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Evaluation of *in vitro* fertilization outcome from very low sperm count and microdissection Testicular Sperm Extraction in patients with non-obstructive azoospermia: Paternal age factor

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Abstract

The study focused on evaluating the results of *in vitro* fertilization from very low sperm count and sperm from testicular microsurgery in NOA patients from different ages. Study subjects were divided into 2 groups: under or above 35 years old group; and each group was divided into 2 subgroups: T-ICSI and E-ICSI. The evaluation criteria mainly focused on the fertilization rates, the good embryo rates and the clinical pregnancy rates. The results showed that, in the under 35 years old group, the fertilization rates in the T-ICSI group were significantly higher than the E-ICSI group. However, the third day good embryo rates, the blastocyst formation and the good blastocyst rates in the both T-ICSI group and the E- CSI group were similar. In the above 35 years old group, the fertilization rates, the third day good embryo and the good blastocyst rates in the T-ICSI group were significantly higher than E-ICSI group The percentage of live births in the T-ICSI group was higher than the E-ICSI group, but there was no statistical difference. Especially, the weight of children born from the group using sperm from microTESE was heavier than that of children born from the group of sperm from Cryptozoospermia. The results of the study showed that sperm extraction from testicular microsurgery improved embryology results in the above 35 years old of males with non-obstructive azoospermia. Regarding clinical treatment results, the group using sperm from testicular microsurgery tended to be higher than the group using sperm from ejaculate samples.

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Introduction

According to statistics, the rate of non-obstructive azoospermia (NOA) is common in about 5% of infertile couples; 10% of male infertility cases and about 1% of the population (Jin *et al.*, 2020). Azoospermia is defined as the absence of sperm in the semen residue after centrifugation in at least two semen analysis (WHO 2021) (WHO, 2021). Cryptozoospermia is defined as a case where no sperm is found in the semen sample, but only a few sperm are found in the pellets after centrifugation (WHO, 2021). Semen from Cryptozoospermia cases have very few spermatozoa, low motility, low survival rates and contain many abnormal sperm. Occasionally, Cryptozoospermia may not found sperm and the literature records such cases as virtual azoospermia (Tournaye *et al.*, 1995). On the day of the wife's oocyte retrieval, when encountering such cases, sperm extraction from testicular microsurgery and performing ICSI is suggested (Ben-Ami *et al.*, 2013),(Cui *et al.*, 2017).

In earlier studies, because of the increased DNA fragmentation rate with the advancing paternal age, concerns about decreased pregnancy rates with ejaculated sperm were appropriate. Until now, the results of ICSI in older men with very low sperm count after using sperm from ejaculate sample or from the testicular have not been well documented. The aim of our study was to evaluate whether sperm origin affects ICSI outcomes at different ages of patients with NOA. To answer the above question, we conducted a study: Evaluation of in vitro fertilization outcome from very low sperm count and microdissection Testicular Sperm Extraction in patients with nonobstructive azoospermia: Paternal age factor.

Material and methods

This is a retrospective and prospective cohort study, which was classified into 2 groups based on the age of men: under 35 years old and above 35 years old group. Each group was divided into 2 subgroups according to the origin of the sperm (obtained from ejaculation or obtained from the testicles), include, group 1: used microdissection Testicular Sperm Extraction (microTESE) sperm to perform ICSI (Testicular-ICSI or T-ICSI) and group 2 used spermatozoa very few spermatozoa in the ejaculate sample (Cryptozoospermia) to perform ICSI (Ejaculated–ICSI or E-ICSI). Subjects with non-obstructive azoospermia were studied with results after ICSI including the following: (i) Assessed background characteristics of patient couples and (ii) Evaluated of the results of embryology and clinical treatment in the two study groups. The evaluation criteria mainly focused on the fertilization rates, the good embryo rates and the clinical pregnancy rates. This study was approved by the Medical ethics council in biomedical research at Hanh Phuc International Hospital (Number: 80b/BVHP-HĐYĐ).

