Human Reproduction vol.12 no.6 pp.1227–1229, 1997

Efficient modification of intracytoplasmic sperm injection technique for cases with total lack of sperm movement

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A rapid, simple and efficient method for selecting living spermatozoa for intracytoplasmic sperm injection (ICSI) in cases with total lack of sperm movement is described. The selection is based on a characteristic deformation of living spermatozoa exposed to hypo-osmotic conditions during short sequential exposures to modified culture medium and polyvinylpyrrolidone solution; the osmolarity of both of these solutions is reduced by one half by diluting them with an equal amount of water. The application of the sperm viability selection step in six ICSI treatment cycles with total absence of sperm movement resulted in a fertilization rate of 41.9% and the establishment of two ongoing clinical pregnancies. The method described for the selection of living spermatozoa makes it possible to reach acceptable fertilization rates and to obtain ongoing pregnancies by ICSI in cases with total lack of sperm movement. Because of its simplicity, this method can easily be improvised when the total lack of sperm movement is an unexpected finding made on the day of the planned ICSI.

Key words: hypo-osmotic swelling test/immotile spermatozoa/intracytoplasmic sperm injection/sperm viability testing

Introduction

Recent technical improvements of intracytoplasmic sperm injection (ICSI) have made it possible to fertilize almost 90% of injected oocytes and to obtain ongoing pregnancies in >50% of started treatment cycles (Tesarik and Sousa, 1995). However, these figures are obtained if motile spermatozoa can be selected for injection. If only immotile spermatozoa are available, the success rate of ICSI drops considerably, and no ongoing pregnancy has been reported in the literature after non-selective ICSI with totally immotile ejaculated spermatozoa (Nagy *et al.*, 1995; Kahraman *et al.*, 1996; Nijs *et al.*, 1996).

Sperm movement by itself is certainly not required for fertilization after ICSI because spermatozoa are immobilized mechanically before injection. In fact, sperm movement, even a very limited one, is the best sign of viability, making it possible to select living spermatozoa for injection even if they represent an extremely small subpopulation of the total sample. On the other hand, the lack of movement does not necessarily mean cell death, and some living spermatozoa are likely to be present even in samples with total lack of motility. Thus, the application of an appropriate sperm viability test can be expected to improve ICSI results in such cases.

From the large variety of sperm viability tests currently available, the hypo-osmotic swelling test (HOST) (Jeyendran *et al.*, 1984) is particularly interesting for application in ICSI for two reasons. First, it does not employ dyes or other chemical reagents that might bear a risk of altering spermatozoa. Second, outcome measures can be made with the same optical system as that used for ICSI. A successful application of the HOST in ICSI has been reported recently (Casper *et al.*, 1996).

Here we describe a simplified and rapid method, based on the same principles as the classical HOST, for selection of living spermatozoa for ICSI from samples with 100% immotile spermatozoa.

Materials and methods

This study deals with six ICSI treatment cycles with ejaculated spermatozoa in which no motile spermatozoa were available. The experimental protocol was realized at the Department of Medical Genetics, Faculty of Medicine, University of Oporto, Portugal, after approval by the institutional ethical authority and after having obtained informed consent from the patients. The methods of ovarian stimulation, gamete recovery and handling, and the basic ICSI procedure were the same as our standard ones (Tesarik and Sousa, 1995). Spermatozoa were treated with 1 mg/ml pentoxifylline (Sigma, St. Louis, MO, USA) during the sperm liquefaction period followed by centrifugation in a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient and washing in medium as described (Tesarik and Sousa, 1995). The use of pentoxifylline augments the chance of finding some slowly moving spermatozoa even in cases with zero motility in the original sample. Other advantages and possible disadvantages of the use of pentoxifylline in assisted reproduction have been discussed previously (Tesarik and Mendoza, 1993). However, this study only deals with cases in which all spermatozoa remained totally immotile even after the pentoxifylline treatment.

To select living spermatozoa among totally immotile ones, sperm samples were subjected to a simplified HOST that was carried out as follows. After the last centrifugation of the sperm washing procedure, the sperm pellet was resuspended in 0.5 ml sperm preparation medium (Medi-Cult, Copenhagen, Denmark) diluted 1:1 with embryo-tested water (Sigma). An aliquot of this suspension was immediately mixed with an equal volume of hypo-osmotic 5% polyvinylpyrrolidone (PVP) solution. The hypo-osmotic 5% PVP solution was prepared by mixing equal volumes of the commercially available 10% PVP in sperm preparation medium (Medi-Cult) and of embryo-tested water (Sigma). A microdrop of the final sperm suspension was then placed on a tissue-culture dish under mineral oil next to another microdrop containing the non-modified preparation of 10% PVP solution in

sperm preparation medium (Medi-Cult) and in the proximity of microdrops containing oocytes to be injected in sperm preparation medium. Spermatozoa showing one of the typical tail deformation patterns characterizing the reaction of living spermatozoa to the hypo-osmotic environment (Jeyendran *et al.*, 1984) were identified in the first microdrop. A swelling pattern characterized by the curvature involving the entire sperm tail was the most common reaction observed in this study (~60% of the reacting spermatozoa), but all the other deformation patterns described by Jeyendran *et al.* (1984) were also seen. No attempt was made to inject spermatozoa showing one particular pattern of deformation. Thus, ~60% of oocytes were injected with spermatozoa with the whole-tail curvature, whereas the remaining oocytes were injected indiscriminately with spermatozoa showing any of the other deformation patterns. Each selected spermatozoon was washed in the microdrop with 10% PVP in sperm preparation medium. During this step, the sperm tail was crushed by the microinjection needle against the dish bottom. Care was taken to crush the sperm tail even in cases where the access to the tail was hindered by its extreme deformation. Each spermatozoon was then aspirated, tail-first, into the microinjection needle and injected into an oocyte. The previously described vigorous aspiration technique of sperm injection (Tesarik and Sousa, 1995) was used in all cases. The sperm tail deformation produced by the previous exposure to hypo-osmotic conditions was elastic and did not represent a serious obstacle during the injection procedure.

