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Strategies for increasing tissue survival in critically perfused musculocutaneous flaps

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1. SUMMARY

Despite a steady improvement of surgical techniques, ischemic tissue damage still remains a relevant problem. This holds true in particular in plastic surgery, as the field deals with extensive soft tissue defects. Reconstructive procedures often undermine large surface areas to mobilize the tissue and create surgical flaps for defect closure. Despite prudent preoperative planning, the necessary dissection can influence the flaps' microcirculation in a detrimental fashion. Due to an impaired microcirculation, tissue areas distal to the arterial inflow are at risk of ischemia and related complications, such as wound breakdown, wound dehiscence or tissue necrosis. Subsequent complex local wound care or surgical revisions prolong hospitalization and increase patient morbidity. This not only has great socioeconomic consequences in general, but also a negative impact for each individual patient.

When a sudden critical restriction of blood supply to an organ or tissue occurs, ischemia forces the cellular metabolism to slow down. Finally, it will stop completely due to a lack of nutrients and oxygen. While a transitory ischemia can be tolerated by the tissue for a certain period of time, it will eventually lead to an increased microvascular permeability, a hypoxia-induced inflammatory reaction and ultimately cell death. Associated tissue edema, immune cell invasion and microvascular thrombosis aggravate the local situation, leading to tissue necrosis.

Besides maintaining microperfusion, several therapeutic strategies have been proposed in the past to increase flap survival. They include the induction of angiogenesis, the reduction of the inflammatory reaction and the improvement of the ischemic tolerance by directly suppressing apoptosis. In the present thesis, three different tissue-protective approaches have been evaluated in a random pattern flap model in dorsal skinfold chambers. Using repeated intravital fluorescence microscopy, blood vessel formation, nutritive blood perfusion and flap necrosis were assessed.

The first study explored dietary restriction in the form of intermittent fasting as a conditioning strategy for musculocutaneous flaps, as it has previously been shown to have protective effects and counteract ischemia-induced necrosis in various tissues. Adult male C57BL/6N mice were separated into a fasting group and a control group with unlimited access to standard pellet chow at random. A perioperative fasting regimen alternating 8 hours of unrestricted access to food with 16 hours of fasting was implemented for the treatment group. Intermittent fasting was performed starting 7 days before surgical flap elevation and was continued up to the third postoperative day. Intravital fluorescence microscopy was carried out 1, 3, 5, 7 and 10 days

after surgery. After the in vivo observation period, additional histological and immunohistochemical analyses were performed on the flap tissue. In comparison with the control animals, the intermittent fasting group exhibited more angiogenic vessel sprouts, a higher density of functional capillaries within the tissue and a significantly lower rate of flap necrosis overall. Furthermore, the analysis of different inflammatory cell subtypes in the flap tissue of fasted animals by means of immunohistochemical staining showed a markedly lower number of myeloperoxidase (MPO)-positive neutrophilic granulocytes invading the tissue as a sign of a decreased inflammatory reaction.

In the second study, the effects of the anti-inflammatory phytochemical bromelain on ischemic flap tissue were assessed. Adult C57BL/6N mice were randomly separated into a control group and a bromelain treatment group. Starting 1 hour before flap elevation, the animals received daily doses of 20 mg/kg bromelain or saline (control) by means of intraperitoneal injection throughout a 10-day observation period. The previously described dorsal skinfold chamber model with musculocutaneous flaps was used and intravital microscopy was performed over a period of 10 days after surgery. Additional histological and immunohistochemical analyses were performed on the flap tissue, which was harvested after the last microscopy. Bromelain was able to increase the survival of the critically perfused flap tissue by ~25%. Moreover, the analyzed musculocutaneous flaps also exhibited a significantly higher density of functional capillaries after bromelain treatment. In addition, more angiogenic vessel sprouts had developed in the transition zone between vital and necrotic tissue of treated animals in comparison with control animals. Subsequent immunohistochemical analyses revealed fewer invading MPO-positive neutrophilic granulocytes, as well as significantly less cleaved caspase (Casp)3-positive apoptotic cells in the transition zone of bromelain-treated musculocutaneous flaps. Thus, bromelain reduces flap necrosis most probably by maintaining nutritive blood flow to the tissue and suppressing ischemia-induced inflammation and apoptotic cell death.

In the third study, adipose tissue-derived microvascular fragments were injected into ischemically challenged random pattern musculocutaneous flaps to study their tissue-protective effects. Wild-type mice were equipped with dorsal musculocutaneous flaps that were mounted into dorsal skinfold chambers. These flaps were subsequently injected with microvascular fragments freshly isolated from green fluorescent protein (GFP)-positive donor mice or with saline solution (control). Due to this GFP crossover design the transplanted microvascular fragments could be tracked after their injection. Intravital fluorescence microscopy was performed to quantitatively assess blood vessel formation, nutritive blood perfusion and flap necrosis rate. The flap tissue was analyzed by means of histology and immunohistochemistry after completion of the in vivo experiments. After the injection of

microvascular fragment, the flaps displayed a markedly reduced rate of tissue necrosis of ~30% when compared to controls (~50%). Flaps injected with microvascular fragments also exhibited a significantly higher functional capillary density and a higher number of angiogenic vessel sprouts in the transition zone between vital and necrotic flap tissue when compared to controls. Immunohistochemical analyses in flaps injected with microvascular fragments showed a significantly lower number of Casp3-positive apoptotic cells in the transition zone, as well as a markedly increased number of CD31-positive microvessels in both the flaps' base and transition zone. Of note, GFP staining revealed that a percentage of up to ~10% of the microvessels was GFP-positive and originated from injected microvascular fragments. These findings indicate that microvascular fragments increase flap survival by improving nutritive blood perfusion and decreasing apoptotic cell death.

Taken together, different therapeutic strategies to decrease flap necrosis in random pattern musculocutaneous flaps were explored in the present thesis. As flap surgery is a versatile surgical technique with various indications, different tissue-protective approaches may be useful in varying clinical scenarios. Dietary restriction and phytochemical treatment may be best suited in an elective setting, as they seem to be most effective when applied before flap elevation. They are very cost-effective and carry few risks for the patient. Injection of microvascular fragments on the other hand, may be more useful in an acute setting, because the short isolation time offers the possibility for an intraoperative single-step application. Further research is warranted to confirm the results of this thesis and translate them into clinical practice. However, the examined approaches show potential for protecting ischemic flap tissue from necrosis and, thus, may help to reduce ischemia-related flap complications in the future.

2. ZUSAMMENFASSUNG

Trotz der stetigen Verbesserung der angewandten Operationstechniken sind ischämische Gewebeschäden vor allem in der Plastischen Chirurgie ein relevantes Problem, da diese Disziplin sich mit der Deckung großer Weichteilschäden beschäftigt. Rekonstruktive Operationen unterminieren hierbei oft größere Flächen, um Gewebe zu mobilisieren und chirurgische Lappen zu heben. Trotz umsichtiger präoperativer Planung kann die notwendige Dissektion die Gewebemikroperfusion negativ beeinflussen. Auf Grund der kritisch eingeschränkten Mikroperfusion sind die Lappenanteile distal der arteriellen Versorgung anfällig für Gewebeischämien und die daraus resultierenden Wundkomplikationen wie Dehiszenzen oder Nekrosen. Nachfolgend notwendige komplexe lokale Wundpflege und chirurgische Revisionen erhöhen sowohl die Hospitalisierungsdauer als auch die Patientenmorbidität. Neben den negativen Auswirkungen für den einzelnen Patienten stellen auch die resultierenden sozioökonomischen Konsequenzen ein großes Problem dar.

Während der Ischämie, welche als plötzlich einsetzende, kritische Restriktion der Blutversorgung eines Gewebes oder Organs definiert ist, verlangsamt sich der Zellstoffwechsel und kommt letztendlich auf Grund des Sauer- und Nährstoffmangels zum Erliegen. Eine zeitweilige Ischämie kann vom Gewebe toleriert werden, führt jedoch langfristig zu einer erhöhten mikrovaskulären Permeabilität, einer Hypoxie-induzierten inflammatorischen Reaktion, sowie schlussendlich zum Zelltod. Ödembildung, die Invasion von Immunzellen ins Gewebe und mikrovaskuläre Thrombosen beeinträchtigen die lokale Situation weiter und führen schließlich zum Gewebeuntergang.

Neben der Aufrechterhaltung der Gewebemikroperfusion sind in der Vergangenheit diverse therapeutische Strategien zur Verbesserung des Lappenüberlebens untersucht worden. Hierzu zählen die die Induktion der Angiogenese, die Reduktion der inflammatorischen Gewebereaktion und die Verbesserung der Ischämie-Toleranz durch die Unterdrückung von Apoptose. Mit der vorliegenden Doktorarbeit wurden drei gewebeprotektive Ansätze in einem randomisiert perfundierten Lappenmodell in der Rückenhautkammer evaluiert. Zur quantitativen Beurteilung der Angiogenese, der nutritiven Blut-Perfusion und der Lappen-Nekrose wurden wiederholte intravitale Fluoreszenzmikroskopien durchgeführt.

In der ersten Studie wurde eine diätetische Restriktion in Form von intermittierendem Fasten als Konditionierungsverfahren für muskulokutane Gewebelappen evaluiert, da in der Vergangenheit gezeigt wurde, dass intermittierendes Fasten verschiedene Gewebe vor Ischämie-induzierter Nekrose schützt. Ausgewachsene männliche C57BL/6N Mäuse wurden

zufällig in eine Fasten-Gruppe und eine Kontrollgruppe eingeteilt. Die Kontrollgruppe hatte unbegrenzten Zugang zu Standard Pellet-Futter, Fasten-Tiere wurden einem perioperativen Fastenregime unterzogen, bei dem 8 Stunden ungehinderter Zugang zu Pellet-Futter mit 16 Stunden Fasten alterniert wurden. Das Fasten-Regime wurde 7 Tage vor der Lappenhebung begonnen und bis zum 3. postoperativen Tag fortgesetzt. Intravitale Fluoreszenzmikroskopien wurden an Tag 1, 3, 5, 7 und 10 nach Lappenhebung durchgeführt. Nach der in vivo Beobachtungsperiode wurde das Lappengewebe asserviert und zusätzliche histologische und immunhistochemische Analysen durchgeführt. Die Fasten-Gruppe zeigte hierbei im Vergleich zu Kontrolltieren eine größere Anzahl neu entstandener angiogener Gefäßsprossen, eine höhere funktionelle Kapillardichte im Lappengewebe und signifikant weniger Lappennekrose. Zusätzlich wurden in der immunhistochemischen Färbung unterschiedlicher Immunzellsubtypen in Fasten-konditionierten Lappen eine deutlich reduzierte Anzahl von ins Gewebe migrierenden Myeloperoxidase (MPO)-positiven neutrophilen Granulozyten nachgewiesen. Dies spricht für eine reduzierte entzündliche Gewebereaktion.

In der zweiten Studie wurde die anti-inflammatorische Phytosubstanz Bromelain und deren Effekt auf ischämisches Lappengewebe untersucht. Ausgewachsene C57BL/6N Mäuse wurden zufällig in eine Bromelain-Behandlungsgruppe und eine Kontrollgruppe unterteilt. Die Tiere erhielten täglich 20 mg/kg Bromelain oder Kochsalzlösung (Kontrollen) mittels intraperitonealer Injektion während der gesamten Beobachtungsperiode. Die erste Injektion wurde hierbei eine Stunde vor der Lappenhebung durchgeführt. Das bereits beschriebene Lappenmodell in der Rückenhautkammer wurde auch für diese Studie verwendet. Dabei wurden bis zum 10. Tag nach Lappenhebung wiederholte intravitale Fluoreszenzmikroskopien durchgeführt. Nach der letzten Mikroskopie wurde das Lappengewebe für histologische und immunhistochemische Untersuchungen asserviert. Bromelain konnte hierbei das Überleben des kritisch durchbluteten Lappengewebes um ~25 % erhöhen. Die behandelten Lappen wiesen im Vergleich zu unbehandelten Kontrollen außerdem eine signifikant erhöhte funktionelle Kapillardichte sowie eine höhere Anzahl neu gebildeter Mikrogefäße in der Transitionszone zwischen vitalem und nekrotischem Lappengewebe auf. Nachfolgende immunhistochemische Analysen konnten außerdem eine deutlich verminderte Anzahl MPO-positiver neutrophiler Granulozyten und cleaved Caspase (Casp)3-positiver apoptotischer Zellen in der Transitionszone Bromelain-behandelter Lappen nachweisen. Diese Untersuchungen zeigen, dass Bromelain die Lappennekrose reduziert und dass dies am Ehesten durch das Aufrechterhalten der nutritiven Perfusion und die Reduktion der Ischämie-induzierten inflammatorischen Gewebereaktion sowie des apoptotischen Zelluntergangs bewirkt wird.

In der dritten Studie wurden aus Fettgewebe isolierte mikrovaskuläre Fragmente in randomisiert perfundierte Lappen injiziert, um deren Effekt auf das Lappenüberleben zu analysieren. Auf dem Rücken von Wildtyp Mäusen wurden randomisiert perfundierte Lappen gehoben und in Rückenhautkammern eingebracht. In die Lappen wurden mikrovaskuläre Fragmente injiziert, welche zuvor aus green fluorescent protein (GFP)-positivem Spenderfett isoliert wurden. Für Kontrolltiere wurde Kochsalzlösung zur Injektion verwendet. Durch die Verwendung von GFP-positiven mikrovaskulären Fragmenten und GFP-negativen Empfängertieren konnte auch der Ursprung der perfundierten Blutgefäße im Lappengewebe untersucht werden. Mit Hilfe intravitaler Fluoreszenzmikroskopien wurden quantitativ die Angiogenese, die nutritive Perfusion und die Lappennekrose analysiert. Die Lappen wurden nach dem Abschluss des zehntägigen Beobachtungszeitraums zusätzlich mittels histologischer und immunhistochemischer Techniken untersucht. Nach der Injektion der mikrovaskulären Fragmente wiesen die Lappen eine deutlich verringerte Gewebsnekroserate von ~30 % im Vergleich zu den Kontrollen (~50 %) auf. Lappen, denen mikrovaskuläre Fragmente injiziert wurden, wiesen im Vergleich zu den Kontrollen auch eine signifikant höhere funktionelle Kapillardichte und eine höhere Anzahl angiogener Gefäßsprossen in der Übergangszone zwischen vitalem und nekrotischem Lappengewebe auf. Mittels immunhistochemischer Färbungen wurde eine deutlich niedrigere Anzahl Casp3-positiver apoptotischer Zellen in der Transitionszone behandelter Lappen nachgewiesen. Die Anzahl CD31-positiver Mikrogefäße war sowohl in der Lappenbasis als auch der Transitionszone im Vergleich zu den Kontrolltieren erhöht. Der immunhistochemische Nachweis von GFP zeigte für bis zu ~10% der Mikrogefäße einen Ursprung aus mikrovaskulären Fragmenten. Diese Ergebnisse zeigen, dass die injizierten mikrovaskulären Fragmente das Lappenüberleben durch die Verbesserung der nutritiven Perfusion und die Reduktion der Apoptose erhöhen können.

In der vorliegenden Arbeit werden unterschiedliche therapeutische Ansätze zur Reduktion von Lappennekrosen in randomisiert perfundierten Haut-Muskel-Lappen untersucht. Da es sich bei der Lappenchirurgie um eine vielseitig einsetzbare Technik mit vielen verschiedenen Indikationen handelt, könnten Gewebe-protektive Strategien in diversen klinischen Szenarios von großem Nutzen sein. Diätetische Restriktion und Phytochemikalien könnten im elektiven Setting nützlich sein, da sie am wirksamsten sind, wenn die Anwendung vor der Lappenhebung begonnen wird. Sie sind hierbei sehr kosteneffizient und mit geringen Risiken für den Patienten verbunden. Die Injektion von mikrovaskulären Fragmenten hingegen könnte in einem akuten Setting besser geeignet sein, da die kurze Isolierungszeit eine intraoperative, einzeitige Anwendung ermöglicht. Weitere Forschung ist erforderlich, um die in dieser Arbeit gewonnenen Daten zu verifizieren und sie in den klinischen Alltag zu übertragen. Die

untersuchten Ansätze haben jedoch das Potenzial, ischämisches Lappengewebe vor Nekrose zu schützen und könnten somit in Zukunft wesentlich dazu beitragen, Ischämie-bedingte Lappenkomplikationen zu vermeiden.

3. INTRODUCTION

Surgical flaps are defined as a unity of tissue consisting of one or more tissue subtypes that is raised on a vascular axis supplying the required blood perfusion (Chase, 1986). The use of tissue flaps for the reconstruction of soft tissue defects is a technique that has defined the field of plastic surgery from its very beginnings. The “father of plastic surgery” Sushruta described a technique for nasal reconstruction using a paramedian forehead flap as early as 1000-800 BC in ancient India (Champaneria et al., 2014). Due to the increased understanding of the human anatomy and the growing need for plastic and reconstructive surgery caused by both world wars and other historic events, surgical techniques continued to be refined. This development resulted in the use of free flaps among other examples, ultimately made possible by the construction and use of surgical microscopes in the 1960ies (Fang & Chung, 2014).

Several classifications for flaps are currently in use, which makes the nomenclature confusing. They can be subdivided according to the contained tissue, such as cutaneous, musculocutaneous or osteocutaneous flaps. A variety of composite flaps have been described to meet the specific needs of the defect (Goodman, 1988), true to one of the basic principles of plastic surgery to replace “like with like” (Gillies, 1957). Depending on the proximity of the donor site to the defect, flaps are also classified into local, regional and distant flaps. A local flap is raised directly adjacent to the defect and remains attached at the donor site on one border or pedicle, thus containing the vessels that supply the flaps’ blood perfusion. A regional flap is another method of reconstruction, whereby tissue is obtained from an area slightly further away from the tissue surrounding the defect, such as the use of tissue from the chest wall to reconstruct a defect in the neck or cheek. Lastly, distant flaps are characterized by a larger distance between the donor and the recipient site. Nowadays, they are usually performed as free flaps, meaning that the blood vessels feeding the flap tissue are completely disconnected from their blood supply and then immediately re-connected to a recipient vessel by surgical anastomosis. The somewhat outdated method of delayed distant flaps requires the flap tissue to be transiently attached to both the donor site and the recipient site. The delayed flap is then detached from the donor site once perfusion from the recipient site is established by newly ingrown vessels. This complex and time-consuming method has become reserved for niche indications and very specific cases, though it is still in clinical use today.

Arguably the most important flap classification is the division according to the blood supply. If no specific blood vessel is included into the flap, blood perfusion must be supplied by the dermal plexus and small musculocutaneous arteries at random and is therefore called a random pattern flap (Fig. 1). Milton (1970) proved that the extent of perfusion captured within

the given flap territory is vital for its survival. Based on these findings, certain width-to-length ratios have been used to plan tissue flaps and minimize the risk of ischemic tissue damage. Once individual subcutaneous vessels with a relatively predictable orientation were identified, the concept of the axial pattern flap was established (McGregor & Morgan, 1973) (Fig. 1). This type of flap allowed for more complex reconstructive surgeries, as slightly larger tissue flaps could be raised. If the axial vessel is not transected, the flap is considered a pedicled flap. If the vessel is severed and microsurgical vascular anastomosis is performed, it becomes a free flap.

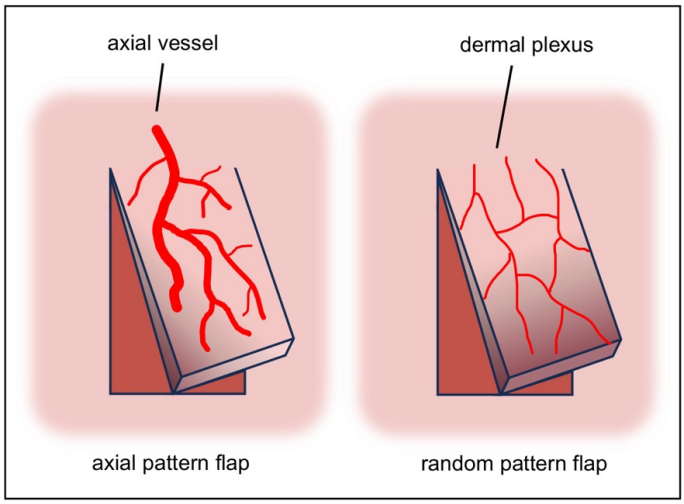

***Figure 1**: Schematic depiction of the blood vessels included in an axial pattern flap and a random pattern flap.*

Despite the development of more sophisticated flap designs, random pattern flaps continue to be relevant due to their availability and ease of use. Local flaps may be utilized across the body for defects following oncologic resection, trauma or burn reconstruction. Furthermore, they provide a very good color and texture match and comply with Gillies' principles of replacing "like with like" due to the immediate proximity to the defect (Gillies, 1957). Moreover, they are associated with a fairly low donor site morbidity. Despite the mentioned advantages, many factors can influence the surgical outcome negatively. Patient characteristics that may affect the blood supply to the surgical site include medications, comorbidities and behaviors (i.e. active tobacco and alcohol use). Diabetes, hypertension, liver failure, renal failure, immune suppression, bleeding disorders and inflammatory skin conditions are just some examples for possibly harmful underlying conditions (Delaney et al., 2011). Moreover, certain anatomic locations like the groin and the lower extremity are more prone to delayed healing or infection,

which increases the risk of an insufficient tissue perfusion. The understanding of the cellular and molecular mechanisms causing ischemic flap complications and strategies for their possible prevention have therefore remained an important area of research over the last decades.

Tissue survival depends on several crucial factors, including the severity of ischemia, the duration before nutrient blood flow is re-established and the extent of the inflammatory tissue reaction. Once the tissue is no longer adequately supplied with oxygen and nutrients, cellular hypoxia causes an insufficient ATP production, which subsequently hinders ion pump function. Accumulating Na^+, Ca^+ and H^+ ions together with the occurring anaerobic glycolysis slowly lead to cellular acidification. Additionally, water follows the osmotic gradient (Kalogeris et al., 2014). As the cells swell and eventually burst, cell organelles start to disintegrate. If the outer mitochondrial membrane is damaged, proapoptotic factors are released into the cytoplasm, where they start the apoptotic cascade. The dying cells release endogenous danger signals called damage-associated molecular pattern molecules (DAMPs) to activate the innate immune system (Tang et al., 2012). The attracted immune cells, in turn, trigger the apoptotic cascade via extracellular receptors, amplifying tissue destruction. On a macroscopic level this manifests as partial or total necrosis of the flap.

