

PERSPECTIVES

Fertility maintenance in male oncological patients: current state and future perspectives

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ABSTRACT

Growing cancer incidence in reproductive age goes hand in hand with a rising survival rate of patients who underwent anticancer therapy. This trend points to the necessity of discussion regarding the fertility maintenance. The patient's future with respect to his reproductive ability has to be addressed properly to achieve a complex approach to cancer management. The germinal epithelium of the testes is highly susceptible to deleterious effects of chemotherapy. After the administration of gonadotoxic chemotherapeutic agents, a patient can develop oligospermia, or even azoospermia. Similarly, radiation exposure can damage spermatogenesis, while higher doses lead to azoospermia. This review brings an overview of the methods of assisted reproduction, which are currently in use for fertility maintenance in oncological patients, but also in those with non-malignant indications. Also, novel, yet still experimental, methods are discussed, which represent promising technologies applicable to prepubertal oncological patients. We also discuss historical milestones in the development of assisted reproduction, summarize the options of semen analysis, and we present a practical guide through the process of sperm cryopreservation and subsequent *in vivo* or *in vitro* fertilisation. We deem that fertility maintenance should be an integral part of the health care in oncological patients in reproductive age (Tab. 1, Ref. 85). Text in PDF www.elis.sk.

KEY WORDS: assisted reproduction technique, sperm cryopreservation, testicular tissue cryopreservation, spermatogenesis, sperm quality in oncological patients.

Historical introduction

Assisted reproduction is a medical field focused on different physiological aspects of male and female gametes and embryos in *in vitro* conditions, while its principal aim is infertility treatment. Although the major goal is female conception and carrying a pregnancy to term, assisted reproduction currently encompasses also the issues of cryopreservation (freezing) of the reproductive cells, and also genetic testing before the embryo is placed into the uterus. Clinical embryology is one of the most recent biomedical fields, whose upswing dates back to 1978, when the first “test-tube” baby was born. A significant contribution to the development of *in vitro* fertilisation method is attributed to Patrick Steptoe, Sir Robert Edwards and Jean Marian Purdy (Brinsden and Brinsden 2009, Gosden, 2018, Steptoe, 2015). In former Czechoslovakia, the first baby, as the result of assisted reproduction, was born in Brno in 1982. This procedure was carried out by the team of specialists,

namely a gynaecologist Professor Ladislav Pilka, in collaboration with embryologists from Masaryk University in Brno, Professors Milan Dvořák, Pavel Trávník and Jan Tesařík. As a method, they used a direct transfer of the *in vitro* fertilized egg into the uterine tube, at that time a unique approach, known today as gamete intrafallopian transfer (Tesařík et al, 1983, Pilka et al, 1984).

Cryopreservation of sperm, oocytes and embryos (colloquially known as “freezing”), plays a central role in assisted reproduction today (Rodriguez-Wallberg, 2015). It can be shortly characterized as a process, which preserves the reproductive cells, embryos, and also tissues from reproductive glands by the means of rapid freezing in liquid nitrogen at its boiling point (minus 196 °C), enabling future usage. The first case of a successful fertilization using cryopreserved and subsequently thawed human sperm was published in 1953 (Bunge a Sherman, 1953). More than 60 years after this ground-breaking event, we can state that the achievements of “cryobiology” have resulted in several million children born from cryopreserved sperm, so far. Good example of an enormous benefit of sperm cryopreservation is the maintenance of fertility in patients with oncological diseases before the onset of chemotherapy and/or radiotherapy (Abram McBride and Lipshultz, 2018, Qu et al, 2019). Not only that, this method has a much broader spectrum of indications, e.g. sperm banking in donation programmes, sperm preservation for future use (for instance as a prevention of such situation when a man is unable to ejaculate because of his distress just before the artificial insemination, or in case of age or disease-

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related worsening of the spermogram). Nowadays, several other alternative circumstances, which motivate patients to undergo sperm cryopreservation are common, e.g. it can be viewed as “a prevention” for men undergoing vasectomy who are afraid of the unchangeable nature of their decision, or as a preventive measure before traveling to areas with the danger of Zika virus infection (Feinberg, 2017). However, new technologies and scientific advancements often bring plenty of unanswered ethical and legal questions, for example the issue of cryopreserved sperm utilization after donor’s death.