The fertilization rates

In vitro, an ovum is considered to show signs of fertilization in the presence of two pro-nucleus. Typically, both progenitors appear from 16 to 20 hours after ICSI with mature oocytes (Hồ Mạnh Tường *et al.*, 2020). Several systems for evaluating fertilized oocytes have been developed, but the Z-score system developed by Scott and Smith in 1998 is the most commonly used (Lan *et al.*, 2003). The evaluation system based on the size of the pro-nucleus, the distribution of nucleic and the position of the nucleus in the zygote is considered an important criterion to evaluate the fertilized oocyte.

The good embryo rates

In the IVF process, the accurate assessment of embryo quality is one of the important factors contributing to the success of infertility treatment. The embryologist will note the features of the embryo under the microscope. In order for the assessment to be carried out correctly, the criteria that were adopted during the Istanbul consensus conference (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) (2011), including: (i) the cleavage-stage embryo, pay attention to the following criteria: number of cell , fragmentation, symmetry, multi-nucleation, vacuoles; (ii) blastocyst stage, the evaluation features include:

cavity enlargement, inner cell mass (ICM) and outer cell mass (Tropholast).

The clinical pregnancy rates

After 14 days of embryo transfer, the patient had serum β-hCG levels measured and transvaginal ultrasound performed on the 28th day post-embryo transfer. Based on the results of these two tests, clinical pregnancy is defined when the presence of amniotic sac, embryo, fetal heart.

Sampling method

Data were collected from couples undergoing IVF treatment at Fertility Center - Hanh Phuc International Hospital, Vietnam from June 2019 to June 2022. Criteria to receive samples: Patients who meet the following conditions will be selected for the study. For the wife: from 18 to 35 years old, number of cycles of IVF treatment under 2 times; the ovarian was stimulated with GnRH antagonist; has no medical conditions related to pregnancy such as ovarian tumor, endometriosis, premature ovarian failure,...; and freeze all embryos as indication. For husband: group 1 (T-ICSI group), male patients with NOA must have at least two Azoospermia, or Cryptozoospermia semen analysis results, performed microTESE technique on the same surgeon; group 2 (E-ICSSI group), NOA male patients with Cryptozoospermia semen analysis results must have at least twice. Morever, samples excluded from this study: donor oocyte cycle; *In vitro* maturation (IVM), and cycle of performing embryo biopsies for preimplantation genetic testing (PGT).

Data processing method

Data were recorded using Microsoft Excel 2010, processed with R version 3.5.1 and IBM SPSS Statistics 26 software. The comparisons of percentage values, mean values between two groups were tested by Student t-test. For categorical data, the chi-square test was used. All tests, when p value < 0.05 was considered to have a statistically significant difference. Value are presented as the Means ± SD (Standard Deviation).

Result

During the sampling period from June 2019 to June 2022 at the Fertility Center - Hanh Phuc International Hospital, we collected a total of 791 cases of satisfactory IVF treatment couples who have the criteria to meet this study. In which, 394 cases belonged to the group using sperm with low quantity in the ejaculate sample (E-ICSI) and 397 cases belonged to the group using sperm from microTESE technique (T-ICSI). All cases were performed by the same team and experienced fertility doctors.

Table 1. The baseline characteristics of young male patients.

Results	E- ICSI group (N=242)	T- ICSI group (N=256)	p-value
Age of wife (years old)	28.2 ± 1.4	27.4 ± 1.6	0.10
Age of husband (years old)	29.3 ± 2.8	29.2 ± 1.7	0.54
BMI of wife (kg/m^2)	21.2 ± 1.3	21.3 ± 1.2	0.91
BMI of husband (kg/m^2)	23.5 ± 2.9	23.4 ± 2.8	0.25
Number of retrieved oocytes	15.8 ± 4.8	16.6 ± 5.7	0.27
Total day of ovarian stimulation	9.9 ± 1.9	9.7 ± 2.2	0.42
Endometrial thickness on the day of embryo transfer (mm)	11.1 ± 0.6	11.2 ± 0.5	0.13

Group of young male patients (under 35 years old)

There were 498 young male patient couples (under 35 years old), accounting for the majority (63%) in the population of patient couples selected for the study and divided into 2 groups. In which, 256 patient couples underwent ICSI using sperm from microTESE (T-ICSI group) and 242 patient couples underwent ICSI using sperm from very small ejaculate samples (E – ICSI group). The baseline of patient characteristics were shown at Table 1.

