Chapter

Advances in Ischemia Reperfusion Injury Prevention in Free Flaps and Vascularized Composite Allotransplantation

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Abstract

In Plastic and Reconstructive Surgery, ischemia reperfusion injury (IRI) prevention is of utmost importance in free flaps and vascularized composite allotransplantation (VCA) to continue increasing accessibility to these advanced reconstructive options. At present, free flaps and VCA undergo irreversible ischemic damage at 3 hours due to the highly metabolic nature of skeletal muscle, and static cold storage (SCS) can only extend this to 4–6 hours. It is important to understand that one of the major challenges with transplanting composite tissues is that each tissue has a unique tolerance and mechanism to ischemia-reperfusion. Research targeting attenuation of IRI can be subdivided into 3 time periods: the pre-ischemic, ischemic, and post-ischemic. In the pre-ischemic period, there are conditioning methods, the delay phenomenon, which is already used clinically, pharmacologic, and stem cell strategies. In the ischemic period, SCS is used clinically, whilst other preservation methods including cryopreservation, vitrification, machine perfusion, and pharmacologic strategies are being studied. Lastly, in the post-ischemic period, our greatest clinical tool is close post-operative monitoring, however conditioning methods, and pharmacologic strategies have been studied. This chapter covers IRI in tissues implicated in free flaps and VCA, and several prevention strategies either currently in use or in pre-clinical studies.

Keywords: reconstruction, free flaps, vascularized composite allotransplantation, ischemia reperfusion injury, static cold storage, pre-ischemic period, post-ischemic period, preservation strategies

1. Introduction

In Plastic and Reconstructive surgery, the focus of ischemia reperfusion injury (IRI) prevention research is in free flaps and vascularized composite allotransplantation (VCA). Free flaps and VCA are at the highest end of reconstructive complexity options available to patients with significant tissue defects. Both free flaps and VCA require the division of blood supply to transfer or transplant the tissue; survival

is dependent on prompt revascularization at the recipient site to minimize total ischemia time. Clinically, we currently have limited options for targeting the pre-ischemic, ischemic, and post-ischemic periods to attenuate IRI. The delay phenomenon is the best described and most validated preconditioning method (see Section 3.1.2), static cold storage (SCS) remains the primary clinical intervention during the ischemic period (see Section 3.2.2), and the mainstay after microvascular anastomosis is close monitoring for microvascular complications (see Section 3.3.2). This chapter will describe several experimental methods and areas of advancement that are expected to be seen in clinical trials and use in coming years.

It is notable that the majority of cutting-edge research in the field is focused on the ischemic period, as simple SCS can no longer meet the demands of preserving tissue as the field advances. Solid organs can withstand varying times in SCS: lungs are considered acceptable for 6–8 hours [1], heart for 4–6 hours [2], kidneys for up to 24 hours but ideally less than 12 [3], and liver for 8–12 hours [4]. Similarly to cardiac muscle, free flaps and VCA include transplantation of highly metabolic tissues which limits ischemia time with no interventions to 3 hours before irreversible ischemic damage takes place. Permissible ischemia time can only be extended to 4–6 hours with the use of SCS which has historically been and continues to be the gold standard for storage. Due to the highly metabolic nature of the tissues, free flaps and VCA are particularly susceptible to IRI, and thus significant advancements in prevention of IRI are targeted at reducing ischemia through various preservation methods described in this chapter.

1.1 Fundamentals of flaps and VCA

A flap is a unit of tissue wherein the blood supply has been maintained. Flaps are transferred from donor to recipient sites, which may be close or distant in proximity [5]. Depending on the indication, flaps containing different tissue types, vascular configurations, or different conformations can be procured. Exploration of donor sites and vascular patterns have led to the categorization of flaps into various subtypes, which are classified based on circulation (blood supply), constituents (composition), contiguity (destination), construction (blood flow), conformation (geometry), and/or conditioning (preparation) of the tissue [6]. The term free flap is used to describe a unit of tissue whose blood supply has been temporarily detached at the pedicle. The free flap can then be anastomosed to a new blood supply at the recipient site to fill and/or cover defects [5]. Thus, free flap transfer is both subject to and limited by an obligatory ischemia time—with ischemic insult between division of the pedicle at the time of flap elevation and eventual microvascular anastomosis. Although heavily criticized at first, technical advances and perfection of this technique has rendered free flap transfer a highly reliable option for reconstructive surgery [7], with reported success rates of 93–98.8% [8]. Free flap transfer is successful when adequate blood supply has been re-established, ischemia time is minimized, and vascular complications such as thrombosis are thwarted [8]. As alluded to, flaps can have varying composition where constituents have different capacities to withstand ischemia.

Skin flaps which are composed of just skin, subcutaneous fat, and superficial fascia are often used to cover wound defects where skin edges cannot be approximated together tension-free, or where there is inadequate vascular supply for a skin graft to survive [9]. Muscle flaps are most often used to fill large oncologic or traumatic defects as they are well vascularized, and if used with overlying skin, a myocutaneous flap, may obliviate the need for an overlying skin graft [10]. Fascial flaps are often used when a

vascular flap is required, but with minimal tissue bulk for good cosmetic outcome [11]. Fasciocutaneous flaps contain skin, subcutaneous fat, and deep fascia, are thin like fascial flaps, but remove need for overlying skin graft [12]. Bone and osteocutaneous flaps are most often used for reconstruction of large bony defects, such as in craniofacial reconstruction [13]. With each different composition, different considerations regarding IRI may play a role and affect viability of the tissue being transferred.

VCA is the most complex and most novel reconstructive option for people suffering from significant tissue loss which offers functional recovery. Unlike solid organ transplantation, VCA refers to the procurement and transplantation of multiple tissues as a functional unit, including skin, nerves, muscle, bone, tendons, ligaments, adipose tissue, and vasculature. Clinically, VCA applications include limb, face, larynx, penile, uterine, and abdominal wall transplants. Since the development of VCA in 1998, the most common form of VCA performed has been hand and upper extremity transplant with 148 total reported between 1998 and 2022 [14]. From 2005 to 2020, 48 face transplants have been described world-wide [15], and since 2005 5 penile transplants have been performed [16]. As well, 42 uterine transplants, commonly performed as male-to-female gender affirming surgery, have been performed with at least 12 live births [17]. Despite significant advances in the field, VCA failure rates continue to be high, and it is thus not a routinely pursued reconstructive option if other options are available. This highlights the need for continued research and innovation in the field [18, 19].

