Cryopreservation of Human Ovarian Tissue: A Review

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\textbf{Keywords}

Ovarian tissue · Fertility preservation · Cancer · Freezing · Vitrification

\textbf{Abstract}

\textbf{Background:} Cryopreservation of human ovarian tissue has been increasingly applied worldwide to safeguard fertility in cancer patients, notably in young girls and women who cannot delay the onset of their treatment. Moreover, it has been proposed to patients with benign pathologies with a risk of premature ovarian insufficiency. So far, more than 130 live births have been reported after transplantation of cryopreserved ovarian tissue, and almost all patients recovered their ovarian function after tissue reimplantation. \textbf{Summary:} This review aims to summarize the recent results described in the literature regarding human ovarian tissue cryopreservation in terms of methods and main results obtained so far. To cryopreserve human ovarian tissue, most studies describe a slow freezing/rapid thawing protocol, which is usually an adaptation of a protocol developed for sheep ovarian tissue. Since freezing has been shown to have a deleterious effect on ovarian stroma and granulosa cells, various research groups have been vitrifying ovarian tissue. Optimization of both cryopreservation strategies as well as thawing/warming protocols is therefore necessary to improve the survival of follicles in cryopreserved ovarian tissue. \textbf{Key Messages:} Human ovarian tissue cryopreservation has been successfully applied worldwide to preserve fertility in patients with malignant or nonmalignant pathologies that have a detrimental effect on fertility. Human ovarian tissue cryopreservation could also be applied as an alternative to postpone pregnancy or menopause in healthy women. Slow freezing and vitrification procedures have been applied to cryopreserve human ovarian tissue, but both alternatives require optimization.

\textbf{Introduction}

The International Agency for Research on Cancer estimated that in 2018 more than 276,000 people under 44 years of age were diagnosed with cancer in Europe \cite{1}. The incidence rates are significantly higher among females (65\%) than among males (35\%) in these younger age groups \cite{1}. While current cancer treatment approaches are frequently able to provide disease remission, prolonging patients’ life expectancy, they may also lead to infertility as chemoradiotherapy can be very harmful to germ cells \cite{2}. Such fertility loss can be psychologically traumatic for women, as most of them may desire to have biological children. It may affect young individuals as well; thus, the improvement of their quality of life during cytotoxic treatment provides hope in terms of future fertility reestablishment and may be considered an extension of cancer care \cite{3}. Scientific and medical communities have therefore given much attention to fertility preservation – even the National Institute for Clinical Excellence in the UK included this topic as part of the guidance for the management of patients diagnosed with cancer \cite{4}.
In addition to fertility loss, effects caused by ovarian failure and decreases in ovarian steroidogenesis may lead to increased risks of osteoporosis and heart disease, as well as to menopausal symptoms including hot flushes, libido loss, and hypertension [4]. The usual approach to managing these symptoms is pharmacological hormonal replacement therapies (pHRTs), usually comprising a combination of drugs. While these are highly efficient treatments, in some patients it may be difficult to establish an adequate hormone dosage and delivery. Moreover, the incidence of stroke, venous thromboembolism, heart disease, and cancer may increase due to long-term pHRT administration [5]. As side effects of pHRT may outweigh its benefits, alternative strategies should be proposed to menopausal patients.

In order to restore fertility and endocrine function, ovarian tissue cryopreservation (OTC) can be proposed to these patients, as it allows maintenance of follicular cell viability and helps maintain reproductive capacity as well as ovarian endocrine potential [6, 7]. This review aims to summarize the recent results described in the literature regarding human OTC.

**Indications for OTC**

OTC has a single objective: maintenance of the ovarian structure and physiology, benefiting multiple target patients in different situations. OTC has been indicated mainly to safeguard fertility in cancer patients at risk of ovarian insufficiency and infertility due to gonadotoxic treatments [8]. Moreover, this is the only fertility preservation alternative for prepubertal patients, since in these cases, protocols for ovarian stimulation and oocyte collection are not possible [9]. However, patients with benign conditions such as recurrent ovarian cysts, ovarian torsion, endocrine disorders, and autoimmune diseases may also benefit from this promising technique [8, 10].

The OTC technique has enabled the birth of more than 130 healthy babies worldwide [11]. When followed by autotransplantation, success rates are high regarding the reestablishment of ovarian activity (63.9%) and natural live births (57.5%), according to a meta-analysis performed in 2017 [12].