Sperm, ejaculate examination and spermogram

Sperm is a haploid cell, highly specialized in a shape, which arises from the process called spermatogenesis, occurring within the convoluted seminiferous tubules of the testes. Spermatogenesis comprises mitotic division of diploid spermatogonia, their subsequent meiotic division, but also the morphological transformation of immotile round spermatids into small motile sperm (Fayomi and Orwig 2018, Nakata 2019, Sharma et al, 2019a). This process takes around 74 days, after which the morphologically mature, yet still immotile sperm cells spend 12 more days travelling within the intratesticular ducts, efferent ductules, but mostly within the epididymal duct. We distinguish two cell populations, which are absolutely essential in the process of spermatogenesis – supporting cells of Sertoli, providing mechanical support, nutrition and immunological protection (formation of the blood-testis barrier) for developing sperm, and interstitial endocrine cells of Leydig, responsible for the production of testosterone (Balko et al, 2017, Staub and Johnson, 2018).

Sperm is about 60 micrometres long, consisting of three parts - head, neck and tail (flagellum). The head includes a condensed nucleus with the paternal genetic information and a membrane-bound cap-like organelle – the acrosome. The acrosome is considered a large secretory vesicle / lysosome that contains several enzymes including glycohydrolases, proteases, esterases, acid phosphatases, and aryl sulfatases. The release of these hydrolytic enzymes degrade the zona pellucida allowing the spermatozoa to penetrate it and join with the oocyte. In human spermatozoa, the acrosome occupies 40 to 70 % of the head (Tello-Mora et al, 2018). The sperm neck contains two centrioles. One centriole corresponds to the basal body of the system of specifically arranged microtubules (the axonemal complex of the flagellum), while the second one is used during the zygote cleavage after the fertilization, since the oocyte doesn’t contain its own centriole. The initial portion of the flagellum, termed the middle piece, is the area of spirally arranged mitochondria forming the mitochondrial sheath, the site of energy production required for the sperm movement. After the fertilization, these mitochondria are actively destroyed by the oocytes lysosomes (Balko et al, 2017). Sperm is a peculiar type of cell, as it doesn’t contain any stored nutritive substances whatsoever, depending totally on diffusion from its surroundings. The sole energy source is glucose, and its proper function also requires calcium ions. Although, the lifespan of sperm cells is limited to several days, their ability to move is exceptional

(1–4 mm/min, what equals approximately 15–70 $\mu\text{m/s}$). Another remarkable feature of the sperm is that their cytoplasm contains proteins essential for the induction of the mitotic division of the fertilized egg, known as sperm-borne oocyte-activating factors (Yeste et al, 2017).

Ejaculate (semen) is an organic fluid, which is formed by mixing of secretory products from various parts of the male genital system, namely the Sertoli cell-derived testicular fluid, and the secretions from the prostate, seminal and bulbourethral gland. Even though the sperm are fundamental components of the ejaculate, they minimally contribute to its total volume. This fact is noticeable after vasectomy, which results in the decrease of the total semen volume by only 10 %. Besides, this figure includes also the testicular fluid produced by supporting Sertoli cells, not only the volume of sperm alone, which is even lower (Weiske, 1994).

Examination of the ejaculate and semen analysis (spermogram) is the most important procedure before sperm cryopreservation takes place (it should be the first examination on the list of infertile couples, which helps to determine the contribution of the “male factor”). Before the semen analysis, 2–5 days of sexual abstinence is recommended. Summarized in a simplified way, the basic laboratory examination of the ejaculate comprises:

- Evaluation of the volume, pH, and liquefaction of the ejaculate – factors reflecting mainly the functional state of the accessory sex glands (the prostate and seminal gland),
- Microscopic evaluation of the sperm count (number of sperm per millilitre), their motility (particularly their progressive movement), and morphology (proportion of the sperm with normal shape and size) – indicators of the functional state of the testes and individual components of the reproductive tract,
- The ejaculate is also microscopically examined for the presence of white and red blood cells (leucocytospermia a hematospermia), epithelial cells and immature forms of sperm (Alipour et al, 2017, Balko et al, 2017, World Health Organisation, 2010).

The evaluation of the sperm concentration is performed using either a counting chamber (e.g. Makler’s, or Bürker’s counting chamber) or an automatic analyser (Alipour et al, 2017, Ber-Hava et al, 2000). These tools enable to count the number of sperm in one millilitre, or even in the whole ejaculate. It is important to note that the interpretation of acquired results should be done exclusively by an experienced expert in the field. The reason is that the spermogram results can considerably vary in a relatively short time span, so no definitive conclusions should be made from a one-time analysis. The best demonstration of this phenomenon are the results of Elzanaty (2008), who concluded that the ejaculate volume and sperm concentration can be strongly influenced by acute stress reaction, which is experienced by some men during the compulsory masturbation in the centres of assisted reproduction.