2. Mechanisms of ischemia reperfusion injury

In ischemia, the loss of oxygenated blood flow results in a mandatory shift from aerobic to anaerobic respiration in all tissue types [20]. At the mitochondrial level, oxygen is required to accept electrons in the electron transport chain (ETC), and in hypoxic conditions, the ability to produce ATP through the ETC is therefore lost [20]. Cells become reliant on glycolysis which can produce 2 ATP per glucose molecule [20, 21]. Glycolysis is dependent on the presence of the coenzyme NAD+ which can be regenerated by lactate dehydrogenase (LDH) catalyzing the reaction of pyruvate to lactate, which simultaneously converts NAD+ to NADH [22]. Notably, as lactate builds up and causes tissue acidosis, this inhibits ATP production through a negative feedback loop [23]. Ultimately, cytosolic ATP becomes depleted through maintenance of membrane potentials, and there is a build-up of intracellular sodium and calcium that draws water into the cells causing swelling [20, 21]. Increased intracellular calcium also activates calpain, a protease which converts xanthine dehydrogenase (XDH) to XO [24]. Phospholipases disrupt cellular membranes, leading to lipid peroxidation and increased circulating fatty acids [23].

Upon reperfusion, XO produces uric acid and superoxide (O_2^-) by degrading hypoxanthine [23]. O_2^- can then be converted to hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) which promotes further lipid peroxidation [23]. Lipid peroxidation releases arachidonic acid which is a primary substrate for production of prostaglandins [23]. In ischemia, prostaglandins typically cause vasodilation to increase blood flow, but in their absence, there is unopposed vasoconstriction leading to worsened ischemia [23]. Uric acid is a damage-associated molecular pattern (DAMP) which bind to inflammasomes and cause a cytokine storm in IRI, recruit neutrophils, and increased effector T-cells. This series of events following IRI demonstrates the role IRI may have in acute and chronic rejection. Lastly, reperfusion normalizes pH in the

extracellular space and removes ions, leaving high intracellular osmolarity. Further water is drawn into cells, leading to further swelling and possible membrane rupture. Mitochondria, which incurred damage during the ischemic phase continue to produce ROS which cannot be eliminated due to antioxidant depletion (**Figures 1** and **2**).

In brief, at the onset of ischemia in the myocardium, reactive oxygen species (ROS) accumulate rapidly from various sources, including the mitochondrial electron transport chain (ETC) and oxidation of ferrous heme (Fe^{2+}) to ferric heme (Fe^{3+}) both of which result in the production of O_2^- [26]. Reperfusion causes additional ROS production by way of xanthine oxidase (XO) [20]. Following ischemia-reperfusion, neutrophils release toxic oxidants, leading to further damage of the myocardium [27]. Mitochondrial permeability transition pore (mPTP) opens in response to elevated ROS, increasing permeability of the membrane, and through various mechanisms leading to calcium overload, apoptosis, and necrosis of cells [28, 29]. It is believed that for this reason, although ischemic necrosis leads to significant cell death, reperfusion may lead to an additional 25–40% cell death in the myocardium [30].

The following sections will discuss differences in IRI in various tissues contained within free flaps and VCA, highlighting differences in mechanism, if applicable.

2.1 IRI in skin and subcutaneous tissue

2.1.1 Tolerance of ischemia in skin and subcutaneous tissue

IRI in the skin has been studied in the context of both VCA and flap surgery. It has been found that intracellular pH changes related to anoxia are reversible up to

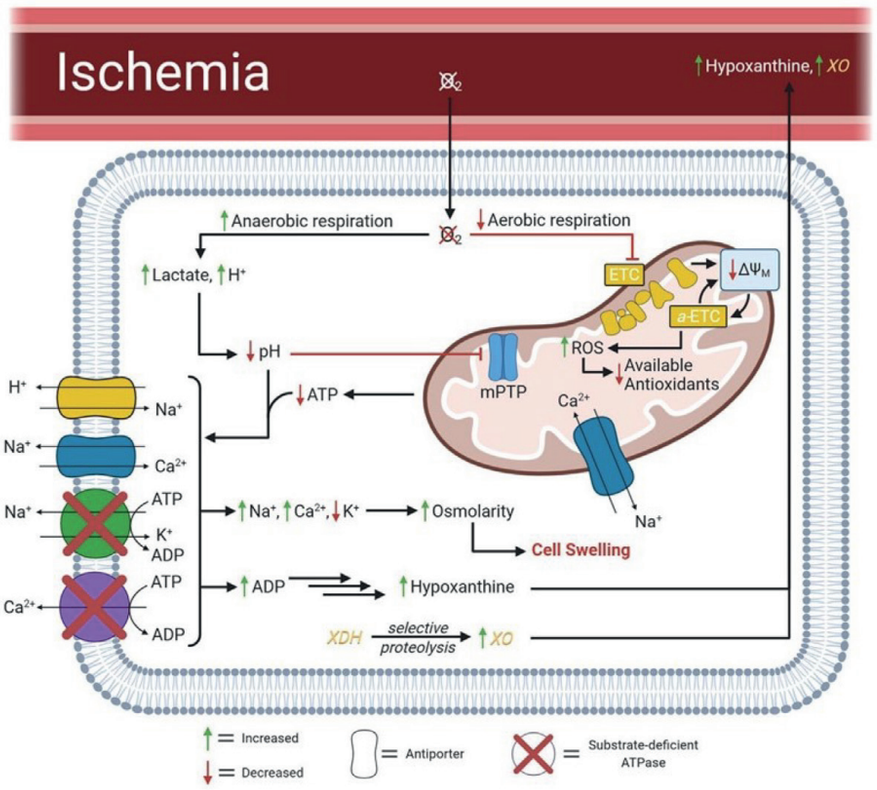

Figure 1.
Pathophysiology of ischemic injury [25].