In the past, insufficient arterial inflow into the flap due to poor preoperative planning alone was made responsible for tissue necrosis of the distal flap tissue. Further insights into flap physiology, however, have shed more light onto other involved pathomechanisms. Vasospasms caused by adrenergic denervation can prevent sufficient tissue oxygenation despite sufficient vasculature being included into the flap base (Richards et al., 1985). Catecholamines are released from the nerve terminal without further catecholamine reuptake when a sympathetic nerve is severed. This hyperadrenergic state then causes vasoconstriction mediated by α-adrenergic receptors in the cutaneous vasculature. Vasospasms may last up to 48 hours until transmitters are depleted and the concentration of norepinephrine declines. Moreover, depending on the affected vessels, arteriovenous shunting can occur and prevent sufficient tissue oxygenation even if sufficient blood flow is present in the distal areas of the flap (Reinisch, 1974). Furthermore, the surviving length of the flap depends on various properties of the supplying vessels including the perfusion pressure. If this pressure drops below the pressure in the interstitial space, microvessels collapse and tissue microperfusion ceases (Lucas, 2017). This effect can be aggravated by an impaired lymphatic drainage as the reduction of lymphatic drainage due to flap elevation results in a further increase of interstitial pressure. This effect is sometimes worsened by the capillary leak caused by the inflammatory reaction. After flap elevation, histamine, serotonin and kinins are released into the extracellular compartment, increasing microvessel permeability. This increases the concentration of

proteins and cells within the extracellular space. The edema increases the diffusion distances between cells and constitutes a direct barrier to diffusion. The pro-inflammatory cytokines released by dying cells also cause an invasion of circulating immune cells into the tissue, creating a vicious circle.

If perfusion is reestablished in time to prevent necrosis, the sudden return of oxygen to the ischemic tissue can paradoxically result in additional tissue damage, the so-called ischemia-reperfusion injury (Eltzschig & Eckle, 2011; Wang et al., 2011). During the reperfusion phase, the return of blood perfusion to ischemic tissue supplies oxygen through erythrocytes. Simultaneously, the production of reactive oxygen species (ROS) escalates due to decreased levels of antioxidative agents within the ischemic cells. Even though ROS are produced to a certain extent during ischemia, the surge of oxygen and the associated sudden increase of ROS contribute to the oxidative stress (Bozkurt et al., 2019). Once the excess of ROS can no longer be eliminated by antioxidant molecules and enzymes, they damage the cell due to endothelial dysfunction and DNA impairment. In addition, the restoration of the microcirculation flushes even more inflammatory cells into the flap. These cells obstruct microvessels, secrete proteolytic enzymes like collagenase or myeloperoxidase (MPO) and produce ROS that add to the existing oxidative stress. Oxidative stress and inflammatory cascades subsequently induce a sudden rush of cytokines, damaging cellular structures and ultimately resulting in cell death.

In random pattern flaps, but also in the border areas of axial pattern flaps, the decline in perfusion pressure intensifies with the growing distance from the base of the flap or the vascular axis, respectively. When perfusion is critically reduced, the adjacent vascular territories provide a low-pressure blood supply through the subdermal plexus to keep the ischemic cutaneous tissue alive. In random pattern flaps the remodeling of these emergency channels into a sufficient vascular system and the formation of new microvessels, i.e. angiogenesis, between the transposed flap and the recipient wound bed can counteract tissue ischemia. However, these processes may take several days and are often too late to prevent tissue necrosis.

Angiogenesis requires the interaction of endothelial cells and perivascular cells and is regulated by several interacting pro- and anti-angiogenic factors (Neri et al., 2015). A hypoxic stimulus causes existing vessels to dilate and become permeable, as it decreases the endothelial junctions between cells, causing them to retract. Proteases then dissolve the basement membrane and the endothelial cells migrate towards the angiogenic stimulus. Capillary sprouts form, as endothelial cells proliferate and pericytes, as well as fibroblasts migrate to stabilize the budding vessels.

Besides maintaining microperfusion, several therapeutic strategies have been proposed in the past to increase flap survival. Concepts include improving the ischemic tolerance by directly suppressing apoptosis, reducing the inflammatory reaction and inducing angiogenesis. One of the earliest known concepts is performing a so-called surgical delay (Reinisch, 1974; Altınel et al., 2019). Flap elevation is performed in a stepwise manner over the course of about two weeks to create a hypoxic stimulus for the induction of angiogenesis. This time-consuming and very invasive form of preconditioning never found its way into clinical routines. However, based on the concept of introducing a sublethal stressor to prepare the tissue for ischemia, several other forms of tissue conditioning have been developed. Examples include remote ischemic preconditioning (Zahir et al., 1998; Küntscher et al., 2005), physical stimuli, such as shock waves (Tobalem et al., 2013) or local heat (Harder et al., 2004a), and pharmacological conditioning using substances like erythropoietin (Schmauss et al., 2019) or anti-neoplastic agents (Askar et al., 2006). Though some strategies showed promising results in preclinical animal studies, they may be associated with significant adverse effects, hindering their implementation in the daily clinical setting. For instance, while anti-neoplastic agents can increase flap survival in rats, they also potentially lead to cardiotoxic, nephrotoxic or hepatotoxic effects (Patil et al., 2008; Hanna et al., 2014; Prasanna et al., 2020). Similarly, the necessity for specialized equipment rendered some of the proposed approaches impractical. Therefore, the search for simple, cost-effective and safe methods to prevent complications related to tissue ischemia remains ongoing.

In the present thesis, three different approaches to protect the ischemic tissue from necrosis were analyzed in a murine random pattern flap model within dorsal skinfold chambers. Following flap elevation, repeated intravital fluorescence microscopy was performed to quantitatively assess blood vessel formation, nutritive blood perfusion and flap necrosis. To further investigate the underlying mechanisms, additional histological and immunohistochemical stainings were performed in each study.

In the first study, intermittent fasting (IF) was chosen as a tissue conditioning strategy. This subtype of dietary restriction (DR) was selected based on previous research that had shown tissue-protective effects induced by short-term fasting interventions. Male C57BL/6N mice were divided into an IF-group undergoing perioperative DR and a control group with free access to standard pellet chow. The fasting regimen applied to IF-animals alternated 8 hours of unrestricted access to food with 16 hours of fasting. IF was performed starting 7 days before flap elevation up to 3 days after surgery. Intravital fluorescence microscopy was performed on day 1, 3, 5, 7 and 10 after flap elevation. At the end of the in vivo observation period, the animals were killed after the last microscopy by cervical dislocation. Flap tissue samples were collected to perform additional histological and immunohistochemical analyses. IF-treated

flaps displayed a significantly lower rate of tissue necrosis, more newly formed microvessels and a higher density of functional capillary vessels when compared to controls. Furthermore, the performed immunohistochemical analysis of different inflammatory cell subtypes showed that fewer MPO-positive neutrophilic granulocytes had invaded the musculocutaneous flap tissue of IF-treated animals as a sign of a decreased inflammatory reaction.

In the second study, the use of phytochemicals as an alternative gentle tissue conditioning strategy was evaluated. Phytochemicals represent a promising approach because they are widely available and rarely cause unwanted side effects. The anti-inflammatory phytochemical bromelain was selected for the potential prevention of ischemia-induced flap tissue necrosis and a bromelain treatment group as well as a control group were analyzed using adult C57BL/6N mice. Bromelain-treated animals received daily intraperitoneal injections of 20 mg/kg bromelain while corresponding saline injections were performed in the control group. Injections were performed starting 1 hour before flap elevation and were continued for the entire 10-day observation period. The previously established dorsal skinfold chamber model with musculocutaneous flaps was used and intravital fluorescence microscopy was performed on day 1, 3, 5, 7 and 10 respectively. After the last microscopy, flap tissue samples were collected for further histological and immunohistochemical analyses. Bromelain treatment was able to increase flap survival by ~25%. When compared to controls, the flaps of bromelain-treated animals displayed a significantly higher density of functional microvessels and more newly formed microvessels in the transition zone between vital tissue and necrosis. Moreover, immunohistochemical staining showed fewer invading MPO-positive neutrophilic granulocytes and a decreased number of Casp3-positive apoptotic cells in the transition zone of bromelain-treated flaps. The enzyme bromelain is therefore capable of reducing flap necrosis, most probably by maintaining the nutritive blood flow and suppressing ischemia-induced inflammation and apoptotic cell death.

In the third study, injection of adipose tissue-derived microvascular fragments (MVF) into random pattern musculocutaneous flaps was performed with the intention of accelerating and increasing angiogenesis in ischemically challenged flaps to possibly prevent tissue necrosis. To trace the fate of the injected MVF after transplantation, a green fluorescent protein (GFP)-positive/negative crossover design in combination with the dorsal skinfold chamber flap model was used. Flaps on the backs of wild-type mice were raised and injected with freshly isolated MVF from GFP-positive donor mice, while control animals received saline injections. Intravital fluorescence microscopy was subsequently performed for the quantitative assessment of microvessel formation, tissue blood flow and flap necrosis. The flap tissue was additionally analyzed by means of histology and immunohistochemistry after the completion of the in vivo experiments. MVF injection reduced necrosis of the ischemically challenged flap tissue by

~20% and was associated with a significantly higher density of functional capillary vessels and number of angiogenic vessel sprouts in the transition zone between vital and necrotic flap tissue in comparison with control animals. A markedly decreased number of Casp3-positive apoptotic cells in the transition zone of MVF-injected flaps and an increased number of CD31-positive microvessels in the flaps' base as well as the transition zone indicated that MVF may reduce flap necrosis by suppressing apoptosis, boosting angiogenesis and improving nutritive tissue perfusion. Of note, up to ~10% of the microvessels were GFP-positive, thus originating from transplanted MVF.

The present thesis explored different therapeutic strategies to prevent necrosis in ischemically challenged random pattern musculocutaneous flaps. Random pattern flaps are a frequently used operating technique in the field of plastic surgery. Moreover, the proposed therapeutic approaches may also be suitable for the prevention of ischemic tissue damage in the border areas of axial pattern flaps or any surgical wound margins with an impaired microperfusion. Thus, the presented approaches may be useful in varying clinical scenarios. Dietary restriction and phytochemical treatment are very cost-effective and carry few risks for unwanted side effects. They may therefore be applicable for a wide range of indications. In the future, the strategies may even be combined for a synergistic positive effect on flap survival. MVF injection might be more suitable in an acute setting or for patients with factors predisposing them for an insufficient angiogenic response to flap ischemia, as the short isolation time offers the possibility for intraoperative single-step application and the fragments are able to boost flap vascularization. Though further research is warranted to confirm the results of this thesis and translate them into clinical routines, the examined approaches showed promising results and may help to reduce ischemia-related flap complications in the future.

4. AIM OF THE THESIS

In the past, several mechanisms including the induction of angiogenesis or the suppression of an inflammatory reaction have been identified and used to increase tissue survival in surgical flaps. The aim of the present thesis was to explore novel potential strategies to increase tissue survival by targeting these tissue-protective mechanisms.

In the first study, published in 2023 in *Plastic and Reconstructive Surgery*, the hypothesis that IF as a form of dietary restriction can decrease flap necrosis was evaluated in a murine dorsal skinfold chamber model. The following questions were addressed:

- Does IF treatment reduce flap necrosis and maintain microperfusion in random pattern musculocutaneous flaps?
- Do IF-treated flaps exhibit a lower amount of invading neutrophilic granulocytes in the transition zone between viable and necrotic flap tissue?
- Do IF-treated flaps show higher amounts of newly formed capillaries in the transition zone between viable and necrotic flap tissue?

In the second study, published in 2022 in *Biomedicines,* the phytochemical compound bromelain was assessed for its efficacy to prevent ischemia-induced flap necrosis in a murine dorsal skinfold chamber model. The following questions were addressed:

- Does bromelain treatment reduce flap necrosis and maintain microperfusion in random pattern musculocutaneous flaps?
- Do bromelain-treated flaps exhibit a lower amount of invading neutrophilic granulocytes in the transition zone between viable and necrotic flap tissue?
- Does bromelain treatment suppress apoptosis within the transition zone between viable and necrotic flap tissue?
- Do bromelain-treated flaps show a higher amount of newly formed microvessels within their tissue?

In the third study, published in 2023 in *Biomedicines,* MVF were injected into musculocutaneous flaps within dorsal skinfold chambers. To clarify the fate of the MVF after injection, GFP-positive donor animals were used. The following questions were addressed:

- Do MVF injections reduce flap necrosis and maintain microperfusion in random pattern musculocutaneous flaps?
- Do MVF-injected flaps exhibit higher amounts of newly formed capillaries in the transition zone between viable and necrotic flap tissue?

- Do the injected MVF survive the transplantation as evidenced by the presence of GFP-positive microvessels within the flap tissue?
- Do MVF injections suppress apoptosis within the transition zone between viable and necrotic flap tissue?
- Do MVF injections influence the inflammatory reaction within the transition zone between viable and necrotic flap tissue?

5. ORIGINAL ARTICLES

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Article

Bromelain Protects Critically Perfused Musculocutaneous Flap Tissue from Necrosis

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Abstract: Bromelain has previously been shown to prevent ischemia-induced necrosis in different types of tissues. In the present study, we, therefore, evaluated for the first time, the tissue-protective effects of bromelain in musculocutaneous flaps in mice. Adult C57BL/6N mice were randomly assigned to a bromelain treatment group and a control group. The animals were treated daily with intraperitoneal injections of 20 mg/kg bromelain or saline (control), starting 1 h before the flap elevation throughout a 10-day observation period. The random-pattern musculocutaneous flaps were raised on the backs of the animals and mounted into a dorsal skinfold chamber. Angiogenesis, nutritive blood perfusion and flap necrosis were quantitatively analyzed by means of repeated intravital fluorescence microscopy over 10 days after surgery. After the last microscopy, the flaps were harvested for additional histological and immunohistochemical analyses. Bromelain reduced necrosis of the critically perfused flap tissue by ~25%. The bromelain-treated flaps also exhibited a significantly higher functional microvessel density and an elevated formation of newly developed microvessels in the transition zone between the vital and necrotic tissues when compared to the controls. Immunohistochemical analyses demonstrated a markedly lower invasion of the myeloperoxidase-positive neutrophilic granulocytes and a significantly reduced number of cleaved caspase 3-positive apoptotic cells in the transition zone of bromelain-treated musculocutaneous flaps. These findings indicate that bromelain prevents flap necrosis by maintaining nutritive tissue perfusion and by suppressing ischemia-induced inflammation and apoptosis. Hence, bromelain may represent a promising compound to prevent ischemia-induced flap necrosis in clinical practice.

Keywords: bromelain; phytochemicals; random-pattern flap; necrosis; angiogenesis; nutrition; microcirculation; dorsal skinfold chamber; intravital fluorescence microscopy

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1. Introduction

The use of phytochemicals has a long-standing tradition in health care. Even nowadays, they play an important role in treating various ailments due to their high therapeutic efficiency and acceptability by patients with different pathologies. Up to 50% of the presently used drugs are based on molecules of natural origins, highlighting the fact that phytochemicals are still a valuable resource in modern medicine [1,2].

The enzyme complex bromelain is obtained from pineapple stems (*Ananas comosus*) and represents one example of a well-known phytochemical that was initially used as an over-the-counter anti-inflammatory agent but has now proven to be useful for various other indications [3–5]. In fact, bromelain has been applied in the therapy of hematomas, rheumatoid arthritis, thrombophlebitis, oral inflammation, diabetic ulcers, rectal and perirectal inflammation, angina pectoris, bronchitis, sinusitis, surgical traumas and pyelonephritis [6,7]. In all these cases, orally administered bromelain could reduce pain and swelling

and facilitate faster healing [6,8]. The possible mechanisms mediating these beneficial effects are the dose-dependent modulation of bradykinin secretion [9], an improved microcirculatory flow due to the fibrinolytic and anticoagulant activity of the enzymes [10], the downregulation of inflammatory mediators [11] and the modulation of leukocyte–endothelial cell interactions [12]. Interestingly, bromelain has also been shown to protect hepatocytes from apoptosis after warm ischemia [13].

Due to the combination of anti-inflammatory, anti-apoptotic and anti-thrombotic properties, bromelain may be a valuable therapeutic compound to prevent ischemic damage in tissue flaps. Such flaps can consist of one or more tissue subtypes and are either raised on a vascular axis or as so-called random-pattern flaps. In the case of the random-pattern flaps, the blood perfusion is provided by the dermal plexus and/or musculocutaneous arterioles that pass through the base of the flaps [14]. The decreased arterial inflow places the tissue zones distant to the flap base at risk for ischemic complications, such as wound breakdown or necrosis. Depending on their thickness, tissue composition and regions of the body, random-pattern flaps are, therefore, planned according to certain length-to-width-ratios [15,16]. Furthermore, comorbidities like diabetes or lifestyle factors, such as active smoking habits, have to be considered in the planning of a flap. Nonetheless, despite careful preoperative planning, the occurrence rates of ischemic flap complications remain high. Pestana et al. [17] reported that in mastectomy flaps, more than 70% of the examined flaps demonstrated poor perfusion, as assessed by an intraoperative fluorescent angiography. Wound breakdown and flap necrosis, which results from such inadequate perfusion, will negatively impact patient morbidity and markedly increase treatment costs. Hence, promoting flap survival and preventing ischemia-induced tissue damage may considerably improve the clinical outcome of flap surgery and eventually benefit patient care in plastic surgery.

In the present study, we, therefore, wanted to analyze whether bromelain exerts beneficial tissue-protective effects on surgical flaps. For this purpose, we prepared random-pattern musculocutaneous flaps in the dorsal skinfold chamber of mice and administered daily 20 mg/kg bromelain or saline (control) by intraperitoneal injection starting 1 h before the flap elevation throughout a 10-day observation period. Vascularization, inflammation and flap necrosis were subsequently analyzed using intravital fluorescence microscopy, histology and immunohistochemistry.

2. Materials and Methods

2.1. Animals

The animal experiments were approved by the local governmental animal protection committee (permit number: 10/2020) and were conducted in accordance with the European legislation on the protection of animals (Directive 2010/63/EU) and the NIH Guidelines on the Care and Use of Laboratory Animals (NIH publication #85-23 Rev. 1985).

A total of 14 male C57BL/6N wild-type mice (Institute for Clinical and Experimental Surgery, Saarland University, Homburg, Germany) were used for the present study. The animals had an age of 12–24 weeks and a body weight of 26–30 g. During the experiments, the animals were kept in individual cages at a room temperature of 22–24 °C, with a relative humidity of 55–60% and a 12 h day–night cycle. Access to standard pellet chow (Altromin, Lage, Germany) and tap water was unrestricted.

2.2. Bromelain Treatment

Based on the study of Juhasz et al. [18], a daily intraperitoneal dose of 20 mg/kg bromelain (Bio Protect BV, Kerkrade, The Netherlands), dissolved in isotonic saline solution, was chosen for the present study because it has been shown to be effective and well-tolerated by animals. Seven mice were each randomly assigned to a bromelain group and a control group, which received daily intraperitoneal saline injections of an equal volume starting 1 h before the flap elevation throughout a 10-day observation period.

2.3. Anesthesia

The mice were placed under general anesthesia for the surgical elevation of the flap, the implantation of the dorsal skinfold chamber, and the subsequent intravital fluorescence microscopy. For this purpose, the animals received an intraperitoneal injection of ketamine (100 mg/kg of body weight; Ursotamin®; Serumwerke Bernburg, Bernburg, Germany) and xylazine (12 mg/kg of body weight; Rompun®; Bayer, Leverkusen, Germany). To prevent postoperative pain, all animals were additionally treated with subcutaneously injected buprenorphine hydrochloride (0.01 mg/kg of body weight; Temgesic®; RB Pharmaceuticals Limited, Slough, UK).

2.4. Dorsal Skinfold Chamber-Flap Model

A musculocutaneous flap was elevated on the back of each animal and mounted into a dorsal skinfold chamber (Irola Industriekomponenten GmbH & Co. KG, Schonach, Germany), as described previously in detail [19]. This model allows for repeated intravital fluorescence microscopy to study the microcirculation within the flap tissue (Figure 1A). In brief, after depilation of the dorsal skin, a musculocutaneous flap, measuring 15 mm (width) × 11 mm (length), was elevated perpendicular to the spine. The lateral wound margins were sutured back to the wound bed, and the dorsal skinfold, including the musculocutaneous flap, was fixed to one chamber frame. The second frame was covered with adhesive insulation foam to adequately seal the chamber and was then mounted to its counterpart. Accordingly, the flap was finally sandwiched between the two chamber frames, making it accessible for microscopic imaging through the observation window of the chamber. The window was subsequently closed with a cover glass that was fixed by means of a snap ring. Due to the chosen width-to-length-ratio, the distal portion of the flap was subject to acute persistent ischemia and the tissue developed roughly 50% necrosis without treatment (Figure 1B). After the preparation, the animals could recover for 24 h from anesthesia and surgery before the first microscopy. All animals tolerated the surgical interventions well, as evidenced by their normal food intake and behavior during the remaining observation period.

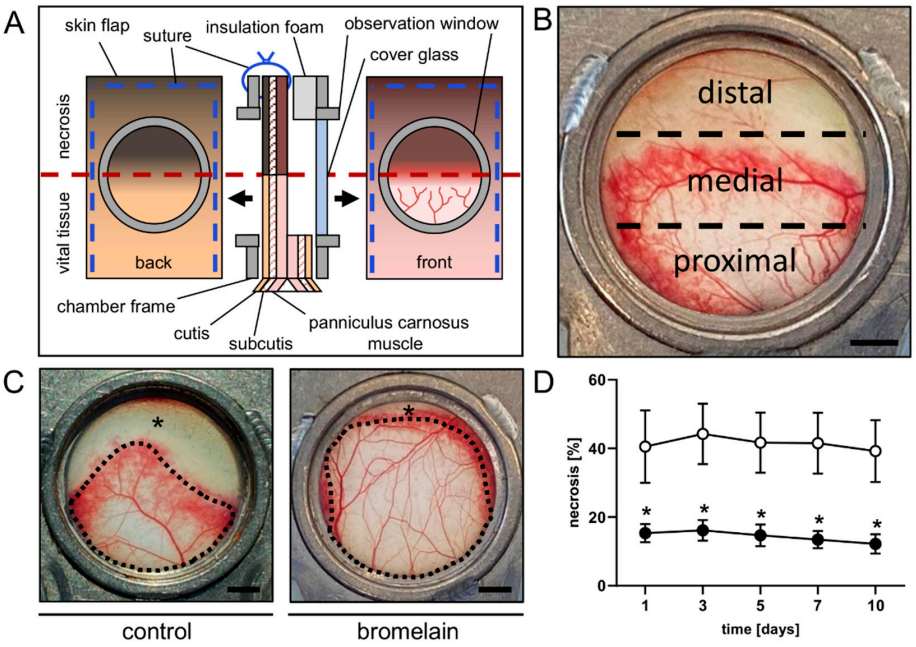

Figure 1. (**A**) Schematic cross-section of the musculocutaneous flap sandwiched between the symmetric titanium frames of the dorsal skinfold chamber. The observation window in one of the chamber frames

provides microscopic access to the flap tissue, consisting of the panniculus carnosus muscle, subcutis and cutis. (**B**) Macroscopic image of the observation window of an untreated control mouse on day 5 after flap elevation, showing three distinct flap zones: a proximal zone consisting of well-perfused vital tissue, a medial transition zone and a distal necrotic zone. Scale bar: 2 mm. (**C**) Macroscopic images of the observation window of an untreated control mouse and a bromelain-treated mouse, displaying a significant difference in vital (borders marked by dotted line) and necrotic tissue (asterisks) on day 5 after flap elevation. Scale bar: 2 mm. (**D**) Necrosis [%] of flaps in bromelain-treated mice (black circles, $n = 7$) and untreated controls (white circles, $n = 7$) on days 1, 3, 5, 7 and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM. * $p < 0.05$ vs. control.

