

# Comparison of Embryo Development between Intracytoplasmic and Piezo-Assisted Sperm Injection after Treating Mouse Sperms by Ca<sup>2+</sup> Ionophore

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## ABSTRACT

**Background:** The purpose of this study was to evaluate the efficiency of intracytoplasmic sperm injection (ICSI) and Piezo-assisted sperm injection after pretreatment with calcium ionophore (CaI) on the mouse embryo development. **Methods:** In this study, the conventional ICSI and Piezo-ICSI procedures were used. The efficacy of the methods was examined after mouse matured oocytes were fertilized with or without CaI-treated sperms. **Results:** Piezo-ICSI demonstrated significantly more favorable results, with a fertilization rate of 64% (conventional ICSI: 42%,  $P<0.001$ ) and a cleavage rate of 73% (conventional ICSI: 58%,  $P<0.05$ ). When the Piezo-ICSI procedure was performed with CaI-pretreated sperms, the cleavage rate significantly increased (92% vs. 73%,  $P<0.05$ ). However, the fertilization rate did not change significantly (64% vs. 56%). **Conclusion:** The Piezo-ICSI accompanies with CaI-treated sperms is more efficient than the conventional ICSI method for fertilizing and thus obtaining more mouse embryos. *Iran. Biomed. J. 11 (4): 245-250, 2007*

**Keywords:** Intracytoplasmic sperm injection (ICSI), Piezo, Sperm activation, Embryo development

## INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a technique that involves mechanical transfer of a single sperm into the oocyte cytoplasm. For mammals, the first report on ICSI was by Uehara and Yanagimachi [1]. Since then, ICSI was established and improved for several different species. For humans, ICSI is regularly used to overcome several types of male infertility [2-4]. For mice and bovine, numerous studies have reported the birth of healthy offspring after transfer of embryos produced by ICSI [5-7].

Piezo-assisted ICSI or Piezo-ICSI consists of two steps, namely penetration of the zona pellucida alone with a Piezo-pulse and then puncturing of the oolemma with a light negative pressure without Piezo, as conventional ICSI. On the other hand, the use of a Piezo-driven pipette instead of a conventional mechanically driven pipette resulted in improved development of ICSI mouse, bovine, and

human oocytes [5, 8-10]. Mouse and human offspring have been successfully obtained from embryos developed after ICSI, using a Piezo micromanipulator (PMM-MB-A: Prime Tech Ltd., Tsuchiura, Ibaragi, Japan) [5, 11].

The acrosome reaction involves the fusion of the outer acrosomal membrane with the plasma membrane and is believed to be essential for fertilization. In mammals, these final changes before fertilization take place at the zona pellucida [12, 13]. Some approaches, such as chemical pretreatment of spermatozoa before ICSI have recently been shown to improve the developmental potential of ICSI oocytes. Treatment of semen with compounds such as heparin, pentoxifylline, bovine serum albumin, caffeine, dithiothreitol, ethanol, and lysophosphatidyl-choline have been used for the induction of an *in vitro* acrosome reaction in mammalian spermatozoa [14-18]. An alternative means of improving the *in vitro* fertilizing capacity of spermatozoa in bovine [19, 20], equine [21],

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caprine [20], ram [22], pig [23] and human [11, 24] is the use of calcium-ionophore (CaI) A23187. Pretreatment of mouse spermatozoa with CaI induces acrosome reaction [25]. As a result of their hydrophobic characteristics, CaIs are able to transport ions across membranes. The increase in free CaI within the cell directly induces the acrosome reaction and bypasses capacitation [12].

However, there is limited information on an *in vitro* induction of the acrosome reaction in semen and the outcome of different methods of *in vitro* fertilization. The present investigation was carried out to study the effect of sperm pretreatment with CaI for fertilization and embryo development following mouse ICSI and Piezo-assisted sperm injection.

## MATERIALS AND METHODS

In this study, we used conventional and piezo ICSI methods. Oocytes were obtained from 7 to 10-week-old female NMRI mouse and fertilized in three groups: group 1, conventional ICSI by non-treated sperms; group 2, Piezo ICSI by non-treated sperms and group 3, Piezo ICSI by CaI treated sperms.

