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REVIEW ARTICLE

WHAT OPTIONS FOR FERTILITY PRESERVATION DO PRE-PUBERTAL BOYS AND GIRLS HAVE (IN CROATIA)?

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Abstract: The latest progress in therapies indicated for the treatment of childhood cancers has enabled the improvement of survival rates in affected patients. However, the treatment can have gonadotoxic effects and sometimes results in infertility. Therefore, strategies for fertility preservation are developed for storage of intact reproductive tissue and possible future fertility restoration when the patient is cured of the initial disease. Currently, only the cryopreservation of immature reproductive tissue is well established. In contrast, the approach to restoring fertility using the stored tissue is, for now, in most cases, only theoretical, especially concerning pre-pubertal boys.
On the other hand, fertility restoration in pre-pubertal girls is much more advanced, but further studies are needed to ensure safety regarding malignancies with a high risk of metastases. In order to overcome the risk of malignant contamination, when the cryopreserved reproductive tissue is intended to be applied (whether it is immature testicular or ovarian tissue), a suitable alternative to reproductive tissue autotransplantation is *in vitro* culture and development of mature gametes. For now, these methods are still in their onset and far from clinical application. Still, their advancement can be expected in the near future, given the rapid development of scientific techniques.

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INTRODUCTION

Significant advances in treatments for malignant diseases have led to a recent decrease in cancer mortality rate in the general population and children, with survival rates higher than 80%.[1, 2] However, this achievement does not end the endless search of an answer to the question: What happens after the treatment? It is well known that anti-cancer therapies, including chemotherapy, radiotherapy, and hematopoietic stem cell transplantation, can have a long-term adverse effect on fertility. The risk of infertility depends on the patient's age, the type of malignancy, and the type, dose, and duration of administered therapy.[3] In pre-pubertal boys, although they do not complete spermatogenesis, a large number of studies have suggested that administrated gonadotoxic treatment can also affect fertility.[4, 5] Likewise, chemotherapy and radiotherapy can destroy ovarian follicles in girls, putting them at risk of delayed or arrested puberty and infertility as well as earlier menopause.[6-8] Therefore, many children and adolescents undergoing aggressive gonadotoxic treatments can benefit from fertility preservation (FP) procedures initiated prior to cancer therapy, so they have become a new priority in reproductive medicine. Currently, options for preservation and restoration of fertility for pre-pubertal cancer survivors who are unable to produce their gametes are still very limited and considered experimental.
Furthermore, these FP strategies involve invasive procedures, and their implementation should be comprehensively discussed, emphasising the individual's risk of fertility loss. An additional challenge for patients and their parents is fertility preservation costs. Insurance does not usually cover experimental therapies, which creates economic distress on top of all other difficulties they have to face. Therefore, currently available fertility preservation options should be offered

to a patient after a careful risk assessment on the potential loss of fertility due to the specific medical treatment to which the patient will be subjected.

Although still in the experimental phase, some fertility preservation approaches are already applied in the clinic, such as ovarian tissue cryopreservation (OTC) in pre-pubertal girls, which can later serve fertility restoration. Conversely, most FP strategies for pre-pubertal boys, such as spermatogonial stem cells[9] or induced pluripotent stem cells[10] application, are still in the research stage and far from clinical implementation. Due to the unique practical and ethical challenges in young patients, a particular emphasis should be put on the multidisciplinary cooperation between oncologists, gynaecologists, urologists, and fertility specialists to reduce stress for patients and their parents/guardians challenging process.

This review discusses the most recent advances in fertility preservation and restoration for pre-pubertal boys and girls, focusing on their current and near-future status in Croatia.

DISCUSSION

Options for fertility preservation and subsequent fertility restoration in pre-pubertal boys