The number of sperm or their concentration represent one of the most important and most commonly monitored parameters. The first, almost 100 years old, reference of the assessment of average sperm concentration is attributed to Macomber and Sanders (1929). According to their observations, the average number of sperm in one millilitre of the ejaculate is 100 million. In the

Tab. 1. Recommended reference values of the spermiogram (normozoospermia), according the World Health Organization (WHO, 2010).

	5 percentile	50 percentile
Ejaculate volume	1.5 ml	3.7 ml
Sperm cell concentration	15 million/ml	73 million/ml
Total sperm cell count	39 million/ejaculate	255 million/ejaculate
Overall motility	40 %	61 %
Progressive motility	32 %	55 %
Normal morphology	4 %	15 %

1950s, several authors, e.g. Danish andrologist Richard Hammen or American physician John MacLeod established the lowest limit of sperm number necessary for successful fertilization to 60 million per millilitre. In 1945, MacLeod examined 100 otherwise healthy medical students, and concluded that as many as 65% of them have more than 100 million sperm per millilitre (MacLeod a Heim, 1945). However, in 1979, MacLeod re-evaluated his previous findings and determined that the cut-off value necessary for natural fertilization to occur should be lowered to 20 million per millilitre (MacLeod a Wang, 1979). The latest World Health Organization recommendation from 2010 (WHO, 2010) (Tab. 1) set the current lowest limit of normal sperm number to 15 million per millilitre. On the contrary, many experts hold the opinion that anything below 40 million, if you like 20 million per millilitre significantly reduces the chance of natural conception, so the WHO recommendations should be revised (e.g. Skakkebaek, 2010). Many authors postulated that the reduction of sperm number is something we must get ready for, considering that over the last 20 years the sperm count in the Western countries have been diminishing by 2.1–2.5 % per year (Auger et al, 1995, Shine et al, 2008). We have been witnessing a similar drop also in the volume of the ejaculate, from 3.4 ml to 2.75 ml (Carlsen et al, 1992). This reduction is known as oligospermia – the medical term for a reduced number of otherwise normal sperm (from the perspective of their motility and morphology). Cryptozoospermia describes a medical condition in which the sperm occurrence in the ejaculate is so sparse that in order to locate at least some, the semen has to be centrifuged (Ku et al, 2018). A complete absence of sperm in the ejaculate is termed azoospermia. According to our research, genetically conditioned disorders such as Klinefelter syndrome or AZF microdeletion of the Y chromosome, account for more than 8 % of all azoospermia cases (Behulová et al, 2011). Contrary to popular belief, abnormally high sperm count, known as polyzoospermia (greater than 250 million spermatozoa / ml) has also detrimental effect on fertility – most of the spermatozoa of polyzoospermic men do not undergo the acrosome reaction *in vitro* (Töpfer-Petersen et al, 1987).

The issue of firm definition of a “normal” sperm count is relatively challenging. Is it the arithmetic mean, or the statistical mode corresponding to the most commonly occurring value? Is it the sperm concentration, which leads to conception, or merely a consensus of professional organizations? One example for all, which demonstrates the challenging and complex nature of this issue, is the research by Professor Shanna H. Swan. She examined the sperm count of American men, who had already fathered an

offspring. The results were remarkable. While the sperm count of men from New York read the value 102.9 million per millilitre, that of Californians showed only 80.8 million, while Missourians and Iowans had the lowest count – 58.7 million and 48 million per millilitre, respectively (Swan, 2006). These geographical differences had been one of the reasons why the WHO recommended, that each individual laboratory should elaborate its own standards grounded in the research on volunteers. This is, however, considerably problematic.

Based on the results of semen analysis, a set of additional specialized tests can be indicated, although many of them are not routinely performed in all assisted reproduction centres across the board (e.g., Abu-Heija et al, 1997, Agarwal et al, 2017, Evenson 2013; Evenson 2016, Losano et al, 2018, Tello-Mora et al, 2018, Sharma et al, 2019b, World Health Organisation 2010):

- The evaluation of sperm vitality (in seemingly dead, immotile sperm) using the hypoosmotic test,
- The evidence of IgA and IgG autoantibodies on the surface of sperm (mixed antiglobulin reaction), indicating a damage of blood-testis barrier. This immunological factor dampens the chance for a successful natural conception,
- Fragmentation of the sperm DNA (sperm chromatin structure assay),
- Diagnosis of aneuploidies by fluorescence *in situ* hybridization,
- Microscopic evaluation of stained semen smear,
- Sperm-cervical mucus interaction test,
- Assessment of sperm oxidative stress, including assessment of sperm mitochondrial function,
- Acrosome reaction test.