Table 2. Embryological results and clinical treatment of young male patients.

Embryo results	E- ICSI group	T- ICSI group	p-value
The fertilization rates (%)	67.3	70.0	0.01
The third day good embryo rates (%)	43.2	44.6	0.35
The blastocyst formation rates (%)	49.2	50.8	0.51
The good blastocyst rates (%)	36.0	37.0	0.75
Pregnancy results	E- ICSI group	T- ICSI group	p-value
The beta hCG positive rates (%)	56.9	57.3	0.14
The clinical pregnancy rates (%)	53.8	54.0	0.74
The on-going pregnancy rates (%)	51.3	52.4	0.80
The miscarriage rates (%)	3.2	3.6	0.96
The multiple pregnancy rates (%)	7.3	5.0	0.65
The embryo implantation rates (%)	48.7	49.7	0.79
Children born result	E- ICSI group	T- ICSI group	p-value
The live birth rates (%)	50.1	50.4	0.83
The full-term birth rates (%)	20.0	22.7	0.58
Child's weight (gram)	3263.4 ± 157.3	3261.5 ± 224.6	0.95

The age of the patient couple between the E-ICSI group and T-ICSI group is quite young and similar (p>0.05), in the wife: the E-ICSI group has an average age of 28.2 (years old) compared to the T-ICSI group had an average age of 27.4 (years old), in the husband: the E-ICSI group had an average age of 29.2 (years old) compared with the T-ICSI group had an average age of 29.3 (years old). The basic indicators of ovarian stimulation results included average number of retrieved oocytes, total day of ovarian stimulation and endometrial thickness on the day of embryo transfer were no significant difference in the parameters between the two groups (p>0.05). This was evident by the fact that the input characteristics of the group of young patients were quite similar.

This made the other outcomes has themore objectivity and high reliability. In general, in embryo results of the male patients under 35 years old group, we found that the fertilization rates in the T-ICSI group were significantly higher than the E-ICSI group (70.0% vs 67.3%, p = 0.01) (Table 2). However, the day 3 good embryo rates, the blastocyst formation and the good blastocyst rates were similar between two groups (44.6% vs 43.2%; 50.8% vs 49.2%; 37.0% vs 36.0%, p > 0.05, respectively). Acorrding to Yu.Y *et al.*, the fertilization rates and high-quality embryo rates in men (under 35 years old) using ejaculated sperm were significantly higher, compared with the testicular sperm subgroup (74.7% vs. 62.4%; p=0.02; 50.5% vs. 36.6%; p=0.03, respectively) (Yu *et al.*, 2019).

It is quite interesting that the results of the authors are completely opposite to our study. If we compare the above results, it is found that the fertilization rate in our study in the T-ICSI group is 70% higher than the results of author Yu .Y is 62.4%.

In our study, we have detailed evaluation of the third day good embryo rates and also the good blastocyst rates instead of just collectively evaluating the good embryo rates like the author. The more detailed the research information, the more information can be provided to help the patient have a more multi-dimensional view.

The results of pregnancy and the outcome of children born in the group of male patients under 35 years old in our study were similar in two groups (p > 0.05). Our results is similar with Yu (Yu, Wang *et al.*, 2019).

But the clinical pregnancy rates in our study was a tendency to be lower than Yu *et al.* in both groups (58, 8% and 55.6%; p>0.05 vs 54.0% and 53.8%; p>0.05; respectively). However, the live birth rates in our study was similar with the author's study, ranging from 45% to 50% in both groups and tended to be higher in the group using sperm of testicular origin.

Group of elderly male patients (above 35 years old)

In our study, there were 293 couples of male patients and older male patients (above 35 years old), accounting for a lower proportion (37%) in the population of patient couples who met the study criteria. Baseline patient characteristics were shown in Table 3.

Table 3. Baseline characteristics of elderly male patients.