Fertility preservation in pre-pubertal boys

Sperm cryopreservation after masturbation is the most established and used method for fertility preservation before gonadotoxic treatments in men.[11] Unfortunately, pre-pubertal boys, for whom spermatogenesis has not yet started, do not have that option. Therefore, the only choice for fertility preservation and future restoration currently available for this group of patients is cryopreservation of testicular tissue containing spermatogonial stem cells (SSCs).[12, 13] SSCs are diploid stem cells within the testes with two abilities: the capacity of self-renewal and the proficiency of differentiation into other cell types.[14] They are responsible for the continuous maintenance of spermatogenesis during adult life and are present in the testes during the whole lifetime, from establishment in the first three months after birth.[15] Complete loss of SSCs caused by a disease or medical treatment has shown to be an important source of permanent infertility. Therefore, the collection of SSCs by a testicular biopsy and their cryopreservation could answer the question of how fertility preservation and restoration can be achieved in pre-pubertal patients. The biopsy obtains testicular tissue before the cancer therapy under local anaesthesia. The main advantage of this technique for FP is a lack of delay in initiating cancer treatment. However, a relatively sizable piece of testicular parenchyma needs to be removed in order to obtain sufficient specimens for SSCs retrieval, which is the major downside of the method. Nevertheless, the rate of reported complications of the procedure is low.[16]

After the sample is collected, immature testicular tissue is cryopreserved for future use either using the slow freezing protocols[17-20] or vitrification.[21, 22] Most centres offering the cryopreservation of pre-pubertal testicular tissue perform slow freezing; however, vitrification has also been demonstrated to be an effective method for tissue preservation.[22] An overview of cryopreservation protocols for human immature testicular tissue is provided in Table 1. Since the primary purpose of fertility preservation and restoration, the production of human functional spermatozoa, has never been achieved for immature testicular tissue, none of the existing cryopreservation protocols can be considered superior over any other reported procedure. According to Picton *et al*[9], at least seven health centres in Europe offer the cryopreservation of testicular tissue as a fertility preservation method to pre-pubertal patients, with more to come in the near future, and University Hospital Center Zagreb is planning to be one of them.

Fertlity restoration after gonadotoxic treatment in male patients

While cryopreservation of testicular tissue has been ethically approved and available for preserving pre-pubertal testicular tissue, methods for obtaining mature spermatozoa with fertilisation capacity are still experimental and have not been successful in achieving their goal so far. The most promising approach for future fertility restoration in previous pre-pubertal cancer patients is based on the fact that SSCs can recover spermatogenesis after the gonadotoxic treatment is completed and the patient is disease-free. The presence of SSCs in pre-pubertal testicular tissue was confirmed in 96% to 100% of samples by studies that included patients who went through the fertility preservation procedure.[23, 24] Consequently, autologous transplantation of testicular tissue containing SSCs (or cell suspension containing SSCs) is recognised as the most promising approach for fertility restoration. It is closest to application in clinical practice. Radford *et al*[25] suggested that obtained and cryopreserved SSCs before the onset of chemotherapy could be reintroduced into the testis after the treatment has been completed. When it comes to cryopreservation, either the pieces of testicular tissue or isolated cells containing SSCs can be cryopreserved. The first of the above, testicular tissue cryopreservation, might be a better option because, using this approach, cell-to-cell contacts are maintained, and the stem niche is preserved. This is vital for the survival and maturation of SSCs.[26] However, the hurdle with transplantation of thawed fragments of testicular tissue into a patient (*in vivo*) is an existing risk of cancer cells introduction. To overcome the risk of malignant contamination, a suitable alternative to testicular tissue autotransplantation is *in vitro* maturation and development of functional spermatozoa that can later be used in intracytoplasmic sperm injection into the oocyte (ICSI) procedure.

Table 1. Overview of cryopreservation protocols for human immature testicular tissue

Cryopreservation protocol type		References
Slow programmed freezing	1.5 M EG + 0.1 M sucrose + 10% HSA	Kvist et al.[17]
	0.7 M DMSO + 5% HSA	Keros et al.[18]; Pietzak et al.[13]
	0.7 M DMSO + 0.1 M sucrose + 10% HSA	Wyns et al.[19]; Poels et al.[20]
	DMEM/F12 + 1.4 M DMSO + 0.15 M sucrose + 10% HSA	Braye et al.[70]
Vitrification	2.8 M DMSO + 2.8 M EG + 25% HSA	Curaba et al.[21]
	2.1 M DMSO + 2.7 M EG + 0.5 M sucrose + 25% HSA	Poels et al.[22]

Legend: EG, ethylene glycol; DMSO, dimethyl sulfoxide; HSA, human serum albumin; DMEM, Dulbecco's modified Eagle's medium

