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Intracytoplasmic Sperm Injection (ICSI) in B6D2F1 and CB6F1 Strains Mice Using Cauda Epididymal Spermatozoa

B6D2F1 ve CB6F1 Irk Farelerde Kauda Epididimal Spermatozoa Kullanılarak İntrastoplazmik Sperm Enjeksiyonu (ICSI)

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ABSTRACT

Objective: Reproductive biotechnology studies focus on the long-term storage of embryos (cryopreservation), embryo cultures, genome editing of embryos and embryo transfer. Micromanipulation techniques in reproduction biotechnologies have an important role, especially in studies investigating assisted reproductive technology in laboratory animals. The aim of the present study was to investigate the effect of epididymal spermatozoa injected to oocyte by intracytoplasmic sperm injection (ICSI) in different mice strains. In this study, we evaluated the *in vitro* development of post-ICSI derived embryos using cauda epididymal sperm.

Material and Method: Female mice (8-10 weeks) were superovulated using pregnant mare serum gonadotropin/human chorionic gonadotropin (PMSG/hCG) and ~14h post hCG, the mice were sacrificed, and the oocytes were collected. Spermatozoa from the cauda epididymal of a 12-week-old were used on the same strain for ICSI and the *in vitro* developmental potential was evaluated. Finally, the embryos were cultured for 120 hours at 5% CO_2 with 37°C.

Results: The results showed that the two-cell embryo of the B6D2F1 strain (79.31%) was significantly higher than the CB6F1 (56.26%) ($p<0.05$). While the blastocyst rate was comparable between both the B6D2F1 strain (68.75%) and CB6F1 strain (69.57%) ($p>0.05$).

Conclusion: ICSI using cauda epididymal sperm is a suitable application for *in vitro* embryo development in B6D2F1 and CB6F1 strains. Finally, ICSI success of the B6D2F1 mice strains was found to be higher than CB6F1 mice strains.

Keywords: Mouse, oocyte, ICSI, development

ÖZ

Amaç: Üreme biyoteknolojisi alanındaki çalışmalar; embriyoların uzun süre saklanması (kriyoprezervasyon), embriyo kültürü, embriyoların genom düzenlenmesi araştırmaları ve embriyo transferi gibi konular üzerinde yoğunlaşmaktadır. Üreme biyoteknolojilerinde mikromanipülasyon teknikleri, özellikle laboratuvar hayvanlarında yardımcı üreme teknolojisinin araştırıldığı çalışmalarda önemli bir yere sahiptir. Bu çalışmanın amacı, farklı fare ırklarında intrastoplazmik sperm enjeksiyonu (ICSI) ile epididimal spermatozoanın oosite enjeksiyonunu araştırmaktır. Bu çalışmada, kauda epididimal fare spermi kullanılarak yapılan ICSI uygulaması sonrasında elde edilen embriyoların *in vitro* gelişimi değerlendirilmiştir.

Gereç ve Yöntem: Dişi fareler (8-10 hafta), gebe kısrak serum gonadotropini/insan koryonik gonadotropini (PMSG/hCG) kullanılarak süperovüle edilmiş, hCG'den ~14 saat sonra fareler sakrifiye edilerek oositler toplanmıştır. 12 haftalık erkek farenin kauda epididiminden alınan spermatozoa, aynı ırk oositle ICSI için kullanılmış ve *in vitro* gelişim potansiyeli değerlendirilmiştir. Son olarak tüm embriyolar 120 saat süre ile %5 CO_2 ve 37°C'de kültüre edilmiştir.

Bulgular: Sonuçlar, B6D2F1 (%79,31) ırkının 2 hücreli embriyo gelişiminin CB6F1 (%56,26) ırklı farelerdeki 2 hücreli embriyo gelişiminden önemli ölçüde yüksek olduğunu göstermiştir ($p<0,05$). Blastosist oranı B6D2F1 (%68,75) ve CB6F1 (%69,57) ırkları arasında karşılaştırılmıştır ($p>0,05$).