2.5. Intravital Fluorescence Microscopy

Intravital fluorescence microscopy was performed on days 1, 3, 5, 7 and 10 after the flap elevation. For this purpose, the anesthetized mice were positioned on a plexiglass stage and received 0.1 mL of 5% fluorescein isothiocyanate (FITC)-labeled dextran (150,000 Da; Sigma-Aldrich, Taufkirchen, Germany) in the retrobulbar venous plexus for plasma staining. The chamber window was then placed under a Zeiss Axiotech fluorescence epi-illumination microscope (Zeiss, Oberkochen, Germany) and the flap microcirculation was recorded on DVD for the offline analysis. The microscopy was performed at a constant room temperature of 22 °C. An overview of the chamber was recorded for the planimetric measurement of the perfused tissue surface area at the beginning of each microscopy. Each flap was divided into three observational zones: proximal, medial and distal to the flap base (Figure 1B). Two regions of interest (ROI) were chosen per zone, containing an arterio-venous bundle that could be identified by its morphology during each microscopy for the repeated measurements. Two adjacent capillary fields were recorded per ROI. The no-longer-perfused ROI were documented with microscopic images for as long as they could be identified. One ROI was recorded additionally within the medial transition zone between the perfused and non-perfused tissue to examine the new vessel formation.

The microscopic images were analyzed offline by means of the analysis system, CapImage (Version 8.5, Zeintl, Heidelberg, Germany). The rate of necrosis expressed in % was determined as 100-(perfused surface area/total chamber surface area ×100). Per capillary field, the functional capillary density (FCD) was measured and expressed in cm/cm^2. Within each ROI, the microhemodynamic parameters were measured in the arterioles, capillaries and venules. The vessel diameters (D) were measured perpendicular to the vessel path in µm. Using the line shift method, the centerline red blood cell (RBC) velocity (V) was assessed [20]. The volumetric blood flow (VQ) was calculated from V and D as $VQ = \pi \times \left(\frac{D}{2}\right)^2 \times \frac{V}{K}$ with K (=1.6) representing the Baker–Wayland factor considering the parabolic velocity profile of the blood in microvessels and expressed in pL/s [21]. Angiogenesis within the transition zone was assessed by quantifying the density of newly formed microvessels, which was expressed in cm/cm^2. The newly formed microvessels could be clearly distinguished by their irregular and entangled configuration from the straight, parallelly arranged capillaries of the panniculus carnosus muscle [22].

2.6. Histology and Immunohistochemistry

Tissue samples of each flap were fixed in formalin and embedded in paraffin. Three-µm-thick sections were then sliced off the processed tissue. Hematoxylin and eosin (HE) staining of individual sections was performed following standard protocol. Sections were subsequently assessed using a BX60 microscope (Olympus, Hamburg, Germany) and the imaging software cellSens Dimension 1.11 (Olympus).

For the immunofluorescent detection of microvessels, sections were stained with a monoclonal rat-anti-mouse antibody against the endothelial cell marker, CD31 (1:100; dianova GmbH, Hamburg, Germany), and with a polyclonal rabbit-anti-human antibody against the microvascular smooth-muscle cell marker, α-smooth muscle actin (α-SMA), (1:100; Abcam, Cambridge, UK) as primary antibodies. A goat-anti-rat IgG-Alexa555

antibody (1:200; Thermo Fisher Scientific, Karlsruhe, Germany) and a goat-anti-rabbit IgG-Alexa488 antibody (1:200; Thermo Fisher Scientific) served as the secondary antibodies. On each section, cell nuclei were stained with Hoechst 33,342 (2 µg/mL; Sigma-Aldrich) to merge the images exactly. The stained sections served for the assessment of the microvessel density (all $CD31^+$ microvessels per high-power field (HPF)) and the fraction of $CD31^+/\alpha$-SMA^+ microvessels (in %) in two randomized HPFs at the flap base (proximal zone) and in the medial transition zone, where the border between the vital and necrotic tissues was detectable.

For the immunohistochemical detection of the myeloperoxidase-positive (MPO^+) neutrophilic granulocytes and cleaved caspase (Casp)-3^+ cells undergoing apoptosis, additional sections were used. Antigens in the samples were demasked by citrate buffer and the unspecific binding sites were blocked using goat serum. Cells were stained by incubation with a polyclonal rabbit antibody against MPO (1:100; Abcam, Cambridge, UK) or a monoclonal rabbit antibody against Casp-3 (1:100; Cell signaling Technology, Danvers, MA, USA) as primary antibodies, followed by a biotinylated goat anti-rabbit IgG antibody (ready-to-use; Abcam) as a secondary antibody. The biotinylated antibody was detected with peroxidase-labeled streptavidin (ready-to-use; Abcam). The used chromogen was 3-amino-9-ethylcarbazole (Abcam). The counterstaining was performed using Mayer's hemalum (Merck, Darmstadt, Germany). The stained cells were counted in two randomized HPFs in the proximal and medial transition zones of the flaps.

2.7. Statistical Analysis

Data were tested for normal distribution and equal variance. Afterwards, the two groups were analyzed for differences using the unpaired Student's t-test (GraphPad Prism 9; GraphPad Software, San Diego, CA, USA). A Mann–Whitney rank-sum test was used in the case of non-parametric data. All values are expressed as means ± standard error of the mean (SEM) and the statistical significance was accepted for a value of $p < 0.05$.

3. Results

3.1. Intravital Fluorescence Microscopy

The flap's survival and perfusion within the dorsal skinfold chambers were analyzed using repeated intravital fluorescence microscopy. The flaps of bromelain-treated animals exhibited a significantly lower necrosis rate of ~12–15% throughout the 10-day observation period when compared to the flaps of untreated controls, which presented with a necrosis rate of ~39–44% (Figure 1C,D). This lower flap necrosis rate was associated with a significantly higher FCD in all flap zones over the entire course of the experiments (Figure 2). In the proximal and medial zones of the bromelain-treated flaps, the FCD was ~250–300 cm/cm^2, whereas the distal zone exhibited an FCD of ~200 cm/cm^2 (Figure 2A–D). In contrast, the FCD in the proximal and medial zones of untreated flaps was markedly reduced (~150–200 cm/cm^2) and only measurable in the distal zone on day 1 (~85 cm/cm^2) (Figure 2A–D).

Additionally, the diameter and centerline RBC velocities in arterioles, capillaries and venules of the flaps were measured to calculate the volumetric blood flow. In both groups, this microhemodynamic parameter slightly increased over the course of the observation period in all vessel types within the proximal and medial zones of the flaps (Table 1). In line with the data on flap necrosis, no perfused ROIs could be detected in the distal flap zone in untreated mice after day 1 (Table 1). Noteworthy, the bromelain-treated animals generally showed higher volumetric blood flows, particularly in the medial zone of the flaps (Table 1).

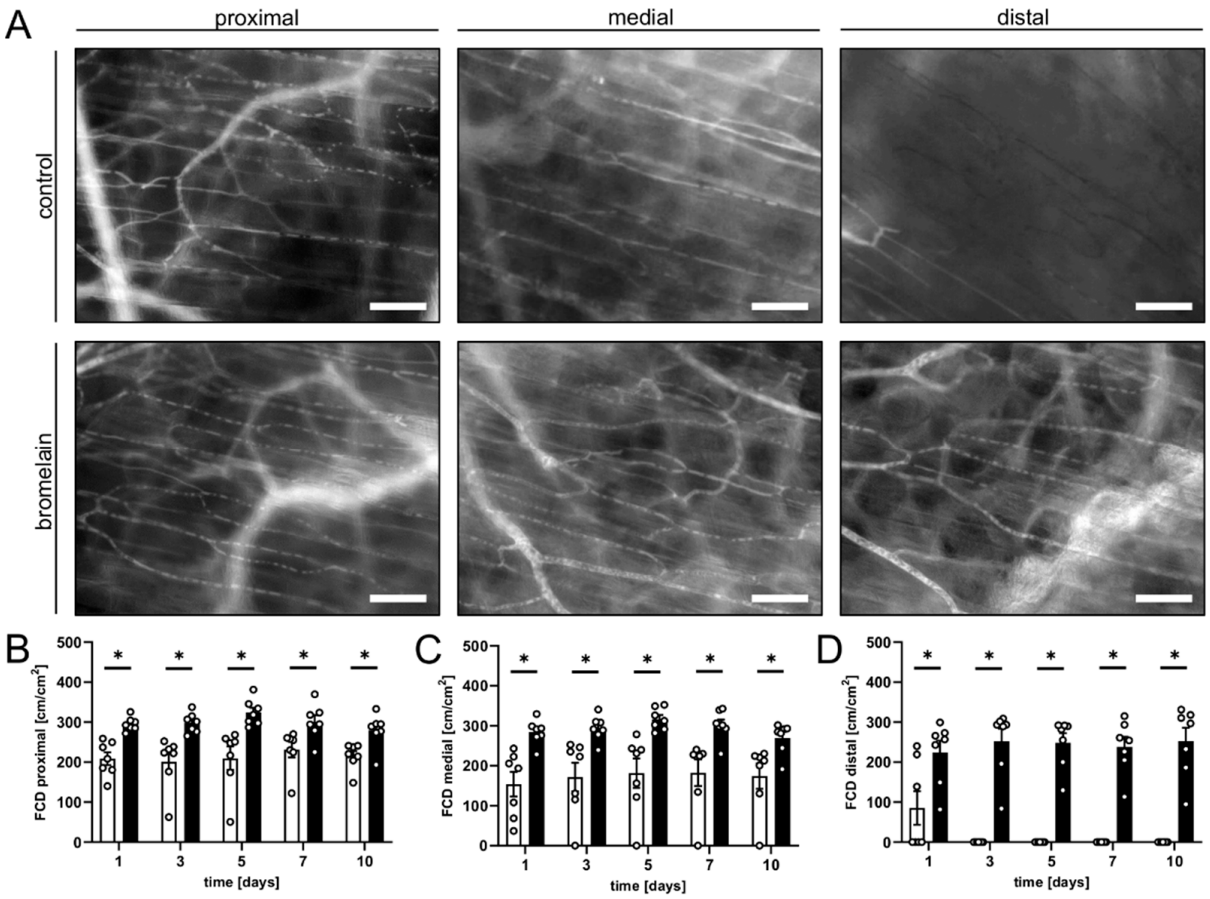

Figure 2. (**A**) Intravital fluorescent microscopic images of the proximal, medial and distal zones of flaps in an untreated control mouse and a bromelain-treated mouse on day 5 after flap elevation. Scale bar: 50 µm. (**B**–**D**) FCD [cm/cm^2] in the proximal (**B**), medial (**C**) and distal zones (**D**) of flaps in bromelain-treated mice (black bars, $n = 7$) and untreated controls (white bars, $n = 7$) on days 1, 3, 5, 7 and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM. * $p < 0.05$ vs. control.

The new blood vessel formation was examined in the transition zone between the vital and necrotic flap tissue throughout the entire observation period. In both groups, the flap tissue displayed typical changes in the capillary architecture within this zone, starting on days 3 to 5. The capillaries dilated and exhibited irregular diameters. In addition, angiogenic sprouts grew out of the pre-existing and horizontally arranged microvessels (Figure 3A,B). Of note, the density of the neovessels was significantly higher in the flaps of bromelain-treated mice on days 7 and 10 when compared to the flaps in control animals (Figure 3C).

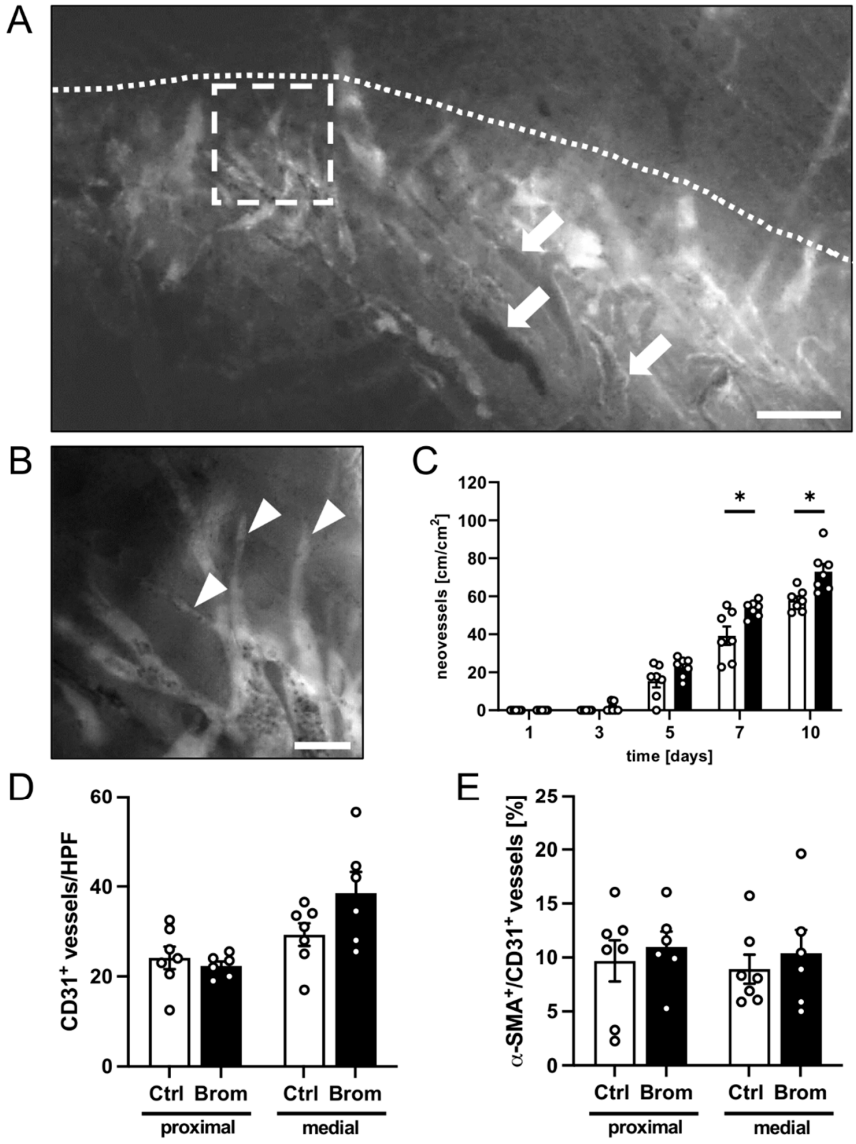

Figure 3. (**A**) Intravital fluorescent microscopic images of the medial zone of a flap in a bromelain-treated mouse on day 10. Note the transition zone with dilated vessels (arrows) adjacent to the necrotic tissue (border marked by a dotted line). Scale bar: 200 µm. (**B**) Higher magnification of insert in image A (borders marked by a broken line), which displays angiogenic sprouts (arrowheads) developing from pre-existing perfused vessels. Scale bar: 50 µm. (**C**) Neovessels [cm/cm^2] in the medial transition zones of flaps in bromelain-treated mice (black bars, $n = 7$) and untreated controls (white bars, $n = 7$) on days 1, 3, 5, 7 and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. (**D**) CD31$^+$ vessels/HPF in the proximal and medial zones of flaps in bromelain-treated mice (Brom; black bars, $n = 6$) and untreated controls (Ctrl; white bars, $n = 7$) on day 10 after flap elevation, as assessed by immunohistochemistry. (**E**) Percentage of α-SMA$^+$ vessels out of all CD31$^+$ vessels per HPF in the proximal and medial zones of flaps in bromelain-treated mice (Brom; black bars, $n = 6$) and untreated controls (Ctrl; white bars, $n = 7$) on day 10 after flap elevation, as assessed by immunohistochemistry. Means ± SEM. * $p < 0.05$ vs. control.

Table 1. Volumetric blood flow [pL/s] of arterioles, capillaries and venules in the proximal, medial and distal zones of flaps in untreated control mice (Ctrl; $n = 7$) and bromelain-treated mice (Brom; $n = 7$) on days 1, 3, 5, 7 and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM. * $p < 0.05$ vs. control.

Volumetric Blood Flow [pL/s]		d1	d3	d5	d7	d10
Arterioles						
proximal	**Ctrl**	458 ± 93	598 ± 158	814 ± 152	1145 ± 230	1272 ± 160
	Brom	790 ± 86 *	807 ± 127	960 ± 146	1459 ± 196	1634 ± 160
medial	**Ctrl**	437 ± 96	603 ± 117	733 ± 90	1013 ± 152	1085 ± 159
	Brom	687 ± 107	1036 ± 91 *	1302 ± 165 *	1574 ± 120 *	1550 ± 188
distal	**Ctrl**	485 ± 149	-	-	-	-
	Brom	538 ± 116	962 ± 237	1141 ± 276	1203 ± 261	1540 ± 248
Capillaries						
proximal	**Ctrl**	3 ± 0	3 ± 0	4 ± 1	5 ± 1	6 ± 1
	Brom	4 ± 0	4 ± 0 *	4 ± 1	6 ± 1	7 ± 1
medial	**Ctrl**	3 ± 0	3 ± 0	4 ± 1	5 ± 1	6 ± 1
	Brom	4 ± 0	4 ± 0 *	4 ± 1	6 ± 1	7 ± 1
distal	**Ctrl**	2 ± 1	-	-	-	-
	Brom	2 ± 0	4 ± 0	5 ± 1	6 ± 1	6 ± 1
Venules						
proximal	**Ctrl**	383 ± 47	502 ± 89	667 ± 100	776 ± 137	1233 ± 517
	Brom	773 ± 87 *	1167 ± 131 *	1570 ± 410 *	1894 ± 536 *	1876 ± 397
medial	**Ctrl**	333 ± 47	452 ± 74	691 ± 140	897 ± 200	779 ± 266
	Brom	833 ± 158 *	1265 ± 146 *	1766 ± 389 *	2009 ± 362 *	1650 ± 433
distal	**Ctrl**	146 ± 41	-	-	-	-
	Brom	472 ± 171	1012 ± 308	1387 ± 326	1268 ± 301	1859 ± 337

3.2. Histological and Immunohistochemical Analysis

At the end of the in vivo experiments, the flap tissue was histologically analyzed to assess the ischemia-induced morphological changes. The HE-stained sections were used for the identification of the transition zone between the proximal and the distal flap tissue. Notably, the distal zone was completely necrotic and, therefore, was excluded from further immunohistochemical analyses.

The quantification of the $CD31^+$ microvessels revealed no significant differences between the groups in the proximal zone and the transition zone of the flaps, even though the observed number of $CD31^+$ microvessels was slightly increased in the transition zone of bromelain-treated flaps (Figure 3D). The percentage of α-SMA^+ arterioles out of all the $CD31^+$ vessels did not show marked differences between the groups in the flaps' proximal zone and transition zone (Figure 3E).

The identification of MPO^+ neutrophilic granulocytes revealed, in contrast to the proximal zone, a massive neutrophilic cell invasion in the medial transition zones of the flaps in both bromelain-treated and untreated mice (Figure 4A,B). Of interest, this inflammatory reaction was markedly reduced in the bromelain-treated animals, as indicated by a significantly lower number of MPO^+ cells/HPF when compared to controls (Figure 4B).

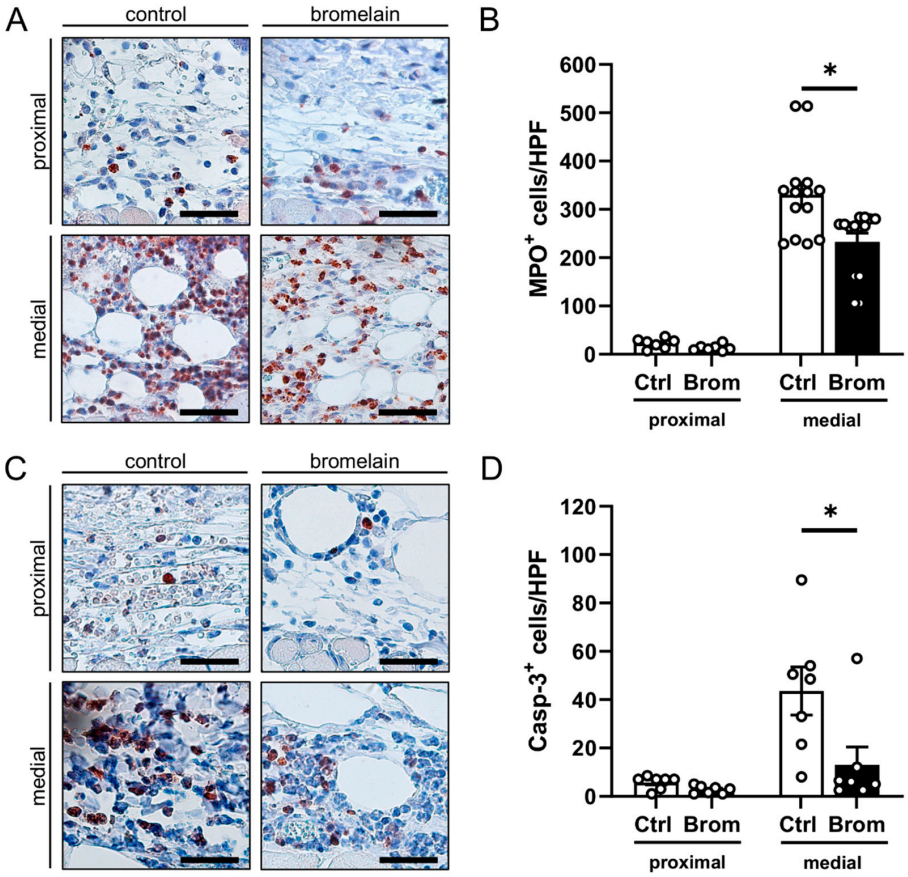

Figure 4. (**A**) Microscopic images of immunohistochemical sections of the proximal and medial zones in flaps of an untreated control mouse and a bromelain-treated mouse on day 10 after flap elevation. The sections were stained with an antibody against the neutrophilic granulocyte marker, MPO. Scale bar: 50 µm. (**B**) MPO^+ cells/HPF in the proximal and medial zones of flaps in bromelain-treated mice (Brom; black bars, $n = 7$) and untreated controls (Ctrl; white bars, $n = 7$) on day 10 after flap elevation, as assessed by immunohistochemistry. (**C**) Microscopic images of immunohistochemical sections of the proximal and medial zones of flaps in an untreated control mouse and a bromelain-treated mouse on day 10 after flap elevation. The sections were stained with an antibody against the apoptotic marker, Casp-3. Scale bar: 50 µm. (**D**) $Casp\text{-}3^+$ cells/HPF in the proximal and medial zones of flaps in bromelain-treated mice (Brom; black bars, $n = 7$) and untreated controls (Ctrl; white bars, $n = 7$) on day 10 after flap elevation, as assessed by immunohistochemistry. Means ± SEM. * $p < 0.05$ vs. control.