The effect of cancer treatment on testes and spermatogenesis

Today’s estimate of male cancer incidence indicates that one in two men will develop an oncological disease at some point in his life, from which 4% will be under 35 years of age. A traditional approach to anticancer treatment is focused on the elimination of the cancer tissue itself, however an increasing efficiency and safety goes hand in hand with the rise of long-term survival rate of young adults who underwent this therapy. Five-year survival rate in patients under 15 years old undergoing anticancer therapy is 75 %. In patients between 15–44 years of age, this rate is estimated at 66 %. Increasing occurrence of these encouraging data clearly indicates that fertility maintenance in oncological patients should belong to the list of issues of major importance before, during and after the cancer treatment. Fertility can be jeopardized by the pathophysiological specifics of the oncological disease, but also by the treatment employed. While some treatment regimens cause “only” reversible infertility, in 50–95 % of malignant oncological diseases, the infertility is permanent. This is the main reason why the discussion concerning the fertility maintenance should be of paramount importance in male oncological patients (Osterberg et al, 2014). It is underlined by the evidence that anticancer treatment is potentially harmful not only to testes directly, but also to

hypothalamo-hypophyseal axis, which controls the function of the male gonads.

The germinal epithelium of the testes is highly susceptible to harmful effects of chemotherapy, regardless of the patient's age at the onset of the treatment. Thus after the administration of gonadotoxic substances, a patient can develop oligospermia, or even azoospermia. The production of testosterone by interstitial endocrine cells of Leydig usually remains unaffected, so secondary sex characteristics develop normally (Wallace et al, 1991). Nevertheless, when the gonadotoxic effect of chemotherapy is evaluated from the perspective of cumulative dose, interstitial endocrine cells of Leydig are no longer able to tolerate this detrimental burden, so their dysfunction is presumably inevitable (Gerl et al, 2001). Some data indicate that the proportion of patients with mild dysfunction of Leydig cells and biochemical, as well as clinical hypogonadism after chemotherapy, can be higher than previously thought (Romerius et al, 2009). Low levels of testosterone result in a number of clinically relevant conditions, including osteoporosis, metabolic syndrome, cardiovascular diseases and erectile dysfunction, so the monitoring of testosterone levels and subsequent hormone replacement therapy (if necessary) is of great importance (Stanworth and Jones, 2008).

Radiation doses of 0.1–1.2 Gy can disrupt spermatogenesis, while doses higher than 4 Gy cause a permanent azoospermia. It was observed that supporting Sertoli cells are more resistant than spermatogonia. Dysfunction of interstitial endocrine cells of Leydig is not detectable, until the radiation exposure reaches 20 Gy in prepubertal patients and 30 Gy in men after sexual maturity (Shalet et al, 1989). Within the paediatric and adolescent age group, the testicular damage occurs after a direct radiation exposure of the testes, e.g. during therapy of leukaemia (Castillo et al, 1990). Patients with leukemic infiltration of the testes usually undergo the radiation therapy with doses of 24 Gy, resulting in a permanent azoospermia (Grundy et al, 1997).

Total body irradiation is the main etiological factor of azoospermia in patients after hematopoietic stem cell transplantation. Patients who underwent total body irradiation at the dose of at least 7.5 Gy are at the greatest risk of infertility (Savani, 2006). The results of a large retrospective cohort study – European Group for Blood and Marrow Transplantation showed that as much as 81 % of patients after total body irradiation manifested with azoospermia, and as little as 1 % of patients had normal sperm count after the therapy (Rovo et al, 2013). Long-term observation in patients with longer than ten-year survival rate, who were under 25 years old at the time of hematopoietic stem cell transplantation and without evident graft-versus-host disease, showed that these patients had a real chance that their spermatogenesis remained preserved (however, only to extent below the official WHO cut-off values), despite receiving total body irradiation in regular dosage (Rovo et al, 2006). Earlier study focused on the influence of graft-versus-host disease on fertility in patients after hematopoietic stem cell transplantation with diagnosed aplastic anaemia, who didn't undergo total body irradiation, only chemotherapy, concluded that the patient's chance to father his own child was around 50 %. In patients with a diagnosed graft-versus-host disease, the rate was

considerably lower, to be specific 29 %. In patients without the graft-versus-host disease, this rate was 62 % (Deeg, 1998). Therefore, it is not completely clear, whether the sterilising effect of this chronic disease is caused by the graft reaction against the host, or it can be considered simply as the result of its chronic course and its consequential repercussions (Tichelli a Rovo, 2013).