Results	E- ICSI group (N=141)	T- ICSI group (N=152)	p-value
Age of wife (years old)	31.8 ± 2.3	32.2 ± 2.8	0.10
Age of husband (years old	36.1 ± 0.7	36.0 ± 0.8	0.31
BMI of wife (kg/m2)	21.2 ± 1.3	21.2 ± 1.4	0.80
BMI of husband (kg/m2)	23.4 ± 1.4	23.5 ± 1.6	0.64
No. of eggs collected	14.6 ± 5.4	16.9 ± 6.4	< 0.05
Total day of ovarian stimulation	9.9 ± 2.4	9.8 ± 1.8	0.22
Endometrial thickness on the day of embryo transfer (mm)	11.2 ± 0.8	11.1 ± 0.9	0.43

There were no significant differences in terms of age wife and husband; body mass indess (BMI) of wife and husband. We found that there was no difference in the basic indicators of ovarian stimulation such as the time of ovarian stimulation in the two groups. However, there was a statistically significant difference in the number of aspirated follicles in between E-ICSI group and T-ICSI group (14.6 oocytes vs 16.9 oocytes, p < 0.05, respectively). For the group of elderly male patients, although we have excluded the cases of couples over 35 years old, but we have some cases that the wife is close 35 years old who responded poorly to ovarian stimulators than younger patients. Therefore, the number of eggs extracted was not similar between the two groups.

The thickness of the endometrium on the day of embryo transfer was guaranteed as required (8-14 mm). In terms of treatment efficacy, the embryonic and clinical results were shown in Table 4.

Table 4. Embryological results and clinical treatment of elderly male patients.

Embryo results	E- ICSI group	T- ICSI group	p-value
The fertilization rates (%)	51.4	59.8	< 0.05
The third day good embryo rates (%)	29.1	35.5	< 0.05
The blastocyst formation rates (%)	55.0	64.3	< 0.05
The good blastocyst rates (%)	35.8	43.0	0.07
Pregnancy results	E- ICSI group	T- ICSI group	p-value
The beta hCG positive rates (%)	48.4	53.8	0.62
The clinical pregnancy rates (%)	45.4	49.0	0.78
The on-going pregnancy rates (%)	43.5	46.9	0.80
The miscarriage rates (%)	4.0	3.7	0.74
The multiple pregnancy rates (%)	8.2	9.1	0.89
The embryo implantation rates (%)	39.9	43.0	0.69
Children born results	E- ICSI group	T- ICSI group	p-value
The live birth rates (%)	36.3	37.6	0.62
The full-term birth rates (%)	26.1	29.1	0.95
Child's weight (gram)	3258.5 ± 226.2	3359.8 ± 384.3	< 0.05

The embryo results in the T-ICSI group were better than the E-ICSI group, specifically as follows: the fertilization rates, the third day good embryos rates and the blastocyst formation rates in the E-ICSI group lower than the T– ICSI group (51.4% vs 59.8%, p<0.05; 35.5% vs 29.1% and 64.3% vs 55.0% , p<0.05, respectively); the good blastocyst rates were no statistically significant differences in two groups, but this rate in T-ICSI group still tends to be higher than E-ICSI group (48% vs 35.8%; p=0.07) Our embryological results were similar with the study of Yu [9]. We found that the embryological advantage was leaning towards the group using sperm from micro-testicular surgery - microTESE in patients above 35 years old.

Regarding clinical results, currently, for older women, infertility centers are tending to reduce the number of embryos transferred to patients in order to reduce the risk of multiple pregnancy as well as reduce the risk of pregnancy. Therefore, the rate of multiple pregnancies in both study groups were less than 10% (in group T-ICSI was 9.1% and in group E-ICSI was 8.2%, p>0.05). This was the desire of the patient as well as the desire of the medical team when treating NOA patients. There was no significant difference in pregnancy rate through tests and ultrasound in the two groups, in which: the positive beta hCG rates, the clinical pregnancy and the ongoing pregnancy rates, but T-ICSI group still has tends to be higher than that of the E-ICSI group. Besides, the percentages of embryos implanted in the T-ICSI group also tended to be higher than that in the E-ICSI group, but there was no statistically significant difference (p>0.05). Another aspect, the miscarriage rate in the T-ICSI group tended to be lower than the E-ICSI group, but there was no statistically significant difference (3.7% vs 4.0%, p>0.05). The percentages of live births in the T-ICSI group was higher than that of the E-ICSI group, but there was no statistically significant difference (37.6% vs 36.3%, p>0.05). Especially, our results showed that there was a difference in the weight of children born in these two groups, the weight of children born from the group using sperm from microTESE was heavier than that of children born from the group of sperm from Cryptozoospermia (3359.8 (gram) vs 3258.5 (gram), p < 0.05).

