

DOCTORAL THESIS

Early and late paternal effects of reactive oxygen species in semen on embryo development after intracytoplasmic sperm injection

(顕微授精後の胚発育における精液中活性酸素の早期および後期父性効果)

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Shinnosuke Kuroda

黒田 晋之介

Departement of Urology
Yokohama City University Graduate School of Medicine

横浜市立大学大学院医学研究科 泌尿器科学

(Research Supervisor: Yasushi Yumura, Associate Professor)

横浜市立大学附属市民総合医療センター生殖医療センター

(研究指導教員：湯村 寧 准教授)

(Doctoral Supervisor: Masahiro Yao, Professor)

指導教員：矢尾 正祐 教授



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Early and late paternal effects of reactive oxygen species in semen on embryo development after intracytoplasmic sperm injection

Shinnosuke Kuroda , Teppei Takeshima , Kazumi Takeshima^b, Kimitsugu Usui^a, Kengo Yasuda^a, Hiroyuki Sanjo^a, Takashi Kawahara^c, Hiroji Uemura^c, Mariko Murase^b, and Yasushi Yumura 

^aDepartment of Urology, Reproduction Centre, Yokohama City University Medical Centre, Kanagawa, Japan; ^bDepartment of Gynaecology, Yokohama City University Medical Centre, Kanagawa, Japan; ^cDepartment of Urology and Renal Transplantation, Yokohama City University Medical Centre, Kanagawa, Japan

ABSTRACT

Although reactive oxygen species in semen are associated with unfavorable results with respect to assisted reproductive technology, their effects based on the detailed stages of embryo development are unclear. We investigated the relationship between reactive oxygen species in semen and the oocyte fertilization rate, cleavage rate, and blastulation rate of intracytoplasmic sperm injection. This retrospective study enrolled 77 couples who underwent intracytoplasmic sperm injection and analyzed 887 eggs from 141 cycles of intracytoplasmic sperm injection. The reactive oxygen species level in semen was compared between the fertilized and nonfertilized groups, between the good-cleavage-embryo and non-developed-embryo groups, and between the good-quality-blastocyst and poor-quality-blastocyst groups. The cut-off level of reactive oxygen species was calculated to predict good-cleavage-embryo and good-quality-blastocyst development. The fertilization rate was 65.4%, and the mean reactive oxygen species levels were not significantly different between the fertilized and nonfertilized groups. The reactive oxygen species level was significantly higher in the non-developed-embryo group than in the good-cleavage-embryo group ($P = 0.0026$) and was significantly lower in the good-quality-blastocyst group than in the poor-quality-embryo group ($P = 0.015$). Cleavage embryos and blastocysts were divided into high- and low-reactive-oxygen-species groups using a cut-off value of 6601 and 4926 relative light units, as calculated from the receiver operating characteristic curve. The rates of good-cleavage embryos and good-quality blastocysts were lower in the high-reactive-oxygen-species group than in the low-reactive-oxygen-species group, which were both statistically significant. To conclude, reactive oxygen species in semen is considered to have an adverse effect on both the early and late stages of embryo development in intracytoplasmic sperm injection.

Abbreviations: GnRH, gonadotropin-releasing hormone; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; LPO, lipid peroxidation; NADPH, nicotinamide adenine dinucleotide phosphate; RLU, relative light units; ROC, receiver operating characteristic; ROS, reactive oxygen species

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Introduction


Reactive oxygen species (ROS) in semen, first detected by Aitken and Clarkson (1987) using chemiluminescence, are known to have adverse effects on semen quality and male fertility. A high ROS level causes DNA damage, lipid peroxidation (LPO), and induction of apoptosis in sperm cells (Agarwal et al. 2003), while a low ROS level is essential and advantageous for fertilization, including capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion (de Lamirande et al. 1997; Du Plessis et al. 2015). Adverse effects of ROS on semen parameters and clinical outcomes have been reported;

Takeshima et al. (2017) demonstrated that logarithmically transformed ROS levels in unwashed semen are inversely correlated with semen parameters, such as sperm concentration, motility, mean head amplitude, and progressive motility. Yumura et al. (2009) reported that the ROS level in semen was significantly higher in nonpregnant compared to pregnant couples with complaints of infertility. In the study, they also set a cut-off value of 4.35 mV/30 min/ 10^6 sperm to predict pregnancy. These findings indicate the adverse effects of ROS on semen quality, and on the outcome of fertility treatment.

