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Rotationally Oscillating Drill (Ros-Drill©) for Mouse ICSI Without Using Mercury

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Abstract

Intracytoplasmic sperm injection (ICSI) is an important assisted reproductive technology (ART). Due to deployment difficulties and low efficiency of the earlier (conventional) version of ICSI, especially in the mouse, a piezo-assisted ICSI technique had evolved as a popular ART methodology in recent years. An important and remaining problem with this technique, however, is that it requires small amounts of mercury to stabilize the pipette tip when piezoelectric force pulses are applied. To eliminate this problem we developed and tested a completely different and mercury-free technology, called the “Ros-Drill©” (rotationally oscillating drill). The technique uses microprocessor-controlled rotational oscillations on a spiked micropipette without mercury or piezo. Preliminary experimental results show that this new microinjection technology gives high survival rate (>70% of the injected oocytes) and fertilization rate (>80% of the survived oocytes), and blastocyst formation rates in early trials (~50% of the survived oocytes). Blastocysts created by Ros-Drill© ICSI were transferred into the uteruses of pseudopregnant surrogate mothers and healthy pups were born and weaned. The Ros-Drill© ICSI technique is automated and therefore; it requires a very short preliminary training for the specialists, as evidenced in many successful biological trials. These advantages of Ros-Drill© ICSI over conventional and piezo-assisted ICSI are clearly demonstrated and it appears to have resolved an important problem in reproductive biology.

Keywords

ICSI; microinjection; assisted reproduction; micromanipulation

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INTRODUCTION

The mouse is an important animal model in biological and biomedical research. Intracytoplasmic sperm injection (ICSI) has been a crucial micromanipulation technique for assisted reproduction in the mouse (Yanagimachi, 2005; Li and Lloyd, 2006). Conventional ICSI employs a spiked micropipette for facilitating penetration of the zona pellucida and piercing of the oolemma to enable penetration into the ooplasm and injection of whole sperm into the ovum. However, the elasticity of the mouse oolemma makes it very difficult to penetrate without rupture, causing irrevocable damage to the ovum. Further, injecting the whole sperm (head, neck, and tail piece) undesirably results in deposition of an excessive amount of medium in the ooplasm. These factors make conventional ICSI very inefficient and difficult to perform successfully in the mouse (Ahmadi et al., 1995; Lacham-Kaplan and Trounson, 1995; Ron-El et al., 1995).

Assisted ICSI using piezoelectric technology (known as *piezo-assisted ICSI*) in the mouse was introduced in 1995 (Kimura and Yanagimachi, 1995). The piezo drill system produces mechanical pulses that travel longitudinally along the ICSI pipette and create vibrations at its tip. Furthermore in ICSI, injection of the sperm head is all that is required to successfully fertilize oocytes and promote subsequent embryo development (Kuretake et al., 1996). Earlier mentioned piezo-induced mechanical vibrations can also be utilized for this objective to effectively separate sperm heads from tails at the neck region. In addition, piezo-assisted ICSI facilitates piercing of the zona pellucida and oolemma and penetration with a flat-tipped (not spiked) micropipette. Although much more efficient than conventional ICSI, in current practice, piezo-assisted ICSI requires the presence of a small amount of mercury to stabilize and suppress undesired lateral vibrations of the pipette tip under mechanical impact (Ediz and Olgac, 2004, 2005; Ergenc and Olgac, 2007). To date, mercury is the only known fluid with sufficiently high density necessary to mitigate these effects and prevent ICSI-induced damage to the ovum, thereby enhancing the drilling efficiency of piezo-assisted ICSI in the mouse.

Unfortunately, mercury is a toxic substance of health and environmental hazard which makes its use in the laboratory very problematic. Despite this, ICSI remains to be a very important technology for researchers in transgenic laboratories around the world. It is broadly applied to maintaining and rescuing valuable genetically-altered mutant mouse strains (Li et al., 2003, 2007; Li and Lloyd, 2006). However, at many institutions, state and local regulations ban the use of mercury for obvious reasons. Since mercury is indispensable in the piezo-assisted ICSI technology especially in the mouse, development of an efficient mercury-free alternative is very much needed. Such a technique must accomplish three goals: It should (1) easily separate the sperm head from the tail; (2) enable the ICSI pipette to efficiently pierce and penetrate the zona pellucida and oolemma, and (3) facilitate injection of the sperm head without damage to the ovum.

