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Laser-induced immobilization and plasma membrane permeabilization in human spermatozoa

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We evaluated the potential use of a non-contact, 1.48 μm wavelength diode laser for immobilization of human spermatozoa and permeabilization of the sperm membrane in different culture media. When we applied a single laser shot near to the middle region of the sperm tail, spermatozoa could be immobilized either temporarily or permanently, depending on the energy used. Above an energy of 2 mJ in polyvinylpyrrolidone and 2–3 mJ in culture medium, a reliable permanent immobilization was achieved by permeabilization of the sperm tail membrane. We then explored the use of a double laser shot technique. Spermatozoa were temporarily immobilized by a first laser shot applied near to the sperm tail followed by permeabilization with a second laser shot aimed directly at the sperm tail. This sequential approach yielded permanent immobilization at much lower energy values compared with the single shot technique. Following the injection of laser-treated spermatozoa, mouse oocytes underwent normal activation and pronuclear formation. We conclude that a non-contact 1.48 μm diode laser system can be used for immobilization of spermatozoa and for permeabilization of the sperm tail membrane. This laser procedure may offer an alternative to currently used sperm pretreatment prior to intracytoplasmic sperm injection.

Key words: ICSI/immobilization/human/laser/spermatozoa

Introduction

In 1992, Palermo and co-workers described for the first time the successful application of intracytoplasmic sperm injection (ICSI) in the human. In regard to previous attempts by other groups (Lanzendorf *et al.*, 1988), it was the implementation of numerous technical improvements which made ICSI successful. Today, most centres all over the world still follow the original procedure. A crucial step is the immobilization of the spermatozoon prior to injection. This is usually achieved by compression of the sperm tail to the bottom of a culture dish with the injection capillary to destabilize and permeabilize the sperm plasma membrane (Dozortsev *et al.*, 1995; Palermo *et al.*, 1995).

Some investigators reported that immobilization and permeabilization improves fertilization rates (Palermo *et al.*, 1993; Fishel *et al.*, 1995; Gerris *et al.*, 1995), presumably because this manipulation facilitates sperm nuclear decondensation after injection of the spermatozoon into the oocyte (Dozortsev *et al.*, 1995), whereas others have reached opposite conclusions (Lacham-Kaplan and Trounson, 1994; Hoshi *et al.*, 1995). However, it was shown that aggressive sperm immobilization, leading to a permanent permeabilization of the sperm membrane, improved fertilization rates and in cases of immature spermatozoa led to higher pregnancy rates (Palermo *et al.*, 1996).

So far, most centres have performed sperm manipulation in a 10% solution of polyvinylpyrrolidone (PVP) in culture medium. The viscosity of PVP reduces sperm motility and facilitates subsequent sperm manipulation (Palermo *et al.*, 1995). However, PVP is a potentially toxic substance and the use of PVP in human tissue culture or ICSI is in debate. Some groups reported successful ICSI without the use of PVP (Feichtinger *et al.*, 1995; Jean *et al.*, 1996; McDermott and Ray, 1996; Butler and Masson, 1997); their results compare favourably to those of centres routinely using PVP (Van Steirteghem *et al.*, 1995). However, sperm handling for ICSI without PVP is more problematic regarding immobilization.

We recently applied a non-contact 1.48 μm wavelength diode laser system for immobilization of spermatozoa prior to cryopreservation (Montag *et al.*, 1999). The ease of this procedure prompted us to investigate in detail the potential use of this laser system for a controlled immobilization of spermatozoa and for permeabilization of the sperm tail membrane in PVP as well as in tissue culture medium.

Material and methods

Sperm preparation

Human ejaculated spermatozoa were washed and prepared by a modified swim-up technique as previously described (Montag *et al.*, 1997).