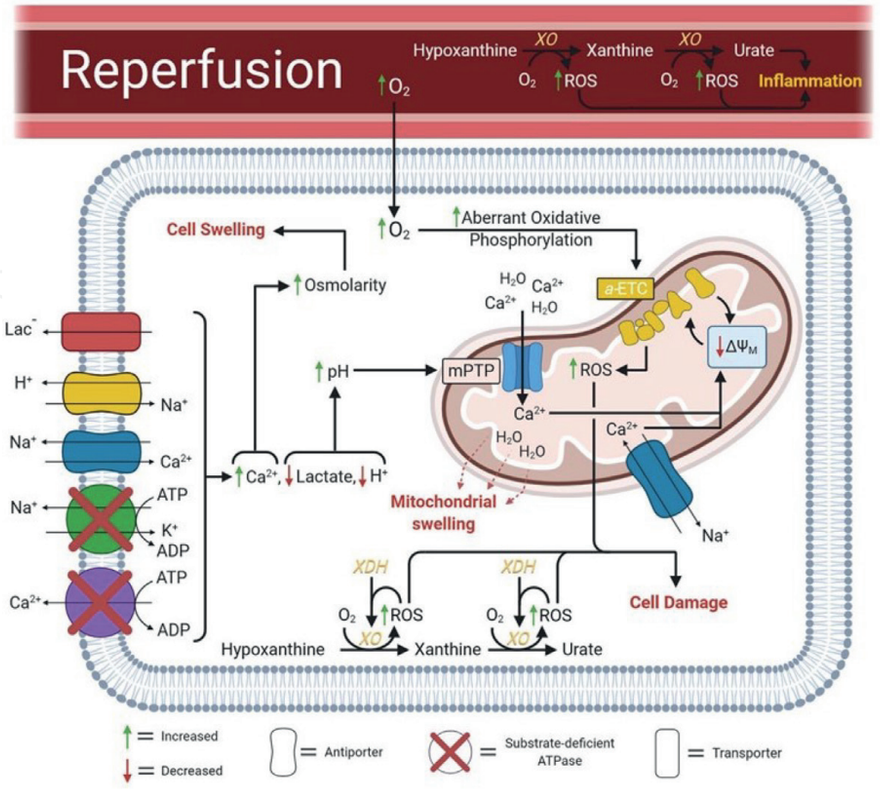

Figure 2.
Pathophysiology of reperfusion injury [25].

24 hours in both skin and subcutaneous tissue [31]. Notably, the effects of anoxia are worsened in warm and mitigated in cold environments [32]—several studies have cited that cooling flaps results in enhanced survival up to 48 hours [33]. In the setting of VCA, ischemic damage to the skin is negligible if warm or cold ischemia time is kept below 6 or 24 hours, respectively. Concern for IRI in skin and subcutaneous tissues is warranted beyond these time points.

2.1.2 IRI mechanistic considerations in skin and subcutaneous tissue

Ballestin, *et al.* showed that rat skin flaps which underwent ischemia-reperfusion (IR) had significantly higher inflammatory cell infiltration and increased necrosis [34]. They did not find significant changes in Th1/Th2 cytokine levels (i.e., IL-2, IL-4, IFN-y), but they did find overexpression of Arginase 1 which is released by M2 macrophages and shifts arginine metabolism to ornithine and urea [34]. This shift in metabolism is also known to happen in cardiac and renal IRI [18, 19]. Wang, *et al.* showed that like the myocardium, Drp1 mediates mitochondrial fission involved in IRI progression and that inhibiting Drp1 improves skin flap function [35, 36].

2.2 IRI in skeletal muscle

2.2.1 Tolerance of ischemia in skeletal muscle

It is widely appreciated that irreversible ischemic damage of skeletal muscle is 3 hours with no preservative intervention. When comparing rat muscle and skin

flaps in cold (4°C) storage conditions, Wagh, *et al.* reported the critical ischemia time of muscle (16 hours) to be less than one third that of skin (3.5 days) [37]. This stark difference is sensible, as skeletal muscle is more metabolically active and will thus expend its energy stores more quickly [7]. Lactate production in skeletal muscle has been shown to occur continuously for up to 6 hours due to anaerobic respiration until it can no longer produce ATP to sustain the tissue [38]. Critical ischemia times in VCA models have yet to be sufficiently studied. Some articles suggest that muscle constituents have a warm critical ischemia time of less than 2 hours, which can be extended up to 8 hours when cooled [39]. The rapid susceptibility of muscle to ischemic insult implies that the ischemia time of VCA replants is largely dictated by skeletal muscle.

2.2.2 IRI considerations in different muscle fibres

IRI has also been shown to have differential effects in different muscle fibre types. In general, it has been found that Type II fibres exhibit more damage and necrosis than Type I fibres after IR—with Type II fast-twitch fibres sustaining the most damage to the mitochondria, sarcoplasmic reticulum, and myofibrils [40]. Type II fast-twitch fibres also demonstrated delayed recovery of function after IR compared to slow twitch fibres, indicating either more profound ischemic damage or a slower course of repair [41]. Hence, the predominant muscle fibre type in each free flap or composite tissue should be considered when estimating critical ischemia time.

2.2.3 Mechanistic considerations in skeletal muscle

The molecular mechanism of IRI in skeletal muscle is quite similar to the myocardium, though where XO is the primary source of ROS in cardiac muscle, NADPH oxidase (NOX) is located in sarcoplasmic reticulum is the most significant source of ROS in skeletal muscle (though both muscle types use both enzymes) [30]. Membrane disruption due to lipid peroxidation is quite pronounced in skeletal muscle as compared to the myocardium [40]. Neutrophils are recruited and release toxic oxidants, leading to changes to permeability of capillaries, increased interstitial fluid pressure and ultimately capillary compression [30]. An additional component of injury in the muscle is mediated by myeloperoxidase from neutrophils, an enzyme which converts hydrogen peroxide (H_2O_2) and chloride into hypochlorous acid (HOCl) which damage myocyte membranes and further peroxidation of lipids [42]. As a result, the muscle cannot get nutrients and is being directly attacked by enzymes. Another subtle difference includes that the subsarcolemmal mitochondria (SSM) are more sensitive to ischemia than interfibrillar mitochondria (IFM) of the myocardium because SSM release cytochrome c more readily in response to elevated calcium levels than IFM, leading to apoptosis of myocytes [43].

2.2.4 Clinical scenarios of IRI in skeletal muscle

Clinically, IRI in skeletal muscle can also be observed in acute compartment syndrome; intracompartmental pressure increases sufficiently to collapse capillaries and ultimately leads to cessation of tissue perfusion [40]. It can also be seen in acute limb ischemia where arterial blood flow is acutely interrupted to a limb. Both acute compartment syndrome and acute limb ischemia scenarios are surgical emergencies.

2.3 IRI in peripheral nerves

2.3.1 Tolerance of ischemia in peripheral nerves

Although not as well studied as some other tissue types, IRI in the peripheral nerve is an important consideration in tissue transplantation. Ischemia induced degeneration of nerve fibers has been reported in several studies—suggesting that reperfusion may result in microvascular or oxidative damage following anoxia [44, 45]. The critical ischemic time for nerve tissue at normal temperature is approximately 8 hours [40]. Iida, *et al.* also report that extended periods of reperfusion (42 days) permitted nerve fibre regeneration [44]. Studies have shown that early inhibition of inducible nitrous oxide synthase exhibited protection/reduction of IRI in the nerve [46].

2.3.2 Mechanistic considerations in peripheral nerves

In mild-moderate ischemia, restoration of blood flow can restore nerve action potentials rapidly [47]. It is hypothesized that the likely mechanism in ischemic fibre degeneration is due to oxidative stress and that in severe ischemia, there is a breakdown of the blood-nerve barrier, also mediated by xanthine oxidase production of hypoxanthine [47]. Nagamatsu, *et al.* showed a large increase in lipid hydroperoxides as well as blood-nerve barrier breakdown with ischemia, and therefore significant endoneurial edema [47]. In reperfusion, they showed worsening edema in the nerve [47]. Nagamatsu, *et al.* suggest that clinically, a bulk ischemia time during surgery (i.e., use of a tourniquet for an entire procedure) is more harmful than if there is periodic reperfusion at regular intervals throughout the procedure [47].