Yet to be mentioned is the growing interest of women in postponing their first pregnancy, owing to education, career planning, or financial instability or even to possible difficulties in finding a partner [13]. The mean age at first pregnancy has increased by 2–4 years in the last 35 years and now is above 30 years [14]. Since both the quality and the amount of follicles decrease considerably with age, cryostorage is an alternative for improving pregnancy outcomes.

OTC can also be indicated for postponing menopause [15, 16]. The rise in life expectancy in the world population is combining with an increase in the number of menopausal women. In some European countries, most girls born in this century will probably live to be over 100 years old [17], which means that a greater part of their lives will unfold after menopause. Side effects linked to menopause (osteoporosis, cardiovascular problems, sexual dysfunction, depression, etc.) can have a dramatic effect on women’s health and quality of life, which has the potential of becoming a significant economic issue. Currently, in the Netherlands, a loss of more than EUR 100 million per year is estimated due to untreated menopausal symptoms [18]. An alternative is the prescription of pHRT. However, pHRT may be unsuitable for some women, including those at increased risk of cardiovascular disease, thromboembolic disease, or some types of cancer. OTC at an early age would safeguard a large population of ovarian follicles that could later be responsible for the production of female hormones, delaying the onset of menopause. In order to avoid the risk of pregnancy and decrease costs associated with ovarian tissue reimplantation, fragments of thawed or warmed ovarian tissue could be heterotopically transplanted.

**How to Cryopreserve Human Ovarian Tissue**

**Conventional Freezing**

Currently, slow freezing is the most frequently used method for cryopreserving human ovarian tissue. This procedure uses programmable freezers to achieve controlled freezing rates. In any slow freezing process, ice crystals form first outside the cells/tissue. As water solidifies, the outside milieu becomes hypertonic. As has long been known, fast cooling rates prevent intracellular water from leaving the cells, resulting in ice crystal formation inside the cells and consequent cell damage. On the other hand, slow cooling rates allow osmotic adjustments between intra- and extracellular fluid, but they may lead to excessive dehydration and shrinkage [19–21].

The addition of cryoprotectant agents (CPAs) to the cryopreservation medium helps to overcome these problems. The role of CPAs is to protect the cells against injuries caused by both ice crystals and hypertonicity during cryopreservation. However, CPAs can have osmotic effects upon the cells during freezing/thawing procedures. When cells are exposed to permeating CPAs, they initially undergo dehydration and shrinkage followed by a return to the original volume as the CPA enters the cell. These changes in volume can cause cell damage or even death, depending on their rapidity and magnitude. Addition of CPAs can cause cell damage also by chemical toxicity [22]. Optimal exposure should aim to minimize osmotic stress while avoiding chemical toxicity and allow
Table 1. Slow freezing methods used by the most prominent groups performing fertility preservation worldwide