Instrumentation

We used a non-contact 1.48 μm wavelength diode laser system (initially developed at the École Polytechnique Fédéral de Lausanne, EPFL, Lausanne, Switzerland). A description of the technical concept of this system and its precision in laser application is described elsewhere (Rink *et al.*, 1994). In short, the InGaAsP laser diode emitting at a wavelength of 1.48 μm is coupled to an inverted microscope (DMIRB; Leica, Bensheim, Germany) and the laser beam is directed along the microscope optical axis. Specially attached

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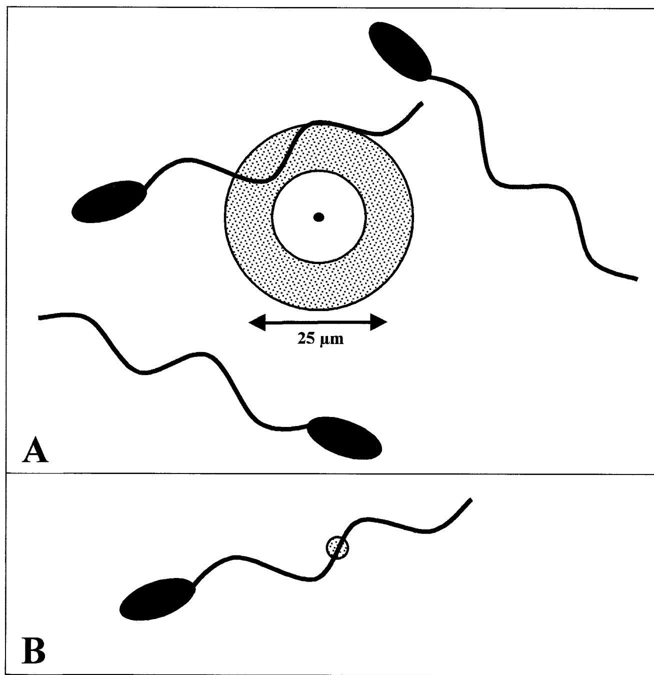

Figure 1. Schematic representation of the orientation of the laser shot using the single shot and/or double shot technique. (**A**) The laser was focused in the centre of the circle which had a 30 μm outer diameter. Only spermatozoa located within this area were affected by the laser irradiation. The laser shot was delivered when the tail of a spermatozoon was located within the grey area which had a width of 5 μm. (**B**) For the double shot technique, a first laser shot at lower energy was applied according to the procedure described in (**A**). The second laser shot for permeabilization of the sperm tail membrane was aimed directly at the tail at the position indicated.

lenses and mirrors within the laser unit allow focusing of the laser beam at the image plane of the microscope objective. The laser energy delivered to the image plane by a single laser shot can be easily adjusted by varying the length of the single laser pulse (Rink *et al*., 1996). The microscope was equipped with a heated stage, a ×40 objective and micromanipulation devices (Narishige, Tokyo, Japan) for sperm manipulation and for intracytoplasmic sperm injection (Montag *et al*., 1998).

Laser-assisted immobilization and permeabilization

For laser treatment, spermatozoa were pipetted into 10 μl droplets of a solution of 10% PVP in culture medium (ICSI 1; Scandinavian IVF Sciences AB, Göteborg, Sweden) or pure tissue culture medium (Gamete 100; Scandinavian IVF Sciences AB) under mineral oil. Each droplet was placed next to another droplet of hypo-osmotic swelling (HOS) test medium (Hypo10; Scandinavian IVF Sciences AB).

We evaluated two strategies for immobilization of spermatozoa and permeabilization of the sperm membrane. In a first series of experiments a single laser irradiation was applied close to the middle of the sperm tail. We used a distance of 10–15 μm from from the tail (see Figure 1A). This distance offered the best choice for the requirements of our field of view. Usually we did not chase spermatozoa but instead caught sperm cells, which, during their forward progression, moved within the laser beam interaction area (Figure 1A). This strategy helped to avoid direct application of the laser pulse to the sperm head, although it is unknown if a direct hit to the sperm head would have biological consequences.

In a second series of experiments, immobilization of spermatozoa and subsequent permeabilization of the sperm tail membrane was performed by two successive laser irradiations. This was intended to reduce as far as possible the laser doses delivered to the spermatozoon and to improve control of the permeabilization process. A first low energy laser shot was applied beside the middle of the sperm tail to induce at least a temporary immobilization, followed by a second shot aimed directly at the sperm tail to permeabilize the sperm tail membrane (Figure 1B).

Both experiments were performed at different energy values in PVP and in culture medium with spermatozoa showing different degrees of motility [World Health Organization (WHO) Type A, B, C; WHO, 1993]. Immediately after laser treatment, each individual spermatozoon was transferred into HOS medium to assess the integrity of the sperm tail membrane. Permeabilized spermatozoa show a negative reaction in the HOS test (Jeyendran *et al*., 1984; Ved *et al*., 1997).

