Microscopic analysis of MTT stained boar sperm cells

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Abstract

The ability of sperm cells to develop colored formazan by reduction of MTT was used earlier to develop a spectrophotometric assay to determine the viability of sperm cells for several mammalian species. It was the objective of the present study to visualize microscopically the location of the formazan in boar sperm cells. The MTT staining process of boar sperm cells can be divided into a series of morphological events. Incubation of the sperm cells in the presence of MTT resulted after a few min in a diffuse staining of the midpiece of the sperm cells. Upon further incubation the staining of the midpiece became more intense, and gradually the formation of packed formazan granules became more visible. At the same time, a small formazan stained granule appeared medially on the sperm head, which increased in size during further incubation. After incubation for about 1 h the midpiece granules were intensely stained and more clearly distinct as granules, while aggregation of sperm cells occurred. Around 90% of the sperm cells showed these staining events. At the end of the staining the formazan granules have disappeared from both the sperm cells and medium, whereas formazan crystals appeared as thin crystal threads, that became heavily aggregated in the incubation medium. It was concluded that formazan is taken up by lipid droplets in the cytoplasm. Further, the use of the MTT assay to test for sperm viability should be regarded as a qualitative assay, whereas its practical use at artificial insemination (AI) Stations is limited.

Keywords: Boar, MTT staining, Sperm, Viability.

Introduction

The histochemical stain 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide is widely used for assessment of cytotoxicity, cell viability, and proliferation studies in cell biology (van Meerloo *et al*., 2011; Stockert *et al*., 2012). The stain is abbreviated as MTT after methyl-thiazolyl-tetrazolium (Mosmann, 1983). When dissolved in water MTT gives a yellowish solution. MTT is readily taken up by cells, most likely by endocytosis (Liu *et al*., 1997). The yellow MTT is turned into a water-insoluble purple colored formazan dye upon reduction by dehydrogenases and reducing agents that are present in metabolically active cells.

A spectrophotometric assay of cell viability has been developed based on extraction of the lipid soluble formazan in an organic solvent and determination of the optical density at a specific wavelength (Denizot and Lang, 1986; Carmichael *et al*., 1987). The assay is widely used for many cell types, including sperm cells. Byun *et al*. (2008) developed a MTT assay for boar sperm and concluded that the test is an effective and simple method to validate sperm viability. A similar MTT assay has been developed for testing the viability of sperm of fowls (Hazary *et al*., 2001), humans (Nasr-Esfahani *et al*., 2002), stallions (Aziz *et al*., 2005), bovine (Aziz, 2006; Al Naib *et al*., 2011), and buffalo (Iqbal *et al*., 2009).

Several authors reported that MTT is reduced by the activity of the mitochondrial enzyme succinate dehydrogenase (Slater *et al*., 1963; Hazary *et al*., 2001;

Aziz *et al*., 2005; Aziz, 2006). Consequently, they assumed that the site of formation and precipitation of the formazan dye were the mitochondria. The amount of formazan formed would then be a measure for the number and activity of the mitochondria. However, recent studies indicated that MTT is mainly reduced in the cytoplasm (Berridge and Tan, 1993; Liu *et al*., 1997; Berridge *et al*., 2005; Diaz *et al*., 2007; Stockert *et al*., 2012). This would then mean that the MTT assay is an indirect method to test for mitochondrial activity. According to these new insights the main source of reducing power in the cell would be the reduced nicotinamide adenine dinucleotide coenzyme (NAD) in addition to many other reducing intracellular compounds such as ascorbic acid, dihydrolipoic acid, cysteine, tocopherols and glutathione (Winterbourn and Hampton, 2008; Stockert *et al*., 2012). These recent insights are derived from microscopic studies of mouse fibroblasts (Diaz *et al*., 2007) and HeLa cells (Stockert *et al*., 2012). But despite the development of MTT assays for sperm cells of several species, studies towards the precise location of the MTT formazan precipitates in sperm cells have not been done so far. Therefore, the present study was undertaken to investigate the MTT staining process in boar sperm cells by direct microscopic observation.

