Are zona pellucida laser drilling and polar body biopsy safe for *in vitro* matured oocytes?

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Abstract

Introduction Preconception diagnosis requires first polar body biopsy. When the hole in the zona pellucida is made with a laser beam, heat propagation could, like the biopsy itself, be deleterious. Our aim was to evaluate the effect of this technique on human *in vitro* matured oocyte and embryo development.

Methods One hundred fifty five retrieved immature oocytes from 75 women, matured *in vitro*, were distributed in 3 groups: 50 oocytes in a control group, without laser drilling and first polar body biopsy, 52 oocytes in a group with only laser drilling, and 53 oocytes in a group with both laser drilling and first polar body biopsy. Safety was evaluated using four criteria: [1] oocyte lysis rate, [2] oocyte activation rate, [3] oocyte development after calcium ionophore treatment, [4] and embryo chromosome breakage incidence after Tarkowski preparation.

Results No difference in the four criteria was observed between the 3 oocyte groups.

Conclusions We did not find evidence of deleterious effect of laser drilling and first polar body biopsy on *in vitro* matured oocytes, according to our criteria.

Keywords Human *in vitro* oocytes · First polar body · Laser · Preconception diagnosis · Safety

Introduction

More than 70% of embryo aneuploidies result from female meiosis and occur during the first meiotic division [1, 2], and the most frequent foetal trisomies are maternally inherited [3], i.e. 93% of trisomy 18, 95% of trisomy 21 and 100% of trisomy 16. Advanced maternal age is the major risk factor for oocyte aneuploidy, mainly due to premature separation of sister chromatids [4, 5]. Preimplantation genetic screening (PGS) performed on blastomeres [6] and preconception genetic screening (PCS) performed on the first polar body [5, 7] or both polar bodies [8] are used to prevent the high rate of embryo aneuploidy in women aged above 35 or 38.

Beside advanced maternal age (AMA), the main indications for PCS or PGS are repeated implantation failure (RIF), repeated miscarriage (RM; usually at least three previous miscarriages) and severe male factor infertility (SMF; usually defined as abnormal semen parameters) [9, 10].

But, whereas non-randomized PGS or PCS studies published worldwide report an increase in implantation rate and/or a decrease in miscarriage rate [11–13], controlled trials of PGS performed for AMA [14–16] and for RIF [17] have shown no benefit of PGS. Implantation and pregnancy rates were similar for women with or without PGS. Even if PGS randomized trials may be

Capsule Safety evaluation of zona pellucida laser drilling and polar body biopsy on *in vitro* matured oocytes: lysis rate, activation rate and oocyte development.

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criticized [18–20], mainly in terms of the methodology of Preimplantation Genetic Diagnosis International Society guidelines, a debate on the usefulness of PGS or PCS is ongoing [21–24]. As yet, no randomized trial has been performed for PCS [25], and the safety of PCS as well as of PGS remains to be demonstrated.

PCS is a viable alternative to PGS [25] in countries where PGS is not allowed for older women, as in France, or is prohibited, as in Austria, or leads to an illegal situation, as in Germany. PCS, on the first polar body, could be considered as a gamete diagnosis, because biopsy was performed before fertilisation. The first polar body is not necessary for embryo development, and extrusion should be less toxic than blastomere biopsy for further embryo development [25].

Initially, polar body or embryo biopsy was done using mechanical methods [26], or chemical digestion using acid Tyrode's solution [27]. The third method, using a non-contact infrared laser, was proposed later for blastomere [28] and polar body [7] biopsy. All those methods have been previously used for assisted hatching. Acid Tyrode's solution is still the most widely used method, but laser zona drilling and thinning are increasingly used. Since the first reported laser systems for zona pellucida micromanipulation [29], many systems have been developed and the 1.48 μm diode non-contact infrared laser is now considered as the most acceptable for laser zona drilling, because it does not use UV lasers, which are associated with mutagenic change in DNA [30].

The impact of non-contact infrared laser dissection on embryo development depends on pulse power and duration. It was first studied after mouse blastomere biopsy [31] and polar body biopsy [32], and secondly after human blastomere biopsy [33]. The use of non-contact infrared laser for zona drilling before embryo biopsy seems to have no impact on preimplantation development to the blastocyst stage, or on pregnancy rate. The situation was the same for human first polar body biopsy, and the PCS results were in accordance with those for ICSI without biopsy, but no study of the impact on oocyte development has yet been done [5, 25].

The aim of our study was to evaluate the safety for subsequent embryo development of first polar body biopsy

after laser zona pellucida (ZP) dissection on donated *in vitro* matured oocytes.