All these experiments were performed with one sperm sample. However, initial studies have shown that there were no variations in the susceptibility of spermatozoa to the laser treatment in different sperm samples (data not shown).

Injection of laser-treated spermatozoa into mouse oocytes

According to federal law of Germany, we obtained permission to perform the animal experimentation presented in this study (K32 147-2737/3203). Superovulation of female mice, isolation of oocytes, denudation by hyaluronidase and incubation was performed as described previously (Montag *et al*., 1998), except that females were not mated and isolation of oocyte–cumulus complexes occurred at 14 h after administration of human chorionic gonadotrophin (HCG).

For injection we used human spermatozoa which were immobilized in culture medium using a double laser shot technique with two successive laser irradiations, as described above. As a control we injected human spermatozoa which were immobilized in PVP using the common ICSI immobilization procedure using a glass capillary.

Injection of mouse oocytes was performed as prevously described (Rybouchkin *et al*., 1996) with the modification that we used HEPES-buffered culture medium (Gamete-100) throughout the manipulation procedure. Mouse oocytes were randomly allocated to the treatment or the control group. When we injected laser-immobilized spermatozoa, which were treated in culture medium, we rinsed the injection capillary prior to the first injection in a viscous medium (PureSperm; Nidacon, Gothenborg, Sweden), followed by several washes in culture medium. This pretreatment avoided sticking of the spermatozoa to the glass capillary. It was not applied for the conventional injection procedure where the use of PVP fulfilled the same purpose. Injected oocytes were cultured for another 12 h and were checked periodically for the extrusion of second polar body and formation of pronuclei.

Statistics

Differences in the effects of treatments were assessed using the χ^2-test.

Results

Immobilization and permeabilization by a single laser shot

Following the application of a single laser shot, spermatozoa showed distinct reactions, which allowed a classification into three categories: (i) spermatozoa retained motility, although progressive motility was reduced (no motility arrest); (ii) spermatozoa stopped both progressive and local motility for a time period of 30 s to 2.5 min, after which a partial recovery of motility was observed, although progressive motility was

Table I. Immobilization and permeabilization of human spermatozoa in polyvinylpyrrolidone by a single laser shot

Sperm type/ laser energy	Total no. spermatozoa	No. permanent motility arrest	No. temporary motility arrest	No. no motility arrest	No. permeabilized (HOS-negative)
WHO A/B					
0.25 mJ	100	12	66	22	0/100
0.5 mJ	100	21	79	0	0/100
1.0 mJ	100	95	5	0	35/100
2.0 mJ	100	100	0	0	100/100[a]
WHO C					
0.25 mJ	100	43	57	0	0/100
0.5 mJ	100	100	0	0	0/100
1.0 mJ	100	100	0	0	60/100
2.0 mJ	100	100	0	0	100/100[a]

[a]$P < 0.001$: permeabilization was significantly greater at the indicated laser energy compared to all previous energy values.
HOS = hypo-osmotic swelling test; WHO = World Health Organization.

never completely restored (temporary motility arrest); (iii) spermatozoa stopped motile movement permanently (permanent motility arrest).

Spermatozoa showing no or only a temporary motility arrest always retained membrane integrity and exhibited a positive response to the HOS test. Spermatozoa with damaged membranes (HOS-negative) were only detected among permanently arrested spermatozoa. This observation was consistent for PVP and for culture medium. The effect of laser treatment in PVP is summarized in Table I. Since no difference could be observed in PVP between the exposed spermatozoa of WHO types A and B, the data concerning these groups were combined. A 100% immobilization and permeabilization of WHO A/B spermatozoa was only achieved at an energy of 2 mJ. Spermatozoa of type WHO C showed a 100% immobilization at 0.5 mJ, but 100% permeabilization only occurred at 2 mJ.

The evaluation of spermatozoa treated in culture medium is shown in Table II. Here we noted clear differences between the different types of motile spermatozoa. In particular, the energy necessary to achieve a combined immobilization and permeabilization was dependent on the degree of progressive motility of the spermatozoon (WHO A: 3 mJ; WHO B: 2.5 mJ; WHO C: 2 mJ).