Moreover, apoptotic cells were identified by means of immunohistochemical Casp-3 staining. In both groups, the proximal vital zone of the flaps only contained a few apoptotic cells (Figure 4C,D). In contrast, apoptotic cell death was markedly increased in the medial transition zone. However, the bromelain treatment significantly reduced the number of $Casp\text{-}3^+$ cells/HPF in this area when compared to the control treatment (Figure 4C,D).

4. Discussion

The development and clinical introduction of novel pharmacological compounds are often hindered by problems, such as low selectivity for target cells or high toxicity against normal cells. Therefore, phytochemicals are continuously explored as possible therapeutics because they generally exert few unwanted harmful side effects and are well-tolerated by

patients [23]. Moreover, they bear the advantages of having a high availability and relative simplicity of acquisition [24].

The enzyme complex, bromelain, is one example of a phytochemical compound with several beneficial effects and few side effects, which have been used for various pathologies in the past. In our study, we demonstrated for the first time, that the anti-apoptotic, anti-inflammatory and anti-thrombotic effects of bromelain can also be observed in the critically perfused musculocutaneous flaps undergoing acute persistent ischemia. In fact, we could show that the rate of flap necrosis is significantly reduced by systemic bromelain administration, as the enzyme complex promotes nutritive tissue perfusion and suppresses inflammation and apoptotic cell death in the ischemically challenged flap tissue.

For our experiments, we used a perioperative administration protocol that would also be feasible under clinical conditions. Because flap surgery is often performed electively, bromelain administration, starting shortly before the flap elevation, could easily be implemented into the perioperative clinical routine. The effect of bromelain on the microcirculation and overall survival of critically perfused flaps undergoing necrosis, if kept untreated, was investigated in a modified murine dorsal skinfold chamber model. By combining the chamber technique with intravital fluorescence microscopy, repeated in vivo analyses of microvascular perfusion and blood vessel formation within a random-pattern musculocutaneous flap with clearly defined dimensions could be performed [19]. The results of the present study, which are consistent with the pleiotropic mode of action of most phytochemicals, suggest that in our experimental setting, bromelain targets not only one cellular mechanism, but rather modulates several pathways at once with synergistic effects, thus, adding to the overall improved outcome.

We observed that bromelain improves the nutritive perfusion of the flap tissue, as evidenced by a higher blood flow in all analyzed vessel types and flap zones in the bromelain-treated animals when compared to the untreated controls. In line with these findings, Bahde et al. [13] proved that bromelain increases the expression of endothelial nitric oxide (NO) synthase, causing vasodilatation and elevated perfusion in a model of warm hepatic ischemia in rats. In addition, bromelain has been shown to decrease the production of reactive oxygen species (ROS) that consume the vasodilator NO [25]. In the present study, the increased perfusion resulted in a maintained microcirculation with a significantly higher FCD in bromelain-treated flaps.

Furthermore, we observed a higher number of newly formed microvessels in the transition zone of bromelain-treated flaps. This effect was confirmed in our immunohistochemical analyses of the flap tissue, where we also observed an increased number of $CD31^+$ microvessels in the medial flap zone, though the difference was not proven to be statistically significant. These findings are in line with several studies suggesting a pro-angiogenic activity of bromelain [26,27]. However, on the other hand, there are also studies reporting that the enzyme exerts an anti-angiogenic effect on cancer cell lines [18,28,29]. Hence, further analyses are necessary to exactly clarify the regulatory function of bromelain in the process of angiogenesis.

In addition, the microperfusion of the flap tissue may have been enhanced by the well-known anti-thrombotic and fibrinolytic effects of bromelain [30,31]. In this context, it should be considered that the lower flow rate and the inflammatory reaction within the elevated flap tissue create a pro-thrombotic environment [32,33]. The resulting microthrombi occlude capillary vessels, which further aggravates the already inadequate tissue perfusion [33]. Of interest, Metzig et al. [30] showed that incubation with bromelain completely prevents the thrombin-induced aggregation of platelets. Furthermore, in high doses, the enzyme complex downregulates both the external and internal pathways of the blood clotting system, inhibits fibrin synthesis and increases serum fibrinolytic activity [10,34]. Taken together, all these bromelain effects may contribute to the prevention of microthrombi and, thus, to the improved perfusion of the flap tissue.

The pro-apoptotic effects of bromelain have been described in the context of cancer cells, where it suppresses proliferation and induces apoptosis through the activation of the

extracellular signal-regulated kinase (ERK)/AKT pathway [35]. In contrast, when used to prevent ischemic cell death, bromelain has been shown to decrease pro-apoptotic signaling pathways. For instance, Juhasz et al. [18] demonstrated an increased phosphorylation of Akt and FOXO3A after bromelain pretreatment in a murine cardiac ischemia model. This resulted in a significantly lower rate of apoptotic cardiomyocytes and a reduced infarct size, leading to an improved cardiac function after the ischemic insult. Similarly, we detected fewer apoptotic cells in the transition zone between the vital and necrotic tissues of bromelain-treated musculocutaneous flaps. This may also explain our observations that the bromelain-treated flaps contained more newly formed microvessels in their transition zones. In fact, it may be assumed that the cytoprotective effects of bromelain also increased the viability of microvessels in the transition zone, which could then serve as the origin for angiogenic vessel sprouts.

Finally, bromelain is also known to ameliorate inflammation, because it downregulates the expression of the transcription factor, nuclear factor (NF)-κB, which controls the expression of various pro-inflammatory enzymes and chemokines, such as cyclooxygenase-2, interleukin-6 or tumor necrosis factor-α [36,37]. Furthermore, the enzyme complex downregulates the expression of different immune cell surface markers that mediate the adhesion of intravascular leukocytes to the endothelium and their subsequent invasion into the surrounding tissue [12,38]. In the present study, the latter anti-inflammatory mechanism could also be observed in the critically perfused flap tissue, where the bromelain treatment resulted in a significantly lower number of invading MPO^+ neutrophilic granulocytes into the transition zone when compared to controls.

Though our findings are promising, further research is warranted to test the efficacy of bromelain for the prevention of flap necrosis in different settings because flap survival is influenced by various factors, such as age and gender [39,40]. As capillary and artery numbers can decline in aging tissue, it may, for example, be interesting to assess whether the positive effects of bromelain on flap tissue are reproducible in aged mice [41,42]. Harder et al. [40] demonstrated that aging decreases the vascular reactivity in tissue flaps. Thus, the beneficial effect of bromelain on angiogenesis may be an interesting approach to compensate for this decreased vascular reactivity in aging patients undergoing flap surgery.

5. Conclusions

The present study demonstrates that perioperative-systemic bromelain administration effectively protects critically perfused flap tissue from necrosis. In fact, the enzyme is able to maintain nutritive tissue perfusion by suppressing ischemia-induced inflammation and apoptosis. When compared to other therapeutic approaches, bromelain may bear the considerable advantage that it can be easily implemented into standard clinical routines without causing severe side effects, making it a possible resource for fragile patients, such as the elderly. Therefore, future clinical trials should evaluate whether the herein observed high effectiveness of bromelain in preventing flap tissue necrosis can also be reproduced in clinical practice. If such clinical trials are successful, bromelain may be a promising therapeutic compound to reduce ischemic flap complications and related patient morbidity.

Author Contributions: A.W. performed the experiments; A.W. and M.W.L. designed experiments and analyzed and interpreted the data; A.W. and M.W.L. prepared the figures and wrote the manuscript; Y.H., D.S. and M.D.M. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Clark, A.M. Natural Products as a Resource for New Drugs. *Pharm. Res.* **1996**, *13*, 1133–1141. [CrossRef]
2. Cheuka, P.M.; Mayoka, G.; Mutai, P.; Chibale, K. The Role of Natural Products in Drug Discovery and Development against Neglected Tropical Diseases. *Molecules* **2017**, *22*, 58. [CrossRef] [PubMed]
3. Braun, J.M.; Schneider, B.; Beuth, H.J. Therapeutic Use, Efficiency and Safety of the Proteolytic Pineapple Enzyme Bromelain-POS®in Children with Acute Sinusitis in Germany. *In Vivo* **2005**, *19*, 417–421. [PubMed]
4. Taub, S.J. The Use of Ananase in Sinusitis. A Study of 60 Patients. *Eye Ear Nose Throat Mon.* **1966**, *45*, 96.
5. Chakraborty, A.J.; Mitra, S.; Tallei, T.E.; Tareq, A.M.; Nainu, F.; Cicia, D.; Dhama, K.; Emran, T.B.; Simal-Gandara, J.; Capasso, R. Bromelain a Potential Bioactive Compound: A Comprehensive Overview from a Pharmacological Perspective. *Life* **2021**, *11*, 317. [CrossRef] [PubMed]
6. Pavan, R.; Jain, S.; Shraddha; Kumar, A. Properties and Therapeutic Application of Bromelain: A Review. *Biotechnol. Res. Int.* **2012**, *2012*, 976203. [CrossRef] [PubMed]
7. Maurer, H.R. Bromelain: Biochemistry, Pharmacology and Medical Use. *Cell. Mol. Life Sci.* **2001**, *58*, 1234–1245. [CrossRef] [PubMed]
8. Taussig, S.J.; Batkin, S. Bromelain, the Enzyme Complex of Pineapple (Ananas Comosus) and Its Clinical Application. An Update. *J. Ethnopharmacol.* **1988**, *22*, 191–203. [CrossRef]
9. Kumakura, S.; Yamashita, M.; Tsurufuji, S. Effect of Bromelain on Kaolin-Induced Inflammation in Rats. *Eur. J. Pharmacol.* **1988**, *150*, 295–301. [CrossRef]
10. Azarkan, M.; González, M.M.; Esposito, R.C.; Errasti, M.E. Stem Bromelain Proteolytic Machinery: Study of the Effects of Its Components on Fibrin (Ogen) and Blood Coagulation. *Protein Pept. Lett.* **2020**, *27*, 1159–1170. [CrossRef] [PubMed]
11. Onken, J.E.; Greer, P.K.; Calingaert, B.; Hale, L.P. Bromelain Treatment Decreases Secretion of Pro-Inflammatory Cytokines and Chemokines by Colon Biopsies in Vitro. *Clin. Immunol.* **2008**, *126*, 345–352. [CrossRef] [PubMed]
12. Fitzhugh, D.J.; Shan, S.; Dewhirst, M.W.; Hale, L.P. Bromelain Treatment Decreases Neutrophil Migration to Sites of Inflammation. *Clin. Immunol.* **2008**, *128*, 66–74. [CrossRef] [PubMed]
13. Bahde, R.; Palmes, D.; Minin, E.; Stratmann, U.; Diller, R.; Haier, J.; Spiegel, H.U. Bromelain Ameliorates Hepatic Microcirculation After Warm Ischemia. *J. Surg. Res.* **2007**, *139*, 88–96. [CrossRef] [PubMed]
14. Deramo, P.; Rose, J. *Flaps, Muscle and Musculocutaneous*; StatPearls Publishing: Treasure Island, FL, USA, 2021.
15. Saint-Cyr, M.; Wong, C.; Schaverien, M.; Mojallal, A.; Rohrich, R.J. The Perforasome Theory: Vascular Anatomy and Clinical Implications. *Plast. Reconstr. Surg.* **2009**, *124*, 1529–1544. [CrossRef] [PubMed]
16. Memarzadeh, K.; Sheikh, R.; Blohmé, J.; Torbrand, C.; Malmsjö, M. Perfusion and Oxygenation of Random Advancement Skin Flaps Depend More on the Length and Thickness of the Flap Than on the Width to Length Ratio. *Eplasty* **2016**, *16*, e12.
17. Pestana, I.A.; Jones, V.M.; Velazquez, C. Breast Reconstruction and Nipple-Sparing Mastectomy: Technical Modifications and Their Outcomes Over Time at an Academic Breast Center. *Ann. Plast. Surg.* **2021**, *86*, e81. [CrossRef]
18. Juhasz, B.; Thirunavukkarasu, M.; Pant, R.; Zhan, L.; Penumathsa, S.V.; Secor, E.R.; Srivastava, S.; Raychaudhuri, U.; Menon, V.P.; Otani, H.; et al. Bromelain Induces Cardioprotection against Ischemia-Reperfusion Injury through Akt/FOXO Pathway in Rat Myocardium. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *294*, H1365–H1370. [CrossRef]
19. Harder, Y.; Amon, M.; Erni, D.; Menger, M.D. Evolution of Ischemic Tissue Injury in a Random Pattern Flap: A New Mouse Model Using Intravital Microscopy. *J. Surg. Res.* **2004**, *121*, 197–205. [CrossRef]
20. De Vriese, A.S.; Verbeuren, T.J.; Vallez, M.O.; Lameire, N.H.; De Buyzere, M.; Vanhoutte, P.M. Off-Line Analysis of Red Blood Cell Velocity in Renal Arterioles. *J. Vasc. Res.* **2000**, *37*, 26–31. [CrossRef] [PubMed]
21. Baker, M.; Wayland, H. On-Line Volume Flow Rate and Velocity Profile Measurement for Blood in Microvessels. *Microvasc. Res.* **1974**, *7*, 131–143. [CrossRef]
22. Menger, M.D.; Lehr, H.A. Scope and Perspectives of Intravital Microscopy-Bridge over from In Vitro to In Vivo. *Immunol. Today* **1993**, *14*, 519–522. [CrossRef]
23. Hikisz, P.; Bernasinska-Slomczewska, J. Beneficial Properties of Bromelain. *Nutrients* **2021**, *13*, 4313. [CrossRef] [PubMed]
24. Che, C.T.; Zhang, H. Plant Natural Products for Human Health. *Int. J. Mol. Sci.* **2019**, *20*, 830. [CrossRef]
25. Neumayer, C.; Fügl, A.; Nanobashvili, J.; Blumer, R.; Punz, A.; Gruber, H.; Polterauer, P.; Huk, I. Combined Enzymatic and Antioxidative Treatment Reduces Ischemia-Reperfusion Injury in Rabbit Skeletal Muscle. *J. Surg. Res.* **2006**, *133*, 150–158. [CrossRef]
26. Shoba, E.; Lakra, R.; Kiran, M.S.; Korrapati, P.S. Fabrication of Core-Shell Nanofibers for Controlled Delivery of Bromelain and Salvianolic Acid B for Skin Regeneration in Wound Therapeutics. *Biomed. Mater.* **2017**, *12*, 035005. [CrossRef]

27. Shoba, E.; Lakra, R.; Kiran, M.S.; Korrapati, P.S. 3 D Nano Bilayered Spatially and Functionally Graded Scaffold Impregnated Bromelain Conjugated Magnesium Doped Hydroxyapatite Nanoparticle for Periodontal Regeneration. *J. Mech. Behav. Biomed. Mater.* **2020**, *109*, 103822. [CrossRef]
28. Rathnavelu, V.; Alitheen, N.B.; Sohila, S.; Kanagesan, S.; Ramesh, R. Potential Role of Bromelain in Clinical and Therapeutic Applications (Review). *Biomed. Rep.* **2016**, *5*, 283–288. [CrossRef] [PubMed]
29. Karlsen, M.; Hovden, A.O.; Vogelsang, P.; Tysnes, B.B.; Appel, S. Bromelain Treatment Leads to Maturation of Monocyte-Derived Dendritic Cells but Cannot Replace PGE 2 in a Cocktail of IL-1β, IL-6, TNF-α and PGE 2. *Scand. J. Immunol.* **2011**, *74*, 135–143. [CrossRef]
30. Metzig, C.; Grabowska, E.; Eckert, K.; Rehse, K.; Maurer, H.R. Bromelain Proteases Reduce Human Platelet Aggregation in Vitro, Adhesion to Bovine Endothelial Cells and Thrombus Formation in Rat Vessels in Vivo. *In Vivo* **1999**, *13*, 7–12.
31. Gläser, D.; Hilberg, T. The Influence of Bromelain on Platelet Count and Platelet Activity in Vitro. *Platelets* **2006**, *17*, 37–41. [CrossRef]
32. Fitzal, F.; DeLano, F.A.; Young, C.; Schmid-Schönbein, G.W. Early Capillary No-Reflow during Low-Flow Reperfusion after Hind Limb Ischemia in the Rat. *Ann. Plast. Surg.* **2002**, *49*, 170–180. [CrossRef] [PubMed]
33. Menger, M.D.; Laschke, M.W.; Amon, M.; Schramm, R.; Thorlacius, H.; Rücker, M.; Vollmar, B. Experimental Models to Study Microcirculatory Dysfunction in Muscle Ischemia-Reperfusion and Osteomyocutaneous Flap Transfer. *Langenbecks Arch Surg.* **2003**, *388*, 281–290. [CrossRef] [PubMed]
34. Errasti, M.E.; Prospitti, A.; Viana, C.A.; Gonzalez, M.M.; Ramos, M.v.; Rotelli, A.E.; Caffini, N.O. Effects on Fibrinogen, Fibrin, and Blood Coagulation of Proteolytic Extracts from Fruits of Pseudananas Macrodontes, Bromelia Balansae, and B. Hieronymi (Bromeliaceae) in Comparison with Bromelain. *Blood Coagul. Fibrinolysis* **2016**, *27*, 441–449. [CrossRef] [PubMed]
35. Romano, B.; Fasolino, I.; Pagano, E.; Capasso, R.; Pace, S.; de Rosa, G.; Milic, N.; Orlando, P.; Izzo, A.A.; Borrelli, F. The Chemopreventive Action of Bromelain, from Pineapple Stem (Ananas Comosus L.), on Colon Carcinogenesis Is Related to Antiproliferative and Proapoptotic Effects. *Mol. Nutr. Food Res.* **2014**, *58*, 457–465. [CrossRef] [PubMed]
36. Insuan, O.; Janchai, P.; Thongchuai, B.; Chaiwongsa, R.; Khamchun, S.; Saoin, S.; Insuan, W.; Pothacharoen, P.; Apiwatanapiwat, W.; Boondaeng, A.; et al. Anti-Inflammatory Effect of Pineapple Rhizome Bromelain through Downregulation of the NF-KB-and MAPKs-Signaling Pathways in Lipopolysaccharide (LPS)-Stimulated RAW264.7 Cells. *Curr. Issues Mol. Biol.* **2021**, *43*, 93–106. [CrossRef]
37. Kasemsuk, T.; Vivithanaporn, P.; Unchern, S. Anti-Inflammatory Effects of Bromelain in Lps-Induced Human U937 Macrophages. *Chiang Mai J. Sci.* **2018**, *45*, 299–307.
38. Hale, L.P.; Greer, P.K.; Sempowski, G.D. Bromelain Treatment Alters Leukocyte Expression of Cell Surface Molecules Involved in Cellular Adhesion and Activation. *Clin. Immunol.* **2002**, *104*, 183–190. [CrossRef] [PubMed]
39. Harder, Y.; Amon, M.; Wettstein, R.; Rücker, M.; Schramm, R.; Menger, M.D. Gender-Specific Ischemic Tissue Tolerance in Critically Perfused Skin. *Langenbeck's Arch. Surg.* **2010**, *395*, 33–40. [CrossRef] [PubMed]
40. Harder, Y.; Amon, M.; Georgi, M.; Scheuer, C.; Schramm, R.; Rücker, M.; Pittet, B.; Erni, D.; Menger, M.D. Aging Is Associated with an Increased Susceptibility to Ischaemic Necrosis Due to Microvascular Perfusion Failure but Not a Reduction in Ischaemic Tolerance. *Clin. Sci.* **2007**, *112*, 429–440. [CrossRef] [PubMed]
41. Chen, J.; Lippo, L.; Labella, R.; Tan, S.L.; Marsden, B.D.; Dustin, M.L.; Ramasamy, S.K.; Kusumbe, A.P. Decreased Blood Vessel Density and Endothelial Cell Subset Dynamics during Ageing of the Endocrine System. *EMBO J.* **2021**, *40*, e105242. [CrossRef]
42. Chen, J.; Sivan, U.; Tan, S.L.; Lippo, L.; de Angelis, J.; Labella, R.; Singh, A.; Chatzis, A.; Cheuk, S.; Medhghalchi, M.; et al. High-Resolution 3D Imaging Uncovers Organ-Specific Vascular Control of Tissue Aging. *Sci. Adv.* **2021**, *7*, eabd7819. [CrossRef] [PubMed]

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Microvascular Fragments Protect Ischemic Musculocutaneous Flap Tissue from Necrosis by Improving Nutritive Tissue Perfusion and Suppressing Apoptosis

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Abstract: Microvascular fragments (MVF) derived from enzymatically digested adipose tissue are functional vessel segments that have been shown to increase the survival rate of surgical flaps. However, the underlying mechanisms have not been clarified so far. To achieve this, we raised random-pattern musculocutaneous flaps on the back of wild-type mice and mounted them into dorsal skinfold chambers. The flaps were injected with MVF that were freshly isolated from green fluorescent protein-positive (GFP^+) donor mice or saline solution (control). On days 1, 3, 5, 7, and 10 after surgery, intravital fluorescence microscopy was performed for the quantitative assessment of angiogenesis, nutritive blood perfusion, and flap necrosis. Subsequently, the flaps were analyzed by histology and immunohistochemistry. The injection of MVF reduced necrosis of the ischemic flap tissue by ~20%. When compared to controls, MVF-injected flaps also displayed a significantly higher functional capillary density and number of newly formed microvessels in the transition zone, where vital tissue bordered on necrotic tissue. Immunohistochemical analyses revealed a markedly lower number of cleaved caspase-3^+ apoptotic cells in the transition zone of MVF-injected flaps and a significantly increased number of $CD31^+$ microvessels in both the flaps' base and transition zone. Up to ~10% of these microvessels were GFP^+, proving their origin from injected MVF. These findings demonstrate that MVF reduce flap necrosis by increasing angiogenesis, improving nutritive tissue perfusion, and suppressing apoptosis. Hence, the injection of MVF may represent a promising strategy to reduce ischemia-induced flap necrosis in future clinical practice.

