RBMO

REVIEW

Ovarian tissue damage after grafting: systematic review of strategies to improve follicle outcomes



BIOGRAPHY

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KEY MESSAGE:

The ovarian follicle pool suffers major depletion after ovarian tissue transplantation. Although strategies using growth factors, hormones and antioxidants are effective at boosting revascularization and decreasing oxidative stress, protecting follicles is proving more challenging. Such protection may be achieved by exploiting the activated tissue response to ischaemia for tissue-healing purposes.

ABSTRACT

Frozen-thawed human ovarian tissue endures large-scale follicle loss in the early post-grafting period, characterized by hypoxia lasting around 7 days. Tissue revascularization occurs progressively through new vessel invasion from the host and neoangiogenesis from the graft. Such reoxygenation kinetics lead to further potential damage caused by oxidative stress. The aim of the present manuscript is to provide a systematic review of proangiogenic growth factors, hormones and various antioxidants administered in the event of ovarian tissue transplantation to protect the follicle pool from depletion by boosting revascularization or decreasing oxidative stress. Although almost all investigated studies revealed an advantage in terms of revascularization and reduction in oxidative stress, far fewer demonstrated a positive impact on follicle survival. As the cascade of events driven by ischaemia after transplantation is a complex process involving numerous players, it appears that acting on specific molecular mechanisms, such as concentrations of proangiogenic growth factors, is not enough to significantly mitigate tissue damage. Strategies exploiting the activated tissue response to ischaemia for tissue healing and remodelling purposes, such as the use of antiapoptotic drugs and adult stem cells, are also discussed in the present review, since they yielded promising results in terms of follicle pool protection.

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KEY WORDS

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INTRODUCTION

In recent decades, anti-cancer treatments have become increasingly effective, yielding significantly improved survival rates in cancer patients. Nevertheless, young women of reproductive age are at high risk of experiencing chemo-/radiotherapy-induced premature ovarian insufficiency and subsequent infertility (*Donnez and Dolmans, 2017*). Ovarian tissue cryopreservation and further ovarian tissue transplantation (OTT) can be offered as a safe and effective fertility preservation approach, having led to more than 200 live births so far (*Dolmans et al., 2020a*, *2020b*) and a live birth rate of around 40% (*Dolmans et al., 2021*; *Meirow et al., 2016*; *Shapira et al., 2020*). However, there is still room for improvement, particularly in terms of the massive follicle loss that occurs soon after grafting (*Dolmans et al., 2020a*). Ovarian tissue is manipulated during cryopreservation and cut into ovarian cortical strips to be stored for future transplantation. It is then thawed and transplanted to the pelvic cavity using an avascular surgical procedure upon disease remission (*Donnez et al., 2018*).

Post-transplantation follicle loss with its accompanying decline in fertility potential may happen (*Nisolle et al., 2000*; *Roness and Meirow, 2019*) for several reasons, including (i) modulation of the mechanical properties of the ovarian tissue due to its fragmentation and the absence of the ovarian medulla, with a consequent impact on follicle dynamics (*Woodruff and Shea, 2011*); (ii) post-transplantation hypoxia due to progressive revascularization and gradual oxygen replenishment (*Van Eyck et al., 2009*, 2010); and (iii) abnormal primordial follicle recruitment (*Roness and Meirow, 2019*). The latter could be sustained by early phosphoinositol-3-kinase (PI3K)/protein kinase B (Akt) signalling pathway activation (*Gavish et al., 2018*; *Masciangelo et al., 2019*, 2020; *Roness and Meirow, 2019*), Hippo pathway disruption related to ovarian cortex fragmentation (*Grosbois and Demeestere, 2018*; *Masciangelo et al., 2020*) and/or follicle pool burnout due to decreased anti-Müllerian hormone (AMH) concentrations in the first few months after transplantation (*Cacciottola et al., 2020a*; *David et al., 2012*). Despite follicle loss, the technique has proved successful thanks to the large follicle reserve in young patients and the ability of ovarian tissue to overcome the trauma of transplantation. Indeed, modulation of hypoxia and ischaemia in the post-transplantation period through revascularization enhancement and oxidative stress reduction is one of the most widely investigated strategies to address large-scale follicle loss, albeit with contentious results (*Demeestere et al., 2009*).

HYPOXIA IN THE OVARY

Primordial follicles reside in the superficial cortical layer, characterized by a sparse vascular network (*Delgado-Rosas et al., 2009*) that is rich in collagen and confers high density (*Laronda et al., 2017*). This tissue architecture is crucial to maintenance of the quiescent state of the follicles, with low metabolic activity and oxygen consumption (*Ishikawa et al., 2014*), and independence from hormone fluctuations occurring within each cycle (*Woodruff and Shea, 2011*). The rigidity of the ovarian cortex is relatively non-permissive to follicle growth through mechanical stimuli that regulate cell proliferation, and therefore appears to be crucial to maintaining primordial follicle quiescence (*Silber, 2015*a; *Woodruff and Shea, 2011*).

On the other hand, the perimedullary zone is characterized by a softer extracellular matrix and richer vascular network (*Delgado-Rosas et al., 2009*; *Woodruff and Shea, 2011*). Recruited follicles move to the medulla to complete their development in a decreasing tissue density gradient (*Silber, 2015*a; 2016) and increasing blood supply (*Delgado-Rosas et al., 2009*). Indeed, growing follicles require greater vascularization to address their growing metabolic needs (*Delgado-Rosas et al., 2009*). This vasculature develops inside the theca layer, separated from the granulosa layer by a basal membrane, which excludes it from the direct vascular supply of oxygen and nutrients during growth (*Thompson et al., 2015*; *van Wezel and Rodgers, 1996*).

As follicles develop, the granulosa layer thickens and oxygen transport becomes more difficult, until they enter a stage beyond which they cannot grow without encountering hypoxia. Formation of the antrum then appears to reduce oxygen demands (as the fluid compartment does not consume oxygen) and facilitate oxygen diffusion towards the oocyte (*Redding et al., 2007*). Granulosa cells also adapt their oxygen consumption during antral follicle growth in order to leave an optimal oxygen supply for the oocyte (*Li et al., 2013*) by up-regulating the HIF1 (hypoxia inducible factor 1) signalling pathway during their rapid growth (*Harris et al., 2007*; *Kind et al., 2015*; *Nishigaki et al., 2020*; *Zhou et al., 2018*).

HIF1 is one of the master regulators of response to hypoxia (*Takahashi et al., 2016*; *Zhou et al., 2018*). It is a highly conserved, heterodimeric transcription factor composed, in its active form, of alpha (HIF1α) and beta (HIF1β) subunits. HIF1α is continuously expressed and degraded in cells under normoxic conditions. Hypoxic signalling blocks proteasome-mediated degradation of HIF1α, allowing its accumulation and binding to HIF1β (*Lendahl et al., 2009*). This dimer binds specific DNA sequences, namely hypoxia response elements, for the transcription of genes responsible for metabolic changes, angiogenesis, inflammation and tissue remodelling (*Tanaka, 2016*). To overcome hypoxia, angiogenesis is enhanced through (i) an increase in proangiogenic growth factors, (ii) recruitment of inflammatory and endothelial progenitor cells, and (iii) migration of fibroblasts.

Of all the proangiogenic growth factors, vascular endothelial growth factor (VEGF) is the master effector of hypoxia-related proangiogenic responses. Upon binding to tyrosine kinase receptors, it triggers endothelial cell proliferation and migration, and promotion of vessel permeability (*Beamer et al., 2010*; *Connolly, 1991*). Basic fibroblast growth factor (bFGF) plays a role in new vessel formation by enhancing both bFGF and VEGF receptors and favouring endothelial cell migration to hypoxic sites for neoangiogenesis (*Murakami and Simons, 2008*; *Tassi et al., 2011*). Angiopoietin family proteins, including Ang-1 and Ang-2, are additional proangiogenic growth factors. Ang-1 has been shown to participate in vessel stabilization in normoxia, while Ang-2 is implicated in angiogenesis during hypoxia (*Gale et al., 2002*). The latter is particularly involved in the wound healing process in hypoxic and ischaemic sites, and in promoting VEGF activity in endothelial cells (*Fiedler et al., 2006*; *Gale et al., 2002*).

During follicle growth, increased VEGF expression not only has a proangiogenic

function, carrying blood around the follicles, but also plays a role in the signalling involved in granulosa cell proliferation (*Nishigaki et al., 2020*; *Yalu et al., 2015*). In fact, VEGF is one of the main regulators of granulosa cell proliferation in hypoxic conditions through the PI3K/Akt signalling pathway, with a subsequent upturn in expression of one of its main effectors, mammalian target of rapamycin (mTOR) (*Shiratsuki et al., 2016*). This signalling pathway is known to be crucial to early follicle activation, triggering the very first cross-talk between granulosa cells, concerning their change in shape and proliferation, and oocytes, regarding their maturation and growth (*Kawamura et al., 2013*; *Maidarti et al., 2020*).