<table>
<thead>
<tr>
<th>Country</th>
<th>Base medium and supplements</th>
<th>CPAs</th>
<th>Equilibrium</th>
<th>Cooling curve</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>Minimum essential medium (MEM) + 4 mg/mL HSA</td>
<td>10% DMSO</td>
<td>30 min at 0°C</td>
<td>–2°C/min to –8°C, manual seeding, –0.3°C/min to –40°C, and –30°C/min to –140°C</td>
<td>67.</td>
</tr>
<tr>
<td>Belgium</td>
<td>Leibovitz L-15 medium</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>30 min at 4°C</td>
<td>–2°C/min to –7°C, manual seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>68–71</td>
</tr>
<tr>
<td>Denmark</td>
<td>PBS</td>
<td>1.5 st EG + 0.1 st sucrose</td>
<td>30 min at 1°C</td>
<td>2°C/min to –9°C, manual seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>72–75</td>
</tr>
<tr>
<td>Portugal</td>
<td>RPMI 1640 medium + Glutamax and 15% FCS</td>
<td>10% DMSO</td>
<td>30 min at 4°C</td>
<td>Overnight in a freezer at –80°C</td>
<td>76.</td>
</tr>
<tr>
<td>Australia</td>
<td>PBS + albumin</td>
<td>1.5 mol PROH + 0.1 st sucrose</td>
<td>30 min at room temperature</td>
<td>–2°C/min to –8°C, manual seeding, –0.3°C/min to –36°C, and –50°C/min to –150°C</td>
<td>77–79</td>
</tr>
<tr>
<td>Australia</td>
<td>Dulbecco’s PBS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>Room temperature</td>
<td>–2°C/min to –8°C, manual seeding, –0.3°C/min to –36°C, and –50°C/min to –150°C</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Leibovitz L-15 medium + 10% decomplemented patient serum</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>81.</td>
</tr>
<tr>
<td>Germany</td>
<td>Leibovitz L-15 medium + 10% FCS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>82.</td>
</tr>
<tr>
<td>Germany</td>
<td>RPMI 1640 medium + 20% fetal calf serum</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –6°C, automatic seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>83.</td>
</tr>
<tr>
<td>Spain</td>
<td>M199 + 5% human serum</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –6°C, automatic seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>84, 85</td>
</tr>
<tr>
<td>Sweden</td>
<td>PBS</td>
<td>1.5 st PROH + 0.1 st sucrose</td>
<td>Room temperature (time not mentioned)</td>
<td>Using the programmable freezing device CTE 920 with automatic seeding at an optimal temperature (detailed curve not described)</td>
<td>87.</td>
</tr>
<tr>
<td>Israel</td>
<td>Ovocyte wash buffer + 15% synthetic serum</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>88.</td>
</tr>
<tr>
<td>Israel</td>
<td>Leibovitz L-15 medium + 10% HSA</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>30 min at 0°C</td>
<td>–1°C/min to –9°C, manual seeding, –0.3°C/min to –36°C, and –5°C/min to –140°C</td>
<td>89, 90</td>
</tr>
<tr>
<td>USA</td>
<td>HEPE5-buffered Dulbecco’s minimum essential medium (DMEM)-F12</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>30 min on ice</td>
<td>–2°C/min to –7°C, manual seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>91.</td>
</tr>
<tr>
<td>USA</td>
<td>Leibovitz L-15 medium + 10% FCS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>92.</td>
</tr>
<tr>
<td>UK</td>
<td>Leibovitz L-15 medium + 10% FCS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>30 min at 0°C</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>93.</td>
</tr>
<tr>
<td>UK</td>
<td>Leibovitz L-15 medium + 10% FCS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>94.</td>
</tr>
<tr>
<td>UK</td>
<td>Leibovitz L-15 medium + 10% FCS</td>
<td>1.5 st DMSO + 0.1 st sucrose</td>
<td>On ice for 15 min</td>
<td>–2°C/min to –7°C, seeding, –0.3°C/min to –40°C, and –10°C/min to –140°C</td>
<td>95.</td>
</tr>
</tbody>
</table>

CPAs, cryoprotectant agents; DMSO, dimethyl sulfoxide; EG, ethylene glycol; FCS, fetal calf serum; HSA, human serum albumin; PBS, phosphate-buffered saline; PROH, 1,2-propanediol.

sufficient permeation and dehydration to achieve protection from freezing injuries.

CPAs are divided into two categories [23]:
- Permeating agents: glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and 1,2-propanediol (PROH); these have a low molecular weight and can pass through the lipid bilayer of the cell membrane, although they do so more slowly than water.
- Nonpermeating agents: sugars (sucrose, trehalose, and raffinose) and macromolecules (Ficoll and polyvinylpyrrolidone), as well as proteins and lipoproteins; these remain in the extracellular solution, for they are large molecules, and help to promote controlled cell dehydration.

In most slow freezing protocols for ovarian tissue, a combination of one permeating agent and one nonpermeating agent is used. CPA concentrations are around 1.5 M for the permeating agent (usually DMSO) and 0.1 M for the nonpermeating agent (usually sucrose). Details on the protocols used by the leading groups working with OTC worldwide are presented in Table 1. Particular care should be taken regarding the permeation of CPAs in ovarian samples, as different types of cell and extracellular matrix compose the tissue. For instance, a higher equilibration period should be proposed for allowing the CPAs to perfuse to the inner tissue areas. Also for this purpose, ovarian tissue biopsies are cut into thin strips.
Although many studies have highlighted the importance of establishing optimal conditions for the use of each cryoprotectant in complex multicellular systems, few differences in protocols have been observed between groups (Table 1). Indeed, slow freezing protocols for human ovarian tissue are based on the protocol described by Gosden et al. [24], which follows a sequence of steps (Fig. 1) with slight variations, such as changes regarding the time and/or temperature of equilibrium with CPAs and the seeding temperature (Table 1).

Gook et al. [25] tested a two-step method for CPA permeation, consisting of 10 min in 1.5 mol PROH and then 30 min in 1.5 mol PROH + 0.1 mol sucrose, versus one-step methods, with both CPAs together, for 30, 60, or 90 min. The authors concluded that single-step dehydration for 90 min allowed the highest proportion of intact and morphologically preserved primordial follicles.