Sperm quality in oncological patients and the influence of sperm cryopreservation on this variable

Many papers have been published so far, confirming the chemotherapy or radiotherapy-induced testicular damage. However, the issue of potentially negative influence of the testicular neoplasm itself (before any treatment) on the spermiogram parameters is still controversial. The majority of current studies are on the same page that, according to the WHO 2010 criteria, the spermiogram is worsened in approximately half of the patients with testicular cancer (e.g. MacKenna et al, 2017, Auger et al, 2016). Luisa Caponecchia, an Italian embryologist, together with her team, performed the spermiogram in 128 men with diagnosed testicular cancer. The sperm concentration in the study group (men with cancer), was significantly lower compared to the results of the control group (healthy volunteers) – the median was 18 million per millilitre in the study group against 46.5 million per millilitre in the control group. Similarly, the sperm motility was also lowered, however, the sperm morphology was not negatively influenced by the cancer. The authors also disproved any deterioration in the spermiogenesis related to the pT1, pT2, or pT3 stages of the cancer (Caponecchia et al, 2016). Similar conclusions were made also by the recent French study, according to which the patients with testicular cancer have approximately one fourth of the sperm number, in comparison to healthy volunteers (average 36.1 ± 48.5 million sperm per millilitre). Approximately half of the patients with testicular cancer had normal spermiogram results (Auger et al, 2016). However, the same study pointed out that apart from testicular cancer, other types of oncological diseases (e.g. leukaemia, lymphoma, brain tumour, sarcoma), but also autoimmune systemic conditions (e.g. *sclerosis multiplex* and Behcet's disease) can also worsen the ejaculate quality. It is necessary to emphasize that many other factors can influence the spermiogram parameters, including age (Eskanazi et al, 2003, Slotter et al, 2006), duration of the sexual abstinence (Sunanda et al, 2014), lifestyle, environmental factors, or even a season (sperm concentration in summer accounts for about 70 % of the value during winter) and geographical location (Jorgensen et al, 2001). Still, the exact mechanism, by which the oncological disease negatively influences the spermatogenesis is not known. Supposed etiopathogenesis is often linked to congenital abnormalities of the reproductive cells, systemic effect of a tumour, or negative influence of cytokines and hormones produced by the cancer tissue (Trottmann et al, 2007).

Global statistics shows that from among men who underwent a treatment due to an oncological disease, approximately 8 % actually use their cryopreserved sperm to artificially inseminate their significant others (Ferrari et al, 2016). Even though the sperm of

oncological patients showed a significantly reduced motility and vitality after thawing, in case of a successful fertilization, no abnormalities were observed during the cleavage of the zygote, and in addition, the rate of embryo implantation into the uterine mucosa was also comparable with the control group (Depalo et al, 2016). The research conducted by the team of specialists from Masaryk University in Brno, Czech Republic (Záková et al, 2014) was in accordance with the abovementioned results, as it showed that patients with testicular cancer whose sperm cells had been cryopreserved before a treatment will have a good chance to father a child someday. According to the archive of this department, between 1995 and 2012; the cohort of 523 patients (mean age 28.5 ± 6.6 years) with testicular cancer had undergone sperm cryopreservation before a treatment, from which 34 individuals later decided to use these sperm in assisted reproduction. This resulted in 16 pregnancies from 46 cycles of artificial fertilization (pregnancy rate was 34.8 %). The average elapsed time from the sperm collection to the patient's decision to actually use these samples in the cycles of artificial fertilization was 22.2 ± 14.7 months.

Laboratory methods of sperm cryopreservation

Cryopreservation is a technique, which enables to preserve tissue components including cells using cryogenic temperatures. Human sperm cryopreservation is an integral and inseparable part of the technology of assisted reproduction, and represents an effective method of male fertility maintenance, e.g. during a treatment of oncological diseases (Stern and Agresta, 2019). This technique of cryogenic preservation is especially important in patients treated with chemotherapy and radiotherapy, both of which can lead to testicular dysfunction or ejaculation impairment (di Santo et al, 2012). In fact, it seems that sperm cryopreservation is the only proven method, which can give patient a chance to have children in future. The cancer treatment can lead to that kind of damage, which results in subfertility or even sterility due to the removal of gonads or because of an irreversible deterioration of the reproductive cells caused by adjuvant therapy. The treatment-associated risks depend on various individual factors such as patient's age at the time of treatment, dosage and type of therapy applied (Jensen et al, 2011). As already mentioned, the benefit of sperm cryopreservation is not the exclusive matter of oncological patients, as there are many non-malignant diseases like *diabetes mellitus* or autoimmune disorders which can also lead to testicular damage. So, in these cases, sperm cryopreservation is also recommended (Anger et al, 2003).