2.4 IRI in bone

2.4.1 Tolerance of ischemia in bone

In VCA, bone constituents may display relative resistance to ischemia. Still, increasing ischemia times result in appreciable changes to bone composition, ultrastructure, mechanical properties, and cellularity [48]. Messner, *et al.* note that on the 10th day following transplantation (cold ischemia = 10 hours), bones subject to ischemia were more brittle compared to controls in the rat model [48]. In addition, they note the formation of a lighter boney layer containing blood vessels and trabeculae superficial to the cortices of bones—the thickness of which was proportional to cold ischemia time [48]. Loss of osteocytes in the lacunar network and changes to the ultrastructure of the bone marrow were also observed 10 days following ischemia [48], though more precise analysis with electron microscopy revealed that osteocytes are irreversibly damaged within 4 hours, and lacunae were near-completely devoid of viable osteocytes within 24 hours [49]. Weiss, *et al.* conducted histomorphometric analysis of bone grafts after 4 and 8 hours of warm ischemia time revealed similar findings, with a drastic decrease in percentage of osteocyte-occupied lacunae [50]. Although osteocytes have been the subject of the aforementioned studies, all bone cell types, such as osteoblasts, osteoclasts, chondrocytes, and bone marrow cells are vulnerable to IRI [51].

Like other tissues, bone IRI is mitigated at decreased temperatures. It was found that after just 3–7 hours of ischemia at 37°C, growth and/or development of bone was effectively stunted [52]. Indeed, another study found that bones subject to just

6 hours of warm ischemia exhibited central areas of disorganization and complete destruction of the growth plate [53]. Decreasing temperatures to 0–5°C, however, can increase the critical ischemia time of bone to over 24 hours [52]. Albeit studies suggest that non-lethal changes can accrue in bone tissue at significantly shorter durations of ischemia [51].

2.4.2 Mechanistic considerations in bone

Information regarding mechanism of IRI in bone has not been extensively studied. It is notable that in contrast to other tissue types and organ ischemia studies, anoxia appears to readily induce bone cell apoptosis in the ischemic phase—rather than necrosis [51, 54]. No bone cell type appears to be particularly sensitive to ischemia compared to the others [51].

2.4.3 Clinical scenarios of IRI in bone

Bone IRI may occur in a variety of clinical circumstances, including bone compression, fractures, transplantation, thromboembolic events, and of course, vascular disruption [39]. Systemic diseases, such as Cushing's Disease and sickle cell anemia, may also result in IRI [55, 56].

2.5 IRI in vasculature

2.5.1 Tolerance of ischemia in vasculature

It is plausible to assume that any condition or event resulting in compromised blood flow will lead to ischemia of distal vessels and structures. The endothelium is particularly sensitive to IRI. Functions of the endothelium include controlling vascular tone and blood flow, participating in coagulation and inflammatory cascades, dictating the permeability of vessels to various macro- and micromolecules, forming new blood vessels, and facilitating immune response pathways [57]. As such, the endothelium is of utmost importance to preserve.

2.5.2 Mechanistic considerations in vasculature

Upon exposure to ischemia, the capacity for endothelium to facilitate vasodilation is particularly compromised [58]. This is a hallmark sign of arteriolar endothelial dysfunction following IRI and is at least in part due to decreased bioavailability of nitrous oxide, which decreases substantially during the reperfusion period [59]. Another potential mechanism for impaired vasodilation includes reduced shear forces or reduced endothelial nitrous oxide synthase activity caused by pH-dependent protein denaturation/proteolysis [59]. Impaired arteriole vasodilation as a result of IRI has been observed throughout the vasculature with varying severities [59]. While animal studies have shown that the renal vasculature is somewhat resilient to ischemia, cerebral vasculature shows signs of endothelial dysfunction even after minor insult [59].

When perfusion is re-established, free radicals are abundantly produced and complement proteins and WBCs are activated. In addition to producing ROS which exacerbate impairment of endothelium-dependent vasodilation, the inflammatory cascade results in activation of the endothelium itself [59, 60]. This leads to marked cell swelling, hypercoagulability, immune cell infiltration, and extravasation of fluid/

proteases into the interstitium [59, 60]. While some posit that cell volume increases in response to disrupted membranes and/or ion channel dysregulation, others suggest that endothelial cell swelling is a direct result of increased cell packing from vasoconstriction [61]. Albeit less relevant in the context of IRI-related arteriolar dysfunction, leukocyte recruitment has also been shown to play a role at the arterial level. Neutrophils localize and become activated in response to enhanced adhesion molecule expression in arterioles, providing an additional source of ROS and resulting in injury upon reperfusion [59].

IRI can also be observed at the level of the capillary. Ischemic injury at this level is characterized by a decrease in the proportion of perfused capillaries and an increase in fluid filtration—a central mechanism of which has not been agreed upon in the literature. Decreased capillary perfusion can be attributed to a combination of 1) vessel congestion caused by platelet and leukocyte recruitment (leukocyte plugging), and 2) narrowing and compression of the vessels caused by barrier dysfunction-related interstitial edema [62]. It is hypothesized that ischemia-related barrier dysfunction is caused by decreased ATP stores and increased ROS production, wherein the endothelial cytoskeleton is interrupted, intercellular adhesion molecules are internalized, intercellular junctions are compromised [63], and finally, vessel permeability increases [64–66]. Barrier dysfunction may also be related to the recruitment and subsequent transendothelial migration of leukocytes, namely neutrophils [67]. The overall result of leukocyte plugging and vessel compression is significantly increased capillary bed resistance, even in the circumstance of restored systemic pressure.

Leukocyte activity in response to ischemic insult is the prominent mechanism of venule dysfunction in vascular IRI. Decreased oxygen in the setting of ischemia results in upregulated expression of adhesion molecules such as P-selectin or ICAM-1 in endothelial cells, promoting localization and activation of neutrophils, which can lead to increased vascular permeability as discussed above [68, 69]. Leukocyte-mediated damage can also be caused indirectly by way of interaction with other blood cells, such as platelets [70].

3. Strategies to combat IRI in flaps and VCA

Strategies to combat IRI in plastic surgery involve efforts that can be applied prior to ischemia (pre-ischemic period), during ischemia (ischemic period) and reperfusion (post-ischemic period). Some strategies are well developed and integrated into clinical practice, whilst others are still experimental and have not been translated into clinical practice (**Figure 3**).