**Animals.** Seven to 10-week-old female NMRI mouse and 10-12-week-old male mouse (Pasture institute of Iran, Tehran, Iran) were used as oocyte donors and sperm donors, respectively. The mice were housed in an environmentally controlled room with a 12 h dark/12 h light cycle at a temperature of  $23 \pm 2^\circ\text{C}$  and humidity of  $55 \pm 5\%$ , and given free access to a laboratory diet and filtered water. The work on animals was performed with the approval of the Ethics Board of our institution.

**Media.** The medium for collection of oocytes from oviducts and for micromanipulation was a modified T6 (HEPES-T6) containing 4 mg/ml BSA. The pH value of medium was 7.4 under 5%  $\text{CO}_2$  in air ( $37^\circ\text{C}$ ). Stock solution (5 mM) of CaI was prepared in dimethyl sulphoxide (DMSO). Working solution was prepared (1: 500) by mixing of stock solution in the medium.

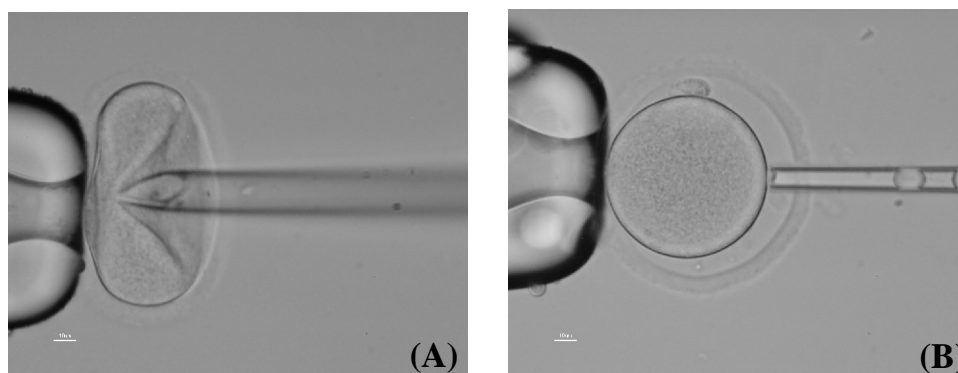
**Preparation of mouse oocytes and sperm.** Female mice underwent ovulation induction by intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotrophin (PMSG Intervet, Netherlands) followed by 7.5 IU human chorionic gonadotrophin (hCG Intervet, Netherlands) hours later. Oocytes were collected from oviducts approximately 14-16

hours after hCG injection. The oocytes were divided into two groups: one group for ICSI and the other group for Piezo-ICSI. The oocytes for ICSI/Piezo-ICSI were treated with 300 IU/ml hyaluronidase until the cumulus masses were dissociated from the oocytes by gentle pipeting. They were rinsed and incubated at  $37^\circ\text{C}$  in T6 under 5%  $\text{CO}_2$  in air before sperm injection. The cauda epididymides of male mice were cut with a pair of scissors, sperm mass was divided into two pieces: one squeezed out into a 5 ml tube containing T6 medium with 15 mg/ml bovine serum albumin (BSA, Sigma A3311, Germany) and other species sperm mass squeezed out into a 5 ml tube containing T6 medium with 15 mg/ml BSA and 10  $\mu\text{M}$  CaI (Sigma A2318-7, Germany). After incubation at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  in air for 45 min, the upper portion of the medium was collected and used for ICSI and Piezo-ICSI.

**Instruments.** An inverted microscope (TE2000-U, Nikon, Tokyo, Japan) equipped with Hoffman modulation optics (Nikon, Japan) was used. For conventional micro injection, the microinjector (IM-4B, Narishige, Tokyo, Japan) was used. A sterilized glass capillary (Borosilicate, B 100-75-10: Sutter instrument Co. Novato, CA, USA) was pulled with heating (setting: ramp Value, 500; conventional ICSI; Heat = 750, pull = 55, Vel = 55, Time = 200, Piezo-ICSI; Heat = 750, pull = 55, Vel = 55, Time = 200) using a micropipette puller (model P-97, Sutter Instrument Co., Novato, CA, USA). The needle holder for injection was attached to the drive unit of a piezo-micromanipulator (PMM-MB-A: Prime Tech Ltd, Tsuchiura, Ibaragi, Japan) was used. The drive unit of the piezo-micromanipulator was driven by a controller (PMAS-CT-140: Prime Tech Ltd).