Discussion

In male, aging can directly affect sperm DNA, increasing methylation through the production of reactive oxygen species (ROS) molecules and free radicals. When ROS levels exceed the body's antioxidant capacity, excess ROS can interact with macromolecules leading to oxidative stress. High concentrations of ROS alter the structure of sperm cell membranes, reduce sperm motility and DNA damage, promote apoptosis, affecting spermatogenesis in men (Elbardisi *et al.*, 2021). Sperm with DNA damage can affect embryonic development and implantation (Wdowiak *et al.*, 2015). Some studies have shown that in men over 35 years of age, high levels of DNA fragmentation and breakage are associated with miscarriages and births with birth defects (Bradley *et al.*, 2016). Sperm DNA fragmentation is one of the important causes of male infertility. The study by Esteves *et al* (Esteves *et al.*, 2020) demonstrated an association between DNA fragmentation and sperm viability in semen. In patients with very few sperm in the ejaculate sample, there will be a high degree of DNA fragmentation, the percentage of dead sperm in the ejaculate sample is also very high. In addition, some studies have demonstrated that sperm from Cryptozoospermia samples have a higher DNA fragmentation index than sperm derived from testes (Watanabe, 2022). The study of Esteves *et al* (2015) (Esteves *et al.*, 2015), when comparing the results of ICSI in the group using sperm derived from the testis and the group using sperm from the ejaculate sample with high DNA fragmentation index. The study was conducted on 147 couples undergoing infertility treatment, divided into two groups: Group 1 used sperm derived from testicles with a DNA fragmentation index of 8.3% and Group 2 used sperm. From ejaculate samples with high DNA fragmentation index of 40.7%. Regarding embryology results, the day 3 good embryos rates in group 1 was 45.2%, which tended to be 41.8% higher than in group 2, this difference was not statistically significant (p>0.05). Meanwhile, the clinical results

showed a statistically significant difference ($p<0.05$) in group 1 compared with group 2: the live birth rates in group 1 was 46.7% higher than in group 2. was 26.4% ($p=0.007$) and the miscarriage rates was lower in group 1 than in group 2 (10.0% vs 34.3%, $p=0.012$). Currently, many substances have been identified that have the ability to prevent free radicals in semen such as vitamin C, vitamin E, glutathione or polyamines, (Sánchez-Rubio *et al.*, 2020). However, their effectiveness has not been significantly improved, many patients still have a high DNA fragmentation index after using them for a long time (Moskovtsev *et al.*, 2010). According to Showell *et al.* (Showell *et al.*, 2014), when comparing ROS levels in the group that used the drug treatment and the group that used placebo or no treatment, the ROS concentration decreased only 10.4%. Therefore, the use of sperm derived from testes by microTESE technique is considered as a reasonable alternative.

A question of patients with Cryptozoospermia is how likely is it to find sperm by microTESE in their cases? In the study of Alkandari *et al* (2021) (Alkandari *et al.*, 2021) when investigating the possibility of finding sperm by microTESE on 223 cases of male patients with NOA. The study was divided into three main groups: group with absolutely no sperm in the testicles (Azoospermia), group with very low sperm count (Cryptozoospermia), group with low density sperm (OligoAsthenoTeratozoosperm). The results showed that the ability to find sperm of the three groups above was 52%, 91% and 100%, respectively. Similarly, in the study of Almajed *et al* (2020) (Almajed *et al.*, 2020), the author performed a study on 103 patients with NOA. Results of successful sperm collection in the group with absolutely no sperm in the testes was 48% and in the group with Cryptozoospermia was 89%. Both studies concluded that: through testicular microsurgery technique, the possibility of finding sperm of very few cases in the ejaculatory sample in NOA patients is over 89%. The study by Thao *et al* (2020) (Huỳnh Thị Thu Thảo and Tuyết, 2020) was conducted at Fertility Center - Hanh Phuc International Hospital, Vietnam have similar results. The rate of sperm finding by testicular microsurgery - microTESE in the group with absolutely no sperm in the testes was 45.5% and in the group with Cryptozoospermia was 89.5%. Therefore, determining the ability to find sperm helps clinicians have a more general view to consult, as well as answer patients' questions.