In this study, we present the successful development of a novel, rotationally oscillating drill (Ros-Drill©) device. It does not require even a small amount of mercury or a piezoelectric force actuator to drive a micropipette for performing ICSI in the mouse. Preliminary results of our testing in mouse embryos showed that Ros-Drill-assisted ICSI gives survival,

fertilization, embryo development, birth, and weaning rates comparable to those of piezo-assisted ICSI using mercury. Another important benefit of the new technology is related to its minimal demand on human expertise, and very short training periods for the operators. These features result from the computer automated nature of the Ros-Drill© methodology as explained in the recent technology article (Ergenc and Olgac, 2007).

MATERIALS AND METHODS

Rotational Oscillatory Drill (Ros-Drill©) Design

It has been observed that mouse zona penetration can be easily accomplished without assistance using a beveled and spiked micropipette. Here, our aim was to build a drill that could rotationally oscillate the micropipette at a desired frequency and angular amplitude both to enable sperm head separation from the tail and to facilitate oolemma piercing and penetration. Due to the fact that a perfectly straight pipette is impossible to be manufactured even using the most advanced fully automatic pullers, some eccentricity and consequent whirling motion during rotational oscillation of the pipette is unavoidable. Having said that, to minimize the eccentricity induced lateral displacement, it (the pipette) was rotationally oscillated with very small angular amplitudes (e.g., 1° peak-to-peak) and at frequencies (e.g., 500 Hz) which are higher than the sensitive natural frequencies of the pipette (e.g., about 100 Hz for the first two modes, as expressed in Ediz and Olgac, 2004). Furthermore, the response characteristics of biological cells to harmonic excitation are expected to decay rapidly for the frequencies exceeding 100 Hz. That is, far the membrane can respond to Ros-Drill© oscillations at frequencies less than 100 Hz, but not as the frequency approaches and exceeds 500 Hz level. Consequently, the relative displacement between the pipette tip and the membrane becomes considerably large for high frequencies enabling better piercing performance (i.e., cleaner, faster piercing with minimum damage to the cell).

A schematic representation of the Ros-Drill© microinjector is shown in Figure 1, and a photograph of the prototype is shown in Figure 2. The pipette holder was placed in precision bearings, which were embedded within the housing. A flexible coupling containing a channel to accommodate injection tubing was attached between the pipette holder and a micro-motor (in our present prototype it is a precision DC-servo motor). The coupling transmits the angular motion from the motor to the pipette holder and also prevents axial misalignment. This DC micro-motor was energized via a linear amplifier (driver). The control signal was generated by a digital controller. The reference signal for rotational oscillations of the pipette tip was harmonic $A \sin(2\pi f t)$, where A (°) is the amplitude, f (Hz) is the frequency of the oscillations, and t is the time (sec). A pure harmonic reference trajectory was purposely selected, so that unnecessary excitation of the natural vibration modes of the pipette was avoided. This trajectory is easy to generate and implement. Detailed technical information about the Ros-Drill© can be found in our recent publication (Ergenc and Olgac, 2007).

The Ros-Drill© was designed to operate in two different modes: (1) high rotational amplitude at low frequency for separating sperm heads from tails, and (2) low rotational amplitude at high frequency for piercing and penetrating the oolemma.

Reagents and Media

KSOM^{AA} medium (Biggers et al., 2000), FHM medium (Lawitts and Biggers, 1993) and fetal bovine serum (FBS) were purchased from Specialty Media (Phillipsburg, NJ). Polyvinyl pyrrolidone (PVP) was purchased from Irvine Scientific (Santa Ana, CA), and mercury from Fisher Scientific (Pittsburgh, PA). All other chemicals including the ones for preparation of Hepes-CZB (HCZB) medium (Nagy et al., 2003), and bovine testis hyaluronidase, pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were purchased from Sigma Chemical Co. (St. Louis, MO). The analgesic Buprenex was purchased from Western Medical Supply, Inc. (Arcadia, CA). Na-EGTA medium was made from Tris-buffered EGTA solution containing 10 mM Tris, 50 mM NaCl, and 50 mM EGTA, pH 8.0.