The tissue response to ischaemic damage is bimodal, directed either at adaptation through the activation of defence mechanisms, or at cell death. It depends on the entity in question and the duration of hypoxia, but also the susceptibility of a given tissue (*Kalogeris et al., 2012*). Indeed, excessive hypoxia-related damage to cell compartments triggers activation of the caspase cascade and consequent cell apoptosis (*Hetz, 2012*).

OXIDATIVE STRESS IN THE OVARY

Reactive oxygen species (ROS) are products of cellular oxygen metabolism originating from incomplete reduction of oxygen, occurring mainly in mitochondrial metabolism during cellular respiration, but also as by-products of cytosolic enzymes such as xanthine oxidase, NADPH oxidase and nitric oxide synthase (*Abramov et al., 2007*; *Caglayan et al., 2017*). With unpaired electrons, such as the anion superoxide (O_{2-}), ROS are very unstable free radicals with high oxidant activity. Conversely, hydrogen peroxide (H_2O_2) is more stable and has fewer reactive free radicals, but it can easily diffuse through cell membranes, also acting as a cell signalling mediator (*Caglayan et al., 2017*).

Cells have developed coordinated mechanisms of sensing and signalling to respond and adapt to ROS. Indeed, at tolerable low concentrations, ROS are second messengers of intracellular signalling pathways related to cell proliferation, differentiation and survival (*Hamanaka and Chandel, 2010*; *McElroy and Chandel, 2017*). Conversely, at high concentrations, ROS can cause damage to cellular macromolecules, such as DNA, lipids and proteins, resulting in the accumulation of mutations, genomic instability and acceleration of disease, ageing and cell death (*Kim et al., 2017*). Cell compartments are protected against biological damage from ROS by a number of antioxidant systems. Some non-enzymatic antioxidants such as vitamin C (ascorbic acid) and vitamin E (α-tocopherol) can neutralize free radicals by donating or accepting electrons. They neutralize free radicals either directly by converting them into more stable ROS species, or indirectly by modulating the activity of antioxidant enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX) (*Hamanaka and Chandel, 2010*).

As signalling molecules in the ovary, ROS are involved in granulosa cell activity, and cumulus and oocyte maturation. Although adequate numbers of ROS are required for follicle growth until ovulation and further progesterone generation by the corpora luteum, oxidative stress is harmful to granulosa cell activity and oocytes, resulting in potential fertility problems. Granulosa cells work actively to maintain ROS concentrations below their antioxidant potential by enhancing antioxidant enzymes such as SOD and CAT to avoid oocyte impairment from oxidative stress during follicle development (*Devine et al., 2012*, *Fu et al., 2014*). These enzymes are not usually expressed in primordial or primary follicles, but have been observed in pre-antral and antral follicles (*Akino et al., 2018*; *Suzuki et al., 1999*). Indeed, the primordial follicle pool may be less sensitive to ROS-mediated damage in the same way it is to hypoxia-related damage, because of its quiescent state in a poorly vascularized portion of the ovary.

EVIDENCE OF HYPOXIA AND OXIDATIVE STRESS IN TRANSPLANTED OVARIAN TISSUE

Ovarian tissue is transplanted orthotopically to the pelvic cavity, either onto the decorticated ovarian medulla if at least one ovary is still present, or inside a specially created peritoneal window (*Donnez and Dolmans, 2015*). As ovarian cortical strips are grafted without vascular re-anastomosis, they undergo a period of hypoxia, whose duration depends on the revascularization process. Massive follicle loss encountered in the early post-grafting period (*Dolmans et al., 2007*; *Gavish et al., 2018*, *Masciangelo et al., 2019*) is estimated to be around 50–90% of the entire reserve (*Baird et al., 1999*; *Nisolle et al., 2000*) and has been found to occur before the re-establishment of revascularization (*Cacciottola et al., 2018*; *Van Eyck et al., 2009*, 2010). When hypoxia-related damage arises, a time-dependent tissue-adaptive response is initiated, towards either cell survival or cell death. In transplanted ovarian tissue, the follicle pool appears to be affected by both, with an increase in follicle death mainly through apoptosis (*Cacciottola et al., 2020a*; *Gavish et al., 2018*) and abnormal primordial follicle activation through modulation of signalling pathways such as PI3K/Akt, involved in cell proliferation and survival (*Cacciottola et al., 2020a*; *Masciangelo et al., 2019*, 2020).

This critical hypoxic period and the reoxygenation kinetics of transplanted ovarian tissue have been identified using electron paramagnetic resonance (*Van Eyck et al., 2009*). Use of a direct continuous measuring method is the gold standard for investigating local oxygen concentrations. Measurement of partial pressure of oxygen (pO_2) by electron paramagnetic resonance is achieved with an external probe, lithium phthalocyanine, which is implanted inside the tissue and interacts with oxygen, but does not consume it (*Carreau et al., 2011*, *Manavella et al., 2018a*, *2018b*).

Using this method, pO_2 was found to be below optimal values for the first 5–10 days after transplantation (*Van Eyck et al., 2009*). Indeed, revascularization of ovarian tissue occurs progressively over days and does not correspond to the mechanisms of reperfusion, as in organ transplantation using vascular pedicles. Here, it occurs through neoangiogenesis, whereby mechanisms of sprouting angiogenesis from existing vessels (*Macchiarelli et al., 2006*; *Rahimi et al., 2010*) and new blood vessel formation from vascular progenitor cells (*Dath et al., 2011*; *Kizuka-Shibuya et al., 2014*) are observed. Both the endothelial graft component already present in the tissue and the host component invading from the periphery contribute to graft revascularization (*Van Eyck et al., 2010*).

The duration of this process varies from species to species. In murine models, neovascularization was found to start after 48 h (*Dissen et al., 1994*; *Israely et al., 2004*), while in human ovarian tissue, evidence of revascularization was detected only from day 5 after grafting (*Van Eyck et al., 2010*). This process occurs progressively, leading to the development of functional vessels, and hence effective tissue reperfusion, after 7 days in murine models (*Israely et al., 2004*) and 10 days in human grafts (*Van Eyck et al., 2010*).

The whole process is modulated by oxygen tension, which, through HIF1 signalling activation, induces proangiogenic growth factor secretion. Responsible for endothelial cell proliferation and migration, VEGF appears to be increased 40- to 60-fold in grafted ovarian tissue soon after OTT) (*Dissen et al., 1994*; *Yang et al., 2008*). During angiogenesis, endothelial cells from existing vessels can acquire migration properties through proteolytic enzyme production (*Marchand et al., 2019*). This mechanism allows endothelial cells to cross the basal membrane, invade the extracellular matrix and proliferate in order to create new capillary vessels (*Macchiarelli et al., 2006*).

Once oxygen is available, other harmful components triggered by ROS generation compound this ischaemic damage, contributing to tissue injury (*Kalogeris et al., 2012*). Although indirect effects of oxidative stress have been widely investigated in transplanted ovarian tissue through the evaluation of scavenger expression and lipid peroxidation (*Demeestere et al., 2009*), the direct quantification of ROS remains technically challenging due to their instability. A xenografting model using microdialysis, an established method to measure different metabolites *in vivo* without altering tissue viability (*Shippenberg and Thompson, 2001*), was developed to characterize metabolic behaviour and quantify oxidative stress in grafted ovarian cortex (*Cacciottola et al., 2018*). ROS kinetics were observed for 3 weeks following OTT. The first ROS peak occurred 10 days after transplantation, once tissue perfusion was re-established, consistent with the initial metabolic shift to aerobiosis. The second peak occurred around 18 days after transplantation, consistent with complete re-establishment of aerobic metabolism. Oxidative stress was therefore observed relatively late, after the most critical period of follicle loss, and persisted until the tissue vasculature stabilized (FIGURE 1).