Materials and Methods

Boar semen samples

Semen samples from 6 different boars were from the AI Stations of Varkens KI Service (Punthorst,

The Netherlands) and Mobiel KI (Dedemsvaart, The Netherlands). Semen was collected and processed as described by van den Berg *et al*. (2014). The semen diluents used were based on the Beltsville thawing solution (BTS) preservation medium developed by Johnson *et al*. (2000). BTS is composed of 37.0 g glucose, 1.25 g EDTA, 6.0 g sodium citrate, 1.25 g sodium bicarbonate and 0.75 g potassium chloride per L. Diluted semen samples at a concentration of about 30×10^6 cells per ml were stored at 17°C until use. For MTT staining, the sperm samples were further diluted to a concentration of about 15×10^6 cells per ml. Concentrations were determined using the CEROS II computer assisted sperm analysis system from Hamilton Thorne (Beverly, MD, USA). All semen samples were analyzed within a day after collection. Only semen samples with a minimal sperm cell motility of 70% were used. Sperm cell motility was assessed using the CEROS II.

MTT staining
The MTT stock solution contained 1 mg/ml MTT in BTS and was filtered through a 0.22 µm Millipore filter. Aliquots of 1 ml were stored at -20°C. MTT and BTS were purchased from Sinus Biochemistry and Electrophoresis (Heidelberg, Germany). For sperm cell staining, 100 µL of the MTT stock solution was added to 1 ml diluted semen in 1.5 ml polypropylene reaction vials. The concentration of sperm cells was adjusted to about 15×10^6 cells per ml. The reaction vials were put in a heat block that was set at 37 °C. The reaction vials were left open to allow for oxidative phosphorylation. For microscopic analysis, samples were taken from the reaction vials at about 2 min time interval during the first 10 min, at about 10 min time interval during the first hour, and about 30 min time interval during the 3 hours thereafter. A final microscopic analysis was done about 24 hours after the start of the staining from samples taken from the reaction vials as well as of the slides from earlier samplings.

Microscopic analysis
For microscopic analysis 5 µL stained cell samples were taken from the incubation medium, pipetted onto microscope slides, and covered with cover glass at room temperature. Microscopic observations and photomicrography were performed using a Nikon Eclipse 50i microscope equipped with phase contrast using a 40x objective (Nikon Instruments Europe, Badhoevedorp, The Netherlands). Digital images were obtained using an Euromex DC5000 digital camera (Euromex, Arnhem, The Netherlands) attached to the binocular tube with a C-mount. The camera has a 1/2.5" CMOS sensor with maximal output resolution of 2592×1944 pixels and pixel size of 2.2 × 2.2 µm. From each MTT staining sample, images of at least 100 sperm cells were captured using the software program ImageFocus (from Euromex, Arnhem, The Netherlands)

and stored for further analysis. For determination of the number of normal and aberrant stained sperm cells 3 ejaculates of each of 3 different boars were analyzed in duplicate by studying at least 200 images per sample after about 1 h staining. Statistical analysis was done using GraphPad Instat (GraphPad Software, San Diego, California, USA) for calculation of standard deviations and significance of differences. Means were considered significantly different with a p-value < 0.05. The data were subjected to pair-wise comparison based on the Tukey method in conjunction with ANOVA to compare the differences.