3.1 Pre-ischemic period

3.1.1 Local and remote ischemic preconditioning

In 1986, Murry, *et al.* showed that inducing multiple brief periods of ischemia by clamping coronary arteries before a sustained ischemic insult reduces infarct size in a canine model [71]. This principle was first tested in skeletal muscle by Mounsey, *et al.* in latissimus dorsi flaps of pigs [72, 73]. In their initial study, a cycle of 30 minutes of pedicle clamping and 30 minutes of reperfusion prior to 4 hours of warm ischemia and 48 hour reperfusion resulted in 20% increased survival when compared to their warm ischemia-only control group [72, 73]. Pang, *et al.* also showed that three cycles

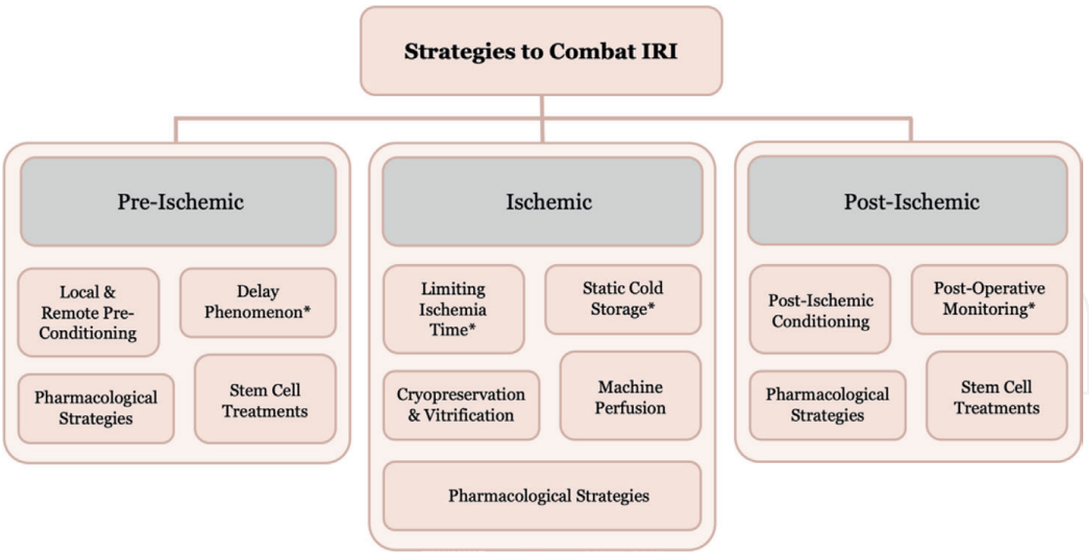

Figure 3.
*Overview of strategies to combat IRI in free flaps and VCA. *Indicates current use in clinical settings.*

of 10 minute ischemia/10 minute reperfusion significantly reduced IRI damage in both latissimus dorsi and gracilis muscles when conducted before the 4 hour sustained ischemia protocol [74]. This principle is called local ischemic preconditioning, and the potential mechanism was elucidated by Pang, *et al.* using adenosine receptor blockers and ATP-sensitive K channel blockers (see in Section 3.1.3, Pharmacological strategies) [74]. Despite the reduction of IRI, local ischemic preconditioning is impractical in clinical conditions because (1) it increases operative time, (2) each clamp cycle carries risk of damaging the pedicle, and (3) the workflow of a free flap procedure is generally already set-up in a way that the ischemia time is limited to time needed to perform anastomoses.

Oxman, *et al.* showed that inducing ischemia on a remote site can also reduce IRI at a distant organ [75]. This principle is called remote ischemic preconditioning. Oxman, *et al.* used a rat model to show that 10 minutes of limb ischemia through tourniquet application could precondition the myocardium against reperfusion injury [75]. Following success in this initial study, similar investigations were performed on skeletal muscle flaps. Addison, *et al.* applied three cycles of 10 minutes IR by applying a tourniquet to the hindlimbs of pigs and testing whether IRI was mitigated on the gracilis, rectus abdominis, and latissimus dorsi after 4 hours of ischemia and 48 hours of reperfusion [76]. Addison, *et al.* showed similar results to what was achieved with local ischemic preconditioning, however non-specific blockage of adenosine receptors did not reduce effects of preconditioning, though could be reversed by naloxone [76]. Furthermore, Moses, *et al.* demonstrated that inhibition of mitochondrial ATP-sensitive K channels resulted in abolition of effects of remote conditioning [77]. Moreover, opening these channels without remote preconditioning showed protective effects [77]. Interestingly, Kolbenschlag, *et al.* used remote ischemic preconditioning on both free and pedicled flap patients on post-operative day 1, 5, and 12 to assess oxygen saturation and flow to the flap after intervention [78]. Significant improvements were seen in free flaps, however effects were insignificant in pedicled flaps [78]. Although this method had shown effectiveness in experimental settings, a recent clinical trial of this technique by Krag, *et al.* in head and neck free flap reconstructions showed no significant effect on the complications and overall survival of the flap [79].

3.1.2 Delay phenomenon

Currently, the most noteworthy strategy in the pre-ischemic period is augmenting viability by surgical or vascular delay. The delay phenomenon is an experimentally and clinically proven form of preconditioning, also known as pre-injury conditioning, achieved by inflicting sublethal ischemia that causes permanent vascular changes in the flap [80]. This can be achieved by (1) surgically incising the flap boundaries without undermining, and/or by (2) dividing other source arteries or perforators that also nourish the intended flap area (vascular delay) 2 weeks before the definitive surgery [81]. Vascular delay results in two main events within the flap: a hypoxic state caused by reduced blood supply, and a hyperadrenergic state caused by severed sympathetic nerve endings [82]. This cascade of events results in hyperplasia, enlargement and re-orientation of the existing choke vessels that link individual skin perforator territories [83, 84]. It also drives angiogenesis within the flap via recruited progenitor cells with the effect of growth factors such as FGF and VEGF [82, 85, 86]. The HIF1a-VEGF (hypoxia) axis plays a central role in the delay phenomenon. An experimental study in rat skin flaps by Jiang, *et al.* showed that application of VEGFR antagonist significantly decreased the vascular enhancing effects of surgical delay [87]. Correspondingly, experimental studies employing VEGF as a pre-treatment showed "delay-like" vascular changes in the flap, albeit they were not as effective as actual surgical delay [88].

The merit of the surgical delay strategy has been known for several hundreds of years, traced back to the sixteenth century work of Tagliacozzi from his descriptions of staged nasal reconstruction using forearm flaps [89]. In current clinical practice, flap delay is used to enhance the flap area intended for transfer, mainly in pedicled flaps. It is used in various locations, including but not limited to the paramedian forehead flap for nasal reconstruction [90], the reverse sural flap for foot and ankle reconstruction [91], and the tensor fascia lata flap to cover extensive defects in the abdomen or lumbar region [92].