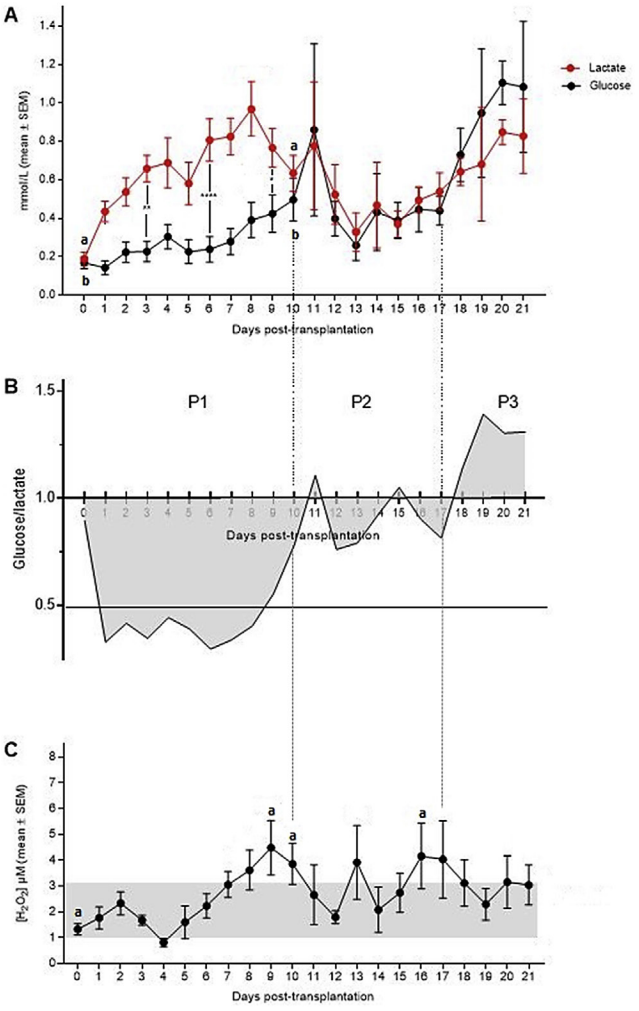


FIGURE 1 Metabolic changes and ROS generation after ovarian tissue transplantation. (A) Daily concentrations of glucose and lactate over 21 days post-transplantation. Differences between glucose and lactate values at each time point are indicated as follows: day 3, $P = 0.0016$; day 6, $P < 0.001$; day 9, $P = 0.037$. Increase in lactate values from day 0 to day 10: $P = 0.0018$. Increase in glucose values from day 0 to day 10: $P = 0.028$. (B) Glucose and lactate concentration ratios. (C) Daily H_2O_2 values over 21 days post-transplantation. Significant differences between time points ($P < 0.05$) are indicated by the same letters: days 0–9, $P = 0.0014$; days 0–10, $P = 0.0117$; days 0–16, $P = 0.015$. The first post-transplantation period up to day 10 (P1) is characterized by anaerobic metabolism and increasing ROS concentrations up to a first ROS peak on day 10. In the second period after transplantation (P2), namely days 10–17, there is a progressive metabolic shift to aerobiosis and ROS values are erratic. The third post-transplantation period (P3), including days 17–21, is characterized by aerobic metabolism and ROS values stabilized within basal ranges (shaded area). ROS, reactive oxygen species. Adapted from *Cacciottola et al. (2018)*.

STRATEGIES TO IMPROVE HYPOXIA-RELATED DAMAGE AND REDUCE OXIDATIVE STRESS AFTER OTT

A number of strategies have been developed to minimize hypoxic tissue damage and restore blood supply sooner, in order to obtain better follicle outcomes in terms of increased follicle survival and enhanced neovascularization (FIGURE 2). Numerous investigations have attempted to identify drugs that are able to increase follicle survival by boosting the revascularization process, such as growth factors, hormones and antioxidants. These drugs interact at different stages of the cascade of events, starting from the initial hypoxia-related response, to the decline in oxidative stress after tissue reperfusion. Various models of administration, doses and treatment protocols have been tested, with contentious findings on their effectiveness.

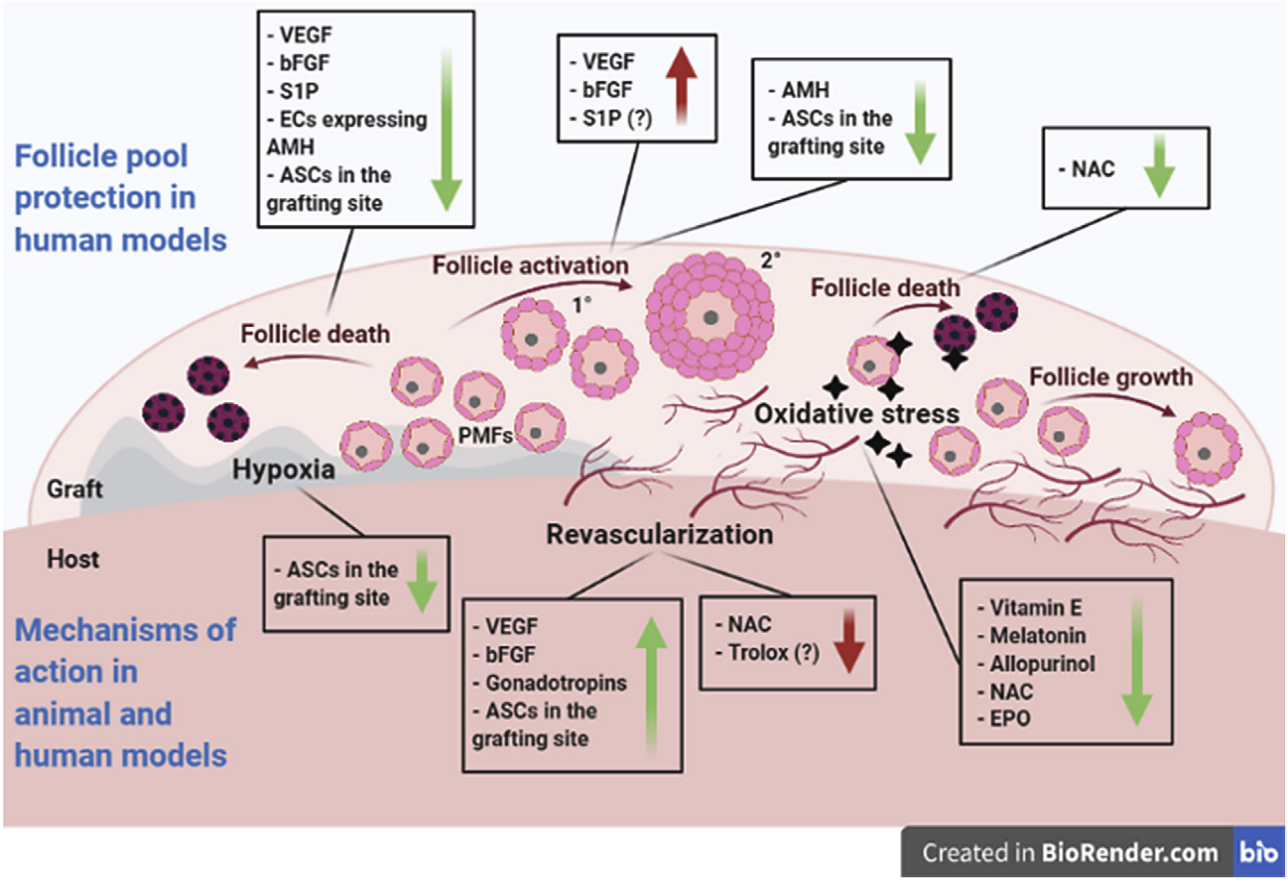


FIGURE 2 Strategies to decrease hypoxia and oxidative stress after ovarian tissue transplantation. Hypoxia and oxidative stress after ovarian tissue transplantation are detrimental to the primordial follicle pool, resulting in either increased follicle death or abnormal follicle activation after transplantation. The upper part of the figure shows adjuvant drugs and transplantation strategies that evidenced an impact on the follicle pool in human xenografting models. The lower part of the figure lists adjuvant drugs and transplantation strategies that had an impact on hypoxia, revascularization or oxidative stress in animal and/or human experimental models. Green arrows indicate favourable effects on ovarian reserve protection (from upper left clockwise: decreased follicle death, reduced follicle activation, decline in oxidative stress, revascularization enhancement, and lower levels of hypoxia). Red arrows indicate detrimental effects on ovarian reserve protection (from upper left clockwise: abnormal follicle activation and decreased revascularization). AMH, anti-Müllerian hormone; ASCs, adipose tissue-derived stem cells; bFGF, basic fibroblast growth factor; EC, endothelial cells; EPO: erythropoietin; NAC, *N*-acetylcysteine; S1P, sphingosine-1-phosphate; VEGF, vascular endothelial growth factor.

MATERIALS AND METHODS

Objectives

The aim of this review was to provide evidence of and information about hypoxia and oxidative stress in the context of OTT. The study explored various strategies undertaking attempts to reduce hypoxia and oxidative stress-related damage in transplanted ovarian fragments to improve follicle outcomes after grafting. To this end, a systematic review of papers that have investigated the use of growth factors, hormones and antioxidants to reduce follicle loss after OTT is presented.

