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HYPOXIA AS A MODULATING FACTOR OF HUMAN SPERM CAPACITATION AND CRYORESISTANCE

Despite increasing interest to the role of hypoxia in reproductive processes, its impact on sperm capacitation and cryoresistance remains insufficiently described. This study investigated the effect of oxygen tension during pre-cryopreservation sperm incubation on the capacitation efficiency and examined the relationship between capacitation and cryoresistance. According to the WHO criteria, ejaculates from 30 healthy normozoospermic men were analyzed. Functional characteristics and cryoresistance of spermatozoa were assessed after density gradient processing and in vitro capacitation under 1, 5 and 21% oxygen concentrations. An incubation of human spermatozoa under moderate hypoxia was found to positively influence the viability, motility, and capacitation activation. For the first time, we have demonstrated that oxygen tension during in vitro incubation is a critical factor determining sperm survival following thawing. These findings suggest that moderate hypoxia may represent a promising biotechnological strategy to improve sperm cryopreservation protocols and enhance the efficiency of assisted reproductive technologies.

Key words: *cryopreservation, hypoxia, human spermatozoa, capacitation.*

The successful assisted reproductive technology programs largely depend on the quality and functional integrity of spermatozoa, in particular their ability to fertilize an egg. One of the key processes that ensure this ability is capacitation, a complex of sequential molecular changes in the membrane and cytoplasm of male germ cells necessary for gaining the hyperactivity and the acrosomal reaction (AR). This process is controlled by a number of signaling cascades, including those involving calcium channels, the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) system, as well as tyrosine kinases sensitive to changes in the microenvironment [2].

In vivo sperm capacitation occurs in the female reproductive tract, particularly the uterus and oviduct [14]. The female reproductive tract of mammals is characterized by hypoxic conditions with O_2 levels ranging from 2 to 8%. The oxygen level in the ovaries is 5—8%, while in the fallopian tubes and uterine cavity it drops down to 2—3% [11]. With the participation of plasma proteins of female secretions, changes in the state of the sperm plasma membrane occur, resulting in the membrane potential hyperpolarization, an increased level of intracellular cAMP and activation of phosphorylation signaling cascades, which contributes to hyperactive motility and readiness of sperm for AR [15].

In vitro capacitation is induced by culturing sperm in special media that mimic the conditions of the female reproductive tract [6]. Sperm in-

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cubation and capacitation media contain carbohydrates, amino acids, lipids, and trace elements. The duration of incubation, concentration of components, and temperature are considered critical for the efficiency of the capacitation process. One of the components of the *in vitro* environment and a critical regulator of sperm function is oxygen. As noted above, *in vivo* capacitation occurs in the hypoxic environment of the female genital tract, but most standard protocols do not take this factor into account. Research results indicate that modification of oxygen levels during sperm culture can affect their biological activity [3]. Thus, oxygenation of the incubation medium is an important regulator of the functional activity of spermatozoa at the *in vitro* capacitation stage.

In modern reproductive practice, various approaches to sperm cryopreservation are used both in normozoospermia and in pathological changed ejaculate [8, 13]. It is known that most manipulations at the stage of preparing sperm for low-temperature storage, including capacitation, are carried out under normoxia conditions [12].

The question about the optimal time for cryopreservation — before or after sperm capacitation — remains debatable and insufficiently studied.

The aim of the study was to assess the effect of the oxygenation level during incubation before cryopreservation on the efficiency of capacitation as well as to find out whether capacitation affects the sperm cryoresistance.