As for assisted reproductive technology, the effects of ROS on semen have been evaluated in a few reports

CONTACT Teppei Takeshima  teppeitalia@gmail.com  Department of Urology, Reproductive Center, Yokohama City University Medical Centre, 4-57 Urafune-cho, Minami-ku, Yokohama City, Kanagawa 232-0024

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(Sukcharoen et al. 1996; Moilanen et al. 1998; Saleh et al. 2003; Zorn et al. 2003). However, these previous studies only evaluated the fertilization and pregnancy rates after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), but not in the detailed stages of embryo. Previous studies have reported that paternal factors, such as unrepaired DNA damage in sperm, are considered to have adverse effects on the late stages of embryo development in IVF or ICSI, which is termed the 'late paternal effect' (Tesarik et al. 2004). Since ROS in semen are reported to induce sperm DNA damage (Barroso et al. 2000; Irvine et al. 2000), there may be a significant correlation between ROS level and embryo development. Determination of the relationship between these two factors may help to predict embryo development based on the ROS level in semen, which can be easily measured in clinical practice. The objective of this study was to investigate the effects of ROS in semen on embryo development during different stages of ICSI among infertile couples.

Results

The patients' characteristics and semen parameters are summarized in Table 1. Data are presented as mean \pm standard deviation. Among 887 eggs, 580 fertilized embryos were obtained thorough ICSI, and the fertilization rate was 65.4%. Among the 580 fertilized embryos, 345 embryos were well-developed on day 3 (59.5%), which were divided into good-cleavage-embryo and non-developed-embryo groups (Figure 1A). Among 326 fertilized embryos in 107 cycles that did not include cleavage embryo transplantation, 58 good-quality blastocysts were acquired (Figure 1B).

The mean (\pm standard error) ROS levels of the fertilized and nonfertilized groups were $54,204 \pm 7,454$ and $52,018 \pm 9,052$ relative light units (RLU), respectively, which were not significantly different ($P = 0.858$) (Table 2, Figure 2A). The ROS level was higher in the

non-developed-embryo group than in the good-cleavage-embryo group, which was statistically significant ($81,451 \pm 15,234$ and $35,645 \pm 6,934$ RLU, respectively; $P = 0.0026$) (Table 3, Figure 2B). As calculated from the receiver operating characteristic (ROC) curve, the cut-off level of ROS was 6601 RLU (area under the curve = 0.539). Using this cut-off level of ROS, the embryos were divided into high- and low-ROS groups. The good-cleavage-embryo rate was lower in the high-ROS group (51.5%) than in the low-ROS group (63.8%), which was statistically significant ($P = 0.004$).

The ROS level was lower in the good-quality-blastocyst group than in the poor-quality-embryo group, which was statistically significant ($27,916 \pm 16,336$ and $81,780 \pm 14,498$ RLU, respectively; $P = 0.015$) (Table 4, Figure 2C). As calculated from the ROC curve, the cut-off level of ROS was 4,926 RLU (area under the curve = 0.608). The blastocysts were divided into high- and low-ROS groups using this cut-off value. Similar to the cleavage embryos, the good-quality-blastocyst rate was lower in the high-ROS group (11.2%) than in the low-ROS group (24.8%), which was statistically significant ($P = 0.001$).

Table 1. Patients' characteristics and semen analyses (n = 77).

