A Comparative Study on the Diagnostic Sensitivity of Rodent Sperm and Embryos in the Detection of Endotoxin in Earle's Balanced Salt Solution

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Purpose: *The bioassay used by most IVF units to assess culture media is the mouse embryo test. The limitations of this assay are well known. The objective of this study was therefore to evaluate the in vitro bioassay potential of rodent sperm, in terms of relative sensitivities to endotoxins, and to compare the results with the routine mouse embryo and human sperm tests.*
Results: *The greater sensitivity of rodent sperm than of mouse embryos was evident in this study. A further advantage in using the mouse sperm test was the time (4–6 hr) in which endotoxins could be detected.*
Conclusion: *This rapid sperm test proved to be inexpensive, convenient, and invaluable for detecting potential sources of cytotoxicity.*

KEY WORDS: bioassay; endotoxins; mouse embryo test; murine sperm test.

INTRODUCTION

The maintenance of optimal conditions for the development of human embryos is fundamental to achieving desirable in vitro fertilization (IVF) results. An objective quality control strategy is therefore imperative in assessing the suitability of culture media. The bioassay used by most IVF units around the world is the mouse embryo assay (1–4). This assay assess the ability of culture media to support the development of mouse embryos to the blastocyst stage. Investigators using this assay conclude that conditions supporting mouse embryo development will also permit human embryo development.

A variety of factors may be monitored by the mouse embryo test including the influence of endotoxins. Bacterial endotoxins have been implicated in increased fragmentation of early cleavage-stage human embryos and decreased pregnancy rates in IVF programs (5). George *et al.* (6) suggested that the mouse embryo bioassay is a crude and insensitive assay that cannot differentiate low toxicity rates. The limitations of this assay are its relative insensitivity, its high cost, and the fact that it takes several days to perform. Preimplantation murine embryos are more resistant than human embryos to bacterial endotoxins. It is therefore possible that mouse embryos will develop under conditions that might retard or inhibit human embryo development (7,8).

Some laboratories formerly made use of human sperm viability assays for testing IVF conditions (9). Unfortunately, human spermatozoa are even more resistant to adverse conditions than mouse embryos. Subsequently, hamster sperm viability assays (HSVA) have recently been devised and show a greater sensitivity to embryotoxic factors and are simpler to use, more defined, less time-consuming, and more economical than the mouse embryo bioassay (10–13).

Being a private unit, we do not house animal breeding facilities. This compels us to obtain laboratory animals from animal breeding centers providing SPF (specific pathogen-free) hamsters. Unfortunately male SPF hamsters are slaughtered due to

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a lack of demand for these animals in research programs in South Africa. They are therefore kept in minimal supply, with the sole purpose of breeding female species for oocyte retrieval. As a result of this and the greater availability of SPF mice, the use of mouse sperm as an alternative to hamster sperm was also investigated.

The purpose of this study was to compare the bioassay potential of mouse and hamster spermatozoa in detecting small amounts of endotoxins in a simplified culture medium, namely, Earle's balanced salt solution (EBSS). Furthermore, we tried to point out the insensitivity of the mouse embryo test compared to the mouse sperm test.

Several authors (10,12) have used stimulants to enhance sperm hyperactivation. We, however, cultured the spermatozoa in EBSS with 10% serum. The addition of protein or other amino acids to the medium may, however, absorb toxic substances and decrease the sensitivity of the bioassay. The trial was therefore duplicated in serum-free EBSS.

MATERIALS AND METHODS

Embryo Recovery

Three- to four-week-old F_1 (C57B1 × CBA) female mice were superovulated with an intraperitoneal injection of 5 IU Pergonal [human menopausal gonadotropins (−hMG); 75 IU luteinizing hormone (LH) and 75 IU follicle stimulating hormone (FSH)], followed by 5 IU Profasi (human chorionic gonadotropins; hCG) 48 hr later. Females were then paired and mating was confirmed by the presence of a vaginal plug. These females were killed 52–58 hr after the injection of hCG. Their oviducts were removed and placed in equilibrated EBSS (24 hr in 5% CO_2 at 37°C). The oviducts were flushed with EBSS and the embryos (two to four cells) were equally divided into five groups and incubated in Nunc four-well dishes at 37°C and 5% CO_2. The first group served as the control and was kept in pure EBSS.