Material and methods

Material

155 oocytes from 75 patients were included in the study. Patients were undergoing routine ICSI infertility treatment for IVF failure [18] or male infertility (57). Multiple follicular growth was induced by exogenous gonadotropins (Gonal F or Puregon) following a desensitisation protocol with GnRH analogues according to a long down-regulation protocol (44 cycles) or a short protocol (20 cycles), or following a desensitisation protocol with GnRH antagonist (11 cycles). Protocol choice was done according to women criteria to maximize matured oocytes retrieval and to reduce immature oocytes number. No reference reported that different protocols differed in the production of immature oocytes and the activation rate. Oocytes were retrieved approximately 36 h after hCG administration. After denudation, oocyte maturity was valued using polar body expulsion. They were considered immature if they were still at the germinal vesicle stage (VG) or if the first polar body was not retrieved (MI).

Patients were informed about the experiments and gave their consent to participate. The protocol was approved by our local ethics committee. All oocytes, still immature just before injection of spermatozoa, were matured *in vitro* using IVF media (Medicult). At day 1, only *in vitro* matured oocytes, after first polar body expulsion, were used. Finally, 90 metaphase I oocytes (without first polar body expulsion at day 0) and 65 germinal vesicles were included in our protocol.

Oocytes were distributed in three groups according to micromanipulation techniques:

- Oocytes without ZP laser dissection ($n=50$), (group 1)
- Oocytes with ZP laser dissection but without polar body biopsy ($n=53$) (group 2)
- Oocytes with both ZP laser dissection and polar body biopsy ($n=52$) (group 3).

Table 1 Lysis and activation in the three groups

Groups		1: Control	2: Laser only	3: Laser/biopsy	All
Oocyte number	Total	50	52	53	155
	VG/MI	20/30	22/30	23/30	65/90
Number of lysed oocytes		0	1 (2%)	2 (4%)	3 (2%)
Number of activated oocytes	Global	32 (64%)	37 (73%)	35 (73%)	104 (68%)
	VG / MI	12/20	15/22	14/21	41/63

Table 2 Cytogenetic analysis in the three groups

Groups		1: Control	2: Laser only	3: Laser/biopsy	Total
Non activated oocytes	Number	18	14	16	48
	Lost	5	5	2	12
	Non interpretable	7	4	11	22
	euploid	4	4	1	9
	aneuploid	2	1	2	5

No randomization was done when allotting oocytes to the different groups, but oocytes from the same patients were distributed equally in the different groups. The mean age of the patients was 33 yr, 34.3 yr and 35.5 yr, respectively, in groups 1 to 3.

Laser dissection and biopsy

Laser-assisted ZP dissection was performed on day 1 with *in vitro* matured oocytes using the Zilos TK laser (Hamilton Thorne Biosciences). Three or 4 laser impacts (180 mW, 0.5 ms pulse) were made in the ZP in groups 2 and 3, as previously described [5]. In group 3, first polar body biopsy was carried out using a biopsy micropipette (Humagen) and an inverted microscope (Nikon, Japan) with a micromanipulation system (Narishige Ltd, Tokyo, Japan).

Oocyte quality evaluation

Oocyte lysis evaluation Oocyte lysis was first observed 15 minutes after laser drilling and/or polar body biopsy and secondly 24 h after.

Activation capacity After ZP drilling and polar body biopsy, all oocytes were exposed for 10 minutes to 10 μ mol/L calcium ionophore A23187 (Sigma, St. Louis, MO) in IVF-50 medium (Scandinavian IVF Science, Gothenburg, Sweden) as proposed previously [34, 35]. After 10 min, oocytes were washed, to eliminate ionophores, and incubated further in the same medium. Oocyte activation was assessed 24 h after calcium ionophore A23187 exposure. An oocyte was

considered to be activated if it displayed one or two pronuclei and/or the second polar body.

Cytogenetic analysis All oocytes, activated or not, were then fixed according to Tarkowski's method [36]. For non-activated oocytes, cytogenetic analysis was performed after 3% Giemsa staining. Chromosome count was only considered if more than 21 chromosomes were observed. If fewer than 21 chromosomes were retrieved, oocytes were deemed uninterpretable and were not considered for cytogenetic evaluation, like oocytes with tangled chromosomes. Chromosome breakage was also evaluated. For activated oocytes, as chromosomes were decondensed, no cytogenetic analysis was done, only oocytes development stage was appreciated.

Statistical analysis

Statistical analysis (χ^2 test, Fisher test) was performed using the Statview program. Differences were considered significant if $p < 0.05$.

