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EXCESS REACTIVE OXYGEN SPECIES IN EJACULATE AFFECT EMBRYO DEVELOPMENT *IN VITRO* WHEN ASSISTED REPRODUCTIVE TECHONOLOGIES ARE USED

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Background. Reactive oxygen species are a necessary condition for the maturation, existence and ability of spermatozoa to fertilize the oocyte. The excess reactive oxygen species in semen can initiate pathological changes in sperm, causing oxidative damage to cell membranes, proteins, and DNA. The aim of the present study was to investigate changes in standard microscopic parameters and the level of ROS in the ejaculate in men with low reproductive function, to determine the influence of paternal age on the mentioned parameters, and to establish a possible relationship between the level of ROS generated in the sperm and an early embryonic development in infertile patients.

Materials and Methods. The development of the embryos from 26 married couples with combined factors of infertility was analyzed. Microscopic analysis of the ejaculate was performed according to the WHO recommendations from 2021. The level of oxidative stress in the ejaculate was analyzed using Oxisperm kits (Halotech DNA, Spain). Donor oocytes were fertilized by the method of intracytoplasmic sperm injection (ICSI). Embryos that reached the blastocyst stage were evaluated by morphological characteristics according to the criteria of D. Gardner (2000). Preimplantation genetic testing of blastocysts for aneuploidy was performed using the next-generation sequencing method. Statistical hypotheses were tested using the chi-square and r_s criteria at significance levels of 0.05 and 0.01.

Results and Discussion. The present study demonstrates the negative effect of the excess of ROS in the ejaculate on early embryo development and embryo ploidy.

Increased ROS level negatively affects both total blastocyst formation rate (r_s = -0.66, *p* = 0.00247) and euploid blastocyst formation rate (r_s = -0.65, *p* = 0.04034). Among the infertile patients the part of men with an excess of ROS in ejaculate is significantly higher compared with sperm donors (*p* = 0.000063). A significant negative correlation was found between sperm motility and paternal age (r_s = -0.54, *p* = 0.01795). No correlation was observed between ROS levels in the ejaculate and sperm motility, concentration, and morphology in infertile patients.

Conclusion. The negative influence of the excess of reactive oxygen species in semen on the early embryo development *in vitro* has been proved. A negative effect of increased ROS levels on male reproductive function has been demonstrated. Excess of ROS in ejaculate results in a decrease of both total blastocyst formation rate (r_s = -0.66, *p* = 0.00247) and euploid blastulation rate (r_s = -0.65, *p* = 0.04034). A negative effect of paternal age on microscopic sperm parameters has been shown. An excess of ROS in sperm does not lead to fertilization failure in ICSI. No correlation was found between microscopic sperm parameters and early embryo development *in vitro.*

Keywords: reactive oxygen species, male infertility, blastocyst formation, aneuploidy, *in vitro* embryo development

INTRODUCTION

According to the literature, in European countries and in Ukraine, male factor is the cause of infertility in couples in more than 50 % of cases (Mazzilli, 2023). Currently, several scientific studies indicate that high levels of reactive oxygen species (ROS) in the ejaculate are one of the causes of low male reproductive function. Reactive oxygen species are a necessary condition for the maturation, existence and ability of spermatozoa to fertilize the oocyte (Mannucci, 2022; Wagner, 2018). The content of ROS at an optimal level is maintained by the antioxidant system. Oxidative stress arises from an imbalance between ROS production and antioxidant defense mechanisms. On the other hand, the measurement of ROS is hampered by the lack of standardization, including the choice of controls and sample selection (Latchoumycandane, 2020). In particular, oxidative stress may cause changes in standard sperm fertility parameters (sperm motility, concentration, and morphology), whereas its effects on the integrity of sperm DNA in the ejaculate are controversial. Indeed, most studies suggest that excess ROS in semen can initiate pathological changes in sperm, causing oxidative damage to cell membranes, proteins, and DNA (Bisht, 2017). However, no effect of ROS on sperm quality was found in some works (O'Flaherty, 2020). The question of whether the excess of ROS in sperm affects human embryo development *in vitro* is still open. Some authors showed that a high percentage of hydrogen peroxide in tne ejaculate reduced the good-quality day 3 embryo rate and the good-quality blastocyst rate (Liu, 2023). On the other hand, S. Kuroda (2020) demonstrated that the reactive oxygen species level in semen did not reduce the fertilization rate in *in vitro* fertilization cycles (IVF) when the method of intracytoplasmic sperm injection (ICSI) was used to fertilize the oocytes. It remains relevant to study the question of whether the level of ROS in sperm leads to the formation of aneuploid embryos when assisted reproductive technologies are used. For example, V. Burruel (2014) showed that high ROS levels in sperm can result in aneuploidy and failure to develop normally to the blastocyst stage. On the other hand, Y. Fu (2025) found that there was no significant difference in blastocysts' aneuploidy rates in groups of patients with different levels of ROS in the ejaculate.

