binding of spermatozoa to the oocyte during fertilization.

Gel incubating film was used to assess the precise location of LDH and MDH in spermatozoa of goat, boar and water buffalo. We provide evidence that MDH is confined to the midpiece, whereas LDH occurs not only in the midpiece, but also in the head and tail. The staining method would also be valuable in studying the in situ activity of these enzymes in spermatozoa under experimental conditions.

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References


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between the heads and tails of bull spermatozoa.


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