Immobilization and permeabilization by a double laser shot technique

From our first series of experiments we identified the threshold energy inducing a temporary arrest of the spermatozoon (Table I). In PVP a threshold laser energy of 0.5 mJ was needed to induce either a permanent or a temporary arrest in all spermatozoa (WHO A, B, C). A similar effect could be obtained in culture medium but at different threshold energies (WHO A: 1–2 mJ, 60–94% temporary arrest; WHO B: 1 mJ/88%; WHO C: 0.5 mJ/100%). Temporary immobilization was achieved in this series using a laser shot at the threshold values listed above, while permeabilization was obtained by a second laser shot of 0.25 mJ aimed directly at the sperm tail (Figure 1B). As shown in Table III, the double laser shot technique caused a reliable immobilization and membrane permeabilization of the sperm tail in PVP and in culture medium.

Laser energy required for the one shot versus the double shot technique

A comparison of the total energy needed to achieve a 100% immobilization and permeabilization in a single or a double shot technique is given in Table IV. The double shot technique allowed us to reduce at least by a factor two the total energy delivered to the spermatozoa for all types of spermatozoa. As expected we found clear differences between PVP and culture medium except for WHO type C spermatozoa. For these, the energy level applied in the single and the double shot techniques was similar in both media.

Activation of mouse oocytes after injection of laser-treated spermatozoa

We injected human spermatozoa which were treated by the double shot technique into mouse oocytes and compared the survival rate as well as the activation rate with control oocytes which were injected by the standard ICSI procedure which uses a glass capillary for sperm immobilization. The survival rate and the activation rate did not differ significantly between both groups. All activated oocytes reached the two-pronuclear stage 7 h after injection (Table V).

Discussion

Strategies of laser-immobilization

Immobilization of spermatozoa is a prerequisite for successful ICSI (Fishel *et al.*, 1995) and it can be achieved by vigorous treatment of the sperm tail with the injection capillary (Palermo *et al.*, 1992) or by piezo-pulses (Huang *et al.*, 1996). We demonstrate here that a 1.48 µm diode laser system can be successfully used for touch-free immobilization of spermatozoa and for permeabilization of the sperm plasma membrane.

As recently demonstrated for zona pellucida drilling (Germond *et al.*, 1995), the use of a 1.48 µm wavelength laser for sperm immobilization provides an increased precision

Table II. Immobilization and permeabilization of human spermatozoa in culture medium by a single laser shot

Sperm type/ laser energy	Total no. spermatozoa	No. permanent motility arrest	No. temporary motility arrest	No. no motility arrest	No. permeabilized (HOS-negative)
WHO A					
0.25 mJ	100	0	0	100	0/100
0.5 mJ	100	0	11	89	0/100
1.0 mJ	100	14	46	40	0/100
2.0 mJ	100	36	58	6	30/100
2.5 mJ	100	100	0	0	75/100
3 mJ	100	100	0	0	100/100[a]
WHO B					
0.25 mJ	100	0	22	78	0/100
0.5 mJ	100	5	41	54	0/100
1.0 mJ	100	17	71	12	10/100
2.0 mJ	100	94	6	0	84/100
2.5 mJ	100	100	0	0	100/100[a]
WHO C					
0.25 mJ	100	11	50	39	0/100
0.5 mJ	100	48	52	0	50/100
1.0 mJ	100	100	0	0	85/100
2.0 mJ	100	100	0	0	100/100[a]

[a]$P < 0.001$: permeabilization was significantly greater at the indicated laser energy compared to all previous energy values.
HOS = hypo-osmotic swelling test; WHO = World Health Organization.

Table III. Immobilization and permeabilization of human spermatozoa ($n = 100$) in polyvinylpyrrolidone and culture medium by a double laser shot technique

Sperm	PVP			Culture medium		
type	1st shot (mJ)	2nd shot (mJ)	HOS-negative spermatozoa (%)	1st shot (mJ)	2nd shot (mJ)	HOS-negative spermatozoa (%)
WHO A	0.5	0.25	100	1.5	0.25	100
WHO B	0.5	0.25	100	1.0	0.25	100
WHO C	0.5	0.25	100	0.5	0.25	100

HOS = hypo-osmotic swelling test; WHO = World Health Organization.