Age of husbands (years, mean \pm SD)	39.4 \pm 7
Age of wives (years, mean \pm SD)	37.4 \pm 4.6
Semen analysis (number of patients)	
Oligoasthenozoospermia	46
Oligozoospermia	25
Asthenozoospermia	3
Normozoospermia	3
Semen parameters (mean \pm SD)	
Volume (mL)	2.65 \pm 1.33
Concentration ($\times 10^6$ /mL)	17.1 \pm 19.6
Motility (%)	18.5 \pm 16.0
VSL (μ m/sec)	17.5 \pm 7.93
VCL (μ m/sec)	45.7 \pm 21.6
Linearity	0.38 \pm 0.15
mALH (μ m)	1.15 \pm 0.65
BCF (Hz)	11.8 \pm 4.10

Abbreviations: BCF: beat-cross frequency; mALH: mean amplitude of lateral head displacement; VCL: curvilinear velocity; VSL: straight-line velocity.

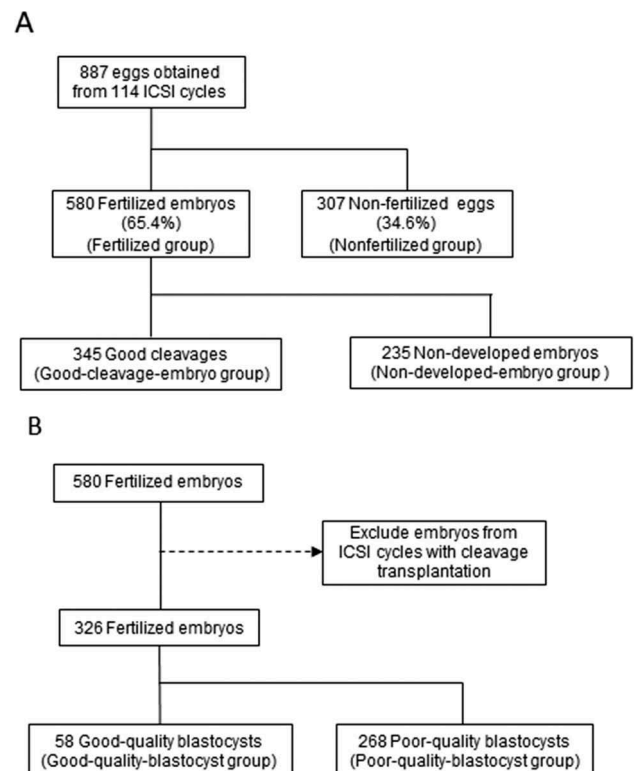


Figure 1. Experimental Design (A) Flowchart of how the eggs and embryos were divided into groups for between-group comparisons; the fertilized and nonfertilized groups and the good-cleavage-embryo and non-developed-embryo groups were compared. (B) Flowchart of how the fertilized embryos were divided to compare the good-quality-blastocyst and poor-quality-embryo groups.

Table 2. Comparison of ROS levels in semen between the fertilized and nonfertilized groups.

	887 Eggs		P-value
	Fertilized group (n = 580)	Nonfertilized group (n = 307)	
Total ROS (RLU)	54,204 ± 7,454	52,018 ± 9,052	0.858

Note: Data are presented as mean ± standard error.

Abbreviations: RLU, relative light units; ROS: reactive oxygen species.

Discussion

One of the common maternal causes of fertilization failure in IVF is hardening of the zona pellucida. Moreover,

oocyte activation failure is regarded to be the main cause of failure in ICSI (Vanden Meerschaut et al. 2014). Thus, paternal factors such as deficiency in oocyte activators (e.g., phospholipase C zeta) can lead to fertilization failure in ICSI (Tesarik et al. 2004). Less common paternal causes of failure in ICSI include a lack of motile spermatozoa, severe forms of teratozoospermia (globozoospermia), and technical problems with sperm injection (Yanagida 2004; Dam et al. 2007). Furthermore, paternal factors such as DNA damage in sperm are considered to have adverse effects on the late stages of embryo development. ROS in semen is known to induce sperm DNA fragmentation