The aim of the present work was to investigate changes in standard microscopic sperm parameters and the level of ROS in the ejaculate in men with low reproductive function to determine the effect of paternal age on sperm indices and to establish a possible relationship between the generated level of ROS in the sperm with infertility factors in men and an early embryonic development.

MATERIALS AND METHODS

The collection of primary information and laboratory studies were conducted at the "Clinic of Professor Feskov O. M." (Kharkiv) during the period of January-August 2025. The study was approved by the Ethics Committee of LLC "Sana-Med" (Center for Human Reproduction "Clinic of Professor Feskov O. M.") in accordance with Protocol No 3 dated December 20, 2024. Each spouse signed the Informed Consent for the Use and Processing of Data.

The control group consisted of 25 sperm donors with an average age of 32 ± 4 years. In the control group, IVF embryos were obtained using donor male and female gametes. The age of the oocyte donors in the control group ranged from 23 to 31 years. The ICSI method was used to fertilize donor eggs with donor spermatozoa.

The treatment group comprised 26 married couples with combined factors of infertility. The study included only couples with both spermatogenesis failures in men and with premature ovarian failure in women. Oocyte donation was used in the studied infertile group. The average male age in the infertile group was 39 ± 4 years. The age of the oocyte in the treatment group ranged from 24 to 30 years. Donor oocytes were fertilized by the method of intracytoplasmic sperm injection due to poor sperm parameters in men in the mentioned group. The effect of the ROS-level in semen on fertilization rates, blastocyst formation rate and blastocyst aneuploidy rate were studied in the infertile group.

Microscopic analysis of ejaculate was performed according to the WHO recommendations from 2021 (Chung, 2024). The ROS levels in th ejaculate were analyzed using Oxisperm kits (Halotech DNA, Spain). The assay is based on the ability of nitro blue tetrazolium and other molecules associated with ROS to be converted by superoxide anions into a water-insoluble blue formazan crystal that changes the color intensity in the reactive gel (from yellow to various levels of violet-blue). The results of the assay were determined by the color scale according to the color intensity. According to the manufacturer's grading, 4 levels of oxidative stress were distinguished (levels 1 and 2 are low ROS exposure; level 3 is medium ROS exposure; level 4 is high ROS exposure) (Castleton, 2022). The principle of determining the level of ROS in the ejaculate is schematically shown in **Fig. 1**.

Controlled ovulation stimulation (COS) of egg donors was performed using the ant-GnRH protocol. Fertilization of the obtained oocytes was done by the ICSI technique. Embryo culture to the blastocyst stage was performed in GAIN medium Single-step (Austria) at a temperature of 36.9 °C–37.1 °C and a CO_2 content of 5.5–5.7 % (Gruber, 2011). Embryos that reached the blastocyst stage were evaluated by morphological characteristics of the intracellular mass (ICM) and trophectoderm (TE) according to the criteria of D. Gardner (Gardner, 2000). Preimplantation genetic testing of blastocysts for aneuploidy was performed using the next-generation sequencing method (Doroftei, 2022).