Table IV. Comparison of the total laser energy needed for immobilization and a 100% permeabilization of human spermatozoa by single versus double laser shot technique in polyvinylpyrrolidone (PVP) and culture medium

Sperm type	PVP		Culture medium	
	Single shot (mJ)	Double shot (mJ)	Single shot (mJ)	Double shot (mJ)
WHO A	2	0.75	3	1.75
WHO B	2	0.75	2.5	1.25
WHO C	2	0.75	2	0.75

WHO = World Health Organization.

Table V. Activation of mouse oocytes after injection with laser-treated versus conventionally treated human spermatozoa

	Laser-treated spermatozoa	Conventionally treated spermatozoa
No. of oocytes injected	28	27
No. of oocytes survived	20/28 (71%)[a]	18/27 (66%)[a]
No. of oocytes activated	12/20 (60%)[b]	10/18 (55%)[b]

[a,b]The survival rate ([a]) and the activation rate ([b]) were not significantly different after injection of laser-treated or conventionally treated spermatozoa.

and added features. Sperm immobilization can be performed according to two simple procedures: in the first, the spermatozoon identified by the operator is placed within the area of laser action, which is typically 10–15 μm wide; in the second, the operator waits until a spermatozoon having the appropriate features enters the laser interaction area. Both approaches allow for selection in real conditions prior to injection. Moreover, as permanent arrest is induced after laser treatment, any spermatozoon can be immobilized for further morphological inspection prior to injection. The laser immobilization procedure maintains the sperm tail aligned with the head, thus facilitating aspiration into the injection pipette. Using either the single or double shot immobilization techniques, permanent immobilization is accomplished within less than 1 min.

Although permanent sperm immobilization can be induced by a single 1.48 μm laser shot, we prefer a double shot technique which allows us to reduce the total laser energy dose delivered to the spermatozoon. The double shot technique allows us to reduce as far as possible the energy used for immobilization, while an even lower energy is sufficient to achieve membrane permeabilization with a 100% success rate due to precise aiming of the laser beam at the tail. The energy necessary for permeabilization of previously immobilized spermatozoa is as low as 0.25 mJ and not dependent on the viscosity of the medium. It should be noted that the dose for laser treatment of spermatozoa is five to 10 times lower than that required for laser zona drilling (Primi *et al.*, 1999) and we can be reasonably confident that it is a safe approach.

The laser energy required for immobilization depends on the degree of progressive motility of the spermatozoa. Spermatozoa with a high progressive speed, which stay for only a short time within the laser interaction zone, require a higher immobilization energy. This is not the case in PVP, where no difference in energy was noticed due to the slow speed of spermatozoa of each category in the viscous medium.

Interestingly, two types of effects have been observed after irradiating the sperm tail. A low laser dose induces only a temporary immobilization, whereas above a certain threshold energy permanent immobilization is obtained. So far we have no detailed information on the type of cellular alteration associated with immobilization in laser-treated spermatozoa. However, we know from previous studies that the mode of action of the 1.48 μm diode laser is thermal (Rink *et al.*, 1996; Hollis *et al.*, 1997). Laser irradiation induces a local temperature elevation within the sperm tail. At low laser doses, the temperature elevation perturbs the thermodynamic processes of the molecular motor driving the tail movements (Khan and Sheetz, 1997): the spermatozoon stops. As a given time is needed to restore the activation mechanisms of the tail, a temporary motility arrest is observed. Above a given threshold temperature, protein denaturation is very likely and permeabilization of the sperm tail membrane is induced, leading to permanent motility arrest. However, the high absorption characteristics of the 1.48 μm wavelength in water, as well as the application of the laser near the end of the spermatozoon, guarantees that the local thermal effect does not affect the DNA within the sperm head. At present we do not know whether a direct application of the laser beam to the sperm head at a high energy could cause any damage to the DNA. This question is under investigation.