The transverse rectus abdominis myocutaneous (TRAM) flap classically demonstrates the concept of delay in the clinical setting [93]. TRAM incorporates the rectus abdominis as well as the overlying abdominal skin and fat. It is mainly used for autologous breast reconstructions. Employing the delay phenomenon by dividing the inferior pedicle and incising the flap boundaries opens the linking vessels between the two systems, thus increasing its vascularity with time, allowing a larger skin paddle to be transferred as if it were based on the inferior pedicle [83, 84]. Pedicled TRAM flaps are less frequently used now, having mostly been replaced by the deep inferior epigastric perforator (DIEP) flap sparing [94]. Even with advanced methods of pre-operative perforator selection [95], some flaps may show vascular compromise during dissection, or in some patients a larger flap may be needed. Delay procedures are helpful to increase vascularity and enhance flap volume (extended DIEP) [96].

3.1.3 Pharmacological strategies

Although pharmacological strategies have not yet seen clinical adoption, many studies have investigated their promise in reducing the effects of IRI. Commonly, the rationale of pharmacological treatment lies in preventing the generation of ROS and other inflammatory modulators, or by inducing signaling pathways that yield cell protection or neovascularization. For instance, free radical scavengers such as edavarone and sodium channel blockers such as riluzole have been investigated as protective

agents for IRI in muscle [97, 98]. Treatment with vitamin C prior to ischemia has also been trialed for moderation of reperfusion injury, with positive findings in animal skeletal muscle [99, 100].

In the early 1990s, Pang, *et al.* demonstrated that, similar to preconditioning muscle with short-duration ischemia, pharmacological methods could be used to improve the tolerance of skeletal muscle to ischemic insult [74]. Preconditioning pig latissimus dorsi with 10 minutes of intravenous adenosine receptor agonist resulted in reduced muscle infarct after 4 hours of global ischemia [74]. This would allow for a more acute approach to achieving ischemic tolerance, and is thus termed acute (pharmacologic) ischemic preconditioning. Notably, the effects of pharmacologic preconditioning are sustained and do not require continuous treatment [74].

Vascular Endothelial Growth Factor (VEGF) is a potent mitogen implicated in the hypoxia signaling cascade [101]. Its expression is upregulated in the presence of HIF-1a, a factor which is constitutively expressed in the cell but otherwise ubiquitinated/degraded under normoxic conditions [101]. In the case of hypoxia, HIF-1a is stabilized and promotes the expression of VEGF, which stimulates vasculo- and angiogenesis [101]. Employing recombinant forms of VEGF for expedited angiogenesis in tissue healing (including myocardium- and ischemia-related applications) has thus been of increasing interest and widely studied in experimental research [102].

3.1.4 Stem cell treatment

Interestingly, treatment with bone marrow-derived and adipose-derived stem cells (BMSC and ADSC, respectively), prior to tissue ischemia may also hold promise in IRI reduction. These stem cells carry the potential to differentiate into endothelial cells [103], which can actively participate in neoangiogenesis and thus improve tissue survival in the ischemic environment [104, 105]. These stem cells also participate in cytoprotective cytokine and proangiogenic factor signaling [106], further potentiating their preventative effects in IRI [107]. Reichenberger, *et al*., for example, suggest that ADSCs may downregulate intracellular TNF-alpha expression, thereby avoiding programmed cell death and promoting cell survival [108]. This claim is complicated by the observed upregulation of HIF-1alpha, VEGF-a, CCL4, and other factors that can also have pro-inflammatory, pro-angiogenic, and/or vasoactive effects [108, 109].

In 2009, Uysal *et al.* described the injection of ADSCs into random skin flaps prior to inducing 6 hours of global ischemia [105]. They found that flaps treated with stem cells showed statistically significant increases in vascular density, number of vessels, flap survival and number of endothelial cells compared to controls [105]. Similar methods and results have been reported by Ichioka, *et al*. [110]. Uysal, *et al*. posit that the observed improvements are a combination of not only enhanced angiogenesis and anti-inflammatory effects, but also upregulation of chemoattractants and other factors that induce in vivo migration, differentiation, and proliferation of ADSCs [105]. Improved blood supply at early time points in the postoperative period promote nutrition/oxygen delivery, sustaining the ischemic tissue until neovascularization is achieved.

Advantageously, adipose tissue containing ADSCs can be harvested in large quantities from the human body in a less invasive manner, with minimal donor site morbidity [105, 106]. It has also been suggested in the literature that ADSCs possess a similar level of potency and proliferative efficiency to that of BMSCs [105, 111]. In the future, this could present an avenue for personalized IRI prevention strategies. Data on this strategy in the context of skin flap protection, however, is limited [108].

3.2 Ischemic period

3.2.1 Limiting ischemia time

Clinically, the best predictor of flap and VCA survival is limiting ischemia time. Current research is targeted at strategies taken to prolong allowable ischemia time by various preservation methods when ischemia time must be extended. In free flap surgeries, the surgeon has control over the onset of ischemia time. The recipient bed, along with the recipient artery and veins are prepared, and molding of the flap is done (when necessary) before pedicle division. Taking this approach permits the surgeon to limit ischemia to the time needed to perform anastomoses. The resultant ischemia in this controlled setting is usually not significant enough to decrease the overall survival of the flap, but it may increase post-operative complications. Chang, *et al.* demonstrated that ischemia time greater than 5 hours was associated with greater complications and flap loss as compared to groups with ischemia time less than 3, 3–4, and 4–5 hours; they did so in osteocutaneous fibula flaps where they had 116 flaps for 114 patients undergoing head and neck reconstruction [112]. Interestingly, overall flap survival was not different across groups [112]. Marre, *et al*. divided their 182 patients who underwent DIEP flaps for breast reconstruction into 4 quartiles (P_{25}, P_{50}, P_{75}, P_{100}) according to intraoperative ischemia time (39–177 minutes) and found that increased ischemia time (above 100 minutes, P_{100}) was associated with increased complications such as venous/arterial thrombosis, skin slough and partial flap loss [113]. It was also found that ischemia time was an independent risk factor for microvascular complications on multivariate analysis [113]. Further, Lee, *et al.* showed that DIEP flap ischemia time over 100 minutes was significantly associated with ultrasound-diagnosed fat necrosis 3 months postoperatively [114].

Clinically, VCA ischemia time is seen in injuries leading to digital amputations. For digital replantations, 12 hours of warm ischemia or 24 hours of cold ischemia are generally considered to be safe [115, 116]. However, successful digital replantations have been reported even after 94 hours of ischemia [117]. As the amputation becomes more proximal, prompt intervention and more stringent ischemia times are necessary due to the proportion of muscle constituents. In major replantations (amputation level above the radiocarpal joint), for example, warm ischemia above 6 hours or expected cold ischemia above 12 hours may be considered a contraindication—especially for cases above the mid-forearm level—to replantation [115, 116]. In these situations, temporary vascular shunting by means of catheters can be performed in an attempt to reduce ischemia time until a stable bone fixation is achieved, radical debridement is completed, and the limb can be properly anastomosed [118]. A major clinical consideration in IRI with replantations is systemic reperfusion syndrome associated with hypotension, metabolic acidosis, hyperkalemia, myoglobinuria, and in some instances cardiovascular collapse and death [119].