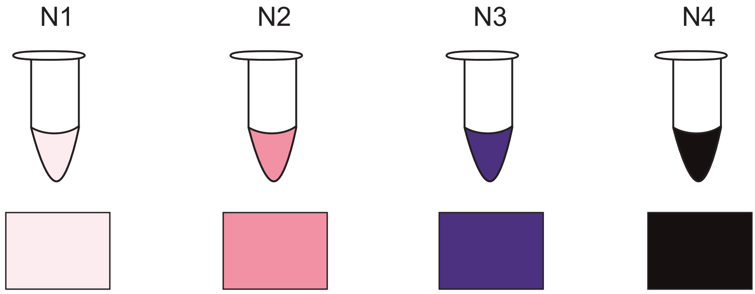

Fig. 1. Scheme for determining the level of oxidative stress in the ejaculate using Oxisperm kits (Halotech DNA, Spain): N1, N2 – low exposure to ROS; N3 – average exposure to ROS; N4 – high exposure to ROS

Comparison of arithmetic means was carried out using the Student's *t*-test. To study the relationships between the features, Spearman correlation analysis was used. The chi-square test was used to assess the significance of the differences in such parameters of the IVF cycle as the fertilization rate and blastocyst formation rate between the group of infertile patients and the control group. Statistical hypotheses were tested using the chi-square and r_s criteria at significance levels of 0.05 and 0.01. (Petrie, 2000). The Apache Open Office 4.0.0 software package (Sana-Light Ltd., Sana-Med Ltd.) was used for calculations.

RESULTS AND DISCUSSION

When studying the effect of paternal age on microscopic sperm parameters, a statistically significant negative correlation was found between sperm motility and the age of the patients (r_s = -0.54, p = 0.01795). There was no correlation between paternal age and sperm concentration and a part of spermatozoa with normal morphology in the ejaculate in the studied group of infertile men. The data obtained are mentioned in **Table 1**.

Table 1. **The correlation between paternal age and classical parameters of the spermogram in men with low reproductive function**

Sperm parameter		r_s	p
Name of parameter	Average mean, $\bar{X} \pm m_x$		
Motility, %	46.5 ± 11.2	-0.54	0.01795
Concentration, ×10^6 cell/mL	49.9 ± 24.2	0.06	$p > 0.05$
Normal sperm morphology, %	7.3 ± 5.0	-0.16	$p > 0.05$

Notes: p – significance level; r_s – Spearman coefficient

The effect of paternal age on the level of ROS in the ejaculate was not proved in the examined group of infertile men. The study did not prove the effect of high levels of ROS in the ejaculate on sperm motility, concentration, and morphology in infertile patients (p > 0.05). Nevertheless, it was found out that among the infertile patients, the part of men with an excess of ROS in the ejaculate was significantly higher compared with sperm donors (61.6 % vs. 8.0 %, df = 1, $\chi 2_{fact.}$ = 15.9966, p = 0.000063, respectively). The obtained results are demonstrated in **Fig. 2**.

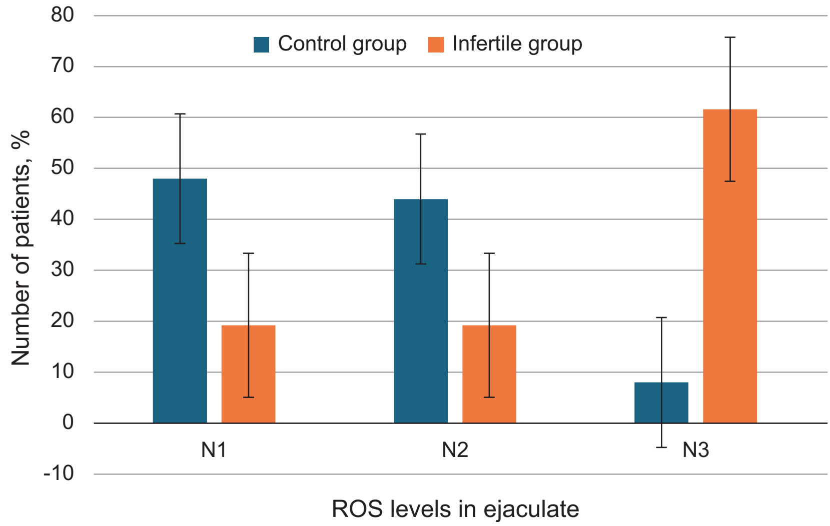

Fig. 2. Proportion of the detected ROS levels in ejaculate among infertile patients and among sperm donors: N1, N2 – low exposure to ROS; N3 – average exposure to ROS