Sperm cryopreservation comprises multiple conventional cryogenic preservation techniques. Slow freezing technique, as suggested by Behrman and Sawada (1966) is based on progressive freezing of the semen, either manually or automatically using a programmable freezer. Principle of the manual technique is the addition of cryoprotective agent, and subsequent lowering of the temperature. The optimal initial speed of the sample cooling from the laboratory temperature to $5\text{ }^{\circ}\text{C}$ was determined to be $0.5\text{--}1\text{ }^{\circ}\text{C}/\text{min}$. After that, the sample is cooled from $5\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ at the speed of $1\text{--}10\text{ }^{\circ}\text{C}/\text{min}$, and is subsequently immersed into liquid nitrogen which freezes it to $-196\text{ }^{\circ}\text{C}$ (Mahadevan a Troun-

son, 1984). However, the reproducibility of the manual technique methodology was showed to be a bit problematic, so despite the reports of successful sperm preservation, it was recommended to use a programmable freezer.

Freezers use a straw-holding plate, in which the straws are frozen by liquid nitrogen stored in a container under the plate. After liquid nitrogen is poured into the container, the machine gradually decreases the temperature from $20\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ at the speed of $1.5\text{ }^{\circ}\text{C}/\text{min}$, the process is then accelerated to $6\text{ }^{\circ}\text{C}/\text{min}$. After the procedure is finished, the straws are stored in liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ (Holt, 2000).

Rapid freezing technique, as put forward by Sherman (1990), requires a direct contact of sperm-containing straws with liquid nitrogen vapours during 8–10 minutes. Next step is immersion in liquid nitrogen at $-196\text{ }^{\circ}\text{C}$. Within the liquid nitrogen vapours, a thermal gradient is formed, depending on the distance from the liquid underneath and its volume. Firstly, the samples are mixed with equal amount of cryoprotective agent, the mixture is put into straws, which are then situated about 15–20 cm above the liquid nitrogen surface. During the freezing process, the best method is to position the straws horizontally to minimize the temperature difference between both ends of the straw. Another technique of sperm cryopreservation is vitrification. In this process, semen is mixed with cryoprotectants, sealed off in a plastic straw and then tossed directly into liquid nitrogen. This method is not routinely used in laboratories, so far.

Despite its undisputed usefulness, cryopreservation can result in deleterious changes in the structure and function of the sperm. While the cryobiology of other cells is well known, scientific literature lacks a consensus regarding the harmful effects on human sperm. Nevertheless, sperm cryopreservation is a crucial technique in the fertility management, what is well proved by its successful application in assisted reproduction (Di Santo et al, 2012).

A practical guide through the process of sperm cryopreservation and subsequent fertilization

Considering the potentially detrimental effect of cancer treatment on quality and quantity of sperm cells, it is of paramount importance that the semen is cryopreserved before the onset of therapeutic intervention. According to the study by American team of researchers, sperm cryopreservation was undergone by 30 % of patients in reproductive age with diagnosed oncological disease. Oncological patients, who are more willing to undergo this procedure, are those of higher socioeconomic status, but also men who do not identify themselves with any religious denomination (Klosky et al, 2009). A physician-oncologist is in charge of the first important step, which is to properly inform a patient about the options of sperm storage for future use. In the United States of America, as many as 91% of oncologists agreed with sperm cryopreservation in oncological patients before treatment, however 48% of them admitted, they hold a proper discussion concerning this method with only less than one quarter of patients. The reasons are assorted, usually it is a shortage of time for a dialogue, but also (disproportionate) assumption of excessive financial demands for a patient (Schover et al, 2002).

Semen collection is not recommended during or shortly after the oncological treatment, so it is all-important to advise a patient to visit a specialized department engaged in assisted reproduction sufficiently ahead of time. A patient should bring his medical chart with properly recorded oncological diagnosis, along with the results from serological testing for HIV, hepatitis B, syphilis and other sexually transmitted diseases. For semen storage out of a quarantine container, the examination for sexually transmitted infections is absolutely essential.