When a new technique is applied clinically, the question is how effective is that technique and how does it affect the patient's health? In Vietnam, most patients diagnosed with NOA underwent at least one testicular biopsy. Finding sperm in patients with NOA usually occurs after multiple biopsies (15-30 tissue samples). Performing microTESE in NOA patients showed that for the sperm finding rate was 56% higher than in patients who had undergone surgery with 1-2 samples. Biopsies (51%) and with 3-4 biopsies (23%) ($p=0.04$) (Ostad *et al.*, 1998). The cause may be due to testicular biopsy damaging blood vessels in the testicle and scar formation, parenchymal fibrosis, testicular hematoma, will affect the spermatogenesis process of surrounding tissue. The more number and size of biopsy sample, the more affect NOA patient can get (Ostad, Liotta *et al.*, 1998). One of the advantages of microTESE is that it minimizes the amount of testicular tissue that is removed. If sperm were found in the first microTESE, the percentage of sperm found in the second microTESE was 96%. Meanwhile, if there was no sperm in the first microTESE, the percentage of sperm found in the next time was only about 33% (Haimov-Kochman *et al.*, 2009). When comparing microTESE with classical-TESE (cTESE), author Vahidi *et al* (Vahidi *et al.*, 2021) recorded sperm collection rates of 47%-63% versus 30%-45%, respectively.

The mean volume of tissue removed was 9.4 (miligram) versus 720 (milligram). His study performed on 555 couples of male patients with NOA, divided into two groups: one using microTESE method to collect sperm and the other group using cTESE method. The results showed that the fertilization rates and live birth rates in both groups had no statistically significant difference. Besides

improving treatment results, the question is whether microTESE implementation is effective in terms of treatment costs? To determine this, Franco *et al.* (Franco *et al.*, 2016) evaluated the cost and effectiveness of treatment of patient couples when performing ICSI from microTESE sperm. The treatment effect of microTESE technique includes the following factors: microTESE procedure average time is about 90 minutes longer than cTESE procedure time is about 30 minutes to 45 minutes. However, the number of sperm obtained from microTESE was 1.5 times more than that of cTESE. The risk of postoperative wound hematoma in patients undergoing microTESE is very low, about 2-3%, while in cTESE it is about 18%-25%. Regarding the cost of the procedure, research shows that the cost of performing a procedure in microTESE technique is 50% higher than in cTESE. The cost of performing a microTESE procedure is higher, but in the event that no sperm is found during cTESE, the chance of obtaining sperm from microTESE from such cases is about 26%-29%. MicroTESE technique helps improve the ability to find sperm compared to cTESE technique from 34% to 51%, thereby reducing the need for patients to have to repeat surgery many times, saving treatment costs for patients. The author's conclusion is that microTESE technique helps to limit risks and ensure safety, as well as patient's health, and it is recommended that the microTESE technique should be prioritized for male patients with nonobstructive azoospermia.

Conclusion

The results of the study showed that the testicular microsurgery sperm extraction technique improved embryology results in the age group of male patients with non-obstructive azoospermia. Regarding clinical treatment results, the group using sperm from testicular microsurgery tended to be higher than the group using sperm from ejaculate samples. Through the above study, clinicians have a database to advise non-obstructive azoospermia male patients to choose the appropriate method to enhance the effectiveness of treatment in *in vitro* fertilization and saves time and money for the patients.

Abbreviation

Beta hCG: Beta humanChorionic Gonadotropin; ICSI: Intracytoplasmic Sperm Injection; MicroTESE: Microdissection Testicular Sperm Extraction; NOA: Non-obstructive azoospermia; PN: Pronuclear, ROS: Reactive oxygen species.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

The study was approved by the medical ethics committee of Hanh Phuc International Hospital.

Availability of data and materials

Not applicable

Authors' contributions

Huyen Nguyen Thi Thuong designed, coordinated this research and drafted the manuscript.

Thong Nguyen Quang; Tu Nguyen Huynh Cam; Khoi Tran Vy; An Vo Thien; Thao Huynh Thi Thu conceived of the study, and participated in research coordination.

The authors read and approved the final manuscript.

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