In total, 317 mature MII donor oocytes were fertilized by the method of ICSI in the infertile group of patients. On average, there were 10 ± 2 MII donor eggs per patient. The fertilization rate in these patients was 72.9 % (231 zygotes with two pronuclei). The total blastocyst formation rate (BFR) was 50.2 % (116 blastocysts) in the examined infertile group.

In the control group, where donor male and female gametes were used, 93 mature MII oocytes were fertilized by ICSI. The fertilization rate in this group was 80.6 % (75 zygotes with two pronuclei). The total BFR was 76.0 % (57 blastocysts), respectively. There was no statistically significant difference between the fertilization rates in the infertile group and in the control group. On the other hand, the total blastocyst formation rate was significantly higher in the control group, compared with the mentioned parameter in the infertile patients (76.0 % vs. 50.2 %, df = 1, $\chi2_{fact.}$ = 15.3173, p = 0.000157, respectively). Such results confirmed the fact that the process of blastocyst formation depends on the general quality of sperm (Piccolomini, 2018). Nevertheless, no correlation was found between microscopic sperm parameters and blastocyst formation rate in the infertile group ($p > 0.05$).

In the infertile group, the preimplantation genetic testing was performed for 105 blastocysts. Biopsy was performed on the fifth day of culture for 63 embryos, and on the sixth day for 42 blastocysts. A photo of a blastocyst before the biopsy procedure is shown in **Fig. 3**. The strong negative correlation between the ROS level and both total blastocyst formation rate and euploid blastocyst formation rate was found (r_s = -0.66, p = 0.00247 and r_s = -0.65, p = 0.04034, respectively). In the treatment group, ROS level did not affect the fertilization rate after the procedure of ICSI. The results obtained are presented in **Table 2**. In the control group, the rate of formation of euploid blastocysts was 56.0 % (42 blastocysts). The euploid blastocyst formation rate was significantly higher in the control group, compared with the mentioned parameter for the infertile patients (56.0 % vs. 11.6 %, df = 1, $\chi2_{fact}$. = 40.2702, $p < 0.001$, respectively). The difference between standard *in vitro* parameters in the infertile men and in the control group is shown in **Fig. 4**.

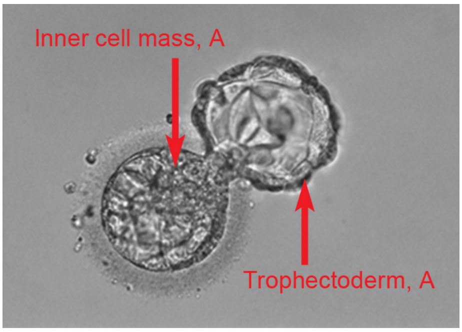

Fig. 3. Blastocyst 5AA according to the D. K. Gardner classification *in vitro*, before the procedure of biopsy. Magnification: ×250. Representing a hatching blastocyst (stage 5) with a tightly packed inner cell mass (A) and a cohesive, high-cell-count trophectoderm (A). It indicates a highly developed embryo ready for implantation

Table 2. **Correlation between the level of reactive oxygen species in semen and standard *in vitro* fertilization parameters**

In vitro fertilization parameters		ROS level	r_s	*p*
Name of parameter	Average mean, $\bar{X} \pm m_x$			
Total number of donor MII-oocytes, N	10 ± 2	N3	-	-
Fertilization rate, %	72.9 ± 7.1	N3	0.05	$p > 0.05$
Total blastocyst formation rate, %	50.2 ± 14.9	N3	-0.66	0.00247
Euploid blastocyst formation rate, %	11.6 ± 7.7	N3	-0.65	0.04034