MATERIALS AND METHODS

The study comprised the ejaculates from 30 healthy men of reproductive age with normozoospermia. All participants provided a written informed consent of using the biomaterial. Inclusion criteria were as follows: no genitourinary tract infections, inflammatory or systemic diseases, as well as no history of reproductive system pathologies. After collection, the ejaculate was incubated for 30 min at 37 °C. Following dilution, a basic spermogram analysis was performed in accordance with the recommendations of the World Health Organization. The following parameters were assessed: ejaculate volume, pH, sperm concentration, total sperm count, motility (progressive/non-progressive, immobile forms) and viability (eosin-nigrosin staining). Afterwards, the ejaculate was layered with 1 ml of SpermGrade density gradient (CooperSurgical, USA) in sterile conical tubes. First, 1 ml of 80% gradient was applied, on top of which 1 ml of 40% gradient was carefully layered, and on top — 1—2 ml of pre-diluted ejaculate. Centrifugation was performed at 300—400 g for 20 min at room temperature. Then, the supernatant was carefully removed, and the pellet was resuspended in Global total for fertilization medium (CooperSurgical). The swim-up method was used to perform capacitation. For this, 0.5 ml of medium was carefully added to the pellet and incubated for 60 min in a SANYO MCO-19M multigas incubator (Sanyo Electric Co., Japan) at a temperature of 37 °C with O_2 concentrations of 1, 5 and 21%. After incubation, the upper layer enriched with motile spermatozoa was carefully removed for further analysis. Thus, the samples were divided into three groups, each of which was incubated under the following conditions: group 1 — 1% O_2 (hypoxic conditions), group 2 — 5% O_2 (physiological norm for the female reproductive tract), group 3 (control) — 21% O_2 (atmospheric level).

The number of spermatozoa that passed throug the AR was determined using FITC-PSA fluorescent labeling (Sigma-Aldrich, USA) according to the manufacturer's protocol. The acrosome was considered intact if uniform intense luminescence of the acrosomal region was observed and completely edited if its luminescence was absent.

For the cryobiological experiment, spermatozoa were collected before and after an hour of *in vitro* cultivation under different oxygenation conditions. The samples were frozen according to the adapted protocol of M. Di Santo *et al.* [4]. Spermatozoa were incubated for 10 min in a 7% glycerol solution (Merck KGaA, Germany) at room temperature. Cryoprotectant was introduced gradually to minimize osmotic shock. After exposure, the cell suspension was distributed into 0.5 ml cryovials (Thermo Fisher Scientific, USA). The containers were labeled and placed approximately 5 cm above the level of liquid nitrogen (–80 °C) for 10—15 min, afterwards the samples were quickly immersed in liquid nitrogen (–196 °C) for a long-term storage.

The samples were thawed by a quick immersion of the cryovial in a water bath heated to 37 °C. In order to minimize osmotic shock, after the complete disappearance of the solid phase, the sample was gradually diluted with a medium without cryoprotectant. The cryoprotectant was removed by centrifugation with subsequent collection of the

supernatant. After thawing, the survival rate of cells was determined by assessing their viability.

The obtained data were statistically analyzed using "GraphPad Prism (10.4.1)" software (GraphPad Software, USA). In all experiments, the normality of the data distribution was checked using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare groups. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

At the first stage of the study, the basic clinical and demographic characteristics of the examined individuals were analyzed. The average age of the participants was (29.8 ± 3.5) years, body mass index made (22.2 ± 4.3) kg/m^2. After assessing the general anthropometric parameters, the spermiological indices were explored: the volume of the ejaculate was (2.67 ± 0.51) ml, corresponding to normal physiological values; the total concentration of spermatozoa was (62.7 ± 19.2) million/ml; motility made (61.9 ± 9.4)%; viability was (77.1 ± ± 5.6)%, which indicated a high number of live cells in the samples.

After 60-min incubation of the samples, the frequency of cell motility and viability changed depending on the incubation conditions (Table).