Sonuç: B6D2F1 ve CB6F1 fare ırklarında, kauda epididimal sperma kullanılarak yapılan ICSI *in vitro* embriyo gelişimi için uygun bir yöntemdir. Sonuç olarak, B6D2F1 farelerde ICSI'nın başarısı, CB6F1 ırk farelere göre daha yüksektir.

Anahtar Kelimeler: Fare, oosit, ICSI, gelişim

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INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is the name given to the method of injecting spermatozoa into the oocytes through microsurgical methods. It is a common approach in clinical practice in the treatment of infertility problems with various causes, and in the production of farm and laboratory animals (transgenic and low-reproductive strains) (1,2). There is a need for a convenient and cost-effective method for long-term sperm preservation of transgenic mice (3).

It is well known that the genetic background of mouse strains affects morphological parameters of sperm (4-6) and *in vitro* produced embryo development (7). Most common mouse strains are used for developing new transgenic lines. And their embryo development results can be compared to the other strains (4-6). Furthermore, using different mouse strains in ICSI can affect embryo development (8-10).

The genetic characteristics of different mouse strains affect embryo development results in mouse embryo studies. Also, no research was found in literature about results of *in vitro* developments of ICSI between oocytes from CB6F1 mouse strain. The present study analyzes the *in vitro* development rates of embryos produced by the application of ICSI in comparison to the mouse strains of CB6F1 (C57BL/6J x Balb/c) and B6D2F1 (DBA2 x C57BL/6J).

MATERIAL AND METHOD

All mice experiments were approved by Koc University Local Ethics Committee for Animal Experiments (approval number: 2014-05). The animals were kept in Koc University, Animal Research Facility of Center for Translational Medicine (KUTTAM) under 12 hours light - 12 hours dark cycle, and a diet of commercial pellet food ad-libitum and automatic water containers were provided according to the Guide for the Care and Use of Laboratory Animals.

Superovulation and Oocyte Collection

10 IU of pregnant mare serum gonadotropin (Sigma G4877-PMSG) was injected by intraperitoneal (IP) to the female mice between 17:00 and 18:00, and 48 hours later, 10 IU of human chorionic gonadotropin (Organon-hCG) was injected by intraperitoneally between 17:00 and 18:00 hours. Fourteen hours after the hCG injection, female mice were sacrificed, and the oocytes were collected by rupturing the oviductal ampullae. The oocytes were washed in HEPES buffered human tubal fluid (HTF) medium with hyaluronidase, and then washed three times in a hepes buffered-M2 medium supplemented with 4 mg/ml BSA (Sigma Catalog, No A-3311, Fraction V). The culture media containing the collected oocytes were kept in an incubator at 37ºC and in 5% CO_2 for 30–60 minutes until the ICSI was performed (10). 10 µl of embryo culture drops were added to a petri dish and covered with mineral oil (Lifeguard-Life Global) in order to avoid the contamination, evaporation and to ensure integrity of the drops. The petri dishes were then placed in an incubator at 37°C, in 5% CO_2 and humidity for gassing at least 2 hours prior to embryo collection.

Preparation of Spermatozoa

The male mice of each strain were sacrificed, cauda epididymis were removed and incubated in 500 µl embryo culture medium (LifeGlobal Media, LGGG-020) at 37°C, 5% CO_2 for 10 minutes. Then, 100 µl was collected from the top of the tube and transferred to the cryotube. The spermatozoa were rapidly frozen in liquid nitrogen which lead to breakage of sperm tail and head (11,12).

Intracytoplasmic Sperm Injection (ICSI)