In preliminary experiments, the percentage of viable spermatozoa in the total and viability-selected sperm populations from eight patients (not included in the present ICSI group) was checked by supravital eosine staining: 100 spermatozoa showing one of the typical tail deformation patterns was compared to 100 randomly chosen spermatozoa from the same sperm preparation. These data showed that 53.8 ± 6.2% (mean ± SEM) of viability-selected spermatozoa excluded the dye as compared to 1.2 ± 0.8% (mean ± SEM) in the total population.

Results

In all cases in which our technique for selection of living spermatozoa was used, there were at least some spermatozoa that reacted to the hypo-osmotic test environment by producing one of the tail deformation patterns characterizing living spermatozoa (Jeyendran *et al.*, 1984). However, the size of this subpopulation was always very small and did not exceed 5% of the total sperm population in any case involved in this study. Only spermatozoa judged to be alive according to their response to the hypo-osmotic environment were injected into oocytes. In view of the small number of positively reacting spermatozoa, no attempt was made to analyse the relative representation of the individual tail deformation patterns.

This method was applied in six ICSI treatment cycles to 43 oocytes. Of these, 40 survived injection, 18 of which (45%) were fertilized normally, as shown by formation of two pronuclei. If all injected oocytes were taken into account, the fertilization rate was 41.9%. Fourteen zygotes cleaved, and all of them were transferred. Two clinical pregnancies (one singleton and one twin) were achieved (Table I).

Discussion

These data suggest that ICSI results in cases with 100% immotile spermatozoa can be improved by selecting the spermatozoa for injection with the use of a viability test. The viability test described in this study is based on the same principles as the previously described HOST (Jeyendran *et al.*, 1984) but is substantially simplified and modified so as to allow the identification of living spermatozoa directly in the sperm suspension from which spermatozoa are aspirated into the microinjection needle. In comparison with another recent study using a similar approach (Casper *et al.*, 1996), our technique is simpler, as it only uses 1:1 dilution of commercially available solutions with embryo-tested water, and faster, as it makes use of an immediate sperm deformation in the hypo-osmotic medium and does not require an additional incubation period.

The fact that only 53.8% of the viability-selected spermatozoa excluded the supravital eosine stain may have different causes. Even the classical HOST (Jeyendran *et al.*, 1984) does not give 100% identical resuls with the supravital eosine staining method (Carreras *et al.*, 1992). The unusually high percentage of false viables observed in our study can be explained by a rapid loss of viability in spermatozoa that are exposed to the relatively aggressive hypo-osmotic condition in this HOST modification. If spermatozoa are dying only during the test, this does not necessarily decrease their fertilizing ability when they are subsequently used for ICSI. In fact, killing spermatozoa shortly before ICSI is a common practice which is even expected to be beneficial for fertilization results.

Table I. Results of six intracytoplasmic sperm injection treatment cycles with 100% immotile spermatozoa

No. oocytes injected			No. zygotes			No. embryos	
Cycle	Survived	Damaged	1 PN	2 PN	3 PN	Cleaved[a]	Clinical pregnancies
1	6	1	0	4	0	1	No
2	7	0	0	1	1	1	No
3	6	1	0	3	1	2	No
4	3	0	0	3	0	3	Yes
5	8	1	0	4	1	4	Yes[b]
6	10	0	1	3	0	3	No
Total	40	3	1	18	3	14	2

PN = pronuclear.
[a]Only embryos developing from 2 PN zygotes are included.
[b]Twin pregnancy.

Thus, the relatively low fertilization rates observed in this study after injection of originally totally immotile spermatozoa are not necessarily due to a high frequency of false viables with this viability selection method but may rather be a consequence of other sperm deficiencies in these extremely poor samples.

The reasons for the total lack of sperm movement were not determined in this study. In some cases, the lack of movement of ejaculated spermatozoa is probably because of sperm degeneration during epididymal transport because spermatozoa with a higher viability can be obtained by testicular sperm recovery in some cases with low viability and total lack of movement of ejaculated spermatozoa (Kahraman *et al.*, 1996). The recourse to testicular spermatozoa for ICSI is thus an alternative treatment possibility in this kind of pathology. The simplicity of the method described in this study makes it particularly suitable for cases in which the total absence of sperm movement is an unexpected finding on the day of ICSI, when testicular sperm recovery has not been planned. Further study is needed to compare the relative efficiency of both methods.

Although the preliminary results obtained with the present method are encouraging, further improvements of this technique still appear possible because the fertilization rates do not reach the standard for ICSI with motile spermatozoa. Analysis of the fertilization performance of spermatozoa with different patterns of tail deformation in response to the hypo-osmotic environment will probably be a promising approach. In fact, different deformation patterns have been reported to be differently associated with sperm fertilizing ability (Mordel *et al.*, 1993). The possibility of further improving the outcomes of ICSI with 100% immotile spermatozoa by preferentially selecting different types of sperm tail deformation in the hypo-osmotic condition is under study in our laboratories.

Acknowledgement

We would like to thank Eng. Antonio Almeida Foundation for library financial support.

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Received on December 17, 1996; accepted on March 26, 1997