Alternatively, if the method of choice is cryopreservation and subsequent autotransplantation of SSCs, they must first be isolated from obtained testicular tissue. Isolation is followed by the procedure of SSCs sorting to remove contaminating somatic cells from the culture, achieve the efficient enrichment of human spermatogonia, and eliminate malignant contamination. Various cell sorting methods have been reported[27–29], but, unfortunately, they have been unsuccessful in the complete elimination of neoplastic cells. Meanwhile, research by Sadri-Ardekani *et al*[30] has indicated that a testicular cell culture system could be used to eliminate malignant cells before SSCs transplantation, in addition to providing the expansion of SSCs. The same group reported *in vitro* propagation of human pre-pubertal SSCs.[31] Several different research groups attempted to develop an efficient *in vitro* culture system for SSCs propagation with the aim of sperm production. For now, there are three established strategies for achieving this goal: 2D culture of testis cell suspension, 3D culture of testis cell suspension and organotypic culture of testicular tissue fragments as the latest one.

A long-term 2D culture system is widely used for *in vitro* proliferation of SSCs, resulting in the production of round spermatids and their differentiation into elongated spermatids.[32] In this culture system, co-culture with feeder cells or extracellular matrix presence is required to obtain attachment, survival, and proliferation of spermatogonia. The major disadvantage of this approach is that it does not mimic structural properties and cellular interactions naturally present in the testes. Therefore, a 3D culture system has been developed that effectively replicates the testis' microenvironment *in vivo* and thus allows more efficient diffusion of nutrients, oxygen, and other essential molecules. With this approach, promising results were obtained, including the development of male germ cells to the level of elongating spermatids.[33] However, the completion of the entire spermatogenic process has not been achieved so far in humans. As the latest strategy for *in vitro* maturation of testicular tissue, the organotypic culture of testicular tissue fragments is the only technique that can preserve the unimpaired structure of seminiferous tubules and intact cell interactions within the tissue. Regardless of its capacity, the completion of spermatogenesis *in vitro* from immature testicular tissue in humans has still not been achieved. The main downside of the testis tissue culture is the limited diffusion rate of the tissue compared to that of monolayer cell cultures, which makes the maintenance of tissue viability in the culture for sufficient time to complete *in vitro* spermatogenesis a challenging task. Also, in the first study on the immature testicular tissue culture, a loss of spermatogonia over the culture period was noticed.[34] In this research, immature testicular tissue was obtained from pre-pubertal cancer patients between 2 and 12 years of age before undergoing gonadotoxic treatments and cultured *in vitro* over 139 days. The advancement of immature testicular tissue toward a pubertal stage with the progression of Sertoli and Leydig cells from an immature to mature state was reported, but, unfortunately, accompanied by a progressive loss of spermatogonia over the culture period. The renewal of spermatogonia and differentiation of haploid germ cells has not been achieved. However, a year later, the same group of researchers provided hope by succeeding in the generation of human haploid germ cells after 16 days of pre-pubertal testicular tissue culture[35] Further research should be focused on epigenetic features of generated germ cells and their ability to complete differentiation into functional sperm.

Besides the testicular tissue culture, thawed fragments of tissue can be xenografted into immunodeficient mice to obtain spermatozoa for ICSI[19], thus avoiding the possibility of malignant contamination as in the case when the tissue containing SSCs is transplanted into a patient. Nevertheless, this technique is in the early experimental stage. Its use is unlikely to be implemented as a fertility restoration strategy in the near future due to the ethical and safety concerns relating to xenografting of human tissue. Additionally, the complete spermatogenesis of immature human testis tissue using this approach has not been achieved.

Options for fertility preservation and subsequent fertility restoration in pre-pubertal girls