Animals

Six to 8 weeks old female and 8–10 weeks old male B6D2F1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as oocyte and sperm donors, respectively. Eight to 10 weeks old CD1 mice from Charles River Laboratories, Inc. (Wilmington, MA) were used to produce vasectomized males and pseudopregnant recipients for embryo transfer. All mice were housed in individually ventilated plastic cages (BioZone, Inc., Fort Mill, SC) with bedding made from reclaimed wood pulp (Absorption Corporation, Bellingham, WA) in a specific pathogen free barrier facility with light cycle 14 hr light and 10 hr dark according to standard operating procedures of the University of California, Davis. Mice were fed ad libitum with food purchased from LabDiet (Richmond, IN), and were allowed free access to deionized and autoclaved water. Mouse euthanasia was carried out by CO₂ asphyxiation followed by cervical dislocation. The care, use, and disposition of all mice used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

There is an important point we wish to raise on the experimental work reported in this article. The tests using Ros-Drill were mostly performed by the second author (a biologist) who was trained on the new technology for less than 2 days. Similar tests (for ICSI only, but not for embryo transfer) were conducted by the first author (a controls engineer) at the University of Connecticut facilities efficiently. These experiments demonstrated the ease of adopting the skills, especially for a biologist which will benefit the scientific community considerably in the long run.

Sperm Head Preparation

Sperm were collected from the caudal epididymids into HCZB or Na-EGTA medium. To compare the effect of the method of sperm head preparation on ICSI outcome, sperm heads were separated from sperm tails using three different methods: (1) freezing 100 µl of sperm suspension in HCZB medium in liquid nitrogen for 1 min followed by thawing in a water bath at 37°C for 1 min; (2) freezing 100 µl of sperm suspension in Na-EGTA medium in liquid nitrogen for 1 min followed by thawing in a water bath at 37°C for 1 min; and (3) applying bi-directional rotational oscillating pulses (pulse duration 6 ms, frequency 50 Hz, amplitude 2°) using the Ros-Drill® in 10% PVP in HCZB medium to the sperm middle piece near the neck region after the sperm tail was aspirated into the ICSI pipette as shown

in Figure 3. Using the latter technique, on average the sperm head was separated from the tail within a few seconds. Sperm heads prepared by freeze-thaw were kept on ice for 1–2 hr before use, and sperm heads (5–10 in each group) isolated using the Ros-Drill© pipette were used immediately after preparation.

Superovulation and ICSI

Female mice were superovulated by the injection of 5 IU PMSG intraperitoneally (i.p.), followed 46–48 hr later by injection of 5 IU hCG, i.p. Mice were sacrificed 14–15 hr later after hCG injection, and oocyte-cumulus complexes (OCCs) were collected from oviducts into warm FHM medium. Cumulus cells were dissociated from OCCs by treatment with 300 U/ml bovine testis hyaluronidase in FHM medium for about 5 min at room temperature. Oocytes were washed in preequilibrated KSOM^{AA} medium, and then cultured in this medium at 37.5°C in humidified 5% CO₂ and 95% air prior to ICSI. Oocytes were used for ICSI within 3 hr after collection.

Ros-Drill ICSI was performed using human ICSI pipettes (diameter 7 µm) from The Pipette Company-TPC (model LICR-ST) on an inverted Nikon TE300 microscope with Nomarski differential interference contrast (DIC) optics at room temperature (Fig. 4). After washing in 10% PVP in HCZB, 5–10 sperm heads prepared using the methods described above were loaded into an ICSI pipette with appropriate spacers of 10% PVP in HCZB. ICSI was performed in a drop of HCZB containing 10% FBS (Suzuki and Yanagimachi, 1997).

The oocyte was held at the 9 o'clock position so that the metaphase II spindle was at either the 12 or 6 o'clock position. The zona could be easily penetrated using the spiked micropipette without the assistance of rotational oscillation, so Ros-Drill© pulses were not applied to penetrate the zona. After zona penetration at the 3 o'clock position, the ICSI pipette was advanced against the oolemma toward the opposite side of the oocyte. The oolemma then was gently aspirated into the pipette tip while a series of rotational oscillations (frequency 500 Hz, amplitude 0.3°) using the Ros-Drill© was applied until the oolemma was pierced and the ovum penetrated. The sperm head then was injected into the ooplasm with a minimum volume of medium. On average, sperm heads could be injected into a group of 10–15 oocytes within a period of 10–20 min. The procedure is shown in Figure 5.