**Method of conventional ICSI.** Just before ICSI, air at the tip of the injection needle was extruded as much as possible. After that, small amounts of mineral oil were sucked. Next, a motile spermatozoon was taken into the injection needle from drops of the sperm suspension. The spermatozoon was immobilized by pipetting. Spermatozoon injection was performed after suction of a small amount of ooplasm to ensure penetration of the oolemma (Fig. 1A).

**Method of Piezo-ICSI.** The tip of needle was flat and inner diameter of the injection needle was 5-6  $\mu\text{m}$ . A captured motile spermatozoon was immobilized by a cutter giving Piezo pulsed (speed: 8-intensity: 6) to sperm tails in 8% PVP drops.



**Fig. 1.** The procedure of conventional ICSI (A) and Piezo-ICSI (B) in mouse oocyte micromanipulation. Since mouse oolemma has high extensibility, the inserted needle may not penetrate the oolemma by ICSI. In contrast, by the vibration of Piezoelectric elements, and the zona pellucida can be penetrated without deformation by vibrating the injection needle fixed on the Piezodriver. Similarly, after the oolemma is extended by inserting the needle, the oolemma can be punctured by applying one Piezo pulse.

A head of spermatozoon was sucked into the injection needle and then the needle was allowed to penetrate only the zona pellucida while Piezo pulses were applied. The speed and intensity of pulses were 6 and 4, respectively. Then the needle was allowed to penetrate deeply into the ooplasm without applying Piezo pulses and when the oolemma extended sufficiently it was punctured with one pulse (speed = 2 and intensity = 1) (Fig. 1B). After insemination and injection (conventional and Piezo-ICSI) with normal and CaI activated sperms, mouse oocytes were incubated in T6 for 6-8 h. Fertilized oocytes were cultured in T6 medium. Embryos were examined for 24 h to evaluate survival, fertilization and cleavage rates.

**Statistical analysis.** Statistical analysis was performed by using the  $X^2$ -test with significance reported when  $P < 0.05$ .

## RESULTS

To assess the efficiency of Piezo procedure on survival and fertilization of mouse oocytes, sperms were injected by conventional ICSI and Piezo-ICSI. Results were recorded in both groups after 6 h and

24 h (Table 1). The survival rate of oocytes after 6 hours was 42% in conventional ICSI, 56% with  $Ca^{2+}$  and 64% without it in Piezo-ICSI groups which this difference was statistically significant between conventional and Piezo-ICSI (with and without CaI treated sperms) ( $P < 0.05$ ).

To evaluate CaI on fertilization and cleavage rate, the sperms were treated with CaI, before Piezo-ICSI. Fertilization rate after Piezo-ICSI was 40% (28/70) in CaI treated group and 39.5% (30/76) in the non-treated group which was not statistically significant ( $P > 0.05$ ). The cleavage rate, 48 hours after hCG injection, was statistically higher in the treated group comparing to the non-treated group in Piezo-ICSI (92.9% vs. 73.3%, respectively) ( $P < 0.05$ ).

## DISCUSSION

In mice, the plasma membrane has high extensibility and ooplasm has low viscosity. As a result, the secure injection of the spermatozoon into the oocyte was considered difficult. In conventional ICSI, an injection needle penetrates the oolemma through the zona pellucida, which causes considerable deformation of the zona.