In some countries, national programs with centralized cryobanks have been created, so that only a few centers with proven expertise perform cryopreservation of ovarian tissue that fulfills certain quality criteria. An example is FertiPROTEKT, a network of fertility preservation programs in Germany, Switzerland, and Austria [26].

Vitrification

More recently, human ovarian tissue has also been cryopreserved by means of vitrification. So far, only 2 live births have been reported after cryopreserving human ovarian tissue using this procedure [27, 28].

Vitrification is a process of converting a supercooled liquid into a glass-like amorphous solid, preventing ice crystal formation [22]. Vitrification processes are based on an ultrafast cooling rate combined with a high concentration of CPAs [29]. However, high concentrations of CPAs have toxic effects on the cells [22]. Because of this, vitrification methods usually use a combination of two or more CPAs [30–33], so that the sum of their concentrations supports vitrification, while the low concentration of each CPA reduces their toxic effects [34]. In theory, any permeating CPA may be used for vitrification. However, ethylene glycol is being established as the best choice [30], because of its low toxicity and rapid diffusion into cells [35].

Another factor that influences vitrification is the volume of the sample. The smaller the sample, the less liquid is required to be cooled, and the lower the probability of ice crystal formation [29]. To achieve low volumes of liquid, different approaches are used, such as medium droplets [30, 32], a solid surface [35], a silver closed vitrification system [33], and plastic straws [36].

Cryopreservation by vitrification is attractive because it is a quick and easy procedure and does not require special and expensive equipment. Although it seems simple to perform, if cooling rates are not fast enough, crystallization may occur. In a successful vitrification, the tissue and surrounding solution become transparent, whereas failed vitrification is characterized by an opaque white sample, meaning ice crystals have formed [28, 29].

Despite the growing popularity of this type of preservation, it is still rarely used for ovarian tissue preservation. Unlike the slow freezing procedure, there is no standard vitrification protocol for ovarian tissue. Apart from the 2 babies reported by the Japanese group [27, 28], promising results using vitrified ovarian tissue were also reported by Kiseleva et al. [37]. In their case study, vitrified ovarian tissue showed recovery of its reproductive potential after autotransplantation [37].

Thawing/Warming and the Risk of Recrystallization

The risk of ice formation during warming is an important factor to be taken into account with slowly frozen or vitrified tissue. Cooling and warming rates interact, and a suitable outcome can be found when both are carefully taken into consideration.

Devitrification is the process of conversion of ultraviscous water into crystalline ice. Recrystallization happens when small ice crystals formed during temperature reduction grow while warming; as a result there is an opaque appearance in a previously clear sample [38]. The risk of recrystallization is greater for vitrified systems [39], as the warming process plays an even greater role than the cooling rate [40]. Water should turn from liquid directly to glass in vitrification. However, during warming, there is a possibility of ice crystal formation rather than "devitra-
**Future Trends in OTC**

Despite the successful results obtained after transplantation of cryopreserved ovarian tissue, studies have shown that freezing negatively affects the ovarian stromal tissue [43–48], granulosa cells [25, 48, 49], and theca cell formation [50], which may be involved in the significant number of empty follicles observed in women with grafted frozen-thawed ovarian tissue [50] and the limited life span of the graft [51]. These findings could be due to ice crystal formation, an inherent step in the freezing procedure and the major source of injury and cell death. In order to optimize a freezing protocol for human ovarian tissue, and to achieve a more homogeneous CPA distribution inside the ovarian tissue samples, Corral et al. [52] decided to modify various parameters in the current slow freezing procedures (Fig. 1). They demonstrated that better CPA permeation and homogeneity in bovine ovarian tissue was observed with a higher DMSO concentration (20%) and lower seeding temperature (–11 °C) [52]. This protocol was then tested on human ovarian tissue, and while ice formation was less likely to occur in the samples frozen with a 20% DMSO concentration due to the higher CPA content inside the tissue fragments, it did not improve follicle survival after short-term xenografting (Fig. 2) [53]. Nevertheless, a longer period of xenotransplantation could eventually show a possible benefit from this optimized protocol.

A few years ago, directional freezing was tested to cryopreserve ovarian tissue [54]. This strategy allows for a precise and uniform cooling rate in samples of different sizes, through a thermodynamic principle where ice crystals are controlled throughout the sample by regulating the sample movement rate through the predetermined temperature gradient [55]. In this way, the tissue sample is cooling while ice crystals are growing in the opposite direction of the sample movement. This procedure successfully allowed the cryopreservation of whole sheep ovaries [54]. Despite these promising findings, directional freezing has not yet been tested on human ovarian tissue.