The findings demonstrate a pronounced effect of the O_2 level under *in vitro* capacitation conditions, on the spermatozoa functional characteristics. The highest motility indices were determined during spermatozoa incubation under moderate hypoxia (group 2), which exceeded the mentioned index in groups 1 and 3. At an O_2 level of 5%, a more pronounced manifestation of hyperactive movements was noted, which demonstrated the potential advantage of moderate hypoxia for activating capacitation processes. An increased viability of spermatozoa of group 2 was noted. In view of this fact, it can be assumed that reduced (5% O_2) hypoxia creates optimal conditions for preserving the functional characteristics of spermatozoa, while excessive hypoxia (1% O_2) and normoxia are accompanied by a decrease in this index, showing the importance of precise control of the oxygen level during manipulations with reproductive cells.

After one hour of incubation of spermatozoa in media with different O_2 concentrations, the level of induced AR was lowest in group 3, while group 2 had the highest one. AR induction is provided by an environment that corresponds to the physiological conditions of the female genital tract [11]. Incubation at 1% O_2 creates an alkaline environment, which may inhibit AR initiation, preserving more spermatozoa with intact acrosome [18]. In addition, it is believed that elevated O_2 levels may cause excessive production of reactive oxygen species (ROS), which leads to membrane damage and premature depletion of the functional potential of spermatozoa [1]. Low O_2 levels provide a moderate level of AR. This is probably due to the limited energy supply of glycolytic and mitochondrial origin [5]. Thus, the obtained data indicate that reduced hypoxia (5% O_2) creates favorable conditions for the course of sperm capacitation *in vitro*, while incubation at 1 and 21% O_2 levels may have a suppressive effect on this process.

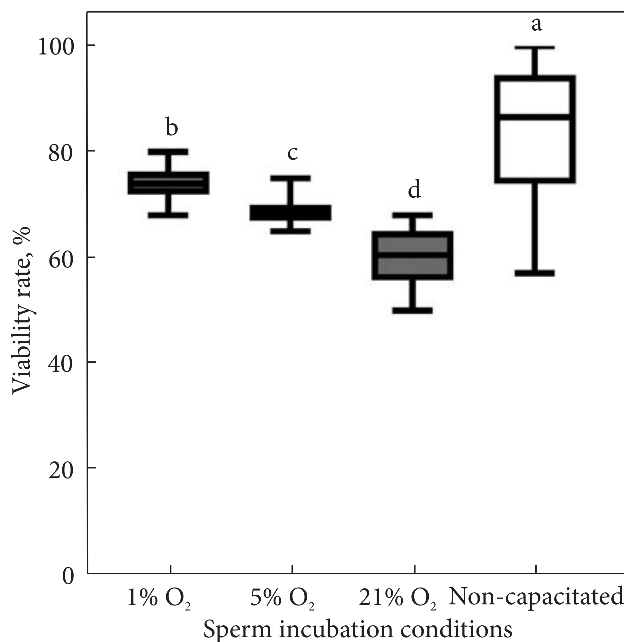
The survival rate of uncapacitated spermatozoa was (84.51 ± 2.5)%, which is consistent with reference data for ejaculates from healthy donors [17]. After capacitation at different levels of oxygenation with subsequent cryopreservation, an evident dependence of the sperm survival rate on the O_2 concentration during incubation was found (Figure).

The highest sperm survival rates after capacitation and cryopreservation were recorded under 1% O_2 conditions, while the lowest ones were found under normoxia. This fact indicates that the oxy-

Functional parameters of human spermatozoa one hour after incubation under various *in vitro* oxygenation conditions

Parameters	Group 1	Group 2	Group 3
Motility, %	50.4 ± 9.3	59.2 ± 4.5	49.2 ± 8.7
Viability, %	70.3 ± 9.4	79.2 ± 9.5 *	66.1 ± 9.5
Number of cells with AR, %	21.6 ± 2.9	26.8 ± 2.3	14.3 ± 2.5 **

Notes: * significant difference compared to group 1, $p < 0.05$; ** significant difference compared to groups 1 and 2, $p < 0.01$.