Results

Oocyte lysis and activation rates (Table 1)

Only 3 oocytes were lysed, one (2%) after laser drilling alone, and 2 (4%) after both laser drilling and polar body biopsy. Lysis rates were similar in the 3 groups.

Activation rates were 64% (32/50), 73% (37/51) and 73% (35/48), respectively, in groups 1, 2 and 3, and were

Table 3 Activated oocyte development

Groups		1: Control	2: Laser only	3: Laser/biopsy
Activated oocytes	Number	32	37	35
	Lost	11	13	6
	MII	5	2	5
	PN stage	10	12	9
	Cleaved or mitosis stage	6	10	15

statistically similar. Activation after VG or MI maturation was similar: respectively, 63% and 70% ($p=0.36$).

Cytogenetic analysis (Table 2)

Of the small part of oocytes that could be analysed, no chromosome break was observed in any oocyte, activated or not. Considering all groups, complete cytogenetic analysis was only possible for 14 oocytes: 9 were euploid and 5 aneuploid. 12 oocytes were lost and 22 were not interpretable.

Oocyte development (Table 3)

For oocyte development after activation, distribution of the pronuclear stage, mitosis and interphase cleaved stage embryo were similar in the 3 groups.

Discussion and conclusion

First polar body biopsy was introduced in 1990 [37] and nowadays is frequently used, especially in countries where preimplantation genetic diagnosis is allowed or restricted, as in Germany, Italy and France. Biopsy can be performed after mechanical partial zona dissection, chemical zona drilling or laser-assisted dissection. Mechanical dissection was first used in diagnosis and is still used [38]. Chemical zona drilling, using acid Tyrode's solution, was suspected to compromise oocyte viability and to interfere with embryo development or implantation [39], but is used with success [40]. Laser drilling is currently the most widely used method for first polar body biopsy, but like the two other procedures no evaluation of the potential deleterious effects has been done and impairment of the developmental potential of the oocyte is possible. Laser heating could have deleterious effects on the oocyte spindle. The laser parameters—pulse power and duration—are important.

Two small studies (25 and 15 oocytes) of human oocytes [41] and one study of mouse oocytes ($n=154$) [32] have been published and suggest that laser ZP drilling is safe. Blastomere viability has been studied [33] and confirmed that the non-contact laser procedure had no impact, using two fluorescent markers of cell viability: CFSE, which binds covalently in the cytoplasm of viable cells, and propidium iodide, which is excluded from viable cells but can enter cells with damaged plasma membrane and bind to double-stranded DNA. Here, as described by Montag et al [32] who studied the impact of first polar body biopsy at the zygote stage, we used 4 criteria of oocyte quality after treatment. Due to French legislation it was impossible to study the impact of first polar body biopsy on matured oocytes after fertilisation. This restriction forced us to use *in vitro* matured oocytes. We first evaluated the impact of

laser drilling and polar body biopsy on oocyte lysis. As previously described [30], we did not find any post-procedure increase in lysis rate (3 oocytes lysed in the whole population). This result was in accordance with prospective, non-randomized studies where lysis rates were very low after treatment [5, 7], but also in accordance to ICSI lysis rate [42], procedure considered as safe for oocytes.

We also evaluated oocyte activation rates and development after calcium ionophore contact. The activation rate was similar in all groups, close to 70% and similar for oocytes after VG and MI *in vitro* maturation.

The developmental capacity of the oocytes was considered normal post-procedure, and laser drilling appeared to have no effect on the chronology of development of activated oocytes. These results are in accordance with the prospective published, non-randomized studies reporting expected fertilisation rates and cleavage rates [5, 7], and with French FIVNAT registry data [43] and data on mouse oocytes [32]. The chronology of development was similar in the three groups, with the same ratio of the pronuclear stage, cleaved and mitosis stages at 20 h.

Laser and biopsy could also interact with the chromosome spindle, via heat or mechanical interactions. Furthermore, temperature increase is known to induce chromosome breaks. Pulse duration and laser power influence these interactions [5, 44]. Even if the sample size was too small to allow assessment of aneuploidy after this procedure, no chromosome break was observed in any oocyte group. A large proportion of the oocytes was not interpretable (22/48, 46%) or lost (12/48, 25%), in accordance with a previous study using Tarkowski's method, where the best success rate of karyotyping unfertilised oocytes was 45.9% [45].

Overall, the laser ZP dissection and first polar body biopsy results suggest an absence of effects on oocyte development. There is still, of course, a need for well-design randomized controlled trials of PCS, in accordance with the Preimplantation Genetic Diagnosis International Society recommendations, and surveys of children conceived using assisted reproductive technology after PCS remain necessary.

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