Results
Sperm cell staining
The first step in the reduction of MTT starts immediately after incubation of the boar sperm cells at 37°C in the presence of MTT. This could be seen from the purple coloration of the incubation medium in the reaction vials. Microscopic analysis revealed already after a few min. of incubation a light purple coloration of the midpiece of the sperm cells. This was a diffuse and even coloration of the midpiece not showing any particulate shape. During longer incubation the color became more intense, but the midpiece was still evenly stained (Fig. 1a). After about 15 min a small purple colored granule medially located on the sperm head could be observed (Fig. 1b). In most cases the location of the sperm head granule was near the ridge of the acrosomal cap. The size of the sperm head granule increased during further incubation (Fig. 1c) from about 0.1 µm to about 1 µm. The increase in size reaches a maximum at about 1 to 2 h of staining, while the multiple granules became
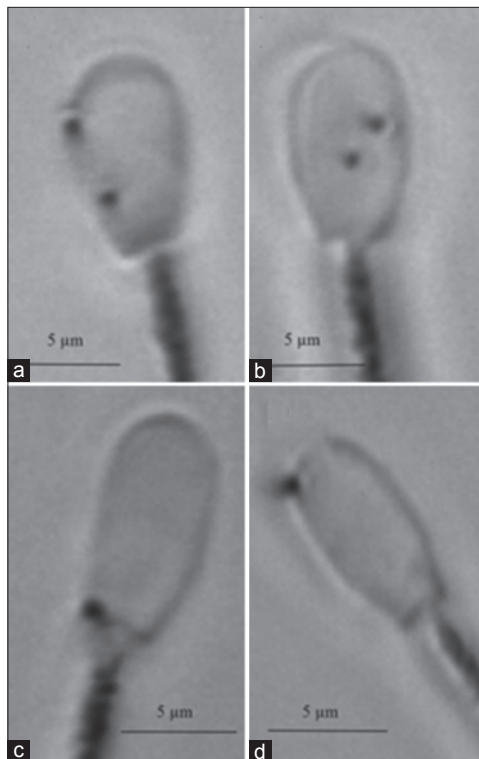

Fig. 1. Microscopic images of MTT stained boar sperm cells. The images show the results of staining during the first 30 min; [(a): at about 5 min; (b): at about 15 min and (c): at about 30 min staining] of incubation of sperm cells at 37°C in the presence of MTT. Note the increase in intensity of the midpiece during the staining process and the appearance of the sperm head granule.

more distinct in the midpiece. Also between 1 and 2 h staining the head granule grew to its maximum size of about 1 µm.

The observations as depicted in Figure 1 were seen for most of the sperm cells of all boar semen samples analyzed. However, in some cases the midpiece remained unstained or lightly stained. Further, in some rare cases two head granules were observed (Fig. 2a and b). Finally, in some cases the sperm head granule appeared near the neck (Fig. 2c) or near the top (Fig. 2d) of the head. These different staining types were considered as aberrant types.

To assess the percentage of normal and aberrant stained cells, 3 ejaculates from each of 3 different boars were analyzed. The percentage of sperm cells with normal stained midpiece and the head granule located median for the 3 different boars was 91.3 ± 4.0, 89.2 ± 3.1 and 85.2 ± 4.2. There was no statistical difference between the results of the analysis for the 3 boars ($P=0.32$). During 2-3 h of incubation the granules of the midpiece became more pronounced and in some cases the granules seemed to be loosely bound to the midpiece or even detached from the sperm cell (Fig. 3). The formation of granules became very clear after 3 h
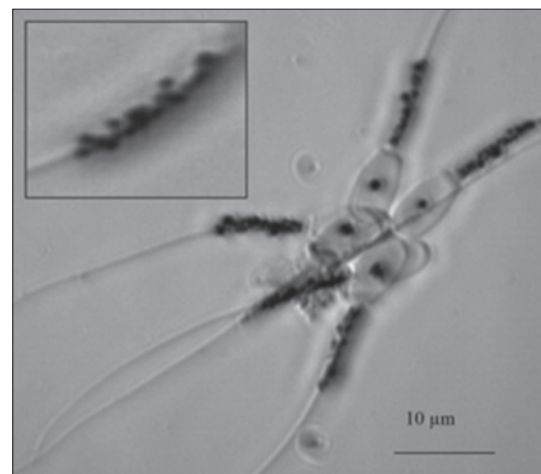
and longer incubation times (Fig. 4a). Granules could then be found not only attached to the midpiece but also in the staining medium and attached to the end piece of the sperm cell.

The formation of formazan crystals could be observed from samples taken after 1 h incubation that were left on the microscope slide for 6 h at room temperature. Crystal formazan threads bound to sperm cells could be observed, while granules had disappeared (Fig. 4b). When the samples remained incubated in the reaction vials for 6 h, similar crystal formation occurred, but due to the many formazan threads and the fluidity of the medium, the threads clogged together forming massive aggregates (Fig. 4c).