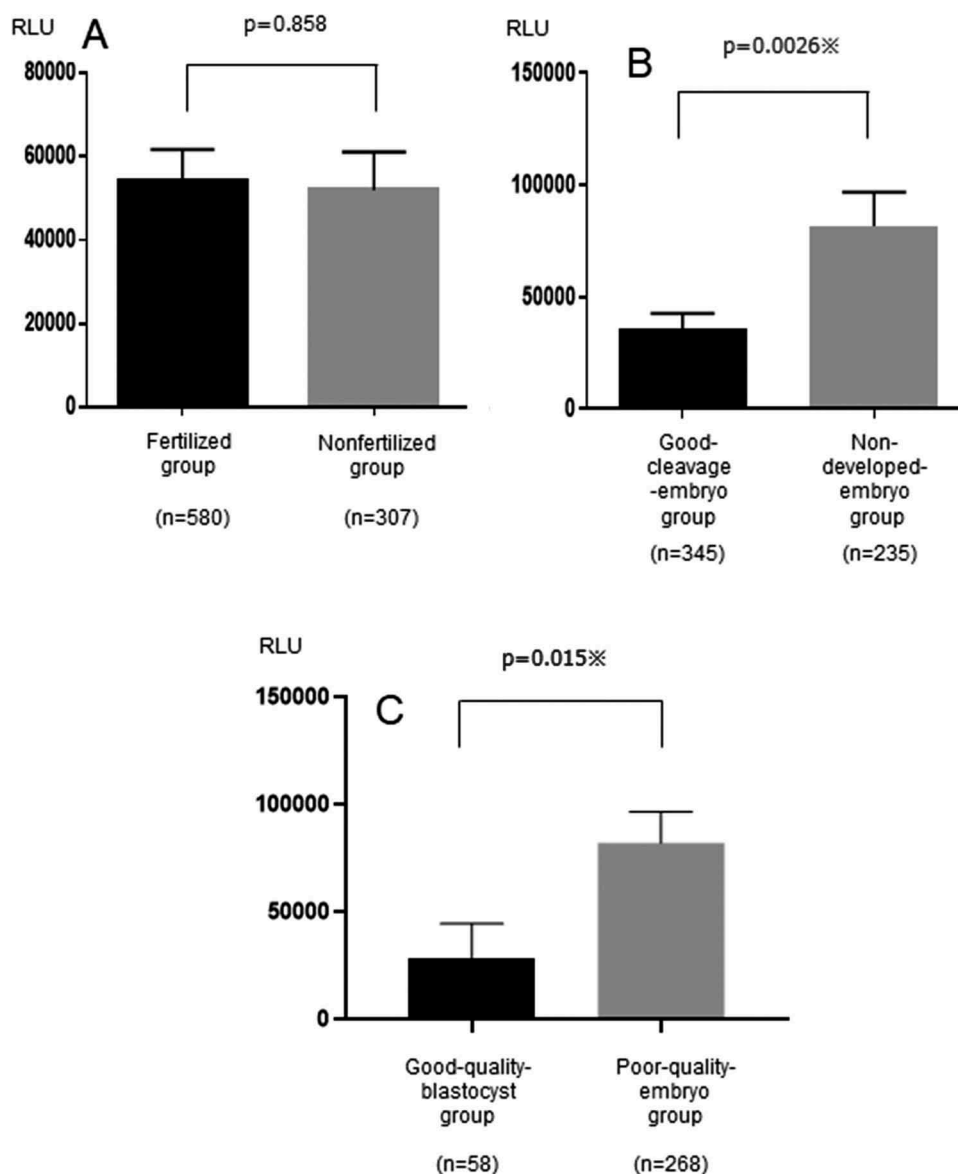


Figure 2. Comparison of reactive oxygen species (ROS) levels (A) Comparison of reactive oxygen species (ROS) levels between the fertilized and nonfertilized eggs. The mean (\pm standard error) ROS levels were not significantly different between the fertilized and nonfertilized groups ($P = 0.858$). (B) Comparison of ROS levels between the good-cleavage-embryo and non-developed-embryo groups. The ROS level was higher in the non-developed-embryo group than in the good-cleavage-embryo group, which was statistically significant ($P = 0.0026$). (C) Comparison of ROS levels between the good-quality-embryo and poor-quality-embryo groups. The ROS level was lower in the good-quality-blastocyst group than in the poor-quality-embryo group, which was statistically significant ($P = 0.015$).

Table 3. Comparison of ROS levels in semen between the good-cleavage-embryo and non-developed-embryo groups.

	580 Fertilized embryos		P-value
	Good-cleavage-embryo group (n = 345)	Non-developed-embryo group (n = 235)	
Total ROS (RLU)	35,645 ± 6,934	81,451 ± 45,234	0.0026

Note: Data are presented as mean ± standard error.

Abbreviations: RLU, relative light units; ROS: reactive oxygen species.

Table 4. Comparison of ROS levels in semen between the good-quality-blastocyst and poor-quality-embryo groups.

	326 Fertilized embryos		P-value
	Good-quality-blastocyst group (n = 58)	Poor-quality-embryo group (n = 268)	
Total ROS (RLU)	27,916 ± 16,336	81,780 ± 14,498	0.015

Note: Data are presented as mean ± standard error.

Abbreviations: RLU, relative light units; ROS: reactive oxygen species.