Ethical approval

Ethical issues approval was not required because this study did not involve clinical trials. All research data came from published papers.

Inclusion criteria

Both animal and human studies were considered. Investigations were conducted into growth factor, antioxidant and hormone administration in the context of OTT compared with no adjuvant treatment.

The primary outcome was evaluation of follicles (survival, count or density, and apoptosis) after transplantation. Secondary outcomes included evidence of enhanced revascularization, decrease in oxidative stress-related damage and modulation of follicle growth.

Data source and research strategy

Following PRISMA guidelines (*Moher et al., 2009*), a PubMed search was conducted up to December 2020 using the following key words: 'growth factors', OR 'hormones', OR 'gonadotropins', OR 'antioxidants', OR 'scavengers', AND 'neovascularization', AND 'oxidative stress', AND 'ovarian tissue transplantation', AND 'ovarian tissue grafts' (344 records). Only peer-reviewed published articles written in English were evaluated.

Study selection and data extraction

First, all screened studies were imported to EndNote (374 records from database research, 10 records from bibliographies of selected papers). Any found to be duplicated were excluded after reading the title and abstract (280 records). Articles were then selected based on their titles and abstracts according to the relevant criteria (71 eligible abstracts; 43 included and 28 excluded). After reading them in full, those conforming to the eligibility criteria were included (43 eligible full texts; 40 included and 3 excluded) (FIGURE 1).

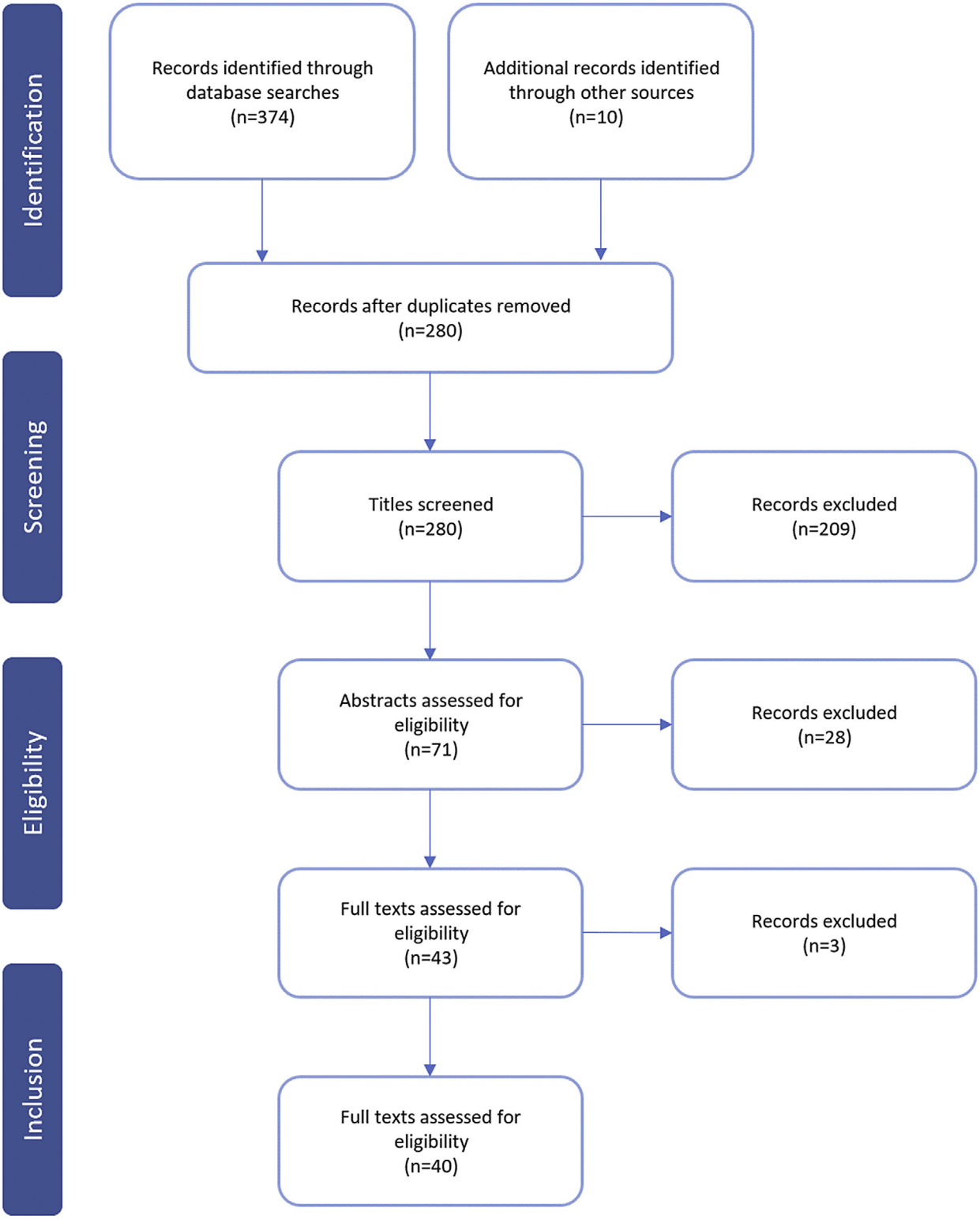


FIGURE 3 PRISMA flow diagram. Literature search methodology for publications investigating the effects of growth factors, hormones and antioxidants on follicle outcomes after ovarian tissue transplantation.

Growth factor administration

Eighteen studies were selected according to the previously mentioned criteria (TABLE 1). Seven studies were conducted with ovarian tissue from multiovulatory rodent species, all seven of them with murine tissue (*Gao et al., 2013*, 2015; *Li et al., 2016*; *Shikanov et al., 2011*; *Skatznik-Wikiel et al., 2011*; *Tavana et al., 2016*; *Youm et al., 2016*). Six studies were performed with ovarian tissue from mono-ovulatory species, three of them with ovine tissue (*Fransolet et al., 2015*; *Henry et al., 2015*; *Labied et al., 2013*), two with bovine tissue (*Kong et al., 2017*;

TABLE 1 GROWTH FACTORS USED TO IMPROVE FOLLICLE OUTCOMES AFTER OVARIAN TISSUE TRANSPLANTATION

		References	Grafting model	Growth factors	Administration route	Time points	Follicle outcomes			Revascularization
							Follicle survival	Follicle apoptosis	Follicle growth	
Rodents	1	*Gao et al., 2013*	Fresh murine OT encapsulated in fibrin hydrogel and subcutaneously autografted	bFGF (75, 100 and 150 µg/ml)	Fibrin hydrogel scaffold	Day 7	Increase in primordial and total follicle count	Decrease in caspase3+ primordial follicles	No difference in growing follicle count Increase in PCNA+ primordial follicles	Increase in vessel number (CD31+)
	2	*Gao et al., 2015*	Fresh murine OT encapsulated in fibrin hydrogel and subcutaneously autografted	bFGF (100 µg/ml) VEGF (75 µg/ml) bFGF (100 µg/ml) + VEGF (75 µg/ml)	Collagen matrix	Days 7 and 21	Increase in primordial and secondary follicle count in bFGF + VEGF group (day 21)	Decrease in caspase3+ follicles with bFGF + VEGF (day 7)		Increase in vessel density (CD31+) (day 21)
	3	*Li et al., 2016*	Vitrified-warmed murine OT subcutaneously autografted	bFGF (9 µg/ml) + VEGF (2 µg/ml)	Added to tissue culture before grafting	Days 7, 14 and 21	Increase in follicle survival rates			No difference in VEGF or bFGF protein expression; increase in vessel number (vWF+)
	4	*Shikanov et al., 2011*	Vitrified-warmed murine OT autografted to ovarian bursa	VEGF-168 (74 µg/ml)	Fibrin hydrogel modified with heparin-binding peptide	Two oestrous cycles (days 17–22)	Increase in primordial follicle count		No difference in growing follicle count	Increase in vessel number (CD31+)
	5	*Skatznik-Wikiel et al., 2011*	Frozen-thawed murine OT autografted to ovarian bursa	VEGF-164 (8 µg/kg per day) VEGF-164 (8 µg/kg per day) +G-CSF (50 µg/kg per day) G-CSF (50 µg/kg per day) + SCF (100 µg/kg per day)	Host injection (day 0–5)	Day 14	Increase in primordial and total follicle count in VEGF + G-CSF group		No difference in growing follicle count	
	6	*Tavana et al., 2016*	Fresh rat OT autografted to back muscle surface	bFGF (2 µg/ml) + VEGF-165 (2 µg/ml)	Hyaluronic acid hydrogel	1 oestrous cycle (days 12–15)	No difference in follicle density			No difference in *VEGF* and *CD31* gene expression; decrease in *CD34* gene expression
	7	*Youm et al., 2016*	Vitrified-warmed murine OT autografted to kidney capsule	Ang-2 (50 and 500 ng/kg)	Host injection (day –1)	Days 2, 7, 21 and42	No difference in follicle density	No difference in TUNEL+ follicles		Increase in vessel number (CD31+)
Ovines/ bovines	8	*Fransolet et al., 2015*	Frozen-thawed ovine OT embedded in a collagen matrix and xenografted to ovaries of SCID or NOD-SCID mice	VEGF-111 (100 nmol/l)	Collagen matrix	Days 3 and 21	Increase in primordial follicle density (day 21)			Increase in functional vascular network (day 3)
	9	*Henry et al., 2015*	Frozen-thawed ovine OT embedded in a collagen matrix and xenografted to ovaries of SCID mice	VEGF-165 (50 nmol/l)	Collagen matrix	Days 3 and 21	No difference in follicle density (day 21)			Increase in functional vascular network (day 3); increase in vessel density (CD31+) (day 21)
	10	*Kong et al., 2017*	Vitrified-warmed bovine OT xenografted to back muscle surface of nude mice	Ang-2 (500 ng) VEGF-164 (200 ng) Ang-2 (500 ng) + VEGF-164 (200 ng)	Host injection (day –1)	Days 7 and 28	Increase in follicle density in VEGF and Ang-2 + VEGF group (day7)	Decrease in TUNEL+ follicles in Ang-2 + VEGF group (day 7)	Increase in growing follicle density in VEGF and Ang-2 + VEGF group	Increase in vessel density (CD31+) in Ang-2 + VEGF group (day 7)