Piezo-assisted ICSI was also performed in HCZB medium containing 10% FBS on the same inverted Nikon TE300 microscope with DIC optics at room temperature using a PMM controller (Prime Tech, Ibaraki, Japan). The diameter of the ICSI pipette is 7 µm loaded with mercury (Li et al., 2007). The zona was penetrated at the 3 o'clock position by applying several piezo-pulses (intensity 2–4, speed 3). The zona piece was expelled into the perivitelline space and the injection pipette was advanced against the oolemma to the opposite side of the oocyte. The oolemma was punctured by applying 1 weak piezo pulse (intensity 1–2, speed 1) and the ovum penetrated, allowing for injection of the sperm head into the ooplasm with a minimum volume of medium.

Embryo Culture and Embryo Transfer

Injected oocytes were washed and incubated in equilibrated KSOM^{AA} medium (50 µl drops under mineral oil) in humidified 5% CO₂ and 95% air at 37.5 C for 4 days. For in vitro experiments, embryos were graded for stage of development every 24 hr after ICSI. For in vivo experiments, blastocysts on Day 4 were transferred into the uteruses (4–6 embryos each horn) of pseudopregnant CD-1 female mice (2.5 days post-coitum with vasectomized males) anesthetized with 2.5% Avertin. Recipients were kept warm on a heating pad until fully recovered from anesthesia. Before the recovery, 0.1 ml of 0.03 mg/ml Buprenex was injected subcutaneously to provide post-operative analgesia.

Statistical Analysis

Unordered and singly ordered contingency tables were analyzed with the Exact Fisher and the exact Kruskal–Wallis test, respectively. Stratified 2 × 2 and 2 × 5 contingency tables were analyzed by the methods described in the manual of StatXact 8. All computations were done using StatXact 8 (Cytel, Inc., Cambridge, MA).

RESULTS

Comparison of Ros-Drill© ICSI and Piezo-Assisted ICSI

To compare results obtained using the Ros-Drill© ICSI and piezo-assisted ICSI techniques, three replicates were performed by the same operator using the same samples of sperm heads isolated by freeze-thaw in Na-EGTA medium. The results from each of the three replicates can be considered to be a set of three stratified contingency tables, the first for the ovum survival data, the second for the fertilization rate data, and the third for the embryo development data.

Ovum survival—A test to determine whether the odds-ratios of the three 2 × 2 contingency tables are the same shows they are homogeneous ($P = 0.288$). The results in the three tables have therefore been combined (Table 1). The percentage of ova that survived when the sperm heads were injected using Ros-Drill© ICSI was 81.9% (95/116), while the percentage that survived using piezo-assisted ICSI was 95.5% (106/11). These two percentages are significantly different (Exact Fisher test: $P = 0.0015$), showing that ovum survival rate was slightly lower when Ros-Drill© ICSI was used.

Fertilization rate—A test to determine whether the odds-ratios of the three 2 × 2 contingency tables are the same shows they are homogeneous ($P = 1$). The results in the three tables have therefore been combined (Table 2). The percentage of injected ova that survived and developed into two-cell embryos (an estimate of the fertilization rate) using Ros-Drill© ICSI was 87.4%, while the fertilization rate when piezo-assisted ICSI was used was 97.2%. The two percentages are significantly different (Exact Fisher test: $P = 0.032$), showing that the fertilization rate was slightly lower when Ros-Drill© ICSI was used.

Embryo development—The Cochran–Mantel–Haenszel test on singly ordered variables of the three stratified 2 × 5 contingency tables shows the distributions of the stages of embryo development reached in each replicate are similar ($P = 0.241$). The results have

therefore been combined in Table 3. Comparison of the distributions of embryos at different stages of development after using Ros-Drill© ICSI or piezo-assisted ICSI just reached significance at the $P = 0.05$ level (Kruskal–Wallis test: $P = 0.050$). Inspection of Table 3 shows that more compacted morulae developed into blastosysts when the piezo-assisted ICSI procedure (76/81; 93.8%) was used while fewer morulae developed when the Ros-Drill© ICSI procedure was used (47/59; 79.7%). These percentages are significantly different ($P = 0.0005$).