Notes: *p* – significance level; r_s – Spearman coefficient

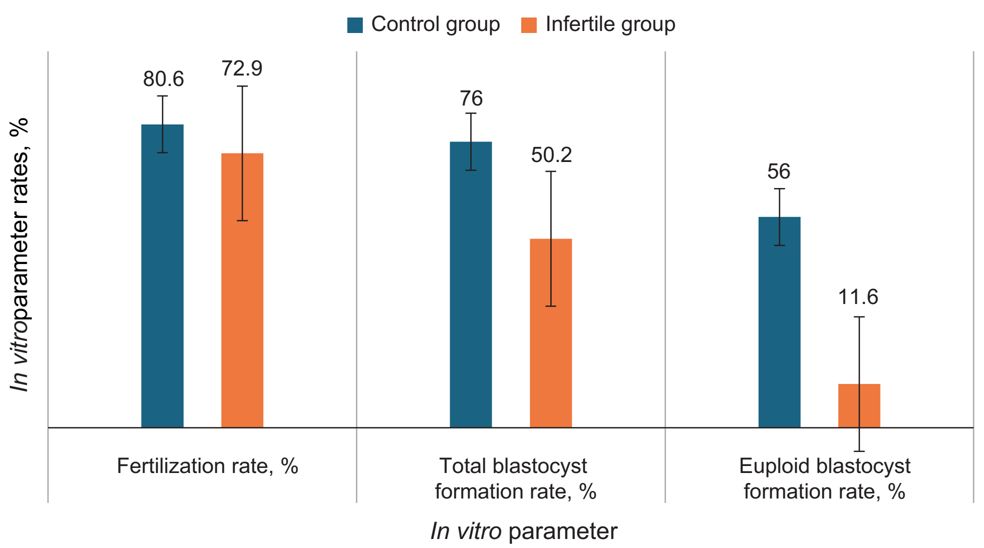

Fig. 4. Standard *in vitro* parameters of embryo development in patients with excess of ROS in ejaculate and in the control group

At this stage of the study, the negative effect of an excess of ROS in the ejaculate on the total blastocyst formation rate and euploid blastulation rate was proved.

As no correlation was observed between ROS levels in semen and sperm motility, concentration, or morphology in the infertile patients, it may be assumed that more complex mechanisms underlie the ROS effects on male reproductive function resulting in early embryo development failures (total blastulation rate and euploid blastocyst formation rate). For example, some authors suggested that high amounts of ROS may impair mitochondrial function, potentially causing sperm dysfunction and, consequently, infertility (Durairajanayagam, 2021). The next possible explanation for such a decreased developmental potential could involve sperm DNA integrity (Kumaresan, 2020). Therefore, further studies are warranted to better understand the influence of the mechanisms of ROS in semen on male reproductive function.

CONCLUSION

The negative influence of the excess of reactive oxygen species in semen on the early embryo development *in vitro* has been proved. A negative effect of increased ROS levels in semen on male reproductive function has been demonstrated. The excess of reactive oxygen species in the ejaculate decreased both total blastocyst formation rate (r_s = -0.66, p = 0.00247) and euploid blastocyst formation rate (r_s = -0.65, p = 0.04034). Among the infertile patients, the part of men with an excess of ROS in the ejaculate was significantly higher compared with sperm donors (p = 0.000063). Paternal age negatively affects sperm motility (r_s = -0.54, p = 0.01795). The level of ROS in sperm does not affect fertilization rate. No correlation was found between microscopic sperm parameters and early embryo development *in vitro* ($p > 0.05$). The study did not prove the effect of the level of reactive oxygen species in the ejaculate on sperm motility, concentration, and morphology in infertile patients ($p > 0.05$). In the future, ROS concentrations may be used as a marker in preconception diagnostic testing in men.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: there is no conflict to declare.

Human Rights: all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards

AUTHOR CONTRIBUTIONS

Conceptualization, [F.O.]; methodology, [Zh.Ye.; F.I.]; validation, [Zh;Ye.]; formal analysis, [Zh;Ye.; Ye.O.].; investigation, [Zh.Ye.; Ch.N.; Ye.O.]; resources, [Zh.Ye.]; data curation, [Zh.Ye.; Ye.O.]; writing – original draft preparation, [Zh;Ye.]; writing – review and editing, [F.O.]; visualization, [Zh.Ye.] supervision, [F.O.; F.I.]; project administration, [Zh.Ye.].