After oocyte collection from the petri dish, a 10 cm petri dish cover was used, the area of which was divided into four sections. Fifteen µl drops were placed in the petri dish and covered with 7-8 ml of mineral oil (Figure 1), and 3 µl of frozen-thawed spermatozoa were placed in M2+3% polyvinyl-pyrrolidone (PVP) drops. ICSI working plate prepared with small droplet of manipulation HEPES buffered HTF medium. The sperm was mixed with five volumes of a 10% solution of PVP (v/v) in HEPES buffered HTF medium. A droplet of piezo pipette prepared for washing in volumes of a 3% solution of PVP in HEPES buffered HTF medium. The spermatozoa were mixed using a manipulation pipette to ensure individual cell formation. ICSI started 16-18 hours after hCG injection and performed by using a Nikon Eclipse and an inverted microscope equipped with Eppendorf TransferMan/Nk 2 micromanipulators. An Eppendorf CellTram Vario embryo holding pipette console and an Eppendorf embryo holding pipette with a 35° angle, 15 µm inner diameter and a 100 µm outer diameter were mounted on the left micromanipulator. An Eppendorf CellTram Air embryo manipulation pipette console and an Eppendorf embryo ICSI piezo drill micropipette (Origio/CooperSurgical, U.S.A, Piezo-6-25) with a 6° angle, 6 µm inner diameter and an 8 µm outer diameter were mounted on the right micromanipulator. The spermatozoa were taken sequentially into the injection pipette (Figure 2A). The injection pipette was transferred into manipulation drops. Using a holding pipette (Optimas, reference code: OMH1202030) with loaded 2-3 µl mercury, the oocytes were fixed at the 6 or 12 o'clock position of the MII (Figure 2A). Using a piezo drill (Prime Tech PMM4G) injection pipette, a single-pulse piezo of 4/4 (intensity/velocity) was made to puncture the zona pellucida of the oocyte (Figure 2B, 2C). Minimum suction of cytoplasm was applied into the ICSI injection pipette (Figure 2D, 2E) and a single-pulse piezo of 2/1 (intensity/velocity) was applied to break the cytoplasm and inject the sperm to oocyte immediately (Figure 2F, 2G). The sperm injection into the cytoplasm was conducted at room temperature (17-18°C) (11,12).

Post-ICSI Embryo Culture

The ICSI-treated oocytes were transferred into a culture medium, and the embryos were washed in at least three times and then incubated at 37º C and in 5% CO_2 for 96–120 hours respectively. The two cell and blastocyst developments were evaluated on 2th day and 5th day, respectively.

Statistical Assessment of Results

IBM SPSS Statistics for Windows (Version 24.0. Armonk, NY: IBM Corp.) was used for the statistical assessment of the results with