Fertility preservation in pre-pubertal girls

Chemotherapy and radiotherapy in female survivors of childhood cancer can destroy primordial follicles,

leading to a reduction in the concentration of anti-Müllerian hormone (AMH), causing the acceleration of surviving primordial follicles recruitment, diminished ovarian reserve with consequential premature ovarian failure as well as a higher risk of infertility.[36, 37] The golden standard for fertility preservation for adult cancer patients is oocyte or embryo cryopreservation after a controlled ovarian stimulation protocol that ensures oocyte collection within two weeks. However, in patients who cannot undergo ovarian stimulation due to the lack of time and require immediate gonadotoxic treatment, or pre-pubertal girls who cannot produce mature oocytes, this approach is impossible. Ovarian tissue cryopreservation is currently the only FP option available for these patients because it does not require ovarian stimulation or egg harvesting, only fragments of normal, functioning ovarian tissue obtained laparoscopically by biopsy retrieval surgery. Ideally, ovarian tissue should be retrieved before the gonadotoxic treatment commences. Still, the practice has shown that tissue fragments from patients who had undergone 1 or 2 regiments of chemotherapy do not differ significantly from those collected from patients who have not received the previous treatment.[38] OTC is not reserved only for women and girls with malignancies but should also be offered for non-malignant conditions when hematopoietic stem cell transplantation (HSCT) is indicated.[39] Additionally, patients suffering from a benign disease that puts them under the risk of POF, such as recurrent benign ovarian cysts, severe endometriosis, systematic disorders requiring gonadotoxic chemotherapy, and even Turner syndrome, could cryopreserve ovarian tissue. On top of that, there were also cases of patients with a family history of early menopause undergoing OTC in order to prolong the period of their fertility.[38]

Biopsy retrieval surgery is in most cases unilateral or bilateral and does not carry a risk of serious complication.[38] However, when the patient is a young pre-pubertal girl, the surgeon usually opts for unilateral oophorectomy to gather a sufficient quantity of quality tissue. Removal of a single ovary could cause premature menopause: 1-2 years earlier menopause in women who had undergone unilateral oophorectomy was reported when compared with women with two intact ovaries.[40] On the other hand, collecting multiple biopsy samples of ovarian tissue from one ovary does not harm future hormone production.[41] The collected tissue is then processed: ovarian cortex should be dissected from the medulla, cut into smaller fragments, washed and immersed into a freezing medium.

Cryopreservation of the obtained tissue is the next step in the procedure. Nowadays, the slow freezing method is still favoured in the majority of centres since it has been tested thoroughly. So far, it has shown remarkable results: more than 95% of live births have been achieved after transplantation of thawed ovarian fragments that were slow-freezed.[38,42-45] The slow freezing protocols for human ovaries are based on the protocol developed by Gosden *et al*[46] for sheep ovarian tissue, which follows a sequence of steps achieving a gradual drop in temperature within predefined time intervals. Different cryoprotectant agents (CPAs) protect cells from injuries caused by crystal formation and hypertonicity.

On the other hand, some researchers claim that vitrification is the way to go because it is quick and demands less expensive equipment. However, in this procedure, high concentrations of CPAs are utilised, and it is known that they can have a toxic effect on cells. Despite its growing popularity, there is still a rather limited amount of reports suggesting its efficiency: only two live births were reported so far.[47]

While the ovarian tissue is being cryopreserved, a sample is sent for histopathological evaluation to determine the follicle count and the possible presence of malignant cells (Figure 1). Additionally, more sensitive methods like immunohistochemistry, PCR, and xenografting are used in any cancer case, especially those with a high risk of metastases (>11%) such as leukaemia, neuroblastoma and Burkitt lymphoma.[48] Due to the increased risk of malignant contamination in patients with those conditions, alternative approaches to obtain mature oocytes should be used.[49] Isolation of primordial follicles and their transfer onto a scaffold could be carried out to create an artificial ovary that could eliminate the risk of malignant cells introduction.[50] However, the difficulty in this procedure is finding appropriate material for the scaffold that will encapsulate and protect the follicles and degrade to allow follicle development, cell migration, and vessel formation.[51] Another option to avoid the risk of malignant cell introduction is *in vitro* development of primordial follicles. This is a multistep culture system that needs to allow the maturation of primordial follicles to the stage of antral follicles.[52] Although further research is required, some promising results have been obtained[53, 54], which give us reason to believe that cryopreserved pieces of ovarian tissue could be matured *in vitro* in the future and, with any luck, clinical pregnancies could be achieved in patients with a high risk of malignant cell transplantation. The controversial discovery of ovarian stem cells able to replace atretic follicles by neo-folliculogenesis in postnatal mammalian ovaries[55] has provided hope that fertility restoration could be achieved by *in vitro* derivation from ovarian stem cells.[56, 57] However, the existence of ovarian stem cells in humans is not fully supported. Further research on this topic is needed, wherefore this method is still far from its application for fertility restoration.