Keywords: microvascular fragments; tissue engineering; random-pattern flap; necrosis; apoptosis; angiogenesis; microcirculation

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1. Introduction

Random-pattern flaps are frequently used to reconstruct tissue defects in plastic surgery. However, the length-to-width ratio of such flaps cannot exceed certain values, because otherwise distal flap areas become vulnerable to necrosis. This is due to an inadequate blood perfusion of the tissue, which causes oxidative stress and inflammation, as well as ischemic and apoptotic cell death [1,2]. Accordingly, maintaining and boosting tissue vascularization is vital to ensuring an optimal outcome in flap surgery [3]. A promising approach to achieve this goal is the use of adipose tissue-derived microvascular fragments (MVF) [4,5].

MVF are functional arteriolar, capillary, and venular vessel segments that can be isolated from adipose tissue using mechanical dissection and short-term enzymatic digestion [6]. These fragments exhibit a length of up to 150–200 µm and an intact vessel

morphology consisting of a central lumen with surrounding endothelial cells and stabilizing pericytes [7]. Moreover, they contain mesenchymal stem cells (MSC) within their physiological niche [8] and are a rich source of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [9,10]. Finally, MVF rapidly reform new blood-perfused microvascular networks after transplantation into tissue defects [11–13].

All these beneficial properties make the use of MVF a promising approach for vascularization with various applications in the field of tissue engineering and regenerative medicine [10,14,15]. In this context, they have also been shown to prevent necrosis of random-pattern skin flaps in rats [4,5]. However, the underlying mechanisms remain incompletely understood. Nakano et al. [5] speculated that the improved survival of the distal part of the flaps may be caused by early patent connections between transplanted MVF and the host microvasculature. Contrary to this assumption, Stone and Rathbone [4] did not detect a difference in the microvessel density between MVF-injected flaps and controls. Hence, they hypothesized that the increased flap survival may be rather promoted by MVF-associated stem cells.

To better understand the beneficial effects of MVF on flap tissue survival, we herein injected freshly isolated MVF from green fluorescent protein-positive (GFP^+) donor mice or saline solution (control) into random-pattern musculocutaneous flaps, which were mounted into the dorsal skinfold chamber of GFP^- wild-type mice. This GFP^+/GFP^- cross-over design allowed us to clarify in detail the fate of MVF after transplantation and to quantitatively assess angiogenesis, nutritive blood perfusion, inflammation, apoptosis, and necrosis of the flap tissue over a 10-day observation period by means of repeated intravital fluorescence microscopy as well as histology and immunohistochemistry.

2. Materials and Methods

2.1. Animals

All animal experiments were approved by the local governmental animal protection committee (permit number: 10/2020). The study was executed according to the European legislation on the protection of animals (Directive 2010/63/EU) and the NIH Guidelines on the Care and Use of Laboratory Animals (NIH publication #85-23 Rev. 1985).

A total of 24 mice were used for the present study. Sixteen animals were C57BL/6 wild-type mice (Institute for Clinical and Experimental Surgery, Saarland University, Homburg/Saar, Germany), and eight mice expressed GFP (C57BL/6-Tg (CAG-EGFP)1Osb/J; The Jackson Laboratory, Bar Harbor, ME, USA). GFP^+ animals were used for the experiments at an age of 30–52 weeks and a body weight of 30–35 g to serve as fat donors for the isolation of MVF. The remaining 16 animals exhibited an age of 12–24 weeks and a body weight of 26–30 g. On each animal, a random-pattern musculocutaneous flap was raised on the back and fixated within a dorsal skinfold chamber. The chamber-bearing animals were housed in individual cages at a room temperature of 22–24 °C, a relative humidity of 50–55%, and a 12 h day/night cycle for the duration of the experiments. The animals had unrestricted access to standard pellet chow (Altromin, Lage, Germany) and tap water.

2.2. MVF Isolation

MVF were isolated from the epididymal fat pads of male GFP^+ donor mice, as previously described in detail [6] (Figure 1A). The epididymal fat pads on both sides were surgically removed and placed into 10% Dulbecco's Modified Eagle's Medium (DMEM; 100 U/mL penicillin, 0.1 mg/mL streptomycin; Biochrom, Berlin, Germany). The fat tissue was then cleaned by rinsing it three times in phosphate-buffered saline (PBS). The excised fat tissue was then diced into small pieces and digested with the enzyme collagenase NB4G (0.5 U/mL; Serva Heidelberg, Germany) under gentle agitation and high humidity (37 °C, 5% CO_2) for 10 min. The digestion process was interrupted by neutralization with PBS containing 20% fetal calf serum (FCS), and the suspension of cells and vessels was subsequently incubated for 5 min at 37 °C. After the supernatant of floating fat was removed, the

remaining suspension was strained through a 300 µm filter, and the MVF were condensed to a pellet by a 5 min centrifugation at 120× *g*. The MVF pellet was suspended once again in 10 µL 0.9% NaCl for flap injection.

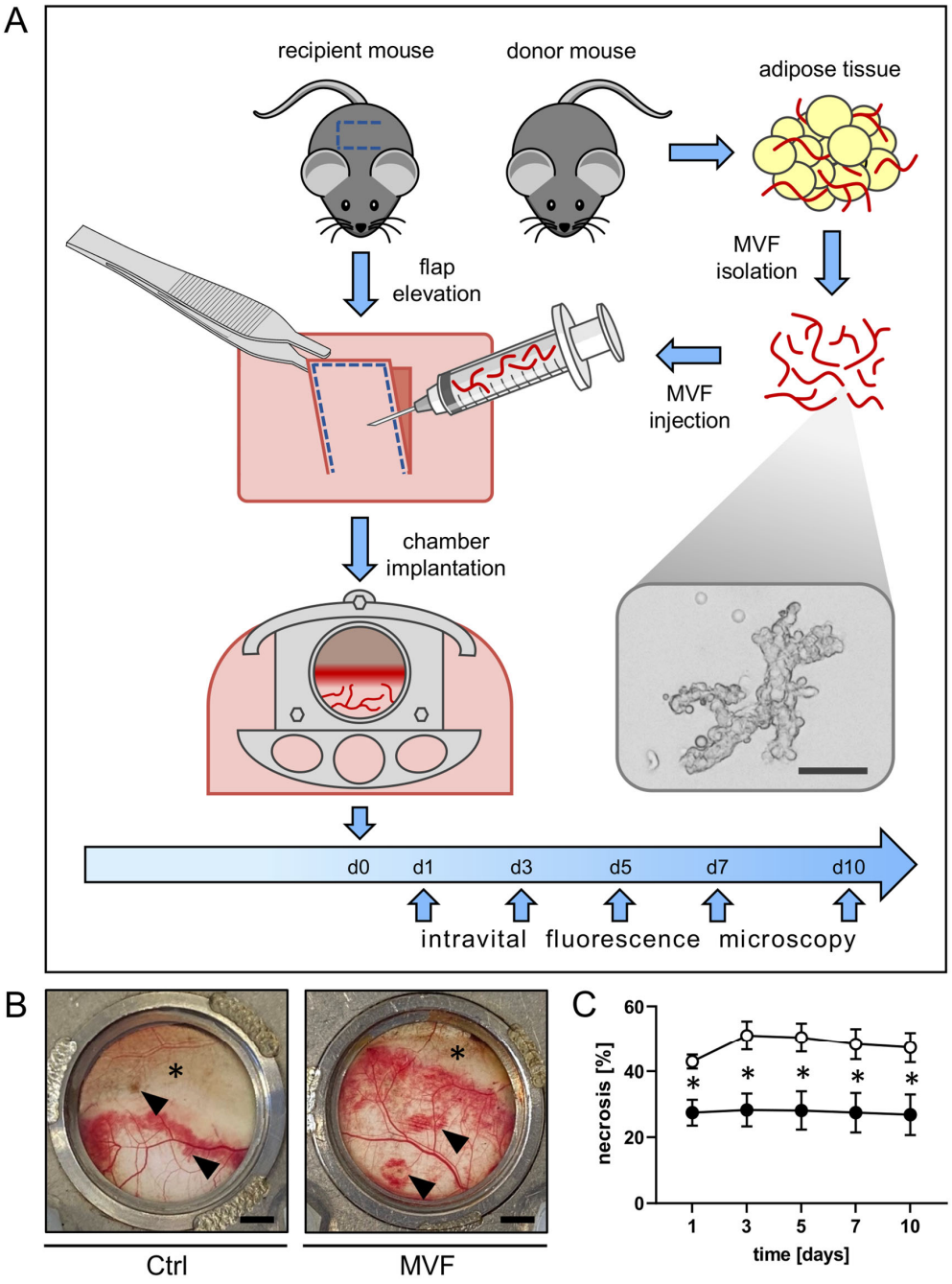

Figure 1. (**A**) Schematic depiction of the experimental setting. On day 0, a random-pattern flap was elevated on the back of a GFP$^-$ wild-type recipient mouse. Microvascular fragments (MVF; inset = microscopic image of a single MVF; scale bar: 20 µm) isolated from the epididymal fat pads of a GFP$^+$ donor mouse were injected into the flap and the dorsal skinfold chamber implantation was completed. Subsequently, repeated intravital fluorescence microscopy of the flap tissue was conducted

on days 1, 3, 5, 7, and 10. (**B**) Macroscopic images of the chamber observation windows of a vehicle-injected control mouse (Ctrl) and an MVF-injected animal (MVF) on day 5 after flap elevation. The distal flap tissue has undergone necrosis (marked by asterisks), and the injection sites have remained visible (marked by arrowheads). Scale bar: 2 mm. (**C**) Necrosis [%] of flaps in MVF-injected mice (black circles, $n = 8$) and vehicle-injected controls (white circles, $n = 8$) on days 1, 3, 5, 7, and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM. * $p < 0.05$ vs. control.

In line with previous studies [16], ~40.000 MVF could be isolated from each donor animal. For the present study, each flap was injected with the MVF from a single donor animal, as suggested by Nakano et al. [5].

2.3. Anesthesia

The surgical flap elevation and dorsal skinfold chamber implantation as well as the subsequent intravital fluorescent microscopic analyses were performed under general anesthesia by means of intraperitoneal injection of ketamine (100 mg/kg body weight; Ursotamin®; Serumwerke Bernburg, Bernburg, Germany) and xylazine (12 mg/kg body weight; Rompun®; Bayer, Leverkusen, Germany). After the chamber implantation, all animals received a subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg body weight; Temgesic®; RB Pharmaceuticals Limited, Slough, UK) to prevent postoperative pain.

2.4. Dorsal Skinfold Chamber

A flap was raised on the back of each animal and mounted into a dorsal skinfold chamber (Irola Industriekomponenten GmbH & Co. KG, Schonach, Germany) [17]. This flap is termed musculocutaneous, as mouse skin contains a thin muscle layer, the so-called panniculus carnosus muscle [17]. Our approach permitted repeated intravital fluorescence microscopy to study the microcirculation within the flap tissue. For the preparation, the anesthetized animal was depilated. Afterwards, a random-pattern musculocutaneous flap measuring 15 mm (width) × 11 mm (length) was elevated at a right angle to the spine on the back of each animal. The lateral flap margins were sutured back to the wound bed. The dorsal skinfold and the attached musculocutaneous flap were then sutured to the superior margin of one chamber frame. Adhesive insulation foam was used to cover the second chamber frame to ensure an air-tight chamber. The second frame was then mounted to its counterpart with screws. The flap was thus secured in between the two frames and was accessible for microscopic imaging through the chamber's observation window.

Within the chamber window, each flap was injected with ~40.000 MVF from one donor animal suspended in 10 µL saline solution ($n = 8$) or vehicle alone ($n = 8$) and distributed into 10 evenly dispersed sites. Injection was performed underneath the panniculus carnosus muscle into the subcutis, as injection into this space is easily reproducible while allowing high-quality visualization by means of intravital fluorescence microscopy [18]. A cover glass secured with a snap ring was used to close the observation window. Due to its conformation with a standardized width-to-length ratio, the flap developed ~50% necrosis without treatment as a result of the acute persistent ischemia (Figure 1B) [17,19].

After surgery, there was a 24 h recovery period for the animals before the first microscopy was performed. All animals showed normal feeding and sleeping patterns during the observation period without any signs of distress. After the last microscopy, the animals were killed by cervical dislocation under general anesthesia. Subsequently, the flap tissue was harvested for histological and immunohistochemical analysis.

2.5. Intravital Fluorescence Microscopy

Intravital fluorescence microscopy was performed on days 1, 3, 5, 7, and 10 after flap elevation. The anesthetized mice were secured on a platform made of plexiglas. To enhance contrast between the blood vessels and the surrounding tissue, the mice received

0.1 mL of 5% fluorescein isothiocyanate (FITC)-labeled dextran (150,000 Da; Sigma-Aldrich, Taufkirchen, Germany) as a blood plasma marker by injection into the retrobulbar venous plexus. The chambers of MVF-injected mice were additionally scanned and recorded before the injection of the fluorescence dye to detect the GFP signal of viable MVFs. A Zeiss Axiotech fluorescence epi-illumination microscope (Zeiss, Oberkochen, Germany) was used to perform the intravital microscopies. During each microscopy, the microcirculation of the flap tissue was recorded on DVD for offline analysis. All microscopies were performed at a constant room temperature of ~22 °C.

At the beginning of each microscopy, a panoramic view of the chamber was recorded for planimetric measurement of the perfused tissue surface. Within each flap, three observational zones were created by subdividing the flap into a proximal, medial, and distal zone. Two regions of interest (ROI) were selected per zone, each containing an arterio-venous bundle. Due to the distinct morphology of the recorded bundles, they could be identified during each microscopy for repeated measurements. Moreover, two capillary fields were recorded in the surrounding tissue of each selected arterio-venous bundle. If an ROI was no longer perfused, the bundle was documented by means of microscopic images throughout the rest of the observation period or as long as it could be identified. Within the medial transition zone between perfused and non-perfused tissue, an additional ROI was recorded to examine the formation of new microvessels.

Microcirculatory parameters were assessed using CapImage (Version 8.5, Zeintl, Heidelberg, Germany) as an offline analysis system. The necrosis rate [%] was determined as 100-perfused surface area/total chamber surface area × 100. The functional capillary density (FCD) [cm/cm^2] was determined as the total length of all perfused capillaries per capillary field. Within the proximal, medial, and distal ROI, microhemodynamic parameters were assessed in arterioles, capillaries, and venules. Vessel diameters (D) [µm] were measured at a right angle to the vessel path. The line shift method was used to measure centerline red blood cell (RBC) velocity (v) [mm/s] [20]. The volumetric blood flow (VQ) [pL/s] was then calculated using parameters v and D as $VQ = \pi \times \left(\frac{D}{2}\right)^2 \times \frac{v}{K}$ where K (=1.6) is the Baker–Wayland factor accounting for the parabolic velocity profile of blood in microvessels [21]. Angiogenesis within the transition zone was assessed by quantifying the density of neovessels [cm/cm^2], which were identified by their irregular and entangled conformation that clearly differs from the straight, parallelly arranged capillaries of the panniculus carnosus muscle [22].

2.6. Histology and Immunohistochemistry

The obtained tissue samples of flap tissue were fixed using formalin, embedded in paraffin, and subsequently cut into 3 µm-thick sections. For the initial evaluation of the tissue, hematoxylin and eosin (HE) staining of individual sections was performed according to standard protocol. A BX60 microscope (Olympus, Hamburg, Germany) and the imaging software cellSens Dimension 1.11 (Olympus) were used for tissue assessment.

For the immunohistochemical identification of microvessels, sections were stained with a monoclonal rat anti-mouse CD31 antibody (1:100; Dianova, Hamburg, Germany) as the first antibody. A goat anti-rat Alexa 555 antibody (1:100; Invitrogen, Waltham, MA, USA) was used as the second antibody. The sections were then stained with a polyclonal goat GFP antibody (1:100; Rockland Immunochemicals Inc., Limerick, DE, USA) and a donkey-anti-goat biotin-labeled antibody (1:100; Life Technologies, Carlsbad, CA, USA) as well as Alexa 488-labeled streptavidin (1:50; Invitrogen). Using this staining, the origin of the contained microvessels could be determined. The nuclei of the cells were stained with Hoechst 33342 (2 µL/mL; Sigma-Aldrich) to superimpose images exactly. The stained sections were used to analyze microvessel density (all CD31$^+$ microvessels per high-power field (HPF)). Furthermore, the fraction of CD31$^+$/GFP$^+$ microvessels [in %] was determined in two randomly chosen HPF at the flap base (proximal zone) and in the medial transition zone, where vital tissue bordered on necrotic tissue.

To identify myeloperoxidase-positive (MPO^+) neutrophilic granulocytes and apoptotic cleaved caspase (Casp)-3^+ cells immunohistochemically, additional sections were used. A citrate buffer was used to demask antigens, and the unspecific binding sites were then blocked with goat serum. Cells were stained by incubating them with either a polyclonal rabbit MPO antibody (1:100; Abcam, Cambridge, UK) or a monoclonal rabbit-anti-mouse Casp-3 antibody (1:100; Cell Signaling Technology, Danvers, MA, USA) as the first antibodies, followed by a biotinylated goat anti-rabbit IgG antibody (ready-to-use; Abcam) as the second antibody. Peroxidase-labeled streptavidin (ready-to-use; Abcam) was used for the detection of the biotinylated antibody. 3-amino-9-ethylcarbazole (Abcam) was used as a chromogen. Mayer's hemalum (Merck, Darmstadt, Germany) was used for the counterstaining. Within two randomized HPF selected in the flap's proximal zone and the transition zone.

2.7. Statistical Analysis

All data were tested for normal distribution and equal variance. Subsequently, differences between the two experimental groups were assessed by means of the unpaired Student's *t*-test (GraphPad Prism 9; GraphPad Software, San Diego, CA, USA). A Mann–Whitney rank sum test was applied if non-parametric data were detected. All given values are stated as the means ± standard error of the mean (SEM). A statistically significant difference was accepted for a value of $p < 0.05$.

3. Results

3.1. Intravital Fluorescence Microscopy

Using repeated intravital fluorescence microscopy, the vascularization and overall survival of the musculocutaneous flaps within the dorsal skinfold chambers were assessed. A significantly lower necrosis rate of ~26–28% was detected in MVF-injected flaps when compared to the flaps of vehicle-injected controls, which exhibited a necrosis rate of ~42–51% over the course of the 10-day observation period (Figure 1B,C). A significantly higher FCD in all observed flap zones was associated with a markedly diminished flap necrosis rate (Figure 2A–E). In fact, in the proximal and medial zone of MVF-injected flaps, the FCD was ~300–350 cm/cm^2. The distal zone still showed an FCD of ~200 cm/cm^2 (Figure 2A–C). Contrastingly, the FCD in the proximal and medial zone of vehicle-injected flaps was markedly decreased (~200–280 cm/cm^2). In the distal zone, it could only be measured on day 1 (~20 cm/cm^2) (Figure 2A–C).

The vessel diameter and centerline RBC velocities in all examined vessel types (arterioles, capillaries, and venules) within the flaps were measured. From the values, the volumetric blood flow was calculated. It increased in all three vessel types in both groups throughout the 10-day observation period, as the flaps adjusted to the changed blood supply (Table 1). Moreover, arterioles and venules of MVF-injected flaps showed a tendency towards a higher volumetric blood flow when compared to the vessels in vehicle-injected flaps (Table 1).

The formation of new GFP^- blood vessels was analyzed in the transition zone between vital and necrotic flap tissue. In both groups, the flap tissue displayed characteristic changes, such as vessel dilation and irregular diameters in the capillary architecture, within this zone starting on day 5. In addition, angiogenic sprouts grew out of pre-existing microvessels (Figure 2F). Notably, the neovessel density was significantly higher in flaps of MVF-injected mice on days 7 and 10 when compared to that of flaps in control animals (Figure 2G).

In addition, MVF clusters could be detected within the flap tissue throughout the observation period. Similar to the newly formed microvessels in the transition zone, their densely cross-linked and chaotic vessel architecture differed from the straight and parallelly arranged muscle capillaries (Figure 3A). Moreover, the emitted GFP-fluorescence signal confirmed their origin from the GFP^+ donor mice (Figure 3B).