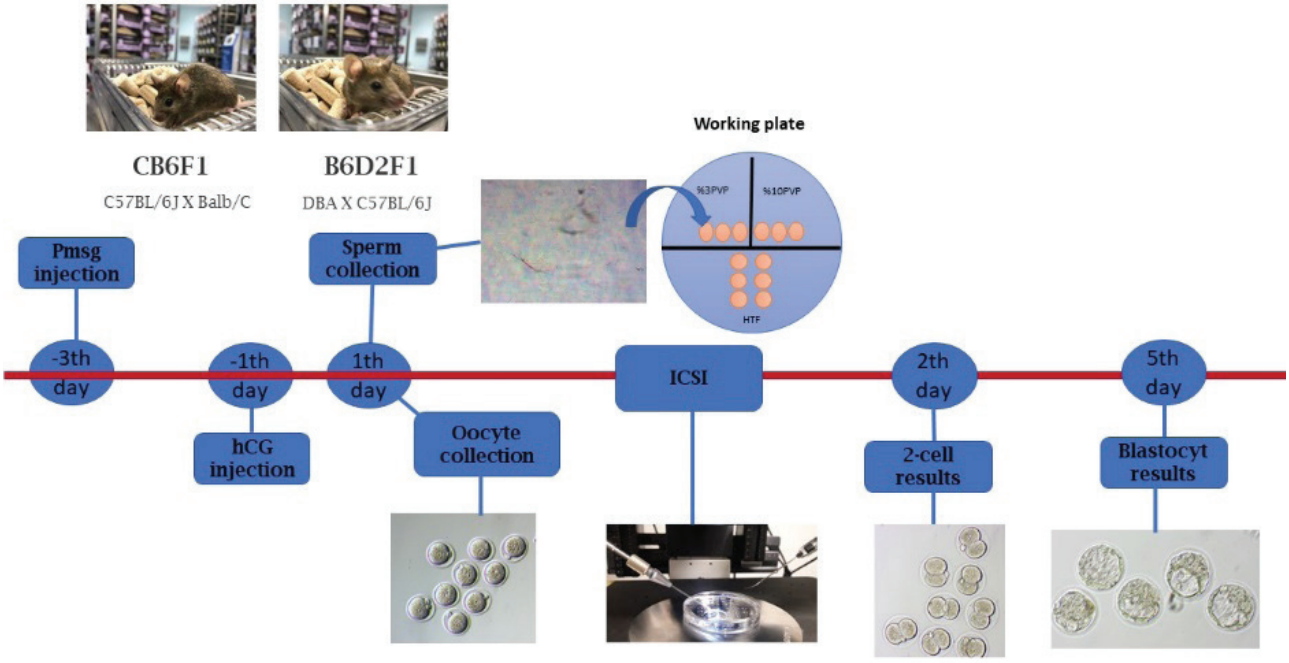

Figure 1. Schematic depicting the experimental timeline in mouse intrastoplasmic sperm injection (ICSI).

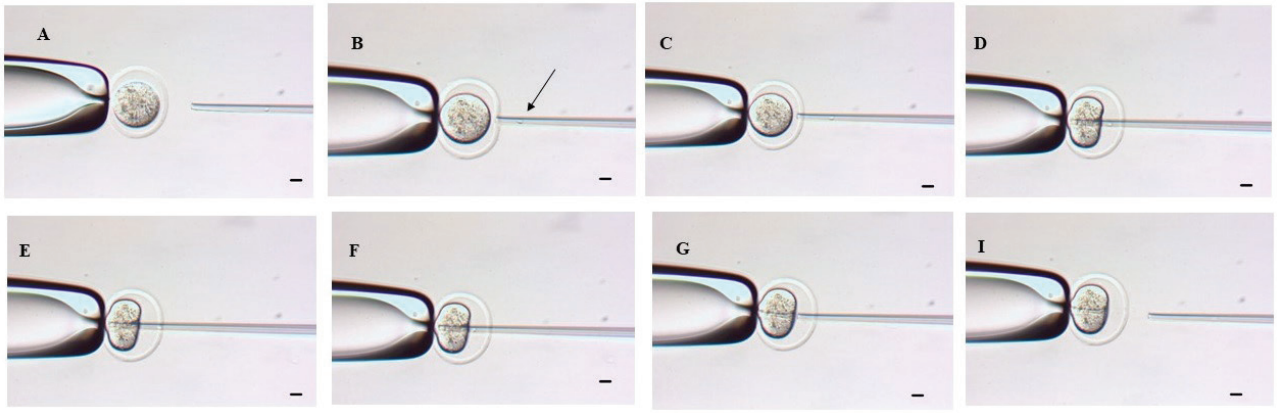

Figure 2. Piezo drill of ICSI on mouse oocyte. (A) The sperm was sucked into the injection pipette, (B) the arrow is sperm in injection pipette and zona pellucida aspirated by injection pipet, (C) a single-pulse piezo was made to puncture the zona pellucida of the oocyte, (D,E) aspiration of cytoplasm is applied into injection pipette and apply a single-pulse piezo to break to cytoplasm, (F) the sperm injected to oocyte, (G,I) when withdrawing the ICSI pipette out of the sperm injected mouse oocyte (20*X*).

sufficient number of repetitions. ANOVA and Chi-square tests were used for the analysis of variance and differences between groups. The p-values <0.05 were considered statistically significant. All experiments were replicated three times.

RESULTS

According to development evaluations done after *in vitro* culture, the two cell development rates were 56.26% and 79.31%, in CB6F1 and B6D2F1 strain groups, respectively p<0.05, Table 1). The differences of two cell development were found significantly important between in CB6F1 and B6D2F1 strain groups (p< 0.05). The blastocyst development rates were 68.75% and 69.57%, in CB6F1 and B6D2F1 strain groups, respectively (Table 1). The differences of blastocyst development were no found significantly between the CB6F1 and B6D2F1 strain groups (p> 0.05, Table 1).