(continued on next page)

TABLE 1 *(continued)*

		References	Grafting model	Growth factors	Administration route	Time points	Follicle outcomes			Revascularization
							Follicle survival	Follicle apoptosis	Follicle growth	
	11	*Labied et al., 2013*	Frozen-thawed ovine OT embedded in a collagen matrix and xenografted to ovaries of SCID mice	VEGF-111 (500 µg/l)	Collagen matrix	Days 3 and 21	No difference in follicle density			Increase in functional vascular network (day 3); increase in vessel density (CD31+) (day 21)
	12	*Langbeen et al., 2016*	Fresh fetal bovine OT xenografted retroperitoneally to nude mice	mVEGF (5 ng/ml)	Added to tissue culture before grafting	Days 14 and 28		Decrease in caspase3+ follicles		Increase in vessel density (vWF+)
Non-human primates	13	*Schnorr et al., 2002*	Fresh and frozen-thawed monkey OT autografted to pelvic cavity	VEGF (1 µg)	Host injection (days 0–14)	Day 14	No difference in follicle count			
Humans	14	*Abir et al., 2011*	Frozen-thawed human OT xenografted to dissected back muscle of BALB/c-nu mice	Vitamin E (dry form, 3.200 IU/l)	Host treatment (days –2 to 2)	Day 14	No difference in follicle count			Increase in VEGF-A protein expression
				VEGF-A (200 ng/ml) + vitamin E (oil form, 400 IU/l)	Added to tissue culture before grafting					
				VEGF-A (200 ng/ml) + vitamin E (oil form, 400 IU/l) + vitamin E (dry form, 3.200 IU/l)	Added to tissue culture before grafting + host treatment (days –2 to 2)					
	15	*Friedman et al., 2012*	Frozen-thawed human OT xenografted to dissected back muscle of BALB/c-nu mice	Melatonin (240 mg/l)	Host treatment (days –14 to 0)	Day 14	No difference in follicle count	Decrease in TUNEL+ follicles		No difference in VEGF-A protein expression
				HA-rich glue + VEGF-A (200 ng/ml) + vitamin E (oil form, 400 IU/ml)	Added to tissue culture before grafting					
				Melatonin (240 mg/l) + HA-rich glue	Host treatment (days –14 to 0) + added to tissue culture before grafting					
				Melatonin (240 mg/l) + VEGF-A (200 ng/ml) + vitamin E (oil form, 400 IU/ml)						
				Melatonin (240 mg/l) + HA-rich glue + VEGF-A (200 ng/ml) + vitamin E (oil form, 400 IU/ml)						
	16	*Kang et al., 2016*	Vitrified-warmed human OT xenografted to back muscle surface of SCID mice	bFGF (150 ng/ml)	Added to tissue culture before grafting	Day 7	Increase in follicle count	Decrease in caspase3+ follicles	Increase in Ki67+ follicles	Increase in vessel density (CD34+)
				bFGF (150 ng/ml) + VEGF (25 ng/ml)						
	17	*Tanaka et al., 2018*	Frozen-thawed human OT xenografted to back muscle surface of SCID mice	bFGF (1 mg/ml)	Biodegradable acidic gelatin hydrogel	6 weeks	Increase in primordial and primary follicles			Increase in vessel density (CD31+)
	18	*Wang et al., 2013*	Fresh human OT xenografted to back muscle of rabbits	VEGF (50 ng/ml)	Added to tissue culture before grafting + host injection (day 7)	6 weeks		Decrease in TUNEL+ follicles in VEGF + bFGF group	Increase in Ki67+ follicles	Increase in vessel density (CD31+)
				bFGF (100 ng/ml)						
				VEGF (50 ng/ml) + bFGF (100 ng/ml)						

Ang-1/2, angiopoietin 1/2; bFGF, basic fibroblastic growth factor; G-CSF, granulocyte colony-stimulating factor; HA, hyaluronic acid; NOD, non-obese diabetic; OT, ovarian tissue; PNCA, proliferating cell nuclear antigen; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor; SCF, stem cell factor; TUNEL, TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling; vWF, von Willebrand factor.

Langbeen et al., 2016) and one with non-human primate tissue (Schnorr et al., 2002). Five studies involved xenografting human ovarian tissue (Abir et al., 2011; Friedman et al., 2012; Kang et al., 2016; Tanaka et al., 2018; Wang et al., 2013).

Growth factors, namely VEGF, bFGF, Ang-2 and granulocyte colony-stimulating factor (G-SCF), were administered at various doses either individually or in combination. These factors have a relatively short half-life. The half-life of VEGF is around 30–40 min in plasma and its mRNA rapidly degrades in normoxic conditions, serving as an endogenous control of its activity. However, VEGF mRNA is also stabilized through HIF1 regulatory action during hypoxia, resulting in a significant increase in its availability (Dibbens et al., 1999). The biological half-life of bFGF in its free form is also very short, around 60 min, and its biological activity is swiftly lost due to diffusion and/or enzymolysis (Andreopoulos and Persaud, 2006; Edelman et al., 1993). The half-life of Ang-2 is somewhat longer, around 18 h, but still much shorter than the hypoxic phase in transplanted ovarian tissue (Youm et al., 2016).

Different administration strategies were chosen: (i) systemic delivery to the host by injection in the days preceding or following grafting; (ii) local administration by culture of tissue strips in enriched medium; or (iii) encapsulation into biocompatible scaffolds transplanted with the tissue itself. Encapsulation in hydrogel scaffolds was shown to provide more extended release of these molecules. In the study by Tanaka and colleagues, ^{125}I-labelled bFGF was encapsulated inside gelatin hydrogel sheets, which still displayed radioactivity up to 14 days after subcutaneous implantation (Tanaka et al., 2018). In the study of Shikanov and colleagues, VEGF was encased in a fibrin hydrogel modified with heparin-binding peptide and heparin to ensure its controlled release over time (Shikanov et al., 2011).

Primary outcome

In terms of follicle outcomes, 5 out of 7 studies demonstrated superior follicle survival after grafting in murine models. Indeed, administration of bFGF, VEGF and G-SCF alone or combined led to an increase in follicle survival, but Ang-2 alone did not yield any improvement in follicle outcomes (Youm et al., 2016).

In mono-ovulatory species, only 3 out of 6 studies showed better follicle survival after grafting. Two of them used VEGF, either loaded inside a collagen matrix, exhibiting maintenance of a larger primordial follicle proportion after 21 days, calculated as follicle density (ovine model) (Fransolet et al., 2015), or added to tissue culture before grafting, with a decrease in apoptotic follicles after 28 days (bovine model) (Langbeen et al., 2016). The third study combined Ang-2 and VEGF administered to the host by injection before xenotransplantation and was found to be effective at protecting follicles from apoptosis for up to 4 weeks after grafting (Kong et al., 2017).