The new laser approach presented here has to be clearly distinguished from other laser sperm manipulations described previously. Laser tweezers have been proposed to catch and move spermatozoa in the context of laser subzonal insemination (SUZI) (Tadir *et al.*, 1989; Schütze *et al.*, 1994). In this application the sperm head is trapped at the centre of a highly focused laser beam due to restoring forces induced by the optical field. The spermatozoon cannot escape, but the movements of the sperm tail are not affected. Although the wavelength of laser tweezers is chosen such that virtually no light is absorbed by the trapped objects, it has been demonstrated that too long an exposure to the optical trap may cause alteration of sperm motility (Tadir *et al.*, 1989) or even cell death (König *et al.*, 1996). In the other attempt, a pulsed UV-laser beam has been proposed to cut the sperm tail before injection or SUZI (Schütze *et al.*, 1994); however, ultraviolet light may bear mutagenic risks (Kochevar, 1989), and for that reason is not recommended.

The benefit and the necessity of permeabilization of the sperm membrane is still a subject of controversy. However, Dozortsev *et al.* (1995) reported that permeabilization is beneficial for subsequent sperm nuclear decondensation. Permeabilization of the sperm plasma membrane may facilitate the influx of a sperm nucleus decondensing factor (SNDF) from the oocyte (Perreault and Zirkin, 1982; Dozortsev *et al.*, 1997). It was postulated that this factor releases a sperm-associated oocyte activation factor (SAOF), whose identity is uncertain at present (Stice and Robl, 1990; Swann, 1990; Kuretake *et al.*, 1996; Parrington *et al.*, 1996; Sette *et al.*, 1997). There might also be differences between the requirements for human ICSI and for ICSI in other species, where sperm pretreatment might be not so important (Yanagimachi, 1998).

Laser immobilization without PVP

The laser technique developed here did not require the addition of a viscous substance to facilitate sperm capture. It offers the potential to avoid PVP, whose use is still controversial. It has been recently shown that PVP has a primary detrimental action on the plasma membrane of spermatozoa (Strehler *et al.*, 1998). Moreover, it is obvious that a certain quantity of PVP is co-injected into the oocyte during the ICSI procedure. A significant increase of degeneration and a reduction of the fertilization rates has been observed, correlated to an increase of the volume of PVP injected during the ICSI procedure, although a direct effect of the volume injected could not be excluded (Payne *et al.*, 1998). It was suggested that chromosomal abnormalities after ICSI could be due to the injection of PVP into the oocyte (Feichtinger *et al.*, 1995). Finally, although cytogenetic mutagenicity tests did not reveal mutagenic effects of PVP (Ray *et al.*, 1995), it is commonly agreed that PVP might be a potentially harmful agent and as a precaution should be avoided (Jean *et al.*, 1997). Recently, Hlinka and coworkers proposed a modified method for ICSI without using PVP (Hlinka *et al.*, 1998). These authors observed a significantly higher rate of normally fertilized oocytes and concluded that this may be associated with the elimination of the potentially harmful effects of the conventional ICSI procedure.

The use of PVP is known to facilitate ICSI procedures by preventing sticking of spermatozoa to the inner wall of the pipette. By contrast, mechanical immobilization in culture medium frequently results in the formation of kinks in the sperm tail, which greatly complicates subsequent sperm pipetting, as kinked spermatozoa tend to stick inside the injection capillary. The 1.48 μm diode laser technique for immobilization and permeabilization bypasses these problems, as we never observed kinked sperm tails following laser treatment. Pipetting of laser-treated spermatozoa in culture medium is thus easy to perform.

During our initial trials with injection of spermatozoa laser-

treated in culture medium, we noted that the inner wall became sticky after some injections although no ooplasmic remnants were detectable inside the injection capillaries. Sticking of spermatozoa usually does not occur with PVP (Palermo *et al.*, 1995). We found that this problem is not related to the laser treatment but to the fact that we used culture medium. It could be prevented by an initial rinsing of the injection capillary in a viscous medium (e.g. a sperm preparation medium) followed by several washes with culture medium prior to starting the injection session.

Further experimentation in an animal model is required to confirm the safety of the laser procedure before its application in human ICSI. However we were able to achieve activation of mouse oocytes and pronuclear formation following injection of laser-treated spermatozoa. This shows that laser-immobilized and permeabilized spermatozoa are at least capable of initiating fertilization.

In conclusion, a novel laser-induced sperm immobilization and permeabilization technique is proposed. The technique is simple and quick and does not require the use of an additional medium to slow down the spermatozoa. As compared to the PVP technique, the laser approach offers the possibility of controlling the sperm motility and behaviour prior to immobilization in representative conditions and allows a final morphological check before injection due to complete arrest of the spermatozoa. However, prior to applying this technique in human IVF, the safety of our approach needs to be investigated in animal experiments with special emphasis on the evaluation of embryonic development, implantation potential and pregnancy outcome.