Table 1. *In vitro* development of post-ICSI derived embryos from different mouse strains.

Strain	Number of oocytes	Number of 2-cell embryos (%)	Number of blastocyst from 2-cell stage embryo (%)
CB6F1	27	16[a] (56.26±11.33)	11[a] (68.75±29.46)
B6D2F1	29	23[b] (79.31±16.16)	16[a] (69.57±8.42)

(a,b)-Differences between the same columns with different symbols (a, b) were found to be significant ($p<0.05$). There was significant difference in B6D2F1 and CB6F1 of two cell development rates.

(a)-Same characters (a) in the same column are not significant ($p>0.05$). There was no significant difference in B6D2F1 and CB6F1 of blastocyst development rates.

DISCUSSION

The mice are appropriate in research of ICSI-derived embryo development for improving of laboratory animal reproductive technologies. ICSI is widely used in both animals and humans as an effective approach to study the fertility (13). Mice are considered as model animals in medical research and ICSI are used to better understand the biology of fertilization (14). It has been shown that both cauda and caput epididymal spermatozoa were used in different mouse strains (13). The aim of this research is to investigate the rates of *in vitro* development of embryos produced by ICSI to different mouse strains. This present study has investigated the rate of *in vitro* culture development in ICSI-derived embryos in CB6F1 (C57BL/6J x Balb/c) and B6D2F1 (DBA2 x C57BL/6J) mice by cauda epididymal spermatozoa.

It is increasingly recognized that ICSI is affected by both age and subspecies, as the study of BALB/c mice demonstrates a relationship between ICSI results and species difference (15). Also, Ogonuki et al. reported about animal strains were significant effect on two-cell embryo development in ICSI (16). The present study also establishes a statistically significant difference in the rates of two-cell embryo development, and we observed strain affect in this study.

In FVB and CD-1 mice, two cell developments are around 80% as a result of piezo ICSI with cauda and epididymal sperm (11). Likewise, in our study, similarly, two-cell development in B6D2F1 mouse race was around 80%. On the other hand, CB6F1 race had a lower rate of two cell development.

Compared intracytoplasmic sperm injections in inbred (C57BL/6) and hybrid mice (B6C3F1 and B6D2F1), and although the fertilization and embryo development rates were found to be similar in the C57BL/6 and hybrid strains, the post-implantation rates were poorer in the C57BL/6 strains than in the other strains, which was believed to be linked to sperm factors (17).

The other study discussed that the reproductive potential of caput epididymal spermatozoa and carried out comparative ICSI applications with caput and cauda spermatozoa in FVB and CD1 strains. Following these applications, the authors established a blastocyst development rate of 57% with caput sperms and 63% with cauda sperms (18).

Mallol et al. reported that blastocyst development rate of 57% in mouse embryos following an ICSI application after a freezing-thawing protocol in hybrid B6CBAF1 (C57Bl/6xCBA/J) mice (12). Moreira et al. reported that achieved a blastocyst development rate of 60% with ICSI application (19). The results of the present study were compared with those of the two commonly used strains, revealing that the two-cell development rate of the B6D2F1 strain was significantly higher, and the blastocyst development rate of the CB6F1 strain, especially after two-cell development, was also significantly higher.

We compared *in vitro* development of ICSI derived embryos with cauda epididymal sperm in two different mouse strains. The two-cell development were found significantly important between in CB6F1 and B6D2F1 strain groups ($p<0.05$). While the blastocyst developments were not found significantly important between CB6F1 and B6D2F1 strain groups ($p>0.05$, Table 1).

Future studies can focus on comparing strains of ICSI derived embryos; *in vivo* development, cryopreservation and embryonic gene expression levels. Based on the results of the present study, a useful model can be developed for veterinary and laboratory animal sciences. Also, these findings of ICSI-derived embryos with biotechnological methods would contribute to the improvement and development of the national livestock. The result of this study showed that ICSI with cauda epididymal sperm is a suitable application for embryo development in both mouse strains. In conclusion, because of its ease of use, this ICSI technique is considered useful in laboratory animals.

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