As previously mentioned, vitrification has been considered an alternative to slow freezing, as it has been shown to preserve the ultrastructure of stromal tissue [47, 56]. However, vitrification is based on three crucial steps: addition of high CPA concentrations and achievement of high cooling and warming rates to decrease CPA toxicity. To avoid the toxic CPA effects, Corral et al. [57] devel-

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**Table 2. Examples of thawing/warming protocols described in the literature**

<table>
<thead>
<tr>
<th>Cryopreservation method</th>
<th>Final CPA concentration</th>
<th>Thawing/warming method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF 1.5 M EG</td>
<td>10-min water bath at 37°C; 10-min baths with lower EG concentrations, sucrose, and PBS at RT</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Vitrification 35% EG</td>
<td>TCM199, SSS, and sucrose at 37°C; 3-min bath with TCM199, SSS, and sucrose at RT</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>SF 1.5 M DMSO</td>
<td>2 min in air at RT; water bath at RT; 3 baths in Leibovitz L-15 medium</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>SF 1.5 M DMSO</td>
<td>30 s in air at RT; 2-min water bath at 37°C; 5-min baths with lower DMSO concentrations and sucrose</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

CPA, cryoprotectant agent; SF, slow freezing; EG, ethylene glycol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; RT, room temperature; SSS, serum substitute supplement.
A procedure called “stepped vitrification,” which consists of gradually decreasing the temperature while CPA is increased. The difference between their strategy and equilibrium vitrification is that the decline in CPA toxicity with temperature and reduction in the system melting point do not need to follow the same curve [57]. Corral et al. [57] tested their new procedure to vitrify bovine ovarian tissue by increasing the DMSO concentration in increments while simultaneously decreasing the temperature in increments, using a device specifically designed for this purpose. Their promising findings revealed follicles with histological integrity after warming similar to fresh controls [57].

Another important concern regarding vitrification is that fast cooling and warming rates are technically difficult to control, especially with bulky samples due to the constraints of heat transfer. Indeed, when the tissue fragment is immersed in liquid nitrogen, the temperature difference induces the formation of gas bubbles around the sample, which shields it from direct contact with the...
liquid nitrogen, negatively affecting the cooling rate. This phenomenon, called the Leidenfrost effect [58], can be avoided by using slush nitrogen, which can increase the cooling rate and decrease CPA concentration and exposure [59]. Recently, Barbato et al. [59] and Talevi et al. [60] tested an ultrarapid vitrification procedure using slush nitrogen to cryopreserve human ovarian tissue and reported an improvement in follicle ultrastructure, viability, and development, as well as preservation of stromal cell integrity.

New approaches have also been developed for improving thawing/warming of cryopreserved tissue. Manuchehrabadi et al. [61] tested a nanowarming technique to warm tissue samples. This idea was raised as an alternative in order to reduce failure during the warming process performed on large samples. Nanoparticles can be suspended in vitrification or freezing solution and used to induce a uniform heat increase in cryopreserved samples. This strategy relies on the concept of nanoparticle oscillations promoted by the laser wave, which produce heat dissipation throughout the sample, allowing for uniform warmth [62]. Iron [63] as well as gold [64] nanoparticles have been cited for this purpose. To induce particle stimulation, radio frequency and light may be used. However, aggregation with proteins or other solutes may occur [65], reducing the heat generation capacity of these systems. These effects can be reduced by coating the particles with silica or polypropylene glycol [66]. A possible concern with nanowarming is the risk of causing toxic effects to the tissue. However, low toxicity was observed after warming vitrified porcine carotid arteries and zebrafish embryos, using iron oxide and gold, respectively [61,63]. Since large fragments of organs can be successfully warmed using nanowarming, this could potentially be applied to human ovarian tissue as well.

Conclusion

OTC has been increasingly applied to preserve fertility of cancer patients and women with benign conditions and, more recently, has been discussed as a strategy for postponing pregnancy and menopause in healthy women. While more robust results have been reported for slow freezing procedures, various centers worldwide have started to test vitrification protocols. Nevertheless, optimization of both cryopreservation strategies and thawing/warming protocols is necessary to improve the survival of follicles in cryopreserved ovarian tissue.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

The authors would like to acknowledge the financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for grant 2016/22947-8 to E.C. Rivas Leonel. C.A. Amorim is a research associate of the FRS-FNRS.

Author Contributions

All authors contributed to the conception and writing of this review article.

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