Retransplantation of ovarian tissue: fertility restoration

When the patient is cured of her initial disease and desires to conceive, their hormonal status, risk of disease's recurrence and overall physical status is carefully considered in collaboration with oncologists before the autotransplantation is performed. The graft can be transplanted either on an orthotopic (pelvic

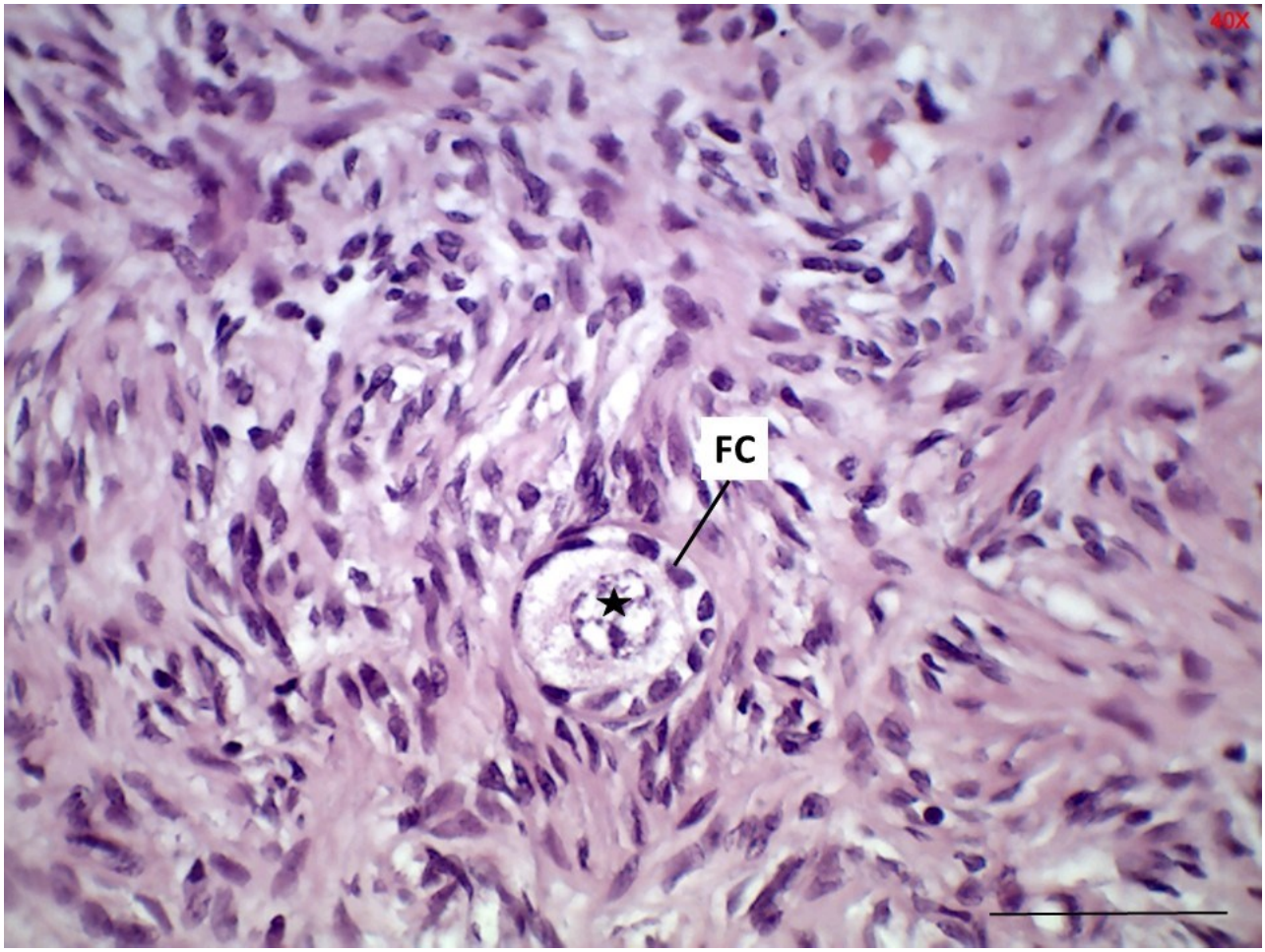

Figure 1. A piece of the ovarian cortex subjected to histopathological analysis. Abundant connective tissue surrounds the monolayered primary follicle. The primary oocyte (★) is typically situated in the middle of the follicle, encircled by follicular cells (FC). (Hemalaun & eosin, x400, scale bar = 50 µm)

cavity) or heterotopic site (outside the pelvic cavity like the forearm, abdominal wall, or rectus muscle).[41, 58]