Discussion

An MTT assay for sperm cell viability has been developed for several species (Hazary *et al.*, 2001; Nasr-Esfahani *et al.*, 2002; Aziz *et al.*, 2005; Aziz, 2006; Byun *et al.*, 2008; Iqbal *et al.*, 2009; Al Naib *et al.*, 2011). It was concluded that the staining intensity is directly proportional to the mitochondrial activity and correlates with the number of sperm cells and their viability (Hazary *et al.*, 2001; Nasr-Esfahani *et al.*, 2002; Aziz *et al.*, 2005; Aziz, 2006; Byun *et al.*, 2008; Iqbal *et al.*, 2009). However, these studies lack extensive microscopic analysis of MTT stained sperm cells. Here, microscopic analysis of MTT stained boar sperm cells is reported to further study the validity of the MTT assay for sperm cell viability.

The microscopic analysis showed that about 90% of the sperm cells of each sample from 6 different boars followed a staining process that can be divided into several events: diffuse staining of the midpiece followed by more intense staining and the appearance

Fig. 2. Microscopic images of MTT stained boar sperm cells. The images show the results of staining after about 30 min to 1 h incubation of sperm cells at 37°C in the presence of MTT. The different images show sperm heads with 2 granules (a and b) and the aberrant position of the head granule near the neck (c) and the top (d) of the sperm head.

Fig. 3. Microscopic images of MTT stained boar sperm cells. The images show the staining after about 2 h incubation of sperm cells at 37°C in the presence of MTT. Note the aggregation of the sperm cells and the formation of granules, especially on the insert image.
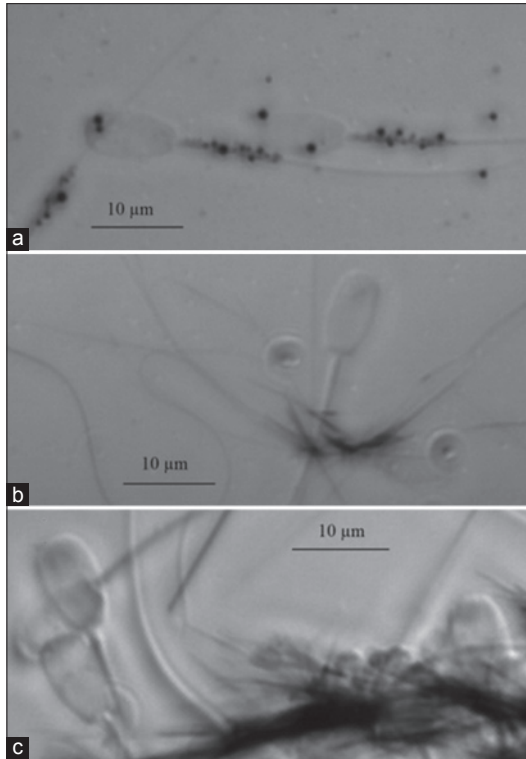
Fig. 4. Microscopic images of MTT stained boar sperm cells. (a) Image showing the formation of granules attached to the sperm head, midpiece and end piece, and granules free from the sperm cells in the medium after about 3 h incubation. (b) Image showing the formation of MTT crystals after about 3 h incubation of boar sperm cells at 37°C and 20 h incubation at room temperature between slide and cover slip. Note the absence of granules. (c) Image showing the heavy formation of MTT crystals after about 24 h incubation of boar sperm cells at 37°C in the presence of MTT.

of formazan granules in the entire midpiece, appearance of a small granule medially on the sperm head that increases in size during further incubation, and finally disappearance of all the formazan granules and formation of formazan crystals. The sperm cells that did not follow the description of the given staining process (about 10%) were considered aberrant types. Whether these aberrant types reflect sperm cells with aberrant functioning or not remains to be determined. To our knowledge this formazan stained head granule is reported for the first time.