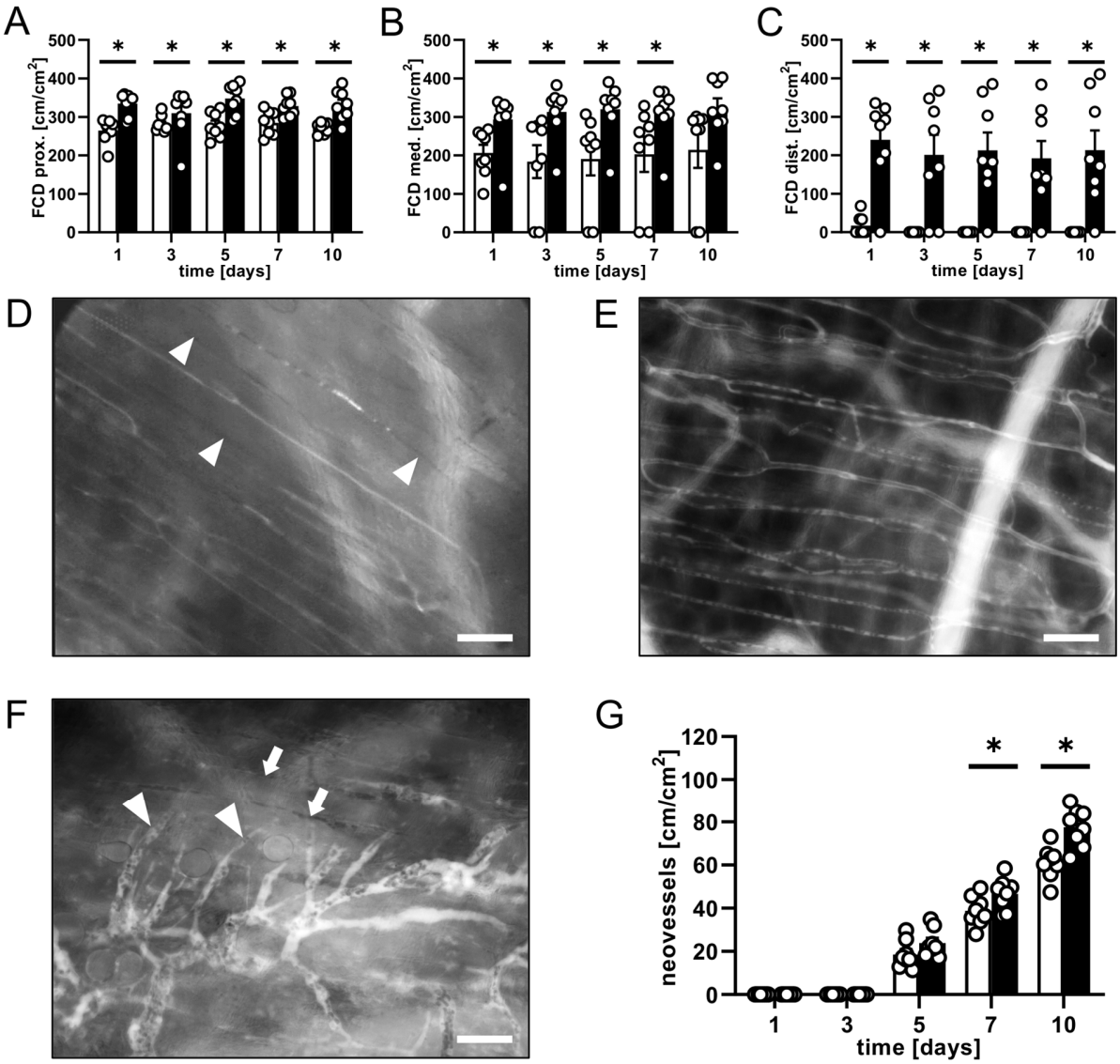

Figure 2. (**A**–**C**) FCD [cm/cm^2] in the proximal (**A**), medial (**B**), and distal zone (**C**) of flaps in MVF-injected mice (black bars, n = 8) and vehicle-injected controls (white bars, n = 8) on days 1, 3, 5, 7, and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM (white circles = individual data points). * $p < 0.05$ vs. control. (**D**,**E**) Intravital fluorescent microscopic images of the capillary fields in the medial zone of flaps in a vehicle-injected control mouse (**D**); no longer perfused capillaries are marked by arrowheads and an MVF-injected mouse (**E**) on day 5 after flap elevation. Scale bars: 50 µm. (**F**) Intravital fluorescent microscopic image of angiogenic sprouts (marked by arrowheads) growing out of pre-existing microvessels. No longer perfused capillaries in the necrotic tissue adjacent to the transition zone can be detected (marked by arrows). Scale bar: 50 µm. (**G**) Neovessels [cm/cm^2] in the transition zone of flaps in MVF-injected mice (black bars, n = 8) and vehicle-injected controls (white bars, n = 8) on days 1, 3, 5, 7, and 10 after flap elevation, as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM (white circles = individual data points). * $p < 0.05$ vs. control.

Table 1. Volumetric blood flow [pL/s] of vehicle-injected control mice (Ctrl; $n = 8$) and MVF-injected mice (MVF; $n = 8$) in arterioles, capillaries, and venules in the proximal, medial, and distal flap zones on days 1, 3, 5, 7, and 10 after flap elevation. The parameter was assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means ± SEM. * $p < 0.05$ vs. control.

Volumetric Blood Flow [pL/s]		d1	d3	d5	d7	d10
Arterioles						
prox.	Ctrl	654 ± 108	871 ± 153	1081 ± 212	1150 ± 223	1503 ± 286
	MVF	773 ± 192	1273 ± 216	1371 ± 272	1741 ± 232	1962 ± 429
med.	Ctrl	367 ± 85	718 ± 168	736 ± 70	980 ± 160	1314 ± 247
	MVF	612 ± 131	983 ± 166	1108 ± 169	1530 ± 167 *	1885 ± 297
dist.	Ctrl	16 ± 3	-	-	-	-
	MVF	340 ± 88 *	765 ± 164	924 ± 228	1282 ± 312	1543 ± 299
Capillaries						
prox.	Ctrl	3 ± 0	4 ± 0	6 ± 0	5 ± 0	7 ± 0
	MVF	3 ± 0	4 ± 0	5 ± 0	5 ± 0	6 ± 1
med.	Ctrl	3 ± 0	4 ± 0	5 ± 0	5 ± 0	6 ± 0
	MVF	3 ± 0	5 ± 0	5 ± 0	6 ± 0	6 ± 1
dist.	Ctrl	0 ± 0	-	-	-	-
	MVF	2 ± 0 *	8 ± 5	4 ± 0	5 ± 0	6 ± 1
Venules						
prox.	Ctrl	518 ± 99	758 ± 160	1300 ± 279	1738 ± 468	1929 ± 782
	MVF	572 ± 76	1027 ± 197	1093 ± 213	2081 ± 640	1500 ± 368
med.	Ctrl	272 ± 88	711 ± 182	908 ± 234	1348 ± 251	1504 ± 291
	MVF	458 ± 102	1034 ± 218	1549 ± 299	2190 ± 443	2232 ± 580
dist.	Ctrl	65 ± 38	-	-	-	-
	MVF	239 ± 31 *	877 ± 272	1123 ± 182	2053 ± 528	2205 ± 515

3.2. Histological and Immunohistochemical Analysis

Additional histological and immunohistochemical analyses were performed at the end of the in vivo experiments to examine morphological changes induced by ischemia within the flap tissue. HE-stained sections were used to identify the transition zone between the proximal and distal flap tissue. The distal zone was completely necrotic and therefore excluded from further immunohistochemical analyses.

In both the proximal and medial zone, a significantly higher number of $CD31^+$ microvessels could be detected in MVF-injected flaps when compared to vehicle-injected controls (Figure 3D). About 5–10% of these microvessels were GFP^+ and, thus, directly originated from the injected MVF (Figure 3E).

Apoptotic cells were identified using immunohistochemical Casp-3 staining. In both groups, the proximal vital zone of the flaps contained only a few apoptotic cells (Figure 4A,B). In contrast, apoptotic cell death was markedly increased in the medial transition zone. However, the number of Casp-3^+ cells/HPF in this zone was significantly reduced in MVF-injected flaps when compared to vehicle-injected controls (Figure 4A,B). The additional identification of MPO^+ neutrophilic granulocytes revealed a massive inflammatory cell invasion in the medial transition zone of both MVF-injected and vehicle-injected flaps without significant differences between the two groups (Figure 4C,D).

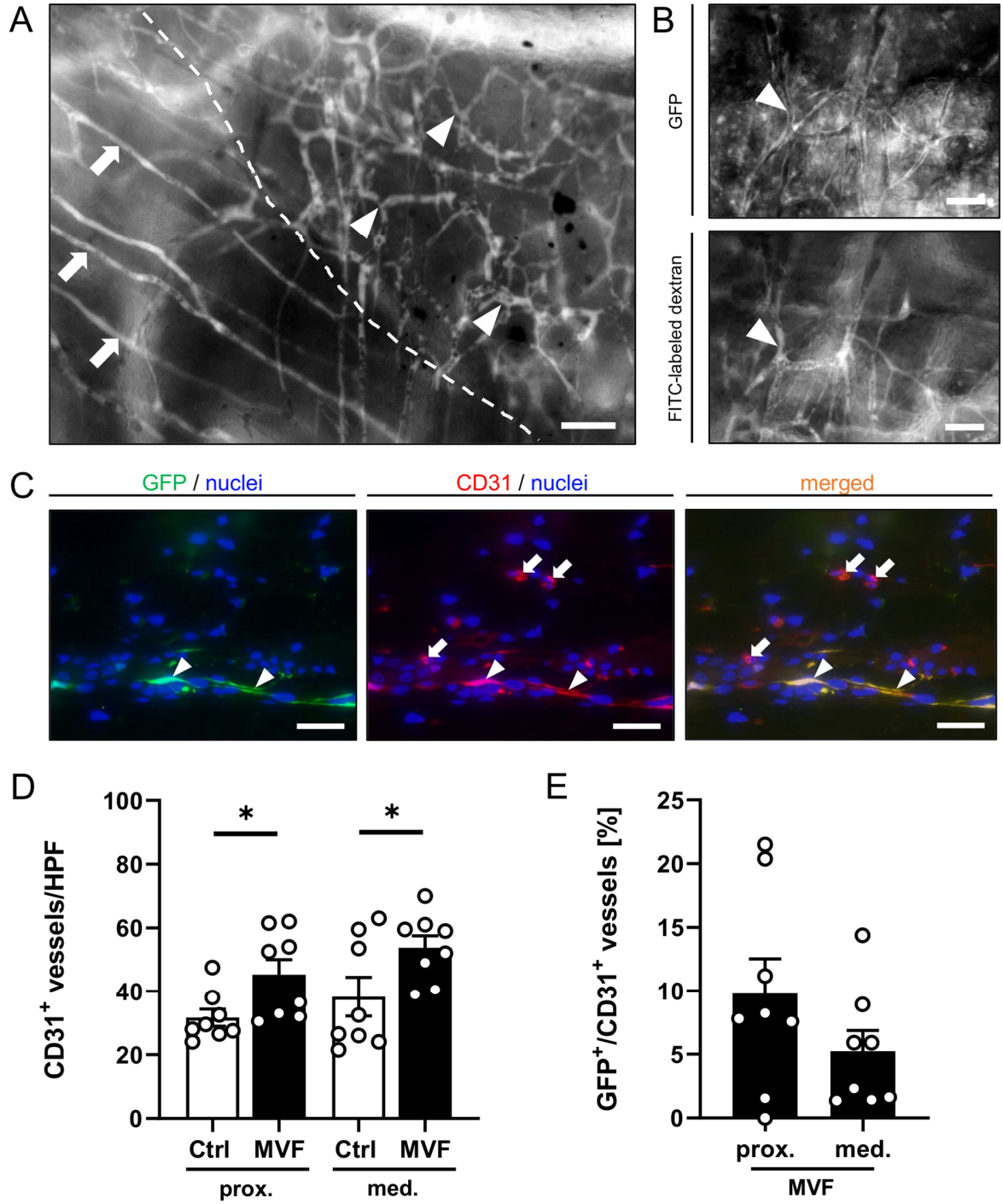

Figure 3. (**A**) Intravital fluorescent microscopic images of an MVF cluster (border marked by broken line). The densely cross-linked and chaotic vessel architecture (marked by arrowheads) can be clearly differentiated from the straight and parallelly arranged muscle capillaries (marked by arrows). Scale bar: 50 µm. (**B**) The GFP fluorescence signal (marked by arrowheads) of the microvessels confirms their origin from the GFP^+ fat donors. In addition, the presence of FITC-labeled dextran within the

vessels indicates their blood perfusion. Scale bar: 50 µm. (**C**) Immunofluorescent CD31/GFP stainings of MVF-injected flap tissue on day 10. $CD31^+/GFP^+$ microvessels (marked by arrowheads) originating from the injected MVF can be distinguished from the $CD31^+/GFP^-$ host microvasculature of the flap (marked by arrows). Scale bars: 25 µm. (**D**) $CD31^+$ microvessels/HPF in the proximal and medial zone of flaps in MVF-injected animals (MVF, black bars, $n = 8$) and vehicle-injected controls (Ctrl, white bars, $n = 8$). Means ± SEM (white circles = individual data points). * $p < 0.05$ vs. control. (**E**): $GFP^+/CD31^+$ microvessels [% of all $CD31^+$ microvessels] in the proximal and medial zone of MVF-injected flaps (black bars, $n = 8$). Means ± SEM (white circles = individual data points).

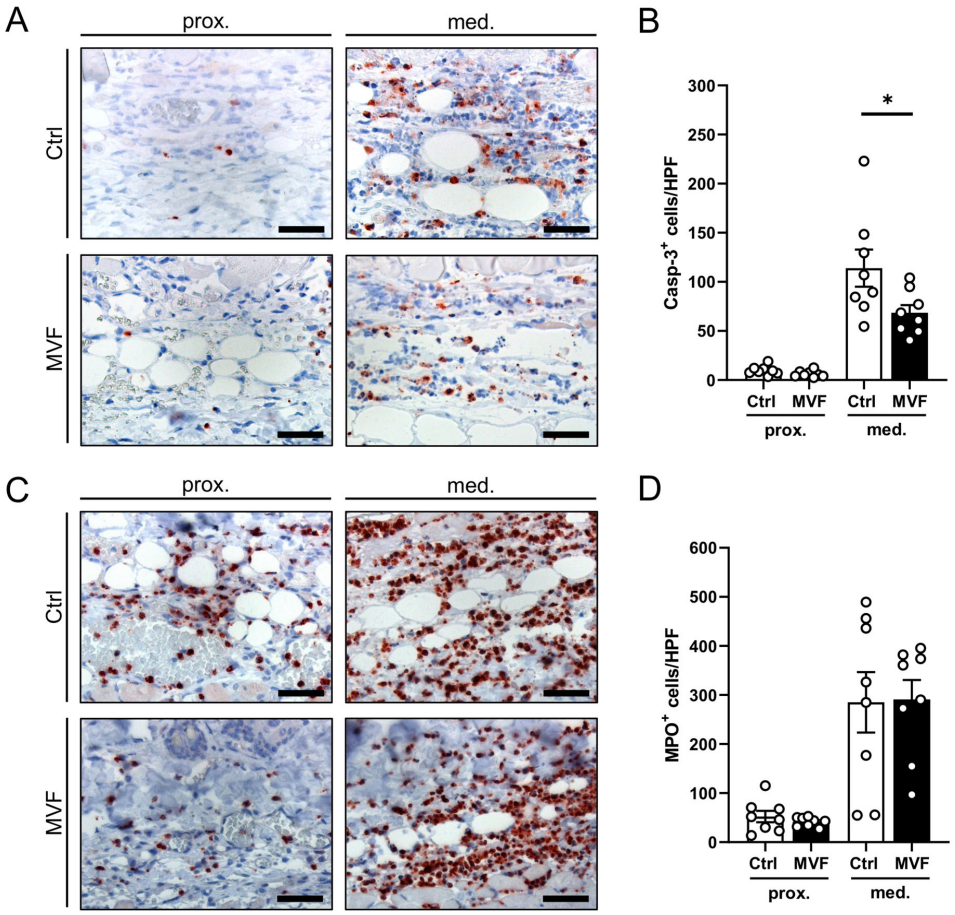

Figure 4. (**A**) Immunohistochemical tissue sections of the proximal and medial zone in flaps of a vehicle-injected control mouse and an MVF-injected mouse 10 days after flap elevation. The sections were stained with an antibody against Casp-3 as a marker for apoptosis. Scale bars: 50 µm. (**B**) Casp-3^+ cells/HPF in the proximal and medial zone of flaps in MVF-injected mice (MVF, black bars, $n = 8$) and vehicle-injected controls (Ctrl, white bars, $n = 8$) 10 days after flap elevation, as assessed by immunohistochemistry. Means ± SEM (white circles = individual data points). * $p < 0.05$ vs. control. (**C**) Immunohistochemical tissue sections of the proximal and medial zone in flaps of a vehicle-injected control mouse and an MVF-injected mouse 10 days after flap elevation. The sections were stained with an antibody against MPO as a neutrophilic granulocyte marker. Scale bars: 50 µm. (**D**) MPO^+ cells/HPF in the proximal and medial zone of flaps in MVF-injected mice (MVF, black bars, $n = 8$) and vehicle-injected controls (Ctrl, white bars, $n = 8$) 10 days after flap elevation, as assessed by immunohistochemistry. Means ± SEM (white circles = individual data points).

4. Discussion

The use of MVF is an established vascularization strategy in the field of tissue engineering. Due to their intact vessel morphology and functionality, MVF have shown a high potential to improve the incorporation of transplanted tissues and increase cell survival by rapidly assembling into blood-perfused networks connected to the host vasculature. As adipose tissue can be harvested from almost all patients in large quantities and the entire procedure for the isolation of MVF lasts only 30–45 min, a single-step intraoperative use of MVF in humans would be clinically feasible. In recent years, MVF from human adipose tissue have already been successfully used to create bioengineered tissues in preclinical studies [23,24]. Though in vivo use in patients has not been attempted yet, the transplantation of MVF represents a promising approach for the treatment of various pathologies associated with an insufficient tissue vascularization.

In this study, we evaluated the application of MVF for increasing the survival of random-pattern flaps. The present work is based on two pioneering studies in rats highlighting the potential of MVF to improve the outcome of flap surgery [4,5]. In line with these studies, we herein found that simple injections of freshly isolated MVF into murine random-pattern musculocutaneous flaps with pre-defined dimensions [17] effectively prevent necrosis of their distal tissue parts. Beyond that, we could demonstrate for the first time that MVF survive the transplantation procedure and reassemble over time into dense vascularization hotspots within the flap tissue.

We injected MVF from GFP^+ donor mice into flaps within the dorsal skinfold chambers of GFP^- recipient animals. In combination with the technique of intravital fluorescence microscopy, this approach did not only enable us to follow the fate of the grafted MVF, but also to prove their final blood perfusion and, thus, their restored original functionality as vessel segments. The latter observation can be explained by the ability of MVF to rapidly reassemble into new microvascular networks, which develop interconnections to the surrounding host microvasculature [25]. Shepherd et al. [11] reported that this already occurs 24 h after MVF transplantation. Because flap necrosis usually demarcates after 3–5 days, this early boost in tissue vascularization seems to maintain nutritive perfusion during the initial critical period after flap elevation, resulting in a significantly lower rate of flap necrosis. In line with this assumption, we measured a markedly higher FCD in all flap zones of MVF-injected animals during the entire observation period when compared to vehicle-injected controls. Additional immunohistochemical analyses confirmed this finding with a significantly increased number of $CD31^+$ microvessels in the proximal and medial flap zones after MVF injection. Most interestingly, only 5–10% of these microvessels were GFP^+ and, thus, originated from the injected MVF. This indicates that MVF not only contribute as direct vascularization units to the nutritive perfusion of the flap tissue but also trigger other mechanisms mediating an improved flap survival.

It should be noted that MVF are a rich source of MSC [6,8,9,13]. Später et al. [13] already proved by flow cytometric analyses that isolated MVF contain 47.5 $\pm$ 4.7%, 7.1 $\pm$ 2.3%, and 9.2 $\pm$ 1% of cells expressing the mesenchymal stem cell markers CD29, CD90, and CD117, respectively. Furthermore, MSC have been shown in previous studies to exert a beneficial effect on the viability of flap tissue [26–28]. Uysal et al. [26] suggested that this effect may be partly traced back to their ability to differentiate into endothelial cells [26]. However, we speculate that in our study, an even more relevant mechanism may be the paracrine stimulation of angiogenesis by MVF. In previous studies, we could demonstrate that MVF secrete various pro-angiogenic factors, including VEGF [9,29]. Moreover, several studies could prevent flap necrosis by the application of VEGF [30–33]. This cytokine does not only stimulate blood vessel formation but also causes vessel dilation and maintains microperfusion via nitric oxide formation [34]. Although we did not perform additional analyses to confirm these findings in the present study, our novel results are consistent with the assumption that MVF exert strong paracrine effects on the microcirculation within the flap tissue. In fact, we detected a significantly higher number of newly formed $CD31^+$ microvessels in the transition zone between vital and necrotic flap tissue of MVF-injected

mice when compared to controls. In addition, we found a higher FCD in all zones of MVF-injected flaps over the course of the experiments.

Our immunohistochemical analyses further revealed a significantly reduced number of apoptotic cells within the medial transition zone of MVF-injected flaps when compared to vehicle-injected controls. This is an interesting finding, considering the fact that the inhibition of ischemia-induced apoptosis has been suggested to be effective in the prevention of flap necrosis [35–37]. We suggest that the herein observed anti-apoptotic effect of MVF injections is most probably caused by the improved nutritive perfusion of the flap tissue. However, we cannot exclude that MVF also directly inhibit apoptosis, which could be another explanation for their high regenerative potential in different experimental settings. This obvious assumption, which should be analyzed in detail in future studies, is supported by the fact that MVF contain MSC and macrophages, which both have the capability of secreting substantial amounts of anti-apoptotic cytokines, such as insulin-like growth factor-1 [38–41].

5. Conclusions

Taken together, the present study sheds more light on the beneficial effects of MVF on surgical flaps. Our experimental findings indicate that MVF protect ischemic musculocutaneous flap tissue from necrosis by improving nutritive tissue perfusion and suppressing apoptosis. Hence, the application of MVF may represent a promising strategy to enhance the success rates of flap surgery. In fact, it is conceivable that in a future clinical scenario, autologous MVF are rapidly harvested from the lipoaspirates of patients by means of automated separation systems, as they are already available for the isolation of stem cells or the generation of platelet-rich plasma [42,43], and then directly retransferred into freshly operated flaps. This intra-operative one-step approach, which would not be associated with additional extensive surgical interventions, could markedly contribute to reducing ischemic flap complications and the related patient morbidity.

Author Contributions: A.W. performed the experiments; A.W. and M.W.L. designed experiments and analyzed and interpreted the data; A.W. and M.W.L. prepared the figures and wrote the manuscript; Y.H., D.S. and M.D.M. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the local governmental animal protection committee (permit number: 10/2020) and executed according to the European legislation on the protection of animals (Directive 2010/63/EU) and the NIH Guidelines on the Care and Use of Laboratory Animals (NIH publication #85-23 Rev. 1985).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data can be obtained in this manuscript.