3.2.2 Static cold storage

Currently, the gold standard method for preserving composite tissues is static cold storage (SCS). SCS decreases the temperature of the tissue, thereby decreasing metabolic demand and preserving energy stores during ischemia. For every 10°C decrease in temperature, a two-fold decrease in metabolic rate is observed [120]. Unlike in free flap surgery, where the workflow of the surgery is centered on minimizing ischemia time to time of microvascular anastomosis, VCA is often

a transplant from donor to recipient and may need to be transported to different sites as is done with solid organ transplantation. As such, expanding ischemia time through various preservation methods is critical in advancement of clinical VCA accessibility. In SCS, the tissue is flushed with a preservation solution and stored at 4°C. The procured tissue is wrapped in moist gauze, then placed in a waterproof bag on ice until transfer to the recipient operating room [116]. The optimal preservation solution for VCA has yet to be defined. Thus, solutions for solid organ preservation are used [25]. To date, University of Wisconsin (UW) solution has been most commonly used for hand and face preservation [121–125]. Rostami, *et al.* compared 4 different preservation solutions, including Perfadex, HTK, UW, and heparinzied saline [126]. Heparinized saline showed the worst outcomes, HTK resulted in higher apoptotic cell count in nerve and skin, and Perfadex and UW were the preferred solutions [126]. Even with SCS, composite tissues used in plastic and reconstructive surgery are limited to the cold ischemia time of skeletal muscle of just 4–6 hours—this has bred the need to move away from SCS as the gold standard, and as such significant advancements have been made in more sophisticated preservation methods described in the following paragraphs.

3.2.3 Cryopreservation

Cryopreservation is a technique aimed at lowering the rate of metabolism, and ultimately function, in tissue using temperatures below −80°C. At this temperature, chemical activity and enzymatic activity are substantially reduced, but structure remains unchanged, allowing for indefinite preservation. Cryopreservation on its own is fatal to biological tissue without the use of cryoprotectant agents such as glycerol, dimethyl sulfoxide (DMSO), or ethylene glycerol [127]. Cryoprotectants reduce ice formation by increasing concentration of solutes in the tissue [127]. Although there are no human studies that have evaluated cryopreservation in VCA, studies have described single tissue and free flap responses to cryopreservation. Rinker, *et al.* show that 9/10 epigastric flaps in Lewis rats cryopreserved with DMSO and trehalose continued to be viable at 60 days and survival after transplant ranged 5–60 days [128]. Arav, *et al.* successfully cryopreserved then transplanted the above-knee rat hindlimb, which remained viable until their endpoint of 3 days [129]. Cryopreservation in free flaps and VCA continue to be limited by their composite nature as each tissue varies in its susceptibility to ischemia and IRI, as well as optimal cryoprotectants.

3.2.4 Vitrification

Vitrification is a process of cryopreservation which avoids the transition from liquid to crystalline structure, but rather shifts to a glass-like phase that behaves like a solid but avoids the ice crystals of cryopreservation [128]. Theoretically, vitrification allows for indefinite storage of tissues in this state. Vitrification contrasts from regular cryopreservation by transitioning from liquid to solid phase very quickly in order to suppress ice nucleation in the process, and in the vitrified state, viscosity prevents ice crystal formation [130]. Arav, *et al.* also vitrified 3 above-knee rat hindlimbs which similarly to their cryopreserved limbs remained viable for 3 days post-operatively, which was their endpoint [129]. Vitrification continues to be very limited due to the significant technical difficulty associated with rapid supercooling.

3.2.5 Machine perfusion

Machine perfusion has become a very popular research avenue in transplant research, particularly following success of ex vivo lung perfusion in the clinical setting. Ex vivo machine perfusion entails the connection of vasculature to an external pump capable of perfusing the tissue with a perfusion with a solution (+/− an oxygen carrier). Machine perfusion allows for constant delivery of oxygen, nutrients, removal of toxic metabolites such as lactate, and tissue viability testing. The first known perfusion of a composite tissue occurred in 1964, when Delorme, *et al.* perfused 6 lower extremities with autologous blood [131]. Since then, several landmark studies have furthered our understanding of the utility of machine perfusion in VCA preservation. In 2011, Constantinescu, *et al.* successfully perfused porcine forelimbs for 12 hours. The forelimbs exhibited retained functional capacity, evidenced by successful electrical stimulation of muscles as compared to loss of stimulation in the SCS control group [132]. Ozer, *et al.* furthered the porcine forelimb work by showing normal single-muscle fibre contractility after 12 hours of perfusion with autologous blood, and successful transplant of limbs to recipient pigs with normalized lactate levels post-reperfusion [133, 134]. Werner, *et al.* perfused 5 human upper limbs with a plasma-based perfusate for 24 hours, which resulted in normal electrical stimulation throughout and no histological myocyte damage [134]. Gok, *et al.* perfused rat hindlimbs for 6 hours with STEEN solution (perfusate optimized for lung perfusion) enriched with RBCs. They found that the muscles remained viable, with no evidence of ischemic necrosis on histology [135]. Krezdorn, *et al.* showed greater 7-day post-operative replantation survival and integrity of porcine forelimbs after 24 hours of perfusion with modified STEEN as compared to SCS control stored for 4 hours [136]. Burlage, *et al.* perfused rat hindlimbs with acellular perfusates (bovine serum albumin (BSA), with either polyethylene glycol or oxygen carrier HBOC-201 added) at subnormothermic temperatures for 6 hours and were successful in heterotopically transplanting the limbs, noting superiority of the group perfused with HBOC-201 in 30-day survival post-operatively [137].

Although the past decade has seen an explosion of research in use of machine perfusion, major limitations are still being explored. Most studies only follow-up post replantation for a maximum of 1 month. Due to heterotopic transplantation, there is a major automutilation confounder as well. A major clinical consideration is the extravasation of perfusate and subsequent edema and weight gain which may result in compartment syndrome necessitating fasciotomies to maintain tissue survival. There is thus a need to develop a perfusate which limits this adverse effect of machine perfusion by providing endothelial protection and adequate balance of hydrostatic and oncotic pressures. To-date, there is no perfusate that has been specifically designed and optimized for use in VCA; no study has been done to directly compare experimental outcomes of various perfusates currently in use for VCA perfusion. Datta, *et al.* reviewed preservation solutions currently used in literature and hypothesized that an ideal solution will reduce ROS production, thus attenuating IRI in VCA, but cautions that the significant diversity within VCA itself will likely give rise to the need to cater solutions to each type of VCA based on tissue subunit composition [25].