Comparison of Sperm Head Isolation Methods for Ros-Drill© ICSI

Freeze-thawing sperm in liquid nitrogen and 37°C water bath is a simple method to prepare mouse sperm heads for ICSI, and even sperm can stand repeated freezing and thawing in normal culture medium for up to 10 times without losing their viability (Aoto et al., 2007). To know if the Na-EGTA medium is better than HCZB medium for protecting sperm during freeze-thaw, sperm were subjected to freeze-thaw in HCZB and Na-EGTA, respectively. Three replicates were done by the same operator for each method of sperm head preparation as described previously. The Cochran–Mantel–Haenszel test on singly ordered variables of the three stratified 2×5 contingency tables shows the distributions of the stages of embryo development reached in each replicate are similar ($P = 0.738$). The three contingency tables have therefore been pooled and the results are shown in the first two lines of Table 4. There is no difference in the distributions of the stages of embryonic development reached after 96 hr of culture resulting from the two methods of sperm head preparation (Kruskal–Wallis test: $P = 0.531$).

After trying different amplitudes and frequencies of the Ros-Drill©, it was found that the drill can also be used to separate the mouse sperm heads (by performing for a duration of 6 ms, controlled oscillations of frequency 50 Hz, and amplitude of 2°). The data are shown also in the third line of Table 4. When these data are included in the above statistical analysis the distributions in embryonic development become statistically different (Kruskal–Wallis test: $P = 0.037$). It must be remembered, however, that the results with the Ros-Drill were obtained in a separate experiment. Nevertheless the results suggest that the Ros-Drill is just as effective, if not better, than other methods in removing sperm heads.

Development of Blastocysts to Term In Vivo

Some of the blastocysts obtained by using sperm heads separated using freeze-thaw in Na-EGTA, freeze-thaw in HCZB and by the Piezo-Drill (Fig. 6) were transferred into the uterus of pseudopregnant CD-1 recipients and allowed to develop to term (Fig. 7). The results are summarized in Table 5. The rates of pups born using the three methods were not significantly different ($P = 0.232$). All (100%) of the born pups in each group of experiments were weaned.

DISCUSSION

In this report, we present the development and testing of a new, mercury-free, assisted-ICSI technology in the mouse using a spiked micropipette which is driven by a microprocessor-controlled rotationally oscillating mechanism, the Ros-Drill©. While the experimental

results presented here are preliminary, they show that this new microinjection technology can result in high survival rate (>70% of the injected oocytes) and fertilization rate (>80% of the survived oocytes), although the blastocyst formation rate (~50% of the survived oocytes) was lower than that using piezo-assisted ICSI (70–80%). Blastocysts derived by Ros-Drill© ICSI successfully developed to term after being transferred into pseudopregnant recipients. All pups born were healthy and successfully weaned, demonstrating that the Ros-Drill© ICSI technique has no untoward or detrimental effects on the health and well-being of mice.

Our preliminary results compare far better than those obtained after conventional ICSI, and similar to the results obtained using piezo-ICSI. For example, while the survival and fertilization rates of injected ova are high (both >80%), the rates are somewhat less than those observed using piezo-ICSI. In addition, the rate of development of compacted morula to blastocysts is slightly less using Ros-Drill© ICSI than that seen using piezo-ICSI. While the reasons for these differences are not fully known at this time, our results strongly justify further refinement and development of this technology-based procedure for application as a reliable, artificial reproduction technique.

Our study also demonstrates that the Ros-Drill© and freeze-thawing can be used to effectively separate sperm heads from the tail at the midpiece. Thus the need for mercury in the injection pipette to isolate sperm heads has been eliminated. So far the effects of freeze-thaw and the Ros-Drill© have not been associated with any developmental defects. Further work on this question is necessary, as well as optimization of the procedures.

In summary, the Ros-Drill© is an easy-to-use and effective device for assisted-ICSI in the mouse. An embryo micro-manipulation specialist can be trained to use this technique in a very short period of time. For example, the lead author (a control engineer) developed the device which was then used and tested by the second author (a biologist) after a very short training period (1.5 days). The authors believe that embryo survival and development rates can be enhanced further with continuing efforts to improve Ros-Drill© assisted ICSI.

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Fig. 1.
The Ros-Drill© assembly and control system. [See color version online at www.interscience.wiley.com.]

Fig. 2.
The Ros-Drill© prototype. [See color version online at www.interscience.wiley.com.]

Fig. 3. Sperm head separation using the Ros-Drill©. [See color version online at www.interscience.wiley.com.]

Fig. 4.
The Ros-Drill©-assisted ICSI setup. [See color version online at www.interscience.wiley.com.]