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References

1. Kalogeris, T.; Bao, Y.; Korthuis, R.J. Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biol.* **2014**, *2*, 702–714. [CrossRef] [PubMed]
2. Lucas, J.B. The Physiology and Biomechanics of Skin Flaps. *Facial Plast. Surg. Clin. N. Am.* **2017**, *25*, 303–311. [CrossRef] [PubMed]
3. Wang, W.Z.; Baynosa, R.C.; Zamboni, W.A. Update on Ischemia-Reperfusion Injury for the Plastic Surgeon. *Plast. Reconstr. Surg.* **2011**, *128*, 685e–692e. [CrossRef] [PubMed]
4. Stone, R.; Rathbone, C.R. Microvascular Fragment Transplantation Improves Rat Dorsal Skin Flap Survival. *Plast. Reconstr. Surg. Glob. Open* **2016**, *4*, e1140. [CrossRef]

5. Nakano, M.; Nakajima, Y.; Kudo, S.; Tsuchida, Y.; Nakamura, H.; Fukuda, O. Effect of Autotransplantation of Microvessel Fragments on Experimental Random-Pattern Flaps in the Rat. *Eur. Surg. Res.* **1998**, *30*, 149–160. [CrossRef]
6. Frueh, F.S.; Später, T.; Scheuer, C.; Menger, M.D.; Laschke, M.W. Isolation of Murine Adipose Tissue-derived Microvascular Fragments as Vascularization Units for Tissue Engineering. *J. Vis. Exp.* **2017**, *122*, e55721. [CrossRef]
7. Laschke, M.W.; Menger, M.D. Adipose Tissue-Derived Microvascular Fragments: Natural Vascularization Units for Regenerative Medicine. *Trends Biotechnol.* **2015**, *33*, 442–448. [CrossRef]
8. McDaniel, J.S.; Pilia, M.; Ward, C.L.; Pollot, B.E.; Rathbone, C.R. Characterization and multilineage potential of cells derived from isolated microvascular fragments. *J. Surg. Res.* **2014**, *192*, 214–222. [CrossRef]
9. Laschke, M.; Kleer, S.; Scheuer, C.; Schuler, S.; Garcia, P.; Eglin, D.; Alini, M.; Menger, M.D. Vascularisation of porous scaffolds is improved by incorporation of adipose tissue-derived microvascular fragments. *Eur. Cells Mater.* **2012**, *24*, 266–277. [CrossRef]
10. Pilia, M.; McDaniel, J.S.; Guda, T.; Chen, X.K.; Rhoads, R.P.; Allen, R.E.; Corona, B.T.; Rathbone, C.R. Transplantation and perfusion of microvascular fragments in a rodent model of volumetric muscle loss injury. *Eur. Cells Mater.* **2014**, *28*, 11–24. [CrossRef]
11. Shepherd, B.R.; Chen, H.Y.; Smith, C.M.; Gruionu, G.; Williams, S.K.; Hoying, J.B. Rapid Perfusion and Network Remodeling in a Microvascular Construct after Implantation. *Arter. Thromb. Vasc. Biol.* **2004**, *24*, 898–904. [CrossRef]
12. Später, T.; Frueh, F.; Menger, M.D.; Laschke, M.W. Potentials and limitations of Integra® flowable wound matrix seeded with adipose tissue-derived microvascular fragments. *Eur. Cells Mater.* **2017**, *33*, 268–278. [CrossRef]
13. Später, T.; Frueh, F.S.; Nickels, R.M.; Menger, M.D.; Laschke, M.W. Prevascularization of collagen-glycosaminoglycan scaffolds: Stromal vascular fraction versus adipose tissue-derived microvascular fragments. *J. Biol. Eng.* **2018**, *12*, 24. [CrossRef]
14. Shepherd, B.R.; Hoying, J.B.; Williams, S.K. Microvascular Transplantation after Acute Myocardial Infarction. *Tissue Eng.* **2007**, *13*, 2871–2879. [CrossRef]
15. Laschke, M.W.; Menger, M.D. Microvascular Fragments in Microcirculation Research and Regenerative Medicine. *Tissue Eng. Part B Rev.* **2022**, *28*, 1109–1120. [CrossRef]
16. Frueh, F.S.; Später, T.; Lindenblatt, N.; Calcagni, M.; Giovanoli, P.; Scheuer, C.; Menger, M.D.; Laschke, M.W. Adipose Tissue-Derived Microvascular Fragments Improve Vascularization, Lymphangiogenesis, and Integration of Dermal Skin Substitutes. *J. Investig. Dermatol.* **2017**, *137*, 217–227. [CrossRef]
17. Harder, Y.; Amon, M.; Erni, D.; Menger, M. Evolution of ischemic tissue injury in a random pattern flap: A new mouse model using intravital microscopy. *J. Surg. Res.* **2004**, *121*, 197–205. [CrossRef]
18. Weinzierl, A.; Harder, Y.; Schmauss, D.; Menger, M.D.; Laschke, M.W. Boosting Tissue Vascularization: Nanofat as a Potential Source of Functional Microvessel Segments. *Front. Bioeng. Biotechnol.* **2022**, *10*, 820835. [CrossRef]
19. Harder, Y.; Schmauss, D.; Wettstein, R.; Egaña, J.T.; Weiss, F.; Weinzierl, A.; Schuldt, A.; Machens, H.-G.; Menger, M.D.; Rezaeian, F. Ischemic Tissue Injury in the Dorsal Skinfold Chamber of the Mouse: A Skin Flap Model to Investigate Acute Persistent Ischemia. *J. Vis. Exp.* **2014**, *93*, e51900. [CrossRef]
20. De Vriese, A.S.; Verbeuren, T.J.; Vallez, M.-O.; Lameire, N.H.; De Buyzere, M.; Vanhoutte, P.M. Off-Line Analysis of Red Blood Cell Velocity in Renal Arterioles. *J. Vasc. Res.* **2000**, *37*, 26–31. [CrossRef]
21. Baker, M.; Wayland, H. On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvasc. Res.* **1974**, *7*, 131–143. [CrossRef] [PubMed]
22. Menger, M.D.; Lehr, H.-A. Scope and perspectives of intravital microscopy—Bridge over from in vitro to in vivo. *Immunol. Today* **1993**, *14*, 519–522. [CrossRef] [PubMed]
23. Porras, M.A.G.; Stojkova, K.; Acosta, F.M.; Rathbone, C.R.; Brey, E.M. Engineering Human Beige Adipose Tissue. *Front. Bioeng. Biotechnol.* **2022**, *10*, 906395. [CrossRef] [PubMed]
24. Xu, X.; Liang, C.; Gao, X.; Huang, H.; Xing, X.; Tang, Q.; Yang, J.; Wu, Y.; Li, M.; Li, H.; et al. Adipose Tissue–derived Microvascular Fragments as Vascularization Units for Dental Pulp Regeneration. *J. Endod.* **2021**, *47*, 1092–1100. [CrossRef] [PubMed]
25. Laschke, M.W.; Später, T.; Menger, M.D. Microvascular Fragments: More Than Just Natural Vascularization Units. *Trends Biotechnol.* **2021**, *39*, 24–33. [CrossRef]
26. Uysal, A.C.; Mizuno, H.; Tobita, M.; Ogawa, R.; Hyakusoku, H. The Effect of Adipose-Derived Stem Cells on Ischemia-Reperfusion Injury: Immunohistochemical and Ultrastructural Evaluation. *Plast. Reconstr. Surg.* **2009**, *124*, 804–815. [CrossRef]
27. Hollenbeck, S.T.; Senghaas, A.; Komatsu, I.; Zhang, Y.; Erdmann, D.; Klitzman, B. Tissue engraftment of hypoxic-preconditioned adipose-derived stem cells improves flap viability. *Wound Repair Regen.* **2012**, *20*, 872–878. [CrossRef]
28. Lee, D.W.; Jeon, Y.R.; Cho, E.J.; Kang, J.H.; Lew, D.H. Optimal administration routes for adipose-derived stem cells therapy in ischaemic flaps. *J. Tissue Eng. Regen. Med.* **2014**, *8*, 596–603. [CrossRef]
29. Laschke, M.W.; Karschnia, P.; Scheuer, C.; Heß, A.; Metzger, W.; Menger, M.D. Effects of cryopreservation on adipose tissue-derived microvascular fragments. *J. Tissue Eng. Regen. Med.* **2018**, *12*, 1020–1030. [CrossRef]
30. Kryger, Z.; Zhang, F.; Dogan, T.; Cheng, C.; Lineaweaver, W.C.; Buncke, H.J. The effects of VEGF on survival of a random flap in the rat: Examination of various routes of administration. *Br. J. Plast. Surg.* **2000**, *53*, 234–239. [CrossRef]
31. Zhang, F.; Oswald, T.; Lin, S.; Cai, Z.; Lei, M.; Jones, M.; Angel, M.; Lineaweaver, W. Vascular endothelial growth factor (VEGF) expression and the effect of exogenous VEGF on survival of a random flap in the rat. *Br. J. Plast. Surg.* **2003**, *56*, 653–659. [CrossRef]

32. Vourtsis, S.A.; Spyriounis, P.K.; Agrogiannis, G.D.; Ionac, M.; Papalois, A.E. VEGF Application on Rat Skin Flap Survival. *J. Investig. Surg.* **2012**, *25*, 14–19. [CrossRef]
33. Vourtsis, S.A.; Papalois, A.E.; Agrogiannis, G.D.; Spyriounis, P.K.; Patsouris, E.; Ionac, M. Improvement of a long random skin flap survival by application of vascular endothelial growth factor in various ways of local administration in a rat model. *Indian J. Plast. Surg.* **2012**, *45*, 102–108. [CrossRef]
34. He, H.; Venema, V.J.; Gu, X.; Venema, R.C.; Marrero, M.B.; Caldwell, R.B. Vascular Endothelial Growth Factor Signals Endothelial Cell Production of Nitric Oxide and Prostacyclin through Flk-1/KDR Activation of c-Src. *J. Biol. Chem.* **1999**, *274*, 25130–25135. [CrossRef]
35. Weinzierl, A.; Harder, Y.; Schmauss, D.; Menger, M.D.; Laschke, M.W. Bromelain Protects Critically Perfused Musculocutaneous Flap Tissue from Necrosis. *Biomedicines* **2022**, *10*, 1449. [CrossRef]
36. Huang, C.; Qing, L.; Pang, X.; Fu, J.; Xiao, Y.; Tang, J.; Wu, P. Melatonin Improved the Survival of Multi-Territory Perforator Flaps by Promoting Angiogenesis and Inhibiting Apoptosis via the NRF2/FUNDC1 Axis. *Front. Pharmacol.* **2022**, *13*, 921189. [CrossRef]
37. Xin, D.; Quan, R.; Zeng, L.; Xu, C.; Tang, Y. Lipoxin A4 protects rat skin flaps against ischemia-reperfusion injury through inhibiting cell apoptosis and inflammatory response induced by endoplasmic reticulum stress. *Ann. Transl. Med.* **2020**, *8*, 1086. [CrossRef]
38. Später, T.; Menger, M.M.; Nickels, R.M.; Menger, M.D.; Laschke, M.W. Macrophages promote network formation and maturation of transplanted adipose tissue-derived microvascular fragments. *J. Tissue Eng.* **2020**, *11*, 2041731420911816. [CrossRef]
39. Qian, Q.; He, W.; Liu, D.; Yin, J.; Ye, L.; Chen, P.; Xu, D.; Liu, J.; Li, Y.; Zeng, G.; et al. M2a macrophage can rescue proliferation and gene expression of benign prostate hyperplasia epithelial and stroma cells from insulin-like growth factor 1 knockdown. *Prostate* **2021**, *81*, 530–542. [CrossRef]
40. Gehmert, S.; Wenzel, C.; Loibl, M.; Brockhoff, G.; Huber, M.; Krutsch, W.; Nerlich, M.; Gosau, M.; Klein, S.; Schreml, S.; et al. Adipose Tissue-Derived Stem Cell Secreted IGF-1 Protects Myoblasts from the Negative Effect of Myostatin. *BioMed Res. Int.* **2014**, *2014*, 129048. [CrossRef]
41. Lee, S.H. Human Adipose-Derived Stem Cells' Paracrine Factors in Conditioned Medium Can Enhance Porcine Oocyte Maturation and Subsequent Embryo Development. *Int. J. Mol. Sci.* **2021**, *22*, 579. [CrossRef] [PubMed]
42. Assmann, A.; Heke, M.; Kröpil, P.; Ptok, L.; Hafner, D.; Ohmann, C.; Martens, A.; Karluß, A.; Emmert, M.Y.; Kutschka, I.; et al. Laser-Supported CD133+ Cell Therapy in Patients with Ischemic Cardiomyopathy: Initial Results from a Prospective Phase I Multicenter Trial. *PLoS ONE* **2014**, *9*, e101449. [CrossRef] [PubMed]
43. Trevisson-Redondo, B.; Becerro-De-Bengoa-Vallejo, R.; Sevillano, D.; González, N.; Losa-Iglesias, M.E.; López-López, D.; Alou, L. Commercial blood cell separation systems versus tube centrifugation methods for the preparation of platelet-rich plasma: A preliminary cross-sectional study. *Rev. Assoc. Médica Bras.* **2021**, *67*, 536–541. [CrossRef] [PubMed]

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6. DISCUSSION

6.1. DISCUSSION OF MATERIALS AND METHODS

6.1.1. DORSAL SKINFOLD CHAMBER FLAP MODEL

The modified dorsal skinfold chamber model in combination with a random pattern musculocutaneous flap is based on a model originally described in hamsters for the repeated intravital microscopic analysis of tissue microperfusion (Endrich et al., 1980). Sandwiching the dorsal skinfold in between two chamber frames, the upper layer of skin and the connective tissue within the observation window are removed to allow microscopic access to the underside of the contralateral striated muscle, subcutaneous and skin layer and visualize tissue perfusion (Menger et al., 2002). The chamber window is hermetically sealed with a cover glass and snap ring to prevent the exposed skin from drying out. Due to the protection and stabilization provided by the dorsal skinfold chamber, microscopy can be performed repeatedly over a period of about two to three weeks. This allows novel insights into biological processes, such as angiogenesis, and the associated functional and morphological changes.

Various modifications of the model exist, for instance to analyze primary and secondary wound healing as well as revascularization of dermal substitutes or skin grafts (Laschke & Menger, 2016). For the modification of the standard model applied in the present thesis, a random pattern flap with clearly defined dimensions was mounted between the chamber frames (Fig. 2) (Harder et al., 2004b; Harder et al., 2014). By elevating the flap, the thoracodorsal artery (TDA) cranially as well as the deep circumflex iliac artery (DCIA) caudally were severed to create the random pattern flap. The used width-to-length-ratio causes acute persistent ischemia in the distal flap zone. Thus, the flap tissue of untreated animals developed ~50% necrosis. The transition zone between vital and necrotic tissue was therefore visible within the observation window to allow for the analysis and quantification of the flap's microcirculation over the course of the observation period. The chamber is well tolerated by the animals, as indicated by undisturbed feeding and sleeping patterns.

The standardized surgical dissection of the flap and the implantation of the chamber is easy to learn, especially for researchers with preexisting surgical proficiency (Fig. 2). However, several possible pitfalls have to be considered when using the dorsal skinfold chamber flap model. Precise chamber and flap placement are crucial, as inexact planning and dissection can lead to an incomplete transection of the TDA or DCIA. This, in turn, leads to false high survival rates of the dissected flap. Moreover, if the chamber is not placed correctly in the midline of the animal's back, it is prone to tilting over the course of the in vivo observation period due to the weight of the chamber and the loss of skin elasticity. If the tilt is excessive, it can restrict flap

perfusion and influence measurements. Furthermore, it can lead to a vertical sliding of the tissue within the observation window and let regions of interest close to the border of the observation window slip under the margin of the chamber frame. These areas are then inaccessible for further microscopic measurements.

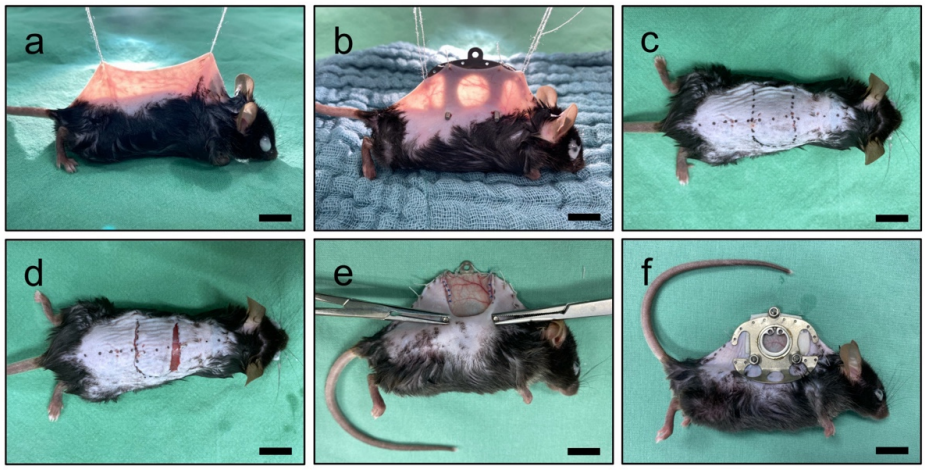

Figure 2: *Surgical preparation of a random pattern flap within the dorsal skinfold chamber. Scale bars: 10 mm.* ***a:*** *Transillumination of the depilated dorsal skinfold is performed to visualize the thoracodorsal and deep circumflex iliac vessels.* ***b:*** *The chamber is temporarily fixed to the skin to create incisions for the screws at the base of the chamber.* ***c:*** *The flap is planned with a width of 15 mm and a length of 11 mm.* ***d:*** *The flap is elevated, transecting the thoracodorsal and deep circumflex iliac vessels. The upper part crossing over the dotted midline is later discarded.* ***e:*** *The flap is sutured back to the lateral wound margins and fixed to the top of the chamber frame with surgical sutures together with the remaining dorsal skinfold cranially and caudally.* ***f:*** *The second chamber frame is placed, sandwiching the flap in between the two chamber frames. Subsequently, the observation window is sealed by means of a cover glass and a snap ring.*

Besides these technical pitfalls, microscopic analysis can also be hindered by biological causes, resulting in insufficient image quality. Examples include infection of the tissue within the observation window, bleeding underneath the cover glass or edema of the flap tissue (Fig. 3). Particular care should therefore be taken to work as sterile as possible and control any occurring bleeding during flap elevation. Moreover, additional animals should be calculated during the planning of each study to potentially replace any animals that might have to be excluded from further analysis due to insufficient image quality.

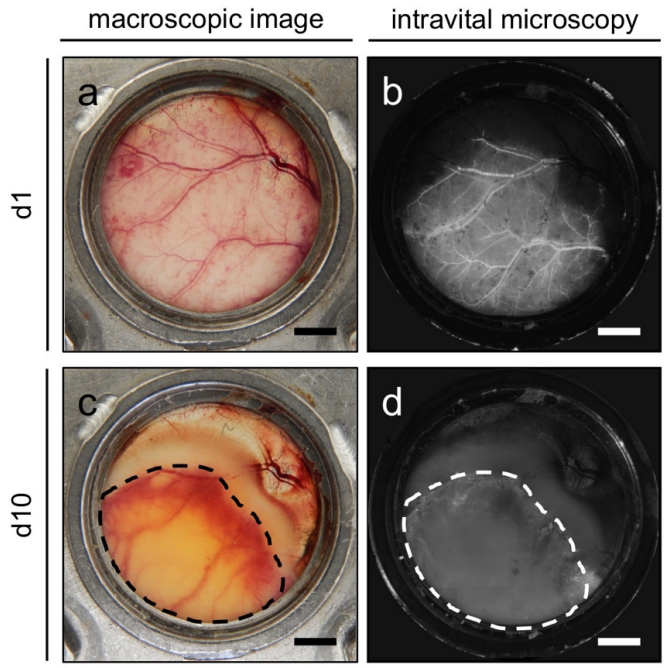

Figure 3: *Chamber edema as a possible pitfall of the dorsal skinfold chamber flap model. Scale bars: 2 mm.* ***a:*** *An exemplary chamber window on day 1 after flap elevation.* ***b:*** *The corresponding intravital microscopic image with vessels visible within the vital flap tissue.* ***c:*** *The same chamber window showing tissue edema with a blurred macroscopic view at day 10 after flap elevation (marked by black broken line).* ***d:*** *The corresponding intravital microscopic image no longer displaying discernable vessels within the vital flap tissue (marked by white broken line).*

6.1.2. *Intermittent fasting*

For this thesis, a standard IF regimen with 8 hours of unrestricted access to standard pellet chow followed by 16-hour fasting intervals was used (Malinowski et al., 2019). As tissue preconditioning before the induction of flap ischemia has been shown to be more effective than postconditioning (Rezaeian et al., 2013), IF was administered starting one week before flap elevation.

Fasting is an important stressor for mice with a visible impact on their activity level. Therefore, care was taken to optimize all remaining conditions. Only male mice were used for fasting experiments, as they were able to reach the target weight faster and more reliable than the female animals. Only mice with a body weight over 26 g were used to conduct the experiments, though generally a body weight of 23-25 g is acceptable for the chamber implantation without

additional flap dissection or fasting interventions. All mice used for the IF study were kept in an incubator at a temperature of 24-25 °C for the duration of the experiments and the body weight was closely monitored. After anesthesia, animals were provided with standard pellet chow soaked in water to ensure an immediate intake of food.

6.1.3. BROMELAIN

After an extensive literature research, bromelain was chosen as a potential therapeutic molecule to reduce flap necrosis. Bromelain decreases ADP-dependent platelet aggregation and thrombogenesis, induces fibrinolytic activity and has also been shown to exert anti-hypertensive and anti-inflammatory effects (Metzig et al., 1999; Maurer, 2001; Braun et al., 2005; Lee et al., 2019). Moreover, it was able to reduce infarct size in myocardial ischemia in a rat ischemia-reperfusion model with anti-apoptotic effects (Juhasz et al., 2008). As it is the case for many other phytochemicals, bromelain induces several different mechanisms that act in a synergistic manner to mediate its overall tissue-protective effect (Maurer, 2001; Pavan et al., 2012; Weinzierl et al., 2022).

After initial experiments, an intraperitoneal administration of bromelain was chosen for the experiments of the present thesis, because oral application by means of gavage resulted in an elevated risk for aspiration during the surgical flap elevation and chamber implantation. The lethal dose (LD_{50}) of bromelain in mice is higher than 10 g/kg and no effective standard dosage has been established so far (Chakraborty et al., 2021). Therefore, a daily dose of 20 mg/kg bromelain, administered intraperitoneally, was used based on the experience of a previous experimental study to repeat the beneficial effects of the substance (Juhasz et al., 2008).

6.1.4. MICROVASCULAR FRAGMENTS

The use of MVF is a well-established technique frequently used in the field of tissue engineering (Laschke et al., 2021). They can be isolated from adipose tissue and are, therefore, abundantly available in most patients or experimental animals. As the isolation takes roughly 45-60 minutes, their use within a single surgical procedure would be clinically feasible. Due to their ability to quickly form blood-perfused networks after their transplantation, they are already in use for a broad variety of biomedical applications. They present a reservoir of endothelial progenitor cells, multipotent mesenchymal stromal cells and fragments of lymphatic vessels and, thus, can enhance tissue vascularization.

For the present thesis, a GFP crossover design with GFP-positive donors and GFP-negative recipient mice was used. Due to this design, the origin of any observed perfused microvessel could be clearly determined, both during intravital microscopy and by means of immunohistochemical staining. MVF were generated using the epididymal fat pads of male C57BL/6-Tg (CAG-EGFP)1Osb/J mice. To avoid contamination of the excised epididymal fat pads, the adipose tissue was dissected with a safety margin of several mm to the epididymidis and testes, as accidental inclusion of these tissues changes the tissues pH level and may disturb the subsequent enzymatic digestion. The isolation process then involved the mechanical mincing, enzymatic digestion, filtration and, ultimately, centrifugation. It was crucial to stop the enzymatic digestion before the fragments were degraded into individual cells (Fig. 4).