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References

Butler, E.A. and Mason, G.M. (1997) Development of a successful ICSI programme without the use of PVP. *Hum. Reprod.*, **12**, 1115.

Dozortsev, D., Rybouchkin, A., de Sutter, P. and Dhont, M. (1995) Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. *Hum. Reprod.*, **10**, 2960–2964.

Dozortsev, D., Qian, C., Ermilov, A. *et al.* (1997) Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 min after injection as a result of the sperm–oocyte interaction. *Hum. Reprod.*, **12**, 2792–2796.

Feichtinger, W., Obruca, A. and Brunner, M. (1995) Sex chromosomal abnormalities and intracytoplasmic injection. *Lancet*, **346**, 1566.

Fishel, S., Lisi, F., Rinaldi, L. *et al.* (1995) Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. *Hum. Reprod.*, **10**, 497–500.

Germond, M., Nocera, D., Senn, A. *et al.* (1995) Microdissection of mouse and human zona pellucida using a 1.48 μm diode laser beam: efficacy and safety of the procedure. *Fertil. Steril.*, **25**, 604–611.

Gerris, J., Mangelschots, K., Van Royen, E. *et al.* (1995) ICSI and severe male factor infertility: breaking the sperm tail prior to injection. *Hum. Reprod.*, **10**, 484–486.

Hlinka, D., Herman, M., Veselá, J. *et al.* (1998) A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone. *Hum. Reprod.*, **13**, 1922–1927.

Hollis, A., Rastegar, S., Descloux, L. *et al.* (1997) Zona pellucida microdrilling with a 1.48 μm diode laser. *IEEE Eng. Med. Biol.*, **16**, 43–47.

Hoshi, K., Yanagida, K., Yazawa, H. *et al.* (1995) Intracytoplasmic sperm injection using immobilized or motile human spermatozoon. *Fertil. Steril.*, **63**, 1241–1245.

Huang, T., Kimura, Y. and Yanagimachi, R. (1996) The use of a piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. *J. Assist. Reprod. Genet.*, **13**, 320–328.

Jean, M., Barriere, P. and Mirallie, S. (1996) Intracytoplasmic sperm injection without polyvinylpyrrolidone: an essential precaution? *Hum. Reprod.*, **11**, 2332.

Jean, M., Barriere, P. and Mirallie, S. (1997) Development of a successful ICSI programme without the use of PVP. *Hum Reprod.*, **12**, 1115–1116.

Jeyendran, R.S., van der Ven, H., Perez-Pelaez, M. *et al.* (1984) Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, **70**, 219–228.

Khan, S. and Sheetz, M.P. (1997) Force effects on biochemical kinetics. *Annu. Rev. Biochem.*, **66**, 785–805.

Kochevar, I.E. (1989) Cytotoxicity and mutagenicity of excimer laser radiation. *Lasers Surg. Med.*, **9**, 440–445.

König K., Tadir Y., Patrizzio P. *et al.* (1996) Effects of ultraviolet exposure and near infrared tweezers on human spermatozoa. *Hum. Reprod.*, **11**, 2162–2164.

Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. (1996) Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.*, **55**, 789–795.

Lacham-Kaplan, O. and Trounson, A. (1994) Micromanipulation assisted fertilization: 'comparison of different techniques'. In Tesarik, J. (ed.), *Frontiers in Endocrinology.* Ares-Serono Symposia Publications, Rome, Vol. 8, pp. 287–304.

Lanzendorf, S.E., Maloney, M.K. Veeck, L.L. *et al.* (1988) A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil. Steril.*, **49**, 835–842.

McDermott, A. and Ray, B. (1996) Intracytoplasmic sperm injection without polyvinylpyrrolidone: an essential precaution? *Hum. Reprod.*, **11**, 2332.

Montag, M., van der Ven, K., Ved, S., Schmutzler, A. *et al.* (1997) Success of intracytoplasmic sperm injection in couples with male and/or female chromosome aberrations. *Hum. Reprod.*, **12**, 2635–2640.