Fig. 5. Intracytoplasmic sperm injection (ICSI) using the Ros-Drill©: (a) alignment of the injection pipette at 3 O'Clock of the oocyte, sperm head is at the tip of the pipette, (b) pipette is advanced to pierce the zona without applying rotational oscillating pulses, (c) pipette is advanced to the opposite side of the oocyte, and Ros-Drill© was activated while a slight negative pressure was applied, (d) oolema was pierced and pipette penetrates the ooplasm, (e) sperm is injected with minimum amount of accompanying medium, (f) pipette is withdrawn gently. [See color version online at www.interscience.wiley.com.]

Fig. 6.
Blastocysts derived by Ros-Drill© ICSI.

Fig. 7. B6D2F2 pups (8 days old) derived by Ros-Drill© ICSI using sperm heads separated by rotational oscillating pulses. [See color version online at www.interscience.wiley.com.]

TABLE 1

Number of Ova That Survived Injection of Sperm Heads Using Either Ros-Drill-ICSI or Piezo-ICSI

Method	Survivors (%)	Nonsurvivors (%)	Total (%)
Ros-Drill-ICSI	95 (81.9)	21 (18.1)	116 (100)
Piezo-ICSI	106 (95.5)	5 (4.5)	111 (100)
Total	201 (88.6)	26 (11.5)	227 (100)

The sperm heads were separated from sperm tails by freeze-thawing in Na-EGTA medium.

Exact Fisher test: $P = 0.013$.

TABLE 2

Number of Two-Cell Embryos That Developed From Ova After Injection of Sperm Heads Using Either Ros-Drill-ICSI or Piezo-ICSI

Method	Developers (two-cells, %)	Nondevelopers (%)	Total (%)
Ros-Drill-ICSI	83 (87.4)	12 (12.6)	95 (100)
Piezo-ICSI	103 (97.2)	3 (2.8)	106 (100)
Total	186 (92.5)	15 (7.5)	201 (100)

Exact Fisher test: $P = 0.013$.

TABLE 3

Development of Embryos (Two-Cell Stage Through Blastocysts) After 96 hr of In Vitro Culture After Injection of Sperm Heads Using Either Ros-Drill-ICSI or Piezo-ICSI

Method	2-Cell embryos (%)	3-4-Cell embryos (%)	Noncompacted morulae (%)	Compacted morulae (%)	Blastocysts (%)	Total (%)
Ros-Drill ICSI	3 (3.6)	9 (10.8)	12 (14.5)	12 (14.5)	47 (56.6)	83 (100)
Piezo ICSI	7 (6.8)	8 (7.8)	7 (6.8)	5 (4.9)	76 (73.8)	103 (100)
Total	10 (5.4)	17 (9.1)	19 (10.2)	17 (9.1)	123 (66.1)	186 (100)

Exact Kruskal–Wallis test: $P = 0.05$.

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TABLE 4

Development of Embryos (Two-Cell Stage Through Blastocysts) After 96 hr of In Vitro Culture After Injection of Sperm Heads Using Ros-Drill ICSI

Method	2-Cell embryos	3-4-Cell embryos	Noncompacted morulae	Compacted morulae (%)	Blastocysts (%)	Total embryos (%)
HCZB	3 (3.8)	7 (8.8)	11 (13.8)	21 (26.3)	38 (47.5)	80 (100)
Na-EGTA	3 (3.6)	9 (10.8)	12 (14.5)	12 (14.5)	47 (56.6)	83 (100)
Ros-Drill	2 (6.5)	1 (3.2)	3 (9.7)	5 (16.1)	20 (64.5)	31 (100)

Sperm heads were isolated by freeze-thaw in HCZB medium, Na-EGTA medium, or using rotational oscillating (Ros-Drill) pulses. Exact Kruskal–Wallis test: $P = 0.037$.

TABLE 5

Number of Pups Born and Weaned After Embryo Transfer of Blastocysts Derived From Embryos Derived by Injection of Sperm Heads Using Ros-Drill ICSI

Sperm head preparation	No. blastocysts transferred	No. pups born (%)	No. pups weaned (%)
Freeze-thaw in HCZB	36	9 (25)	9 (100)
Freeze-thaw in Na-EGTA	47	20 (43)	20 (100)
Rotational oscillations	20	6 (30)	6 (100)

Sperm heads were isolated by freeze-thaw in HCZB medium, Na-EGTA medium, or using rotational oscillating (Ros-Drill) pulses.

Exact Fisher test: $P = 0.232$.