Endotoxins (*Pseudomonas aeruginosa*) were added at an increasing concentration in the four remaining groups (group 2, 0.1 ng/L; group 3, 1.0 ng/L; group 4, 5.0 ng/L; group 5, 10.0 ng/L). These concentrations were confirmed by the chromogenic *Limulus* amebocyte lysate (LAL) test (E-Toxate, Sigma). This trial was duplicated in 10% serum-enriched medium.

The embryos were examined 72 hr later and the percentage blastocyst formation was calculated. Fifty-eight hours after the first examination (130 hr after flushing the embryos) the development and morphological appearance of the embryos were recorded.

Sperm Preparation

Spermatozoa were obtained from the cauda epididymis of adult male mice and 3- to 6-month-old hamsters. After killing the animals by cervical dislocation, the epididymis and a small part of the vas deferens were removed, placed in a Falcon culture dish containing EBSS, and taken to the sterile laboratory. The epididymis was rinsed to remove excessive blood. Using forceps the spermatozoa were forced via the vas deferens into the culture medium by pressing on the cauda epididymis. The sperm suspension was incubated for 10 min (37°C and 5% CO_2) to allow dispersing of sperm before any assessment was made. Where sperm clumping (20% or more) was still present after this period of incubation and the motility was lower than 60%, the sample was discarded. The spermatozoa were then diluted into 5 million sperm/ml, divided into five groups, and incubated in four-well Nunc trays. Each treatment was triplicated. These groups represent those of the murine embryos.

Sampling of the sperm suspension was performed at 2-hr intervals (2, 4, and 6 hr) after obtaining the murine sperm. The average proportion of motile spermatozoa and the average forward progression quality (FPQ) were assessed. The FPQ represents six groups with the following classifications:

0 immotile sperm;
1 twitching with no FPQ;
2 slow FPQ;
3 good purposeful FPQ;
4 rapid, good, purposeful, FPQ without hyperactivation; and
5 hyperactivation.

The average FPQ presents the motility across the total population of spermatozoa. Four to six visual fields per well were used for estimates. A sperm motility index (SMI) value was calculated for each well at each observation interval, where SMI = $(FPQ)^2$ × percentage motile cells (10). A mean SMI was calculated for each treatment from the triplicated wells. Data were analyzed by Student's *t* test.

RESULTS

We evaluated the potential of mouse and hamster spermatozoa to detect endotoxins in EBSS. Al-

though the study was not designed to evaluate the effects of protein in rodent sperm, the results do agree with previous observations (10) that the sensitivity of these bioassays is greatly increased when protein or other amino acids are excluded from the culture medium. The exclusion of protein from EBSS in the bioassay is recommended since it increases the sensitivity of the test and thereby decreases the completion time from 6 to 4 hr. The mean SMI values for EBSS achieved for each of the species at 2, 4, and 6 hr in the presence of serum are presented in Table 1. Table II presents the mean SMI values for each species at each endotoxin concentration where serum-free EBSS was used.

The SMI observed from the different species shows the hamster spermatozoa to be more sensitive to endotoxins and in vitro conditions (Tables I and II). These results may also reflect factors such as culture conditions or the ability of the medium to induce capacitation of the spermatozoa in the strain of hamster and mouse used.

The spermatozoa of both species were incubated prior to the assay to allow the cluster of sperm to disperse. At the first examination (2 hr) the sperm were moving more vigorously and showed a forward progression of 1–2 for the hamster and 2–3 for the mouse, respectively. Virtually all the clusters had broken up and the sperm of both species exhibited hyperactivation after the 4-hr incubation period. At this time motility scores often reached their peak for mouse spermatozoa and an average SMI of 318 was established for pure EBSS in the absence of serum (Table II). The more sensitive hamster control sperm reached their motility peak within 2 hr of incubation. From the results published in Table II a significant reduction in the SMI at 4–6 hr was noticed in the tests where endotoxin was present compared to the control samples. Through the endotoxin concentration range of 0.1–10 ng/ml, at the 2-hr as well as the 4-hr incubation period the mouse spermatozoa showed a significant decrease in SMI values. Although the SMI values of the hamster spermatozoa were lower than those of the mouse, the hamster SMI values decreased significantly only at the higher endotoxin concentrations of 5 and 10 ng/ml. After an incubation period of 6 hr, no motility was seen at any of the endotoxin concentrations.