Montag, M., van der Ven, K., Delacrétaz, G. *et al.* (1998) Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy. *Fertil. Steril.*, **69**, 539–542.

Montag, M., Dieckmann, U., Rink, K. *et al.* (1999) Laser-assisted cryopreservation of single human spermatozoa in cell-free zona pellucida. *Andrologia*, **31**, 49–53.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.

Palermo, G., Joris, H., Derde, M.P. *et al.* (1993) Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil. Steril.*, **59**, 826–835.

Palermo, G., Cohen, J., Alikani, M. *et al.* (1995) Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. *Fertil. Steril.*, **63**, 1231–1240.

Palermo, G.P., Schlegel, P.N., Colombero, L.T. *et al.* (1996) Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum. Reprod.*, **11**, 1023–1029.

Parrington, J., Swann, K., Shevchenko, V.I. *et al.* (1996) A soluble sperm protein that triggers calcium oscillations in mammalian oocytes. *Nature*, **379**, 364–368.

Payne, D., Goodwin, J., Flaherty, S.P. *et al.* (1998) The effect of polyvinylpyrrolidone on fertilization, embryo quality and implantation rate. *Hum. Reprod.*, **13**, (Abstract Book 1), 219.

Perreault, S.D. and Zirkin, B.R. (1982) Sperm nuclear decondensation in mammals: role of sperm-associated proteinase *in vivo*. *J. Exp. Zool.*, **224**, 253–257.

Primi, M.P., Senn, A., Rink, K. *et al.* (1999) Assisted hatching. *Giornale Italiano di Ostetricia e Gynecologia*, **11**, 513–516.

Ray, B.D., Howell, R.T., McDermott, A. and Hull, M.G.R. (1995) Testing the mutagenic potential of polyvinylpyrrolidone and methyl cellulose by sister chromatid exchange analysis prior to use in intracytoplasmic sperm injection procedures. *Hum. Reprod.*, **10**, 436–438.

Rink, K, Delacrétaz, G., Salathé, R.P. *et al.* (1994) 1.48 μm diode laser microdissection of the zona pellucida of mouse zygotes. *Proceedings SPIE*, **213A**, 412–422.

Rink, K., Delacrétaz, G., Salathé, R. *et al*. (1996) Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48 μm diode laser. *Lasers Surg. Med.*, **18**, 52–62.

Rybouchkin, A., Dozortsev, D., Pelinck, M.J. *et al*. (1996) Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. *Hum. Reprod.*, **11**, 2170–2175.

Schütze, K., Clement-Sengewald, A. and Ashkin, A. (1994) Zona drilling and sperm insertion with combined laser microbeam and optical tweezers. *Fertil. Steril.*, **61**, 783–786.

Sette, C., Bevilacqua, A., Bianchini, A. *et al*. (1997) Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development*, **124**, 2267–2274.

Stice, S.L. and Robl, J.M. (1990) Activation of mammalian oocytes by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.*, **25**, 272–280.

Strehler, E., Baccetti, B., Sterzik, K. *et al*. (1998) Detrimental effects of polyvinylpyrrolidone on the ultrastructure of spermatozoa (Notulae seminologicae 13). *Hum. Reprod.*, **13**, 120–123.

Swann, K. (1990) A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development*, **110**, 1295–1302.

Tadir, Y., Wright, W.H., Vafa, O. *et al*. (1989) Micromanipulation of sperm by a laser generated optical trap. *Fertil. Steril.*, **52**, 870–873.

Van Steirteghem, A., Tournaye, H., Van der Elst, J. *et al*. (1995) Intracytoplasmic sperm injection three years after the birth of the first ICSI child. *Hum. Reprod.*, **10**, 2527–2528.

Ved, S., Montag, M., Schmutzler, A. *et al*. (1997) Pregnancy following intracytoplasmic sperm injection of immotile spermatozoa selected by the hypo-osmotic swelling test: a case report. *Andrologia*, **29**, 241–242.

World Health Organization (1993) *Laborhandbuch zur Untersuchung des menschlichen Ejakulates und der Spermien-Zervikalschleim-Interaktion*, 3rd edn. Springer, Berlin.

Yanagimachi, R. (1998) Intracytoplasmic sperm injection experiments using the mouse as a model. *Hum. Reprod.*, **13** (Suppl. 1), 87–98.

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