Cryoresistance of capacitated and non-capacitated spermatozoa. a, b, c, d — significant difference between groups, $p < 0.05$

gen level during capacitation is a critical factor determining subsequent cell viability. Presumably, the decrease in oxygenation reduces oxidative stress, which under normoxic conditions can be exacerbated and cause membrane and mitochondrial damage and disruption of sperm homeostasis [16].

Some authors believe that hypoxia can induce autophagy to conserve energy and recycle damaged structures in sperm [7]. Autophagy may play an important role in preparing sperm for AR, in particular by regulating membrane remodeling, mitochondrial utilization and mobilization of energy resources. Impaired hypoxic response mechanisms or autophagy dysfunction may interfere with the implementation of AR, which can reduce the fertilizing ability of sperm. The results obtained have practical significance for creating conditions for preparing sperm for fertilization by insemination, ICSI or IMSI [10].

Since increased ROS production during capacitation can trigger apoptosis or cause membrane and DNA damage, the reduced survival rate of cryopreserved sperm after capacitation under normoxic conditions is likely to be related to oxidative stress. This is supported by the research results that revealed increased levels of lipoperoxidation and DNA fragmentation of sperm depending on their functional state [19]. Hypoxia, which is characteristic of both the physiological environment of the epididymis and certain stages of cryopreservation, can modulate the activity of HIF-dependent signaling pathways, affect energy metabolism, and lower the activation threshold of capacitation mechanisms [9]. In combination with hypoosmotic stress, which also initiates cascade changes in membrane structure, there is a risk of premature or inadequate activation of sperm after thawing, which may reduce their fertilizing potential.

The results obtained emphasize the need to optimize capacitation and cryopreservation conditions assuming the physiological oxygen level and may contribute to increasing the survival rate of sperm after thawing. Further studies should be directed to a detailed investigation of the interaction of HIF-dependent mechanisms with cryoresistance indices, that opens up prospects for a personalized approach in reproductive medicine.

CONCLUSIONS

1. The oxygen level during *in vitro* incubation affects the sperm functional characteristics. It has been shown that hypoxia at 5% O_2 supports sperm motility and viability as well as stimulates the capacitation activation.

2. The cryoresistance of non-capacitated sperm has been established to be higher than that of capacitated sperm.

3. An increased oxygen concentration during induced capacitation of sperm leads to its decreased survival rate after cryopreservation.

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ГІПОКСІЯ ЯК МОДУЛЮЮЧИЙ ЧИННИК КАПАЦИТАЦІЇ ТА КРІОРЕЗИСТЕНТНОСТІ СПЕРМАТОЗОЇДІВ ЛЮДИНИ

Попри поглиблений інтерес до ролі гіпоксії у репродуктивних процесах, її вплив на капацитацію та кріорезистентність сперматозоїдів досі недостатньо з'ясовано. У роботі досліджено вплив рівня оксигенації під час інкубації сперматозоїдів перед кріоконсервуванням на ефективність капацитації, а також визначено зв'язок між капацитацією та кріорезистентністю. Відповідно до критеріїв ВООЗ проаналізовано еякуляти 30 здорових чоловіків із нормозооспермією. Оцінено функціональні характеристики та кріорезистентність сперматозоїдів після обробки градієнтом щільності та капацитації в умовах *in vitro* за концентрацій кисню 1, 5 та 21%. Встановлено, що інкубація сперматозоїдів людини в умовах помірної гіпоксії позитивно впливає на життєздатність, рухливість та активацію капацитації. Вперше показано, що кисневий режим в умовах інкубації *in vitro* є критичним фактором, який визначає рівень виживання сперматозоїдів після розморожування. Отримані результати дають підстави розглядати помірну гіпоксію як перспективну біотехнологічну стратегію для покращення протоколів кріоконсервування сперматозоїдів та підвищення ефективності допоміжних репродуктивних технологій.

Ключові слова: кріоконсервування, гіпоксія, сперматозоїди людини, капацитація.