3.2.6 Pharmacological strategies

As discussed previously in pre-ischemic treatment methods, treatment with various agents during tissue ischemia may also improve survival upon reperfusion. In one study, bone grafts were exposed to varying durations of warm ischemia time

prior to reperfusion. Just prior to anastomosis, the experimental group was treated with superoxide dismutase—which functioned to prevent the generation of reactive oxygen species when blood flow was re-established. Bone grafts treated with superoxide dismutase exhibited enhanced survival compared to controls [50]. A study by Tamura, *et al.* also achieved pronounced protection from IRI by administering a derivative of vitamin C prior to reperfusion in a rat hind limb model [53].

3.3 Post-ischemic period

3.3.1 Post-ischemic conditioning

The principle outlined in Section 3.1.1 (Local and remote ischemic preconditioning) also has a protective effect when applied just before sustained reperfusion—this is known as post-ischemic conditioning. This strategy was first described in myocardial tissue by Zhao, *et al.* where they showed three cycles of 30 second coronary artery clamping and 30 seconds of reperfusion applied just before the 3 hour reperfusion period following a 50 minute ischemic period resulted in significant reduction of infarct size compared to their control group [138]. Moreover, the protective effect was similar to the pre-conditioning group that they included in their study [138]. This strategy was again applied to muscle flaps. Park, *et al.* showed that six cycles of 15 second IR periods at the end of 3 hours of ischemia result in the attenuation of IRI in rat extensor digitorum longus [139]. The possible mechanistic explanation of this effect was studied by McAllister, *et al.* on pig latissimus dorsi muscle flaps [140]. They showed that intravenous injection of cyclosporine A, an inhibitor of the opening of mPTP after 4 hours of ischemia results in a similar effect to four cycles of 30 second IR [140]. They also found this effect could be reversed by actractyloside, an mPTP opener [140]. Moreover, McAllister, *et al.* showed that the application of cariporide, a Na^+/H^+ exchange inhibitor, effectively reduces the mitochondrial Ca^{2+} content and has protective effects like cyclosporine A [140]. Despite promising results, ischemic post-conditioning is currently not common clinical practice.

3.3.2 Monitoring for microvascular complications post-operatively

The reperfusion period starts upon the completion of proper anastomosis of vessels. It is critical to monitor the transplanted tissue hourly, particularly in the first 24 hours, as this is the period where most microvascular complications will arise due to arterial and/or venous insufficiency. Capillary refill time, temperature, color, and turgor are monitored [141, 142], blood flow is confirmed with a doppler, and oxygenation with a tissue oximeter [143, 144]. Short capillary refill time (<2 seconds), purple skin color, marked edema, and increased turgor are indications of venous insufficiency [142]. Long capillary refill (>3 seconds), pallor and cool temperature are indicators of arterial insufficiency [142]. Arterial insufficiency usually results from thrombosis within the anastomosis whilst global venous insufficiency may result from venous thrombosis, torsion in the pedicle, or hematoma around the pedicle [145, 146]. When these complications arise, the flap returns to an ischemic state and blood flow must be restored. Failure to recognize these changes, or late intervention are risks of losing the tissue entirely. As expected, time from the initial operation until salvage attempt negatively correlates with salvage success [147, 148].

3.3.3 Pharmacological strategies

Pharmacological treatment is usually initiated in the OR and continued post-operatively to decrease risk of thrombosis. Low molecular weight heparin, unfractionated heparin and aspirin are the most commonly used anticoagulant and anti-platelet agents [149]. However, there is no consensus on the optimal regime, and it is questionable whether they actually have any effect on overall survival [149, 150]. Treating muscle with copolymer surfactants—which have been shown to adsorb and seal damaged cell membranes—post-injury has also seen success in improving the survival of muscles in the rat [151]. Orfany, *et al.* describe a significant reduction of IRI using a mouse acute limb ischemia model with mitochondrial transplantation, leading to decreased apoptosis, infarct, and increasing viability and function post-IRI [152].

Treatments targeting IRI in the vasculature have also been explored. In a study by Ward, *et al.,* superoxide dismutase or Trolox (antioxidants) were used to prevent morphological endothelial cell changes characteristic of ischemic injury, such as cell swelling and bleb formation [153]. Another study found that incubating arterioles in sepiapterin, a BH4 precursor, or MH4, a BH4 analog, could restore endothelium-dependent vasodilation following a period of ischemia in the pig [154]. Although this work primarily sought to explain the mechanism by which endothelial function is blunted in IRI (depletion of cofactor BH4), this work paves the way for future reagent-based approaches to IRI prevention [154].

Although not a pharmacologic measure per se, Haapaniemi, *et al.* also suggest that by increasing the partial pressure of oxygen in the tissue, treatment with hyperbaric oxygen can effectively reduce IRI [155].

3.3.4 Stem cell treatment

Similarly discussed in the pre-ischemic section of this chapter, stem cells have also been investigated as a post-ischemic treatment strategy for IRI. Studies have shown that intravenous treatment of skin flaps with adipose derived stem cells exhibited enhanced flap survival, flap perfusion, and expression of pro-angiogenic and inflammatory genes in the rat [108]. Another study by Nakagami, *et al.* found enhanced blood flow and capillary density 4 weeks post-transplantation of adipose derived stem cells co-cultured with human aortic endothelial cells in the ischemic mouse hindlimb [156].

4. Conclusion

In conclusion, ischemia reperfusion injury is of critical concern in Plastic and Reconstructive Surgery as the highest complexity reconstructive options offered to patients are subject to ischemia. In contrast to ischemia of solid organs for transplant, Plastic Surgery must uniquely manage composite tissues with varying tolerances to ischemia and IRI. Various measures are employed to mitigate the adverse effects of IRI, some of which are present in clinical settings today whilst others have yet to be translated to human clinical trials. Firstly, in the pre-ischemic period, great effort is made to work with well-vascularized tissues through pre-operative planning whenever possible and strategies such as delay can be employed. Significant benefits have been described through local and remote pre-conditioning methods, and with pharmacological and stem cell treatments in the pre-clinical setting. Secondly, the

ischemic period occurs after division of the pedicle which is currently the target of most research efforts. Currently, SCS is the gold standard of storage, but it has limits and research has shown promising outcomes with freezing methods and machine perfusion. Thirdly, in the post-ischemic, or reperfusion period, rigorous post-operative monitoring of both the patient and the transplanted tissue to promptly address any complications that may arise. Some post-conditioning and pharmacological treatments have been described in the pre-clinical setting with promise to enhance the reperfusion period. Altogether, there have been significant advances in IRI implications in Plastic and Reconstructive Surgery, and in order to continue increasing accessibility to these advanced reconstructive options for patients with significant tissue loss, research must be continued with ultimate translation to clinical studies in the near future.

Conflict of interest

The authors declare no conflict of interest.

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