All authors have read and agreed to the published version of the manuscript.

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НАДМІРНА КІЛЬКІСТЬ АКТИВНИХ ФОРМ КИСНЮ В ЕЯКУЛЯТІ ВПЛИВАЄ НА РОЗВИТОК ЕМБРІОНІВ *IN VITRO* ЗА ВИКОРИСТАННЯ ДОПОМІЖНИХ РЕПРОДУКТИВНИХ ТЕХНОЛОГІЙ

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Обґрунтування. Активні форми кисню є необхідною умовою для дозрівання, існування та здатності сперматозоїдів запліднювати ооцит. Надлишок активних форм кисню у спермі може ініціювати патологічні зміни в сперматозоїдах, спричиняючи оксидативне пошкодження клітинних мембран, білків і ДНК. Метою даної роботи було дослідити зміни стандартних мікроскопічних параметрів і рівень АФК в еякуляті у чоловіків зі зниженням показників репродуктивної функції, з'ясувати вплив віку батька на зазначені параметри та встановити ймовірний зв'язок між рівнем генерації АФК у спермі чоловіків із фактором безпліддя та раннім розвитком ембріона.

Матеріали та методи. Проаналізовано розвиток ембріонів 26 подружніх пар із комбінованими факторами безпліддя, з використанням донорських ооцитів. Мікроскопічний аналіз еякуляту проводили відповідно до рекомендацій ВООЗ від 2021 р. Рівень активних форм кисню в еякуляті визначали за допомогою наборів Oxisperm (Halotech DNA, Іспанія). Донорські ооцити запліднювали методом інтрацитоплазматичної ін'єкції сперматозоїдів (ІКСІ). Ембріони, що досягли стадії бластоцисти, оцінювали за морфологічними характеристиками згідно з критеріями Д. Гарднера. Преімплантаційне генетичне тестування бластоцист на анеуплоїдію проводили методом секвенування наступного покоління. Статистичні гіпотези перевіряли за допомогою критеріїв хі-квадрат та r_s за рівнів значущості 0,05 та 0,01.

Результати. У цьому дослідженні продемонстровано негативний вплив надлишку активних форм кисню в еякуляті на ранній розвиток і плоїдність ембріонів *in vitro*. Збільшення АФК у спермі негативно впливає як на загальну частоту формування бластоцист (r_s = -0,66, p = 0,00247), так і на частоту формування еуплоїдних бластоцист (r_s = -0,65, p = 0,04034). Серед безплідних пацієнтів частка чоловіків із надлишком активних форм кисню в еякуляті значно вища порівняно з донорами сперми (p = 0,000063). Виявлено значну негативну кореляцію між рухливістю сперматозоїдів і чоловічим віком (r_s = -0,54, p = 0,01795). Не виявлено жодної кореляції між надлишком АФК в еякуляті й рухливістю, концентрацією та морфологією сперматозоїдів у пацієнтів з безпліддям.

Висновки. Доведено негативний вплив надлишку активних форм кисню у спермі на ранній розвиток ембріона *in vitro*. Продемонстровано негативний вплив підвищеного рівня активних форм кисню на чоловічу репродуктивну функцію. Надлишок активних форм кисню у спермі призводить до зниження як загальної частоти формування бластоцист (r_s = -0,66, p = 0,00247), так і частоти формування еуплоїдних бластоцист (r_s =-0,65, p = 0,04034). З’ясовано, що збільшення віку чоловіка негативно впливає на мікроскопічні показники еякуляту. Рівень активних форм кисню у спермі не впливає на частоту запліднення у разі використання методу ІКСІ. Не виявлено кореляції між мікроскопічними параметрами спермограми та раннім розвитком ембріона *in vitro*.

Ключові слова: активні форми кисню, чоловіче безпліддя, формування бластоцист, анеуплоїдія, розвиток ембріона *in vitro*

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