In human ovarian tissue xenografting studies, 2 out of 5 studies detected an improvement in follicle outcomes after OTT. Administration of bFGF inside a biodegradable scaffold was able to protect early-stage follicles for 6 weeks post-grafting (Tanaka et al., 2018). A combination of bFGF and VEGF added to tissue culture before grafting and injected into the host over the first week after transplantation also led to a decrease in apoptotic follicles.

It is known that follicle distribution and time to revascularization differ between mice and humans. These differences may explain why superior follicle outcomes were obtained with growth factors in murine tissue transplantation (TABLE 1). When looking at follicle outcomes according to growth factor release strategy, it appears that the use of biocompatible scaffolds for controlled release of growth factors may yield better follicle outcomes than other administration strategies. Nevertheless, not all groups using biocompatible scaffolds achieved an advantage in terms of follicle survival, probably due to differences between scaffold composition and growth factor doses. Moreover, while VEGF and bFGF proved their effectiveness in some models, Ang-2 failed to significantly enhance follicle survival when used alone.

Secondary outcomes

Sixteen out of 18 studies evaluated neovascularization among their outcomes and evidenced a positive impact of growth factors on tissue neoangiogenesis potential and revascularization, namely increased vessel density, greater proangiogenic growth factor expression and/or enhanced vessel function and perfusion. Regarding follicle growth after transplantation, the variability of time points between studies does not allow conclusions to be drawn. However, none of the studies investigated whether boosting revascularization could curb early primordial follicle activation after grafting.

Conversely, in-vitro culture of human ovarian tissue with growth factors (bFGF alone or combined with VEGF) prior to xenotransplantation showed increased follicle proliferation (Kang et al., 2016). This is not surprising, since growth factors like VEGF are known to stimulate early follicle growth by activation of the PI3K/Akt pathway. Moreover, VEGF and bFGF are able to promote follicle development by regulation of a number of cytokines, which exert effects on both granulosa and theca cells (Lu et al., 2015; Nilsson and Skinner, 2004).

Abnormal follicle activation and growth represent an additional risk of large-scale follicle depletion soon after grafting and may be due to the chosen method of delivery. Further investigations are needed to establish whether enhancement of angiogenesis by direct administration of growth factors could have a significant impact on preservation of the ovarian reserve after transplantation, or whether this effect would be lost due to the massive concomitant follicle activation that these factors also promote.

Hormone administration

Six studies were selected (TABLE 2), four of which involved gonadotrophin administration by injection at the time of OTT (Imthurn et al., 2000; Yang et al., 2008), *in vitro* during tissue vitrification (Yang et al., 2015) or after thawing (Wang et al., 2012). All research groups investigating the impact of gonadotrophins used ovarian tissue from multiovulatory species (rodents). The two remaining studies attempted administration of AMH in human xenografting models as a strategy to protect the follicle pool (Detti et al., 2018; Man et al., 2017). The AMH-mediated signalling pathway was shown to play a role in primordial follicle pool preservation in AMH knockout mice (Durlinger et al., 2002), but this was not confirmed in other mono-ovulatory species (Campbell et al., 2012) or humans. However, AMH receptors were detected in human primordial follicles (Cacciottola et al., 2020b; Kristensen et al., 2015),

TABLE 2 HORMONES USED TO IMPROVE FOLLICLE OUTCOMES AFTER OVARIAN TISSUE TRANSPLANTATION

		References	Grafting model	Hormones	Administration route	Time points	Follicle outcomes			Revascularization
							Folliclesurvival	Follicle apoptosis	Folliclegrowth	
Rodents	1	*Imthurn et al., 2000*	Fresh murine OT autografted to kidney capsule or peritoneal pocket	rhFSH (3 IU) + rhLH (3 IU)	Host injection (days −4 to 2)	Day 7	Increase in follicle number with rhFSH + rhLH (days −4 to 0 and days −2 to 2)			
	2	*Wang et al., 2012*	Fresh murine OT autografted to kidney capsule	HMG (0.15, 0.30 and 0.60 IU/ml)	Added to tissue culture before grafting	Days 1, 2 and 28	Increase in follicle count (day 28) with 0.15 IU/ml HMG			Increase in VEGF protein expression in tissue culture before grafting with 0.60 IU/ml HMG
	3	*Yang et al., 2008*	Frozen-thawed rat OT autografted to back muscle surface	PMSG (10 IU)	Host injection (every second day)	Days 2, 7 and 30	No difference in follicle count			No difference in VEGF protein expression
	4	*Yang et al., 2015*	Vitrified-warmed mouse OT autografted to ovaries and kidney capsule	FSH (0.3 IU/ml)	Added to vitrification solution	Day 2	Increase in normal follicle proportion	Decrease in caspase3+ follicles		Increase in *VEGF* and *VEGFR2* gene expression
Humans	5	*Detti et al., 2018*	Vitrified-warmed prepubertal human OT xenografted to nude mice	rhAMH (1.23 µg/day)	Released by an osmotic pump in the peritoneum	Day 7	No difference in primordial follicle density		Decrease in follicle growth (Ki67)	No increase in *VEGF* gene expression
	6	*Man et al., 2017*	Frozen-thawed human OT xenografted to muscle surface of immunodeficient mice	AMH-ECs, AMH-MSCs	Encapsulation in fibrin clot with AMH-ECs or AMH-MSCs	14 days, 14–22 weeks	Increase in relative follicle count (14 days) with AMH-ECs		Increased positivity for ovulatory markers (14–22 weeks)	

AMH-ECs,: endothelial cells expressing anti-Müllerian hormone; AMH-MSCs, mesenchymal stem cells expressing anti-Müllerian hormone; HMG, human menopausal gonadotropin; OT, ovarian tissue; PMSG, pregnant mare serum gonadotropin; rhLH, recombinant human LH; rhAMH: human recombinant anti-Müllerian hormone; rhFSH: recombinant human FSH; VEGF, vascular endothelial growth factor.

TABLE 3 ANTIOXIDANTS USED TO IMPROVE FOLLICLE OUTCOMES AFTER OVARIAN TISSUE TRANSPLANTATION

			References	Grafting model	Antioxidants	Administration route	Time points	Follicle outcomes			Revascularization	Antioxidant effect
								Follicle survival	Follicle apoptosis	Follicle growth		
EPO	Rodents	1	*Kolusari et al., 2018*	Fresh rat OT autografted to abdominal wall	EPO (5000 IU/kg)	Host injection (days 0 and 28)	8 weeks			Increase in antral follicles	Decrease in VEGF protein expression	Increase in CAT and GPX activity. Decrease in SOD and LPO protein expression
		2	*Mahmoodi et al., 2014*	Fresh murine OT autografted to back muscle surface	rhEPO-α (500 IU/kg)	Host injection (days –1 to 7)	Days 7 and 28	Increase in follicle count (day 28)	Decrease in TUNEL+ follicles (day 7)			Decrease in MDA levels (day 7)
	Dogs	3	*Commin et al., 2012*	Frozen-thawed canine OT xenografted to ovaries of SCID mice	EPO (500 IU/kg)	Host injection (day 0–3)	1, 8 and 16 weeks	No difference in follicle count				
		4	*Suzuki et al., 2008*	Frozen-thawed canine OT xenografted to ovaries of	rhEPO (400 IU/kg) Asialo-EPO	Haemostatic gelatin sponge	4 weeks	Increase in follicle survival rates with				
		5	*Wasaka et al., 2017*	Vitrified-warmed canine OT xenografted to kidney capsule of SCID mice	Asialo-EPO (400 IU/kg)	Haemostatic gelatin sponge	4 weeks	Increase in follicle count				
NAC	Rodents	6	*Amorim et al., 2014*	Fresh rat OT autografted to ovaries	NAC (150, 300, 600 and 1200 mg/kg)	Host injection (day 0)	Days 4 and 15	Increase in primordial follicle count (day 14) with 1200 mg/kg NAC				
		7	*Danehpoya et al., 2017*	Fresh rat OT autografted to back muscle surface	NAC (150 mg/kg)	Host injection (days –2 to 7)	Day 28	No difference in follicle count			Increase in vessel density	No difference in MDA concentrations
		8	*Mahmoodi et al., 2015*	Fresh murine OT autografted to gluteal muscle surface	NAC (150 mg/kg per day)	Host injection (days -1 to 7)	Day 28	Increase in follicle count				Decrease in MDA concentrations
		9	*Tuncer et al., 2018*	Fresh autografted rat OT	NAC (150 mg/kg per day) Benfotiamine (70 mg/kg per day)	Host injection (days 1–21)	Day 21	Increase in primordial follcle count	Decrease in TUNEL+ follicles with NAC		Decrease in VEGF protein expression	Decrease in MDA concentrations
	Humans	10	*Olesen et al., 2021*	Frozen-thawed human OT xenografted to subcutaneous pockets in nude mice	NAC (150 mg/kg)	Host injection (days 0–7)	Days 7 and 29	No difference in follicle density	Decrease in caspase3+ follicles		No differences in *VEGF* or *Angptl-4*gene expression (day 7), decrease in vessel density (CD31+) (day 29)	Increase in *CAT*, *HMOX1* and *SOD1* gene expression (day 7)