Orthotopic ovarian tissue transplantation was first described by Donnez *et al*.[42] This type of transplantation involves grafting of ovarian cortical fragments to the exposed medulla of the present denuded ovary or creating the peritoneal site in case both ovaries are absent.[59] Thus, it requires a more invasive surgical approach in the form of abdominal surgery under general anaesthesia. However, the efficiency of the method was reported by many independent researchers in terms of patients renewing their hormonal cycles, conceiving, and giving birth successfully. More than 85 live births have been achieved worldwide with this approach in addition to the restoration of endocrine function.[60] Despite the success of this method, strategies for the improvement of transplantation outcomes are needed to reduce the challenge of early post-transplantation hypoxia, which remains the primary cause of follicle loss during the first few days after grafting. Various approaches are used to increase vascularisation in grafted tissue, including the supply with angiogenic and antiapoptotic factors[41] or stimulation of neovascularisation with adipose tissue-derived stem cells in an experimental model.[61] The latter is called a two-step transplantation procedure. It has been demonstrated to increase rates of oxygenation accompanied by a boost in vascularisation of ovarian tissue, which results in lower apoptosis and a higher survival rate of follicles.[62]

Heterotopic ovarian tissue transplantation is preferred in cases of changed pelvic anatomy, pelvic adhesions, or insufficient vasculature when orthotopic transplantation is impossible. Frozen-thawed cortical tissue can be placed in the abdominal wall, into the subcutaneous tissue of the abdomen, to the subcutaneous space of the forearm, beneath the peritoneum, or even in the rectus muscle. The method offers some advantages compared to orthotopic transplantation, including avoiding invasive surgical procedures with general anaesthesia, effortless retrieval of mature oocytes, and more accessible and cheaper repetition of transplantation procedures when needed. However, since it does not provide an environment as beneficial as orthotopic transplantation, pregnancy rates of heterotropic transplantation are significantly lower when comparing

these two methods, with only two pregnancies reported so far.[63] Furthermore, natural conception in heterotopic reimplantation is impossible, and for patients to conceive, *in vitro* fertilization (IVF) is required.
To summarize, the exact approach of ovarian tissue reimplantation should be discussed and selected individually for every patient. Nevertheless, no matter which method for reimplantation is used, patients should always be transplanted with ⅓ to ½ of their preserved ovarian tissue in case the transplantation procedure has to be repeated in the future.

Ovarian activity restoration: pregnancy and live birth rates

The first pregnancy and birth achieved by frozen-thawed ovarian tissue transplantation were reported in 2004.[42] Since then, the number of live births has increased to 130 in 2017[41], and has probably reached more than 200 by now.[64] When it comes to estimating the pregnancy rate, it is hard to provide the exact percentage since it is unknown how many reimplantations have been carried out worldwide. Still, an assessment could be conducted based on the reported work of leading centres for OTC. In 2017, pregnancy and live birth rates of 33% and 25% respectively were reported in a cohort of 74 women 65, while in a very recent paper, the pregnancy and live birth rates were even higher: 50% of 60 women achieved pregnancy, and 41% of them gave birth to live babies.[66] Regarding the hormonal function of the graft, renewed ovarian activity was reported in 95% of women, with the mean life span of the graft of 4-5 years.[43] Nevertheless, in some cases, ovarian function after transplantation can persist to 10 years.[67] Interestingly, the initiation of puberty after the transplantation is also possible in patients who underwent OTC before starting their hormonal maturation; thus, the auto-transplantation of frozen-thawed ovarian tissue could be useful for the restoration of endocrine function both before and after puberty.[68]