(Barroso et al. 2000; Irvine et al. 2000); hence, in this study, we investigated the effects of ROS on fertilization and embryo development.

ROS are categorized as free radicals (e.g., superoxide anions, hydroxyl radicals, peroxy radicals) and nonradical species (e.g., hydrogen peroxide, hypochlorous acid) (Agarwal et al. 2003; Sanocka and Kurpisz 2004). Immature spermatozoa and seminal leukocytes are considered to be the sources of ROS in semen. Spermatozoal cytoplasm deposits contain large amounts of glucose-6-phosphate dehydrogenase enzyme, which produces nicotinamide adenine dinucleotide phosphate (NADPH) (Aitken and Clarkson 1987; Sharma et al. 2001; Sakkas et al. 2003; Koppers et al. 2010). NADPH generates ROS through an NADPH oxidase enzyme complex (Williams and Ford 2004). Owing to this mechanism, immature spermatozoa with large amounts of cytoplasm are considered to generate higher ROS levels. Furthermore, ROS generation from leukocytes is attributable to an inflammation or infection in the male genital tract, producing ROS through the myeloperoxidase system, which is an essential immune system for pathogens (Iwasaki and Gagnon 1992). High ROS levels in semen cause LPO of the sperm cells, resulting in the loss of fluidity and integrity, which are essential for the fusion of the sperm and oocyte (Aitken et al. 1993). Zabludovsky et al. (1999) reported that LPO has adverse effects on sperm concentration and motility.

Unrepaired DNA damage in sperm has a negative effect on embryo development and implantation, as well as on pregnancy, which is termed the 'late paternal effect' (Tesarik et al. 2004). The late paternal effect occurs because embryo development is dependent on maternal factors at the time of fertilization, but switches