**Table 1.** Results of conventional and Piezo-ICSI.

Groups	No. of injected oocytes	Survived oocytes after 6 h	No. of survived Fertilized oocytes	No. of 2 to 4-cell embryos after 24 h
Conventional ICSI	88	37 (42) <sup>b*</sup>	12 (32)	7 (58) <sup>b*</sup>
Piezo-ICSI (non-treated sperms)	117	76 (64) <sup>a*</sup>	30 (39)	22 (73) <sup>c*</sup>
Piezo-ICSI (CaI treated sperms)	125	70 (56)	28 (40)	26 (92)

<sup>a</sup>comparison between Piezo-ICSI (non-treated sperm) and conventional ICSI; <sup>b</sup>comparison between conventional ICSI and Piezo-ICSI (CaI treated sperm); <sup>c</sup>comparison between Piezo-ICSI (non-treated sperm) and Piezo-ICSI (CaI treated sperm); \*,  $P < 0.05$ . Values in parentheses are percentage.

This deformation may increase the internal pressure of the oocyte, induce the emission of ooplasm from the oocyte after extraction of the needle, and contribute to oocyte death. Since oolemma has high extensibility, the inserted needle may not penetrate the oolemma [5]. Here, we report Piezo-ICSI as a better procedure than conventional ICSI for mouse oocyte fertilization to have 2 to 4-cell embryos. First report on Piezo-ICSI was published by Kimura and Yanagimachi [5], who used mice as subjects and then by Huang *et al.* [26] to humans. Yanagida *et al.* [11] applied this method to human ICSI, in comparison to conventional ICSI and observed a significant increase in survival and fertilization rates.

Our improvements in survival rate were, according to Kimura and Yamagimachi [5] due to the fact that the extended oolemma agglutinates more securely after extraction of the needle and the internal pressure of the ooplasm is lowered due to less deformation. In addition, oocytes are sufficiently activated by sperm factor brought in by an injected spermatozoon [27, 28]. Improvements in the fertilization rate were considered due to the elimination of failures in the injection of spermatozoa, as spermatozoa were injected only after the puncture of oolemma by the needle was confirmed. An injection needle with a flat tip is used in Piezo-ICSI. Recently, microdynamics of the Piezo-driven pipettes in ICSI were reported [29].

The mechanism of smooth puncture of the zona pellucida and oolemma by the needle seems to be as follows: the needle vibrates back and forth by taking Piezo pulses. At this point, since inertia of mercury at the tip of the needle is high, rapid variation in internal pressure is caused in the lumen from mercury to the tip, and by pressing the flat tip of the needle against the zona pellucida, the zona is perforated in the shape of the tip lumen. Kawase *et al.* [10] used partial zona pellucida incision by Piezo-micromanipulator to increase the *in vitro* fertilization of frozen-thawed mouse sperms. Deep insertion of the needle into the ooplasm by hand causes no rupture of the oolemma in ~82% of metaphase II oocytes [11], but applying one Piezo pulse to such oocytes causes rupture of the oolemma by variation in the internal pressure at the tip of the needle. Once the needle breaks the oolemma, vibration is transmitted to an oocyte.

Because the vibration added is one Piezo pulse and no suction of ooplasm is conducted at the sperm injection, it is thought that the damage caused by Piezo-ICSI is much less than that by conventional ICSI. Grinding and spiking of the tip of the needle

are not necessary in Piezo-ICSI, which is considered an advantage because it reduces the amount of preparation required by researchers. In conventional ICSI, immobilization of motile spermatozoa to be injected is conducted by abrasion of sperm tails or pipetting, while in Piezo-ICSI it can be conducted relatively easily by applying several Piezo pulses to sperm tails.

With regard to embryonic development after ICSI, since no suction of ooplasm is conducted in Piezo-ICSI, it was observed more 2 to 4-cell embryos in Piezo-ICSI due to slight influence on the cytoskeleton. However, no difference was recognized between the two groups by grade classification of the embryos after injection in human [11]. On the other hand, pregnancy rates were improved by Piezo-ICSI, which is considered due to the higher number of transferred oocytes per human embryo transfer that were obtained from higher survival and fertilization rates in Piezo-ICSI [11].