The number of semen collections required for the storage of sufficient amount of material depends on various factors such as ejaculate quality, health status of a patient, type of cancer, time elapsed from the onset of an oncological treatment, and last but not least the method of assisted reproduction, for which the sample will be used (Nangia et al, 2013). Most of the departments recommend to store at least three ejaculate samples with a minimum of two day gap between collections, if the patient's health status is satisfactory and the postponement of treatment is acceptable. For semen collection, a patient can use a special isolated room within the premises of assisted reproduction centre. Some departments even allow a significant one to be present during the collection, if it is a wish of the patient. If a centre of assisted reproduction is located nearby a patient's domicile, there is a possibility to collect the sample at home into fit-for-purpose container. Methodology of the following semen usage must be in accordance with the type of procedure employed during prospective assisted reproduction. We distinguish three general approaches to oocyte fertilization: intrauterine insemination (IUI), classic *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

Intrauterine insemination is the easiest method of assisted reproduction, but its usage has certain limitations. There are several prerequisites, which ought to be fulfilled. Success rate of this method is dependent on the patency of the uterine tubes. At the commencement of IUI, sperm washing including the removal of any possible microbial contaminants or foreign substances must be performed. Later on, only motile sperm cells are selected, which are subsequently injected, using a special catheter, directly into the uterine cavity during ovulation. Thus, from the perspective of cryopreservation, the cardinal requirement is that the preserved sample contains properly motile sperm in a high concentration, as these two factors are of utmost importance when it comes to the success of this method (Nangia et al, 2013). The main advantages of IUI are that, neither oocyte retrieval, nor general anaesthesia are required. In case of inadequate quality of thawed sperm cells, an attending gynaecologist, with specialty in reproductive medicine normally recommends a more efficient method of infertility treatment, generally *in vitro* fertilization.

In vitro method is one of the basic approaches of external fertilization. It consists of the following individual steps:

- Stimulation of the growth of ovarian follicles (via the administration of hormone therapy drugs),
- Transvaginal oocyte retrieval – aspiration of the oocyte from the ovary using a special puncture needle through the vaginal wall (“oocyte puncture”), in short general anaesthesia under transvaginal ultrasound guidance,
- Semen collection and processing (sperm washing – separation of sperm from the seminal fluid, removal of potential bacteria, blood cells, as well as dead and immotile sperm), or thawing of cryopreserved sperm,
- Co-incubation of the sperm and oocyte in a culture medium,
- Embryo cultivation during different stages of early embryogenesis (zygote, cleavage stages (morula), blastocyst) after successful fertilization,
- Placement of the embryo into the uterine cavity, also known as embryo transfer. In the presence of multiple embryos, some of them can be cryopreserved and used later on (Řezábek, 2014).

However, classic *in vitro* fertilization also requires, similarly as intrauterine insemination, a higher sperm concentration with an adequate motility, so it is necessary to cryopreserve samples of high quality. In case of suboptimal spermogram results, sperm cryopreservation should be repeated.

Intracytoplasmic sperm injection into the oocyte is another technique used in assisted reproduction. This method is considered as a partial solution of male infertility, as it is feasible also in patients with low sperm count and inadequate sperm motility. This method even allows sperm harvesting directly from the tissue of the testis or epididymis. ICSI is based on injection of one particular sperm using a special needle directly into the oocyte cytoplasm. This whole process is performed using a specialized inverted microscope with two “arms” attached to electronically-assisted device (micromanipulator), which enables high precision of movement. One arm bears a holding micropipette, stabilizing the oocyte in place, while the other contains an injection micropipette, which is used for the sperm transfer into to the oocyte cytoplasm. Firstly, the sperm which is used in this procedure is immobilized by detaching the sperm tail with a micropipette. This is an inevitable step, as the sperm movement could damage the inner structure of the oocyte. Moreover, this procedure triggers a discharge of special proteins from the sperm known as sperm-borne oocyte-activating factors, which facilitate the fertilization process (Neri et al, 2014; Amdani et al, 2015). The advantage of ICSI is that the number of sperm cells needed equals the number of oocytes obtained during the puncture. It follows that, ICSI represents a suitable method even for those couples, in which the female body produces antibodies against the partner's sperm. On top of that, this method is feasible even with a minimum number of cryopreserved sperm.