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(continued on next page)

TABLE 3 *(continued)*

			References	Grafting model	Antioxidants	Administration route	Time points	Follicle outcomes			Revascularization	Antioxidant effect
								Follicle survival	Follicle apoptosis	Follicle growth		
Vitamin E	Rodents	11	*Nugent et al., 1998*	Fresh murine OT autografted to ovaries of SCID mice	Vitamin E (5 mg/day)	Host injections (days 0–7)	Days 3 and 7	Increase in follicle count (day 7)				Decrease in MDA concentrations (day 3)
	Non-human primates	12	*Scalercio et al, 2015*	Fresh autografted non-human primate (squirrel monkey) OT	Trolox (vit E) (50 µmol/l)	Added to tissue culture before grafting	Day 7	No difference in follicle count	No differences in TUNEL+ primordial follicles, decrease in TUNEL+ secondary follicles	No increase in Ki67+ primordial follicles	No increase in vessel density (CD31+)	
Melatonin	Rodents	13	*Hemadi et al., 2009*	Vitrified-warmed autografted newborn murine OT	Melatonin (200 mg/kg per day)	Host injection (days 0–2)	Days 1–2, 3, 7–8, 10–12 and 32	No difference in follicle count	No difference in TUNEL+ follicle count	No difference in growing follicle count		
		14	*Hemadi et al., 2012*	Vitrified-warmed auografted newborn murine OT	Melatonin (20, 50, 100 and 200 mg/kg per day)	Host injection (days 0–32)	Days 1, 8 and 32	Increase in healthy primordial follicle rates with 20 mg/kg per day melatonin	Increase in TUNEL+ follicles with melatonin at 50, 100 and 200 mg/kg per day			
PEG-SOD	Rodents	15	*Kim et al., 2015*	Vitrified-warmed murine OT autografted to kidney capsule	PEG-SOD (100, 1000 and 10,000 IU/ml)	Host injection (every 48 hours)	Days 2, 7and 21	Increase in primordial follicle proportions (days 2 and 7)	No difference in TUNEL+ follicle proportions			
Allopurinol		16	*Abedi et al., 2013*	Fresh mice OT autografted to back muscle surface	Allopurinol (5 mg/kg per day)	Host injection (days 0–7)	Day 21	Increase in follicle count				Decrease in MDA levels with allopurinol + FSH
	Rodents				rhFSH (1 IU/day)	Host injection (days 7– 14)						
					Allopurinol (5 mg/kg per day) + rhFSH (1 IU/day)	Host injection (days 0 – 7 and days 7– 14)						

Q13 Asialo-EPO, desialated erythropoietin; CAT, catalase; EPO, erythropoietin; GPX, glutathione peroxidase; LPO, lipid peroxidation; MDA, malondialdehyde; NAC, *N*-acetylcysteine; OT, ovarian tissue; PEG-SOD, polyethylene glycol-superoxide dismutase; rhEPO, recombinant human erythropoietin; SCID, severe combined immunodeficient; SOD, superoxide dismutase; TUNEL, TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling; VEGF, vascular endothelial growth factor.

suggesting at least partial sensitivity of this follicle subgroup to AMH inhibition of development to the growing stage.

Primary outcome

Three studies investigating gonadotrophins revealed an increase in follicle numbers with host administration around the time of grafting (*Imthurn et al., 2000*) or addition to tissue culture before grafting (*Wang et al., 2012*). They also showed lower proportions of apoptotic follicle 2 days after grafting with addition of gonadotrophin to the vitrification medium (*Yang et al., 2015*). Despite promising results in rodent models, large differences in folliculogenesis, gonadotrophin action and sensitivity (*Barnett et al., 2006*), and revascularization dynamics do not allow meaningful conclusions to be drawn about any potential benefits of gonadotrophin administration for follicle survival after human OTT.

The potential role of AMH post-grafting was investigated by co-transplantation of human ovarian endothelial cells (engineered to constitutively express AMH) (*Man et al., 2017*). This approach, where AMH was released locally in the lumen of newly created vessels, showed increased retention of primordial follicles 2 weeks after OTT. Pump-mediated intraperitoneal infusion of recombinant AMH also had a protective effect on follicle outcomes 7 days after grafting, not by reducing follicle loss, but by mitigating abnormal follicle activation and growth (*Detti et al., 2018*).

Secondary outcomes

Enhancement of neoangiogensis was evaluated in three studies investigating the impact of gonadotrophins by quantification of VEGF at the gene and protein levels in grafted tissue, and was evidenced in two of them. None of the studies analysed neovascularization by assessing vessel density or function.

Both studies on AMH demonstrated an effect of its paracrine function on the follicle pool, but there was no evidence of any significant proangiogenic activity, as proved by an absence of increased VEGF gene expression in the study of Detti and co-workers (*Detti et al., 2018*).

Antioxidant administration

Sixteen studies were selected based on the aforementioned criteria (TABLE 3). Eleven of them were performed on rodent models, three with canine tissue, one in non-human primates and one in human xenografted tissue. In five studies, the potential effect of erythropoietin (EPO) was investigated in the context of OTT. To this end, two research groups developed a rodent model (*Kolusari et al., 2018*; *Mahmoodi et al., 2014*), while the others used canine ovarian tissue (*Commin et al., 2012*; *Suzuki et al., 2008*; Wasaka et al., 2017). This low molecular weight glycoprotein hormone EPO was able to stimulate erythropoiesis and angiogenesis by increasing VEGF expression and recruiting endothelial progenitor cells (*Kimáková et al., 2017*). It also has pleiotropic effects as an antioxidant, antiapoptotic and anti-inflammatory cytokine (*Nairz et al., 2012*).

Five studies evaluated the impact of *N*-acetylcysteine (NAC) on OTT: four in rodents (*Amorim et al., 2014*; *Danehpoya et al., 2017*; *Mahmoodi et al., 2015*; *Tuncer et al., 2018*) and one in humans (*Olesen et al., 2021*). NAC is a precursor of intracellular cysteine and reduced glutathione (*Sun, 2010*), with the ability to scavenge ROS and up-regulate various antioxidants, such as SOD, CAT and GPX (*Zafarullah et al., 2003*).

Another non-enzymatic antioxidant used to improve follicle outcomes in grafted ovarian tissue was vitamin E, tested in mice (*Nugent et al., 1998*) and non-human primates (*Scalercio et al., 2015*). It was also investigated in combination with VEGF in human xenograft models (*Abir et al., 2011*; *Friedman et al., 2012*). Vitamin E contains alpha-tocopherol, with eight recognized and chemically distinct isomers (*Ahsan et al., 2014*). All of these isoforms exert antioxidant action *in vitro*, but less is known about their scavenging ability *in vivo*. Only Scalercio and colleagues used trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (*Scalercio et al., 2015*), which is an analogue of vitamin E with potent scavenger action (*Alberto et al., 2013*).

The potential effects of melatonin, known for its antioxidant and antiapoptotic activity, were also explored in a murine autografting model by another team (*Hemadi et al., 2009*, *Hemadi et al., 2012*) and in human xenografts in combination with both VEGF and vitamin E (*Friedman et al., 2012*).

One research team investigated the possible benefits of using polyethylene glycol-SOD (PEG-SOD) by repeated intraperitoneal injection in an autografting murine model (*Kim et al., 2015*). PEG-SOD is a modified version of SOD, and has shown promising results as a scavenger in in-vivo models, as its half-life of over 30 h is much longer than that of native enzyme forms (*Galiñanes et al., 1992*).

Allopurinol, an inhibitor of xanthine oxidase with antioxidant activity, was also tried on a murine model to protect follicles from oxidative stress (*Abedi et al., 2013*).

Primary outcome

Four out of five studies investigating EPO administration demonstrated an improvement in follicle outcomes after OTT. In two studies, desialylated EPO (asialo-EPO), which exhibited more efficient activity *in vitro* (*Imai et al., 1990*), was administered locally inside a haemostatic gelatin sponge placed next to the grafted tissue, yielding increased follicle survival rates 4 weeks after grafting in canine models (*Suzuki et al., 2008*; *Wakasa et al., 2017*). In the other three studies, EPO was delivered by intraperitoneal injection repeated at different time points. In the study by Commin and colleagues daily injections given for the first 3 days post-transplantation were not able to prevent follicle loss in canine tissue. Indeed, the proangiogenic effects of EPO seem to be insufficient to counteract ischaemia-related follicle loss, but EPO treatment does appear to enhance follicle morphology and development, as observed after 8 and 16 weeks (*Commin et al., 2012*). In the study of Mahmoodi and co-workers EPO injection was repeated after 7 days in a murine model and better follicle outcomes were obtained up to 4 weeks post-grafting (*Mahmoodi et al., 2014*). Kolusari and colleagues administered a second EPO injection after 4 weeks and saw an increase in murine antral follicles after 8 weeks (*Kolusari et al., 2018*).