to the embryo's own genomes on days 2 and 3 (Tesarik et al. 1986). However, recent studies have shown that DNA damage in sperm also influences the early stages of embryo development (Simon et al. 2014; Neyer et al. 2015). In our study, a significantly higher ROS level in semen was confirmed in the non-developed-embryo and poor-quality-embryo groups; nonetheless, there was no significant difference in fertilization. These findings support those of earlier studies, indicating that a high ROS level in semen leads to damage during the late stages of embryo development, as well as the early stages of embryo development through DNA fragmentation. We did not measure the ROS level in prepared sperm (the condition of chosen sperm for ICSI); however, we did measure the ROS level in raw semen samples during the patient's closest visit before ICSI. Measuring ROS levels in raw semen prior to ICSI may predict embryo development; we found significantly higher ROS levels in the non-developed-embryo and poor-quality embryo groups. Additionally, we found significant cut-off values of ROS in semen for good-cleavage (6,601 RLU) and good-quality blastocysts (4,926 RLU). This suggests that acquisition of a good-quality blastocyst requires a lower ROS level than acquisition of a good-cleavage embryo. These values may aid in predicting embryo development in ICSI by ROS level. Furthermore, the ROS levels did not significantly differ between the fertilized and non-fertilized groups in the present study, implying that maternal factors may act as a balance against sperm LPO and DNA fragmentation induced by ROS in semen at the time of fertilization.

In this study, we analyzed the fertilization, good-cleavage-embryo, and blastulation rates for each oocyte in order to assess the effects of ROS on embryo development. Several reports have described the negative effects of ROS in semen on the outcomes of IVF and ICSI and compared the outcomes according to the cycles of IVF or ICSI (Zorn et al. 2003; Das et al. 2008). In this manner of investigation, a '50% fertilization rate' can mean 50 good embryos out of 100 per cycle, as well as one good embryo out of two. Thus, the fertilization rate should not be considered to be reflective of embryo development. Therefore, we analyzed embryo development by each oocyte, which is more accurate.

This study had some limitations. First, we focused on fertilization and embryo development in this study and did not hence evaluate the pregnancy rate, which includes several maternal factors (e.g., uterine factors). Second, although a previous study reported that ROS were positive in 111 out of 243 patients with idiopathic infertility (45.6%) and 105 out of 206 patients with varicocele (51.0%) (Yumura et al. 2017), we did not

measure the ROS level in each background of male infertility. Third, the total antioxidant capacity in semen, which has been reported to be useful for evaluating the oxidant stress status (Sharma et al. 1999), was not estimated in this study. The balance between ROS and total antioxidant capacity is worth investigating, and our data imply that ROS has an adverse effect on days 3 and 5 of embryo development. Fourth, not analyzing embryo development by cycles of ICSI, but rather by each oocyte, is not a standard method of assessment. However, we consider it to be a novel and more accurate way to assess embryo development. In order to determine the adverse effect of ROS, not only ROS level measurement but also further testing of sperm DNA fragmentation or chromatin integrity should be conducted in future research.

In conclusion, ROS in semen adversely affected the circumstances of embryo development in ICSI with respect to the development of both good cleavage and good blastocysts, rather than fertilization. As expected, ROS in semen were associated with both early and late paternal effects. These findings may aid in predicting the development of good cleavage and blastocysts from the ROS level in semen.

Materials and methods

Population

A total of 77 infertile couples who underwent ICSI at the Reproduction Centre of Yokohama City University Medical Centre between March 2013 and December 2016 were enrolled. All husbands visited the urology department and underwent measurement of ROS levels in semen at least once. Subjects were included based on the wife's age (≤ 43 years) and the presence of at least one metaphase II oocyte for ICSI. A total of 887 eggs acquired from 141 cycles of ICSI procedures were analyzed. Eggs were first divided into two groups – namely, the fertilized group (embryos fertilized through ICSI) and the nonfertilized group (non-fertilized eggs) (Supplemental Figure 1A, B). Among the fertilized embryos, those on day 3 were subsequently divided into the good-cleavage-embryo group (successfully developed embryos) and the non-developed-embryo group (unsuccessfully developed embryos). Good cleavage was defined as an embryo with more than 7 grade 3 cells according to the Veeck classification on day 3 (Supplemental Figure 1C, D).

Among the fertilized embryos, blastocysts were also divided into good-quality and poor-quality blastocysts. Good-quality blastocysts were defined as blastocysts $>3BB$ according to the Gardner classification on day 5 (Supplemental Figure 1E, F). To avoid selection bias when evaluating blastocyst development, we excluded

the ICSI cycles with cleavage transplantation on day 3 (Supplemental Figure 1B).