ICSI also ensures a precise selection of mature sperm, which are used during fertilization. It is also an advantage, because mature sperm were demonstrated to be significantly less susceptible to chromosome abnormalities (Žáková et al, 2012). An embryologist has several options at disposal to select “the best”, i.e. mature sperm. Firstly, he can focus on the sperm morphology, which can be examined using differential interference contrast microscopy (Nomarski interference contrast/Nomarski microscopy). At high magnification, this approach is suitable for a study of low-contrast details of the sperm. This method is called “intracytoplasmic morphologically selected sperm injection” (IMSI). However, the recent randomized controlled trial has not demonstrated any statistically significant increase in the live birth rate using

IMSI, when compared to “classic” ICSI (Teixeira et al, 2013). Second alternative is the usage of hyaluronic gel, which has the ability to mimic the oocyte surface along with the cells of *corona radiata* (supporting follicular cells). Arguably, only mature sperm possess hyaluronic-binding receptors, so the utilization of such sperm rises the chance of successful fertilization (Žáková et al, 2010). This method is termed “preselected ICSI” (PICSI). Another option is to use presumably one of the newest methods – magnetic-activated cell sorting (MACS). Sperm cells with a high rate of DNA fragmentation are tagged using superparamagnetic nanoparticles, which are subsequently trapped inside a separation column. Intact sperm cells without DNA fragmentation are free to pass through this special column, so they can be used in ICSI.

Future perspectives: testicular tissue cryopreservation in prepubertal patients

Sperm cryopreservation is a relatively simple and effective method for fertility maintenance in oncological patients before the start of gonadotoxic cancer treatment. However, the main challenge are the cases of patients in prepubertal age, as the absence of spermatogenesis makes it impossible for them to benefit from this procedure. Experiments using animal models have indicated that cryopreserved spermatogonia can be successfully used in regeneration of damaged spermatogenesis. However, the application of such experiment to human medicine is dubious. To the best of authors' knowledge, only one department (Wake Forest Institute for Regenerative Medicine, USA), is currently active in testicular tissue cryopreservation of prepubertal oncological patients obtained from biopic samples (Sadri-Ardekani et al, 2016). Up to now, researchers from this department were able to cryopreserve samples from 14 boys, however they themselves describe this procedure as “experimental”. Nevertheless, these experts assume that in near future, this preserved tissue will be usable (if necessary) for the purpose of fertility restoration in these child patients. However, until that time many individual issues must be resolved, e.g. elimination of potential cancer cells “contamination” of the cryopreserved tissue prior to auto-transplantation, development of methodology for re-implantation of harvested testicular tissue after treatment, perhaps even development of laboratory modalities for *in vitro* development of sperm from spermatogonia. Similar methodologies and recommendations already exist for cryopreservation of tissue from ovarian cortex in women before the initiation of oncological treatment. Even though more than 35 children have already been born after the auto-transplantation of the ovarian tissue (Stoop et al, 2014), the exact fate of the transplanted tissue still remains unknown, moreover it is also only partially understood for how long this transplanted tissue will be able to constitute an adequate ovarian reserve.

Conclusion

Thanks to advances in chemotherapy, radiotherapy and surgical treatment, we have been witnessing a significant increase in five-year survival rate of the patients with testicular cancer. Indeed, these exceptional results should go hand in hand with

an increased demand for sperm cryopreservation (Caponecchia et al, 2016). Gonadotoxicity of chemotherapy is a variable influenced by multitude of factors like age, cumulative dosing, or past testicular surgeries. In any case, some chemotherapeutic agents definitely cause an irreversible damage of the reproductive glands. Similarly, radiotherapy performed in specific locations can also cause a dose-dependent direct damage of testes or hypothalamo-hypophyseal axis. Ergo, it is necessary to keep in mind that the loss of fertility during oncological treatment is an up-to-date issue, which represents also a source of serious chronic psychosocial deprivation. According to several studies, 50–70 % of oncological patients would like to have their own children in the future (Trottmann et al, 2007). Whereas a lot of patients are still uninformed or at least inadequately informed about these possibilities of fertility maintenance (this is also the case of women in reproductive age with diagnosed oncological disease), many internationally-renowned professional medical organizations, e.g. *American Society of Clinical Oncologists*, *American Society for Reproductive Medicine*, or *National Comprehensive Cancer Network*, have already published their recommendations. Fertility maintenance should be “personalized” for each patient according to many variables such as patient's age, type of oncological disease, character of prescribed therapy, or time remaining until the initiation of given treatment.

Despite these problems, we think that fertility maintenance should be an integral part of health care provided for oncological patients. International guidelines recommend that physicians should discuss, as early as possible, with all patients of reproductive age their risk of infertility from the disease and/or treatment and their interest in having children after cancer, and help with informed fertility preservation decisions. This complex management has a potential to comfort the patient, who is given a chance to father his own child, and this hope alone has a great power to help overcome all the difficulties associated with the cancer treatment (Lambertini et al, 2016, Martinez et al, 2017).

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