Reproductive (immature) tissue banking in Croatia

Cryopreservation and storage of reproductive tissue in pre-pubertal patients have not yet been implemented as an option for preservation and future restoration of fertility in Croatia. However, cryopreservation of testicular tissue in adult men is an established method that has been practised since 2013 in the Testicular Tissue Bank, which operates within the Clinical Department of Transfusion Medicine and Transplantation Biology, Clinical Hospital Centre Zagreb. The testicular tissue of patients with azoospermia or patients with testicular cancer is collected at the Clinic of Urology, University Hospital Center Zagreb by bilateral or unilateral biopsy (Figure 2). So far, the testicular tissue of more than 340 men has been stored, thus providing extensive experience in testicular tissue processing and controlled slow freezing, which should facilitate the introduction and implementation of a similar procedure for immature testicular tissue. Since the collection and processing do not differ significantly for adult and immature testicular tissue, this part of the protocol does not need to be changed. A different cryopreservation protocol adapted to immature testicular tissue should be implemented using the existing equipment for slow tissue freezing.
In 2020, a new tissue bank within the Clinical Department of Transfusion Medicine and Transplantation Biology was established, the Reproductive Tissue Bank, which is a functional part of the Croatian Tissue and Cell Bank and includes the Testicular Tissue Bank as well as the future Ovarian Tissue Bank. Ovarian tissue bank will conduct processing, cryopreservation and long term storage of mature and immature ovarian tissue that would be collected in the operating room of the Department of Gynecology and Obstetrics, University Hospital Center Zagreb. With that in mind, a workshop on ovarian tissue cryopreservation was conducted in 2019 for the employees of the Croatian Tissue and Cell Bank. Preparations for the establishment of the bank have begun: required documentation to obtain a licence has been prepared. The container for the transport of ovarian tissue and cold plates for tissue processing has been validated. However, all the necessary equipment has not yet been procured.

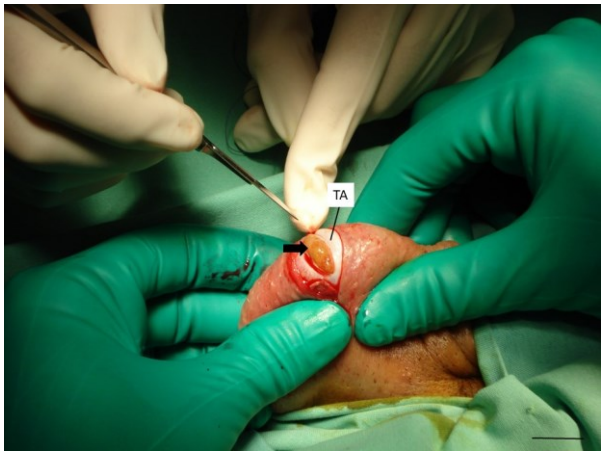

Figure 2. Performing a testicular biopsy for cryopreservation of the testis tissue. After a small incision into the tunica albuginea (TA), testicular parenchyma spontaneously protrudes (➡) and can be easily removed by microscissors. (Scale bar = 1 cm)

CONCLUSION

Significant progress has been made in fertility preservation techniques for children and adolescents with cancer in the past few years. Nevertheless, despite significant advances in preserving reproductive tissue, methods to restore fertility are still at a research stage, especially when it comes to pre-pubertal boys: cryopreservation of testicular tissue is widely used, but the proper strategy for its utilisation is not yet entirely

clear. Ovarian tissue cryopreservation, a promising approach for fertility preservation in young girls who cannot delay gonadotoxic treatment, was considered experimental until recently when the American Society for Reproductive Medicine removed the experimental label from the procedure and enabled its wider clinical application.[69] This progress was encouraged by promising results, with several achieved pregnancies and a high rate of renewed ovarian activity in young cancer survivors. However, if tissue retransplantation is used as a fertility restoration method, particular caution should be undertaken in case of malignant indications with a high risk of metastases.

Ethical considerations in experimental interventions for children are more challenging and thus more strict than those for adults. Therefore, currently available fertility preservation and restoration options should be considered in children only when treatments with a high risk of infertility are indicated, always taking into account the best interest of the young patient.

As for the situation with fertility preservation and restoration options in Croatia, pre-pubertal patients could very soon be offered with the option of immature reproductive tissue cryopreservation before the gonadotoxic treatment is initiated, thus providing them hope for a normal (reproductive) life in the future, once their primary disease is cured.

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