In addition, our results indicated the rate of developed embryos by using Piezo-ICSI with CaI-activated sperms, was statistically higher than untreated sperms. These findings are in agreement with others [30, 31]. Calcium Ionophore is a known inducer substance for acrosome reaction and it significantly enhanced acrosome reaction rates in culture dishes in a concentration dependent manner without any effect on sperm motility [32, 33]. Also, we did not observe significant enhancement in total motility of sperms in presence of CaI in comparison to control group in our previous work (unpublished data). Induction of acrosome reaction after CaI treatment has been reported on many mammals as mentioned before. Moreover, CaI was the most effective acrosome reaction inducer as compared with progesterone and follicular fluid [34]. Better embryo development with CaI-treated sperms may be due to the effect of CaI in enhancement of sperm capacitation and accelerating in forming male pronucleus.

In conclusion, stable and favorable fertilization results can be obtained by Piezo-ICSI which is a promising method for obtaining improved ICSI outcome especially by using CaI-treated sperms which may increase the fertilization rate.

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## REFERENCES

1. Uehara, T. and Yanagimachi, R. (1976) Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol. Reprod.* 15: 467-470.
2. Dozortsev, D., De Sutter, P., Rybouchkin, A. and Dhont, M. (1998) Timing of sperm and oocyte nuclear progression after intracytoplasmic sperm injection. *Hum. Reprod.* 10: 3012-3017.
3. Palermo, G.D., Joris, H., Devroey, P., Van Steirteghem, A. (1992) Pregnancies after intracytoplasmic sperm injection of a single spermatozoon into oocytes. *Lancet* 340: 17-18.
4. Suttner, R., Zakhartchenko, V., Stojkovic, P., Muller, S., Alberio, R., Medjugorac, I., Brem, G., Wolf, E. and Stojkovic, M. (2000) Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pretreatment and injection technique. *Theriogenology* 54 (6): 935-948.
5. Kimura, Y. and Yanagimachi, R. (1995) Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* 52: 709-720.
6. Perry, A.C.F., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y. and Yanagimachi, R. (1999) Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 284: 1180-1183.
7. Horiuchi, T., Emuta, C., Yamauchi, Y., Oikawa, T., Numabe, T. and Yanagimachi, R. (2002) Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: a methodological approach. *Theriogenology* 57 (3): 1013-1024.
8. Katayose, H., Yanagida, K., Shinoki, T., Kawahara, T., Horiuchi, T. and Sato, A. (1999) Efficient injection of bull spermatozoa into oocytes using a Piezo-driven pipette. *Theriogenology* 52 (7): 1215-1224.
9. Takeuchi, S., Minoura, H., Shibahara, T., Shen, X., Futamura, N. and Toyoda, N. (2001) Comparison of Piezo-assisted micromanipulation with conventional micromanipulation for intracytoplasmic sperm injection into human oocytes. *Gynecol. Obstet. Invest.* 52 (3): 158-162.
10. Kawase, Y., Aoki, Y., Kamada, N., Jishage, K. and Suzuki, H. (2004) Comparison of fertility between intracytoplasmic sperm injection and *in vitro* fertilization with a partial zona pellucida incision by using a Piezo-micromanipulator in cryopreserved inbred mouse spermatozoa. *Contemp. Top Lab. Anim. Sci.* 43 (1): 21-25.
11. Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Konnai, K. and Sato, A. (1999) The usefulness of a Piezo-micromanipulator in intracytoplasmic sperm injection in humans. *Hum. Reprod.* 14 (2): 448-453.
12. First, N.L. and Parrish, J.J. (1987) *In vitro* fertilization of ruminants. *J. Reprod. Fertil.* 34: 151-165.
13. Bleil, J. (1991) Sperm receptors of mammalian eggs. In: *Element of Mammalian Fertilization*. (Wassermann, P.M. ed.), CRC Press, Boston, USA, pp. 133-151.
14. Aparicio, N.J. (1979) Therapeutical use of pentoxiphylline in disturbed male fertility. *Singapore Med. J.* 20 (suppl 1): 43-51.
15. Niwa, K. and Ohgoda, O. (1988) Synergistic effect of caffeine and heparin on *in vitro* fertilization of cattle oocytes matured in culture. *Theriogenology* 30: 733-741.
16. Ikumi, S., Asada, M., Sawai, K. and Fukui, Y. (2003) Effect of activation methods for bovine oocytes after intracytoplasmic injection. *J. Reprod. Dev.* 49 (1): 37-43.
17. Fiedler, K. and Wurfel, W. (2004) Effectivity of heparin in assisted reproduction. *Eur. J. Med. Res.* 9 (4): 207-214.
18. Tian, J.H., Wu, Z.H., Liu, L., Cai, Y., Zeng, S.M., Zhu, S.E., Liu, G.S., Li, Y. and Wu, C.X. (2006) Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from *in vitro*-matured oocytes and intracytoplasmic sperm injection. *Theriogenology* 66 (2): 439-448.
19. Smith, M., Peterson, R.N. and Russel, L.D. (1983) Penetration of zona-free hamster eggs by boar sperm treated with ionophore A23187 and inhibition of penetration by antiplasma membrane antibodies. *J. Exp. Zool.* 225 (1): 157-160.
20. Pereira R.J., Tuli, R.K., Wallenhorst, S. and Holtz, W. (2000) The effect of heparin, caffeine and calcium ionophore A23187 on *in vitro* induction of the acrosome reaction in frozen-thawed bovine and caprine spermatozoa. *Theriogenology* 54 (2): 185-192.
21. Zhang, J.J., Muzs, L.Z. and Boyle, M.S. (1991) Variations in structural and functional changes of stallion spermatozoa in response to calcium ionophore A23 187. *J. Reprod. Fertil.* 44: 199-205.
22. Shams-Borhan, G. and Harrison, R.A.P. (1981) Production, characterization, and use of ionophore induced, calcium dependent acrosome reaction in ram spermatozoa. *Gamete Res.* 4: 407-432.
23. Garcia-Rosello, E., Matas, C., Canovas, S., Moreira, P.N., Gadea, J. and Coy, P. (2006) Influence of sperm pretreatment on the efficiency of intracytoplasmic sperm injection in pigs. *J. Androl.* 27 (2): 268-275.
24. Heindryckx, B., Van Der Elst, J., De Sutter, P. and Dhont, M. (2005) Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte

- activation following diagnostic heterologous ICSI. *Hum. Reprod.* 20 (8): 2237-2241.
25. Lacham-Kaplan, O. and Trounson, A. (1995) Intracytoplasmic sperm injection in mice: increased fertilization and development to term after induction of the acrosome reaction. *Hum. Reprod.* 10: 2642-2649.
  26. Huang, T., Kimura, Y. and Yanagimachi, R. (1996) The use of Piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. *J. Assist. Reprod. Genet.* 13: 320-328.
  27. Parrington, J., Swann, K., Shevchenko, V.I. Sesay, A.K. and Lai, F.A. (1996) Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* 379: 364-368.
  28. Palermo, G.D., Schlegel, P.N., Colombero, L.T., Zaninovic, N., Moy, F. and Rosenwaks, Z. (1996) Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum. Reprod.* 11: 1023-1029.
  29. Ediz, K. and Olgac, N. (2004) Microdynamics of the piezo-driven pipettes in ICSI. *IEEE Trans. Biomed. Eng.* 51 (7): 1262-1268.
  30. Olds-Clarke, P. and Segó, R. (1992a) Calcium alters capacitation and progressive motility of uterine spermatozoa from +/+ and congenic  $t^{w32}$  /+Mice. *Biol. Reprod.* 47: 629-635.
  31. Olds-Clarke, P. and Wivell, W. (1992b) Impaired transport and fertilization *in vivo* of calcium – treated sperm from +/+ and  $t^{w32}$  /+mice. *Biol. Reprod.* 47: 621-628.
  32. Aitken, R.J., Ross, A., Hargreave, T., Richardson, D. and Best, F. (1984) Analysis of human sperm function following exposure to the ionophore A23187. Comparison of normospermic and oligozoospermic men. *J. Androl.* 5: 321-329.
  33. Tesarik, J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability *in vitro*. *J. Reprod. Fertil.* 74: 383-388.
  34. Jaiswal, B.S., Eisenbach, M. and Tur-Kaspa, I. (1999) Detection of partial and complete acrosome reaction in human spermatozoa: which inducers and probes to use? *Mol. Hum. Reprod.* 5: 214-219.