From the staining of the midpiece it cannot be concluded whether the formazan is located in the cytoplasm or the mitochondria, since the mitochondria are located in the midpiece arranged in a helical pattern around the longitudinal fibrous elements of the tail (Bakst and Howarth, 1975; Thurston and Hess, 1987). This structure makes a detailed visualization of the location of the staining impossible using light microscopy. However, as the description of the staining

events as described here is strikingly similar to those reported by others, it can be concluded that we might deal here with similar cellular location of the MTT reduction. The formation of formazan stained granules, their disappearance and the concomitant formazan crystal formation has been described for other cell types (Nikkhah *et al*., 1992; Shearman *et al*., 1995; Liu *et al*., 1997; Molinari *et al*., 2005; Diaz *et al*., 2007; Stockert *et al*., 2012). The disappearance of the granules from the cells is likely caused by a process called exocytosis (Liu *et al*., 1997; Molinari *et al*., 2005). Further, it is reported that the MTT staining occurs outside the mitochondria but in the cytoplasm by the NADH coenzyme (Liu *et al*., 1997; Diaz *et al*., 2007; Stockert *et al*., 2012). Diaz *et al*. (2007) suggested, based on literature and the result of different staining techniques, that formazan is located in lipid droplets. This was later confirmed by Stockert *et al*. (2012) using direct microscopic observation and colocalisation analysis with fluorescent probes. Thus, the similarity of the staining process as described by Diaz *et al*. (2007) and Stockert *et al*. (2012) with the staining process of boar sperm cells may indicate that we deal here with similar molecular events, namely uptake of MTT in the sperm cell, followed by reduction to formazan in the cell plasma of the main piece, and uptake of the formazan in lipid droplets leading to the granule formation.

It is not known whether sperm cells contain lipid droplets, but boar sperm cells are able to synthesize lipids that can be used as energy source, while different pools of 1,2-diacylglycerols were identified (Vazquez and Roldan, 1997; Santoro *et al*., 2013). Further, recent proteome studies indicated that peroxisomal and mitochondrial lipid metabolism might be more active than generally assumed (Amaral *et al*., 2013a). Thus, endogenous pools of lipids might possibly serve as a way to store formazan and may fit well with the hypothesis of Diaz *et al*. (2007).

The appearance of the MTT stained sperm head granule seems not to be an artefact. Further, it cannot be explained in a similar way as the granule formation in the midpiece, since the occurrence of cell plasma and production of reducing NADH by glycolysis has never been reported for the sperm head. But the staining events for the head granule are similar to those for the midpiece granules. The specific position of the head granule near the edge of the acrosomal cap may suggest a structure in the sperm head with reducing capacity that may be related to the acrosomal reaction. The stained head granule, as well as the staining process as described above, was also observed for sperm cells of stallion and rabbit sperm cells (B.M. van den Berg, unpublished observations).

As outlined above, reduction of MTT in the midpiece is assumed to be caused mainly by the action of the NADH coenzyme, the latter being the product of glycolysis.

Glycolysis and respiration in the sperm cells has been the object of many studies (Storey, 2008). Until recently the issue of sperm energy metabolism related to motility showed many conflicting reports (Ramalho-Santos *et al*., 2009). But recent evidence indicates that glycolysis may be the main provider of energy for motility (Mukai and Okuno, 2010; Amaral *et al*., 2013b; Takei *et al*., 2014) and that mitochondrial activity is related to other metabolic functions of the sperm cell, among others the production of reactive oxygen species (Aitken *et al*., 2012; Amaral *et al*., 2013b). This indicates that the product of glycolysis in the sperm cytoplasm is the major reducing agent responsible for MTT reduction. Consequently, the MTT assay for viability of sperm cells should be regarded as a qualitative measure of glycolytic activity. The MTT assay for sperm has been claimed to be a reliable test method and practical for routine evaluation of semen (Hazary *et al*., 2001; Aziz *et al*., 2005; Aziz, 2006), but the qualitative character makes the method unreliable. Therefore the method cannot be used by AI Stations to determine whether a sperm sample should be accepted or rejected for commercial purposes. Only in cases when one may choose between different semen samples for AI, a quick visual assay would perhaps be of value. Thus, for routine semen inspection, the value of the assay seems to be restricted to comparative analysis of semen samples.

Acknowledgements

Conflict of interest

The author declares that there is no conflict of interest.

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