ROS assessment

For the measurement of ROS levels, the unprocessed semen sample at the time closest to oocyte retrieval was used. The ROS levels in semen samples were measured using Monolight 3010™ Luminometer (BD Biosciences Pharmingen, Ltd., San Diego, CA, USA). Before and after adding 40 μ L of 100 mmol/L luminol to 500 μ L of the raw semen sample, the subtraction of the integrated chemiluminescence was measured between 0 and 200 seconds (Supplemental Figure 2). The chemiluminescence value was expressed in RLU.

The ROS levels in semen were compared between the fertilized and nonfertilized groups, between the good-cleavage-embryo and non-developed-embryo groups, and between the good-quality-blastocyst and poor-quality-blastocyst groups. The cut-off value of ROS was calculated from the ROC curve to predict good-cleavage-embryo and good-quality-blastocyst development.

ICSI procedures

Female partners underwent ovarian stimulation using either a short or long protocol of gonadotropin-releasing hormone (GnRH) agonist, or GnRH antagonist, which was chosen depending on her age and serum anti-Müllerian hormone level. Human chorionic gonadotropin (10,000 IU) was administered until the follicles were 18–20 mm in diameter. Oocytes were retrieved with transvaginal ultrasound assistance at 36 hours after human chorionic gonadotropin injection. After retrieval, oocytes were pre-incubated for 4 hours prior to sperm injection (37.0°C, 6.0% CO₂, 5.0% O₂). Cumulus was removed from the oocytes just before ICSI using hyaluronidase (ICSI Cumulase®; CooperSurgical Inc., Trumbull, CT, USA).

Spermatozoa were prepared for ICSI with the two-layer concentration gradient method using SpermGrad®, SpermRinse® (Vitrolife, Göteborg, Sweden) and centrifugation at 500 g for 20 min. After washing (centrifugation at 162 g for 10 min), the swim-up method was used. A metaphase II oocyte was aspirated, and one selected spermatozoon was injected into the ooplasm. After confirmation of fertilization, the embryo was cultured for 3 days in Sydney IVF Cleavage Medium® (Cook Medical, Bloomington, IN, USA). Subsequently, the cleavage embryo was cultured to a blastocyst in IVF Blastocyst Medium® (Cook Medical, Bloomington, IN, USA).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences version 21 (IBM Corp., Armonk, NY, USA). Unpaired t-tests were used to compare each group. For all tests, P-values ≤ 0.05 were considered statistically significant.

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Ethical approval

The study was approved by the Yokohama City University Review Board. Written informed consent was obtained from each couple prior to study initiation. All procedures were conducted in accordance with the World Medical Association's Code of Ethics (1964 Declaration of Helsinki, as revised in 2003, and the 1975 Declaration of Tokyo, as revised in 2006).

Data availability statement

The data that support the findings of this study are available upon request from the corresponding author, TT.

Disclosure statement

The authors report no conflict of interest.

Authors' contributions

Wrote the manuscript: SK; Conceived and designed the experiments: SK, TT, KT, YY; Performed the experiments: SK, TT, KT, KU, KY, HS; Analyzed the data: SK, KT, TK; Contributed reagents/materials/analysis tools: KT, HU, MM, YY.

ORCID

Shinnosuke Kuroda  <http://orcid.org/0000-0001-8890-0297>
Teppei Takeshima  <http://orcid.org/0000-0003-2733-5487>
Yasushi Yumura  <http://orcid.org/0000-0003-0909-478X>

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論文目録

I 主論文

Early and late paternal effects of reactive oxygen species in semen on embryo development after intracytoplasmic sperm injection

Kuroda S., Takeshima T., Takeshima K., Usui K., Yasuda K., Sanjo H., Kawahara T., Uemura H., Murase M., Yumura Y.:

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