Table I. Mean SMI Values for Hamster and Mouse Spermatozoa in EBSS with 10% Serum Supplementation for the Time Interval 0–6 hr

Endotoxin (ng/ml)	Mean SMI score			
	0 hr	2 hr	4 hr	6 hr
	(A) Hamster			
0.0	85	272	180	65*
0.1	85	142	146	0*
1.0	85	165	124	0*
5.0	85	177	140	0*
10.0	85	142	146	0*
	(B) Mouse			
0.0	135	469	594	375*
0.1	135	406	419	288
1.0	135	400	375	289
5.0	135	549	388	184*
10.0	135	450	350	170*

* $P < 0.05$.

Table II. Mean SMI Values of Hamster and Mouse Spermatozoa in EBSS (Without Serum) for the 0- to 6-hr Interval

Endotoxin (ng/ml)	Mean SMI score			
	0 hr	2 hr*	4 hr*	6 hr*
	(A) Hamster			
0.0	106	122	107	45
0.1	113	85	45	0
1.0	126	64	40	0
5.0	126	52	2	0
10.0	126	60	6	0
	(B) Mouse			
0.0	303	302	318	186
0.1	299	138	75	0
1.0	299	98	78	0
5.0	298	107	22	0
10.0	295	84	28	0

* $P < 0.05$.

The use of these rodent sperm has proved to be a sensitive bioassay for quality-control testing of EBSS. The bioassay is simple to perform and has the advantage that it can be completed in 4 to 6 hr.

It is customary to supplement medium with serum for successful culture of mouse embryos. To sensitize the assay we cultured the embryos in pure culture medium (EBSS). It was also suggested by Bavister and Andrews (10) that the inclusion of protein in the culture medium seemed to eliminate contamination problems. The blastocyst formation of the mouse embryos in serum-free medium did not exceed 30% in the control as well as the test group. There was no development to postblastocyst stages from the embryos cultured in serum-free medium. Alternatively, when optimizing the mouse embryo bioassay by incubating the embryos in serum-enriched medium, at least 80% of the embryos de-

Table III. Mouse Embryo Development Over a Period of 120 hr in the Presence of Endotoxin

Developmental rate	Pure EBSS		Serum-enriched EBSS	
	Control	Endotoxin	Control	Endotoxin
Blastocyst formation (%)	66.7	65.5	87	85
Hatching postimplantation (%)	45	38	82	80
Vacuolization (%)	N/D[a]	N/D[a]	20	75

[a] The embryo did not develop any further than the hatching stage.

veloped into blastocysts. No statistical differences could be found between blastocyst formation and embryo hatching in the control group (0.0 ng/ml endotoxins) and those cultured in 10 ng/ml endotoxins. The percentage vacuolization present in the endotoxin group at 120 hr reached statistical significance (Table III). In our experience the routine mouse test was shown to be sensitive only when embryos were cultured past the blastocyst stage and when attention was given to the percentage vacuolization.

Since the human sperm bioassay is routinely used as a quality-control test, we cultured human spermatozoa in the presence of 10 ng/ml endotoxins. This high dosage of endotoxins had no effect on the motility or forward progression of the spermatozoa after 48 hr compared to the control (0.0 ng/ml).

CONCLUSION

Bacterial endotoxins are derived from the outer membrane of Gram-negative bacteria and are macromolecular structures containing polysaccharide, phospholipid, and small quantities of protein. Decreased pregnancy rates in humans have been correlated with the presence of endotoxins in IVF medium (14).

Randall and Gantt (7) reported that the level of endotoxin required to decrease murine preimplantation development is significantly higher than that reported for human embryos and concluded that the mouse embryo test should not be used to verify the quality of culture medium for human IVF. The hamster sperm motility assay has been described previously (10–13) as a valuable component and a sensitive assay for quality control in an IVF laboratory. According to Bavister and Andrews (10) the reduction of the SMI at 4 or 6 hr indicated the presence of endotoxins. The sperm bioassay described in this study is capable of detecting even low endotoxin concentrations at as early as 4 hr.

Some authors have reported (10–13) higher SMI values than obtained in this study. The SMI values acquired in this study are unique to our laboratory conditions; other laboratories might obtain different minimum SMI values, related to their laboratory conditions, culture medium, supplements, and incubation methods. The lower SMI values in our studies probably reflect the absence of sperm motility stimulants from the culture medium; other contributing factors might also have been the lack of proximity between the sterile and the nonsterile working area and the nonuse of oil in the culturing technique. Although the hamster sperm assay is more sensitive, we prefer the mouse sperm assay because of the higher SMI values. These findings therefore suggested that the mouse sperm motility bioassay described in this report can be developed into an alternative very sensitive and inexpensive bioassay procedure which can be completed in 1 day but which demands a certain degree of expertise.

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