NAC was administered systemically by injection for up to 9 days after OTT. Two of the three studies in rodents showed higher follicle survival rates at 15 and 28 days post-transplantation. Investigations into the impact of NAC in human xenografts did not reveal any clear positive effect on follicle outcomes because, while suggesting a decrease in apoptosis, they did not result in a

significant upturn in the follicle count after grafting (*Olesen et al, 2021*). The same was true of vitamin E. Indeed, although an increased follicle count was detected in a murine model (*Nugent et al., 1998*), experiments using non-human primate models (*Scalercio et al., 2015*) and human xenografts (*Abir et al., 2011*; *Friedman et al., 2012*) did not demonstrate any significant protection of the follicle pool after OTT.

Melatonin was tested in rodents, either by administration to the host for a month, yielding an increase in follicle survival (*Hemadi et al., 2012*), or by in-vitro ovarian tissue pretreatment, which made no difference to follicle outcomes (*Hemadi et al., 2009*). This research group highlighted potentially negative effects, including increased follicle apoptosis, with extremely high melatonin doses (200 mg/kg). Further studies need to be conducted to establish the optimal dose and mode of administration of this drug, in order to reap the benefits of its antioxidant properties in the context of OTT.

Both allopurinol and PEG-SOD proved effective in protecting the follicle pool in a murine model after 21 days of grafting. However, despite encouraging findings in rodents, the small number of studies performed with human ovarian tissue and lack of conclusive evidence on follicle outcomes leave questions on the protective role of antioxidants on the follicle pool after OTT still wide open.

Secondary outcomes

Investigations into EPO suggest a late impact of this hormone on ovarian tissue quality (as beneficial effects were seen with both repeated administration and use of a haemostatic gelatin sponge for controlled delivery), which may be explained by its antioxidant and anti-inflammatory activity. In both studies using rodent models, lower levels of oxidative stress-related damage were detected by targeting malondialdehyde concentrations (*Mahmoodi et al., 2014*), and greater expression of scavengers (*Kolusari et al., 2018*) was noted. As oxidative stress occurs after revascularization, and hence later than massive ischaemia-related follicle loss, and persists throughout all vasculature remodelling (*Cacciottola et al., 2018*), there may be a protective effect of EPO on follicles surviving the hypoxic period in terms of their further development.

When assessing oxidative stress modulation by the use of NAC, 2 out of 3 studies detected a decrease in malondialdehyde concentrations (*Mahmoodi et al., 2015*; *Tuncer et al., 2018*), while one showed an increase in antioxidant activity by enhanced action of scavengers like CAT and SOD1 (*Olesen et al, 2021*). Looking at potential impacts on neovascularization, some investigations into NAC revealed findings to the contrary, namely a fall in VEGF expression (*Tuncer et al., 2018*) and a drop in vessel density (*Olesen et al, 2021*). A possible explanation could be that the anti-inflammatory and antioxidant effects of NAC may diminish the tissue response to neoangiogenesis by decreasing the expression of proangiogenic growth factors like VEGF (*Licks et al., 2015*; *Moazzen et al., 2014*). Indeed, NAC was shown to decrease such expression in a dose-dependent manner in in-vitro ovarian tissue culture (*Olesen et al, 2021*). Moreover, ROS themselves might act as signalling factors to promote neovascularization by increasing expression of growth factors such as VEGF (*Kim and Byzova, 2014*; *Oshikawa et al., 2012*), and production of lactates as by-products of anaerobic metabolism occurring during hypoxia, which have proangiogenic effects (*Morland et al., 2017*; *Porporato et al., 2012*). Negative modulation of neovascularization sustained by NAC antioxidant activity may have a double impact on the ovarian reserve, protecting part of the follicle pool from oxidative stress, but not safeguarding it from hypoxia-related follicle depletion occurring early after grafting.

Other strategies to reduce hypoxic damage after transplantation

The cascade of events driven by ischaemia in tissue is extremely complex and involves various players. Activation of signalling pathways in response to hypoxia and oxidative stress not only allows cells to adapt to this stress, changing cell metabolism and promoting survival, but also triggers many subsequent tissue events, such as inflammation, angiogenesis and tissue remodelling, which seek to overcome tissue injury. Moreover, cell death mechanisms such as apoptosis participate in the cascade of events to select cells able to recover from damage for better healing of the tissue itself.

Following this principle, sphingosine-1-phosphate (S1P), a lysophospholipid acting as a potent signalling molecule with roles in many processes including angiogenesis, immunomodulation, cell survival and growth (*Chun et al., 2020*), was found to enhance human ovarian neoangiogenesis after transplantation (*Soleimani et al., 2011*). Follicle survival also increased 10 days after grafting thanks to a decrease in follicle apoptosis. This latter effect was observed in other studies developing strategies to protect the follicle pool from chemotherapy-mediated damage (*Li et al., 2014*). However, such antiapoptotic impacts on human follicles were found to be associated with follicle activation and growth *in vitro* (*Guzel et al., 2018*; *Pors et al., 2020*). For this reason, further studies need to be conducted to assess the long-term effect of S1P on the follicle pool, whose maintained quiescence is crucial to ovarian graft function and longevity.

Other research groups developed strategies to shorten the hypoxic period using a more holistic approach. Israely and collaborators observed a drop in primordial follicles when transplanting ovarian cortex into wound-healing granulation tissue in a murine autografting model (*Israely et al., 2006*). Angiogenesis is already accelerated in granulation tissue, which is rich in growth factors and mobilized endothelial cells, so in the presence of a previously triggered endogenous process of neoangiogenesis, inflammation and tissue remodelling to reduce tissue damage, ovarian tissue could benefit in terms of its own survival thanks to faster vessel reconnection. Further studies subsequently confirmed the advantage of implanting ovarian tissue in a previously activated grafting site. Indeed, transplantation to a freshly decorticated ovarian site showed greater revascularization and better tissue remodelling than the standard procedure in a non-human primate model (*Dolmans et al., 2015*).

In recent years, attempts have been made to improve OTT outcomes using mesenchymal stem cells (*Shojafar et al., 2018*; *Xia et al., 2015*), which are known to have beneficial effects when applied to wound-healing models investigating anti-inflammatory and proangiogenic behaviour (*Au et al., 2008*; *Moon et al., 2006*). A transplantation strategy was developed to enhance follicle outcomes using adipose tissue-derived stem cells (ASCs) to prepare the implantation site

prior to grafting. The potential of ASCs to boost angiogenesis and vascularization could then be activated by their transplantation inside a fibrin scaffold (*Manavella et al., 2018a*, *2019*). This approach was able to reduce follicle loss soon after transplantation by decreasing both follicle death and abnormal primordial follicle activation (*Cacciottola et al., 2020a*; *Manavella et al., 2018b*), and was sustained in the long term (*Cacciottola et al., 2020b*).

All these findings lend support to the idea that positive modulation of ischaemia and hypoxia in the context of OTT can be achieved by evaluation of and response to its complexity.

CONCLUSIONS

Follicle development and oocyte maturation depend on the ability of all cell compartments in ovarian tissue to adapt and evolve in hypoxic conditions, and modulate their own metabolism in order to maintain a balance in ROS generation. These endogenous abilities are crucial to good ovarian function, namely preservation of the ovarian reserve throughout the entire fertile period in women, along with active folliculogenesis that results in follicle development up to ovulation of mature oocytes.

After OTT, the ovarian cortex is subjected to a series of stressful events that cause large-scale follicle loss. The avascular nature of transplantation appears to be the main stress inducer, as it leads to prolonged hypoxia and only gradual revascularization after grafting. A number of authors have developed experimental xenotransplantation models in attempts to improve follicle outcomes by boosting revascularization with growth factors or hormones, or decreasing oxidative stress-related damage with antioxidants. Although such approaches suggest a potentially positive impact on follicle survival after grafting, the findings have not identified a clear advantage of one technique over another. Indeed, the complexity of events resulting in tissue healing after transplantation-related ischaemia does appear to be slightly altered by specific intervention with single molecules. This is why strategies addressing this complexity using a previously triggered endogenous response to ischaemia have yielded such promising results in terms of follicle survival after transplantation.

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