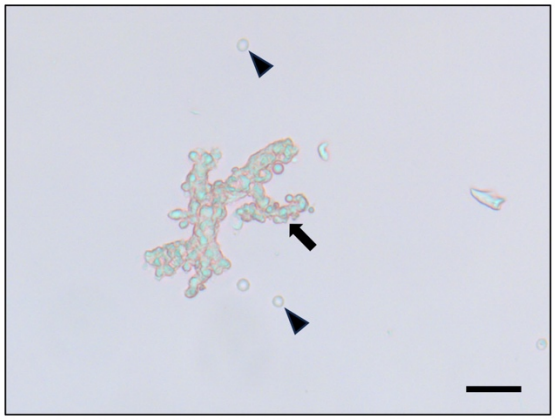

Figure 4: *Microscopic image of an MVF (arrow) surrounded by individual cells (arrowheads). Scale bar: 20 µm.*

After the isolation, the MVF were injected into the flap tissue. Though the injection caused additional trauma to the flap tissue, this approach was chosen, because initial experiments with a mere topical application of the MVF underneath the cover glass resulted in the absence of traceable viable fragments by day 3. This was likely caused by insufficient MVF attachment to the host tissue, resulting in vertical sliding of the MVF underneath the margin of the chamber frame as well as necrosis of the fragments.

6.2. Discussion of results

6.2.1. *Intermittent fasting*

Research on DR was initially conducted in the context of life span extension and healthy aging. These potential benefits of long-term DR are not considered relevant in the current clinical setting, as patient compliance to adhere to a restricted diet rapidly declines over time (Ravussin et al., 2015). Moreover, time-intensive preoperative fasting protocols would heavily complicate the scheduling of elective surgeries. More recent research shows however that even short-term DR increases acute stress resistance (Robertson & Mitchell, 2013; Walsh et al., 2014).

On a cellular level, several mechanisms have been proposed to induce the tissue-protective effects of fasting. These mechanisms are evolutionary conserved remnants of strategies that organisms used to strive even in environments with limited resources and nutrition. They include increased autophagy, lower insulin levels under fasting conditions, as well as decreased resting energy consumption and oxidative stress within the tissue (Il'yasova et al., 2018; Redman et al., 2018). Autophagy, for instance, has also been shown to be very beneficial in the context of flap surgery (Martinez-Lopez et al., 2017; Li et al., 2019). Acting as a cellular recycling program, it eliminates misfolded proteins, defect organelles and other aggregates from the cell (Madeo et al., 2019). Fasting therefore seems to improve the overall cellular function, resulting in an increased ischemic tolerance. It has thus been proposed as a potential strategy to act against surgical stress and ischemia (Brandhorst et al., 2017). In the present thesis, it could be shown that perioperative IF significantly increased the rate of flap survival, as it suppressed ischemia-induced inflammation and maintained nutritive blood flow to the tissue.

The beneficial effects of IF on ischemically challenged tissue seem to consist of several mechanisms that act in a synergistic manner, ultimately preventing tissue necrosis. DR induces a resistance to the stress of nutrient deprivation, while also causing a cross-resistance to various other stressors, including oxidative insults (Michalsen & Li, 2013). The expression of cytoprotective genes, including hemeoxygenase (HO)-1 and components of the glutathione detoxification system, is increased to mediate this effect (Mitchell et al., 2010). Moreover, under fasting conditions cells seem to shift from proliferation into survival programs to slow down their metabolism, in turn protecting them from ischemic stress (Raffaghello et al., 2008). Cell division decreases during the fast. To become more stress-resistant, cells utilize degradation products from the breakdown of organelles, proteins and fats. This effect has also been analyzed in the context of chemotherapy. Cancerous cells are unable to enter a non-dividing state due to their altered growth signaling. Therefore, they remain sensitive to chemotherapy under fasting conditions, while the tolerance of healthy cells can be drastically increased by

applying DR (Raffaghello et al., 2008; Tinkum et al., 2015; Brandhorst et al., 2017). This improved cellular resistance was also detected in the present thesis, as indicated by a significant decrease in apoptotic cell death in the transition zone of IF-treated flaps, as well a reduced rate of flap tissue necrosis overall.

The reduction of oxidative stress and inflammation through DR also seems to be beneficial in the context of flap surgery (Walsh et al., 2014). In a previous study, various biomarkers for oxidative stress in cardiac, renal and cerebral tissue in rabbits were decreased after short-term IF (Hardiany et al., 2022). Fastened rats also exhibited a reduced expression of inflammatory cytokines and chemokines in their liver and kidney tissue, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1(Chiba & Ezaki, 2010). In line with these findings, significantly less neutrophilic granulocytes invading the transition zone between vital and necrotic flap tissue were detected in IF-treated animals in comparison with controls. Suppressing the tissue invasion of immune cells is crucial for flap survival, as these cells may be responsible for parenchymal cell dysfunction and a disturbed blood rheology (Carden et al., 1990; Puhr-Westerheide et al., 2019).

Moreover, DR has been shown to stimulate angiogenesis. Under DR, the revascularization in an ischemic hindlimb model in mice was increased via the activation of the enzyme endothelial nitric-oxide synthase (eNOS) mediated by adiponectin (Kondo et al., 2009). DR also increases the expression of stress response proteins, including key mediators of angiogenesis such as hypoxia inducible factor (HIF)-1α (Soñanez-Organis et al., 2013; Zimna & Kurpisz, 2015). An increased angiogenic response to the hypoxic stimulus of surgical flap elevation could also be observed in the present thesis, as more newly formed microvessels were recorded in the transition zone between vital and necrotic flap tissue.

The discussed effects also seem to be mediated by shifts in the gut microbiome, which regulates nutrient bioavailability and intestinal physiology (Cignarella et al., 2018; Nakai et al., 2021; Richards et al., 2022). Fasting can influence the differentiation of regulatory T-cells and the body's overall cytokine profile by changing the composition of the gut microbiome (Zheng et al., 2018; Rinninella et al., 2020). A fasting intervention of a 4-day 30% CR was already able to alter the composition of the microbiome and increase the presence of bacteria that produce short chain fatty acids (SCFA) (Anderson et al., 2022). Among them were the primary producers of butyrate, a molecule that suppresses the pro-inflammatory nuclear factor (NF)-κB pathway and the differentiation of regulatory T-cells (Zhu et al., 2018; Nakai et al. 2021; Richards et al., 2022).

The concept of using fasting to counterbalance surgical stress is particularly promising for clinical use, as the tissue-protective effects of DR have already been examined in humans. For

instance, multiple studies have tested the safety and feasibility of a DR intervention before vascular surgery (Kip et al., 2019, 2021; Mitchell et al., 2013). Similarly, the incidence of acute kidney injury after cardiac surgery was significantly reduced after a 40% reduction of the caloric intake for 7 days prior to surgery (Grundmann et al., 2018). Based on preclinical research in aged or overweight mice, where DR still showed protective effects against renal ischemia-reperfusion injury (Jongbloed et al., 2014), preoperative DR has also been tested in morbidly obese individuals without causing major side effects (Jongbloed et al., 2016). The possible clinical use of DR conditioning for other indications including flap surgery is therefore likely to be feasible as well. The anti-inflammatory effect of DR has been studied extensively in human subjects by examining fasting individuals undergoing the religious Ramadan fast. These studies showed beneficial immunomodulatory effects, such as a reduced secretion of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 after fasting (Faris et al., 2012; Adawi et al., 2017).

Currently, the only recommendation in human patients regarding fasting prior to surgery is an overnight fast to reduce the risk of regurgitating and aspirating food under anesthesia. However, extending preoperative DR, for instance in the form of short-term IF, may decrease the incidence of ischemia-induced complications in flap surgery and could optimize the surgical outcomes for this type of operation in the future.

6.2.2. *Bromelain*

Phytochemicals are compounds derived from plants that are not considered essential to the human diet (Liu, 2013). Existing research has proven the efficacy of phytochemical compounds to prevent and cure various pathologies (George et al., 2021; Ismaeel et al., 2021). However, their usefulness and versatility for clinical applications is often underestimated. The medicinal use of phytochemicals is dating back several thousand years. Though often overlooked, today they remain an important health resource, as they are more readily available than prescription drugs, making them irreplaceable in many developing countries (Aggarwal et al., 2007). Accordingly, a large percentage of people worldwide is still actively using herbal medicines (Ekor, 2014). Nowadays, phytochemicals seem to slowly gain importance in Western medicine as effective therapeutic substances due to their favorable risk-benefit-profile with only few unwanted side effects (Siddiqui et al., 2015; Wahab et al., 2021). Moreover, they have become a valuable resource for drug development (Clark, 1996; Cheuka et al., 2017). The phytochemical compounds can modulate anti-oxidative, anti-inflammatory and anti-apoptotic signaling pathways (Haidarali et al., 2015; Zhang et al., 2015; Al-Ishaq et al., 2020) and

promote angiogenesis (Thangapazham et al., 2016). Thus, they represent a highly promising approach to prevent ischemic damage in tissue flaps. In fact, they can influence the tissue's microenvironment (Cheng et al., 2016), meaning the exact cellular and molecular composition regarding cell types, extracellular matrix, growth factors and cytokines (Vogt et al., 2005; Pei et al., 2020).

The phytochemical bromelain is an enzyme complex obtained from pineapple stems and represents an example for a naturally occurring compound with several tissue-protective effects. It has been applied for the therapy of inflammatory diseases, such as rheumatoid arthritis (Pavan et al., 2012; Maurer, 2001) and can reduce pain and swelling during wound healing (Taussig & Batkin, 1988; Pavan et al., 2012). With the present thesis we were able to show that the anti-inflammatory, anti-apoptotic and anti-thrombotic effects of bromelain effectively increase the survival of critically perfused musculocutaneous flaps. This was associated with a maintained microperfusion, a decreased invasion of immune cells and a lower rate of apoptotic cell death.

Bromelain may have positively influenced the microvasculature in the flaps through several pathways. Under bromelain treatment, an increased blood flow in all analyzed vessel types and flap zones was detected. This may be explained by an increased expression of the vasodilator nitric oxide (NO), as detected for instance in hepatic tissue undergoing warm ischemia (Bahde et al., 2007; Gwozdzinski et al., 2023). In humans, a combination of bromelain and anthocyanins reduced systolic blood pressure and improved oxygen utility capacity, resulting in an improved tissue saturation in muscle tissue (Pekas et al., 2021). This enhanced vascular function may have led to a maintained microperfusion with higher functional capillary density values in the present thesis, particularly in the distal flap zones, when compared to untreated controls.

In addition, bromelain has been shown to exert fibrinolytic and anti-thrombotic effects in several studies (Gläser & Hilberg, 2006; Azarkan et al., 2020). The formation of microthrombosis is particularly detrimental to random pattern flaps, as it frequently occurs in still perfused fringe areas, where the blood flow has begun to slow down (Fitzal et al., 2002; Menger et al., 2003). These areas subsequently become necrotic. Other anti-thrombotic substances have been used successfully to improve flap survival (Takase et al., 2006). As microthrombosis is strongly aggravated by tissue inflammation and the secreted cytokines, the fact that bromelain exerts multifaceted effects that also include an anti-inflammatory component is highly beneficial. The compound decreases the transcription factor NF-κB and, thus, the expression of various pro-inflammatory chemokines and enzymes (Kasemsuk et al., 2018; Insuan et al., 2021). Bromelain has also been shown to decrease the production of ROS (Neumayer et al., 2006)

and to downregulate the invasion of immune cells into the tissue by altering the expression of immune cell surface markers which mediate the interaction of leukocytes with the endothelium (Hale et al., 2002; Fitzhugh et al., 2008). In line with these results, the administration of bromelain resulted in a significantly lower invasion of MPO-positive neutrophilic granulocytes into the tissue within the transition zone of the analyzed flaps.

Of interest, an increased number of newly formed microvessels could be detected by means of intravital fluorescence microscopy in the transition zone between vital and necrotic flap tissue after bromelain treatment. Though the difference was not statistically significant, an increased number of CD31-positive microvessels was detected in the medial zone of bromelain-treated flaps. This pro-angiogenic activity of bromelain has been suggested in other studies in the past (Shoba et al., 2017; Shoba et al., 2020). However, conflicting data shows an anti-angiogenic effect of bromelain on cancer cell lines (Juhasz et al., 2008; Karlsen et al., 2011; Rathnavelu et al., 2016). Similarly, pro-apoptotic effects of the enzyme have been described for malignant cells. Bromelain decreases their proliferation and activates the extracellular signal-regulated kinase (ERK)/AKT pathway, inducing apoptosis (Romano et al., 2014). By contrast, other studies show anti-angiogenic effects, such as an increased phosphorylation of Akt and FOXO3A under bromelain treatment in a mouse model for cardiac ischemia (Juhasz et al., 2008). This led to a significantly lower rate of cardiomyocytes undergoing apoptosis, decreasing the overall infarct size. In the present thesis fewer apoptotic cells were detected in the transition zone between vital and necrotic tissue of musculocutaneous flaps after bromelain administration. This conflicting data suggests that bromelain might play a regulatory function in several cellular processes that may differ depending on other factors, such as the tissue's microenvironment or the applied dosage. This observation emphasizes how difficult it is to identify all involved pathways and factors after the application of phytochemicals, as they tend to act in a pleiotropic manner.

The present thesis shows that perioperative systemic bromelain administration effectively decreases flap tissue necrosis. Bromelain may bear the considerable advantage that it does not cause severe side effects. This characteristic makes it a possible resource for optimizing the clinical outcome of flap surgery in fragile, e.g. elderly, patients. Therefore, future research should evaluate if the beneficial tissue protective effects of bromelain can also be reproduced in human patients. If successful, the perioperative application of bromelain may help prevent ischemic complications and improve the overall outcome of reconstructive flap surgery.

6.2.3. *MICROVASCULAR FRAGMENTS*

A particularly innovative strategy to prevent ischemia-induced flap complications is the approach to create flaps with a surgically altered or enhanced blood perfusion. The technique of “prefabricating” flaps has been described as early as 1973 (Bakamjian & Holbrook, 1973). For this approach, a vascular axis is surgically isolated and buried in the anatomical area designated for subsequent flap elevation (Yao, 1982). Neovascularizing tissue by implanting a vascular axis is thus supposed to create a flap with a robust perfusion for pedicled or free tissue transfer. However, the technique has proven to be unreliable in clinical practice (Pribaz & Fine, 1994). A more recent approach to optimize flap microcirculation and to possibly create a superior flap is the use of vascularization techniques from the field of tissue engineering. Freiman et al. (2018) implanted scaffolds seeded with endothelial cells and mesenchymal stem cells (MSC) around the femoral artery of rats to create vascularized engineered flaps for the reconstruction of full-thickness skin defects. However, the engineered flaps required a prolonged cell culture, as well as at least two surgical steps for defect coverage. In fact, the scaffolds needed to be implanted in a first procedure with a subsequent vascularization period, before the created flap could be used for defect coverage in a second surgical step. This makes an implementation into clinical routines relatively cumbersome. In contrast, the use of MVF to enhance flap tissue vascularization is clinically feasible as the isolation procedure for MVF lasts only 45-60 minutes, making a single-step intraoperative use in clinical practice possible. Moreover, the fragments are isolated from adipose tissue, which is abundantly available in almost all patients. For many years, MVF have been used to study basic mechanisms of angiogenesis, endothelial cell function, as well as microvascular network formation. Based on this promising research, MVF have been used to counteract flap necrosis and improve the surgical outcome of flap surgery in two pioneering preclinical studies in rats (Nakano et al., 1998; Stone & Rathbone, 2016). These studies effectively used the characteristic features of MVF to prevent necrosis and increase cell survival due to their ability to assemble into blood-perfused networks connected to the surrounding host vasculature (Laschke & Menger, 2015; Laschke et al., 2021; Laschke & Menger, 2022).

With the present thesis, the involved mechanisms and associated morphological changes after MVF transplantation into ischemically challenged flap tissue were elucidated further, using a murine dorsal skinfold chamber flap model. MVF injections into the flap tissue were able to significantly decrease flap tissue necrosis. Moreover, the present thesis conclusively demonstrates that MVF survive the transplantation and reorganize into vascularization hotspots within the host tissue. Flap necrosis usually demarcates 3-5 days after flap elevation and the occurring neoangiogenesis is usually too late to counteract it effectively. MVF-injected

animals showed a higher density of functional capillaries in all flap zones when compared to vehicle-injected controls. This indicates that MVF seem to effectively support microperfusion by quickly reassembling into new blood-perfused vessel networks as early as 24 hours after transplantation (Shepherd et al., 2004),

Using a GFP-positive / GFP-negative crossover design in combination with repeated intravital fluorescence microscopy, the survival and engraftment of transplanted MVF could be tracked. Additional immunohistochemical analyses revealed a markedly higher number of CD31-positive microvessels in the proximal and medial flap after MVF injection, from which 5-10% were positive for GFP. Thus, these microvessels originated from the transplanted GFP-positive MVF. These results also suggest that besides acting as direct vascularization units, the use of MVF induces additional mechanisms that improve flap survival. Immunohistochemical analyses demonstrated a notably decreased quantity of cells undergoing apoptosis within the medial transition zone of MVF-injected flaps as opposed to those injected with the vehicle. While the improved tissue perfusion may have led to a decrease of ischemia-induced apoptosis, it is tempting to speculate that MVF may also inhibit apoptosis directly. The fragments contain both MSC and macrophages. These cells can secrete large amounts of anti-apoptotic cytokines, such as insulin-like growth factor-1 (Gehmert et al., 2014; Später et al., 2020; Lee, 2021; Qian et al., 2021). It has been demonstrated in previous studies that MSC exert beneficial effects on flap viability (Uysal et al., 2009; Hollenbeck et al., 2012; Lee et al., 2014). Even though there is a certain amount of stem cells undergoing endothelial differentiation that contribute to the beneficial effects, they mostly increase skin flap survival through growth factor secretion (Foroglou et al., 2016). The stem cells stimulate angiogenesis by secreting vascular endothelial growth factor (VEGF), a central molecule for pro-angiogenic signaling (Laschke et al., 2012; Laschke et al., 2018). Besides directly stimulating blood vessel formation, the cytokine also causes vasodilation (He et al., 1999), which seems to have contributed to a maintained microperfusion despite the ischemic insult.

In conclusion, the present thesis provides a more comprehensive understanding of the positive impacts of MVF on surgical flaps. These novel findings indicate that the application of MVF may be a promising strategy for optimizing surgical flaps by boosting tissue vascularization. MVF could be directly transplanted into flaps during their surgical elevation, using an intra-operative one-step isolation and transplantation approach without additional surgical procedures. Thus, their use may represent a successful bench-to-bedside application of tissue engineering techniques with a possible clinical implementation. Hence, future research should focus on translating the approach into human patients and evaluating the effectiveness of MVF transplantation into flap tissue in a clinical setting.

6.3. CONCLUSION

In the present thesis, three different approaches to increase the survival of ischemically challenged musculocutaneous flap tissue have been evaluated. All three approaches were able to improve tissue survival by inducing various tissue-protective effects, including maintaining nutritive tissue perfusion, increasing angiogenesis, suppressing an inflammatory reaction or decreasing apoptosis, in a synergistic manner. Dependent on patient characteristics and the specific requirements of the planned surgical procedure, individual or combined approaches may be used in future clinical practice to optimize the outcome of flap surgery. Though future research, e.g. the development of automated separation systems to generate MVF, may be necessary to facilitate effective clinical use, the explored strategies bear the potential to counteract flap necrosis and reduce the associated patient morbidity. Clinical studies should therefore evaluate their effectiveness in patients. If successful, these strategies may increase the success rates of elective flap surgery and contribute to the solution of a relevant clinical problem.

7. REFERENCES

1. **Adawi M, Watad A, Brown S, Aazza K, Aazza H, Zouhir M, Sharif K, Ghanayem K, Farah R, Mahagna H, Fiordoro S, Sukkar SG, Bragazzi NL, Mahroum N.** Ramadan fasting exerts immunomodulatory effects: Insights from a systematic review. *Frontiers in Immunology* 8:1144, 2017.

2. **Aggarwal BB, Sundaram C, Malani N, Ichikawa H.** Curcumin: The Indian solid gold. *Advances in Experimental Medicine and Biology* 595:1-75, 2007.

3. **Al-Ishaq RK, Overy AJ, Büsselberg D**. Phytochemicals and gastrointestinal cancer: Cellular mechanisms and effects to change cancer progression. *Biomolecules* 10:105, 2020.

4. **Altınel D, Serin M, Erdem H, Biltekin B, Huseyinbas O, Toplu G, Kurt Yazar S.** Comparison of incisional delay patterns on a rat random flap model. *Journal of Plastic Surgery and Hand Surgery* 53:247-253, 2019.

5. **Anderson EM, Rozowsky JM, Fazzone BJ, Schmidt EA, Stevens BR, O'malley KA, Scali ST, Berceli SA**. Temporal dynamics of the intestinal microbiome following short-term dietary restriction. *Nutrients* 14:2785, 2022.

6. **Askar I, Oktay MF, Gurlek A, Bac B.** Protective effects of some antineoplastic agents on ischemia-reperfusion injury in epigastric island skin flaps. *Microsurgery* 26:193-199, 2006.

7. **Azarkan M, González MM, Esposito RC, Errasti ME.** Stem bromelain proteolytic machinery: Study of the effects of its components on fibrin(ogen) and blood coagulation. *Protein & Peptide Letters* 27:1159-1170, 2020.

8. **Bahde R, Palmes D, Minin E, Stratmann U, Diller R, Haier J, Spiegel HU**. Bromelain ameliorates hepatic microcirculation after warm ischemia. *Journal of Surgical Research* 139:88-96, 2007.

9. **Bakamjian VY, Holbrook LA**. Prefabrication techniques in cervical pharyngo-oesophageal reconstruction. *British Journal of Plastic Surgery* 26:214-222, 1973.

10. **Bozkurt M, Sezgic M, Karakol P, Uslu C, Balikci T**. The effect of antioxidants on ischemia-reperfusion injury in flap surgery. Antioxidants [eBook], *IntechOpen*, London, 2019.

11. **Brandhorst S, Harputlugil E, Mitchell JR, Longo VD**. Protective effects of short-term dietary restriction in surgical stress and chemotherapy. *Ageing Research Reviews* 39:68-77, 2017.

12. **Braun JM, Schneider B, Beuth HJ**. Therapeutic use, efficiency and safety of the proteolytic pineapple enzyme Bromelain-POS® in children with acute sinusitis in Germany. *In Vivo* 19:417-421, 2005.

13. **Carden DL, Smith JK, Korthuis RJ**. Neutrophil-mediated microvascular dysfunction in postischemic canine skeletal muscle. Role of granulocyte adherence. *Circulation Research* 66:1436-1444, 1990.

14. **Chakraborty AJ, Mitra S, Tallei TE, Tareq AM, Nainu F, Cicia D, Dhama K, Emran T Bin, Simal-Gandara J, Capasso R**. Bromelain a potential bioactive compound: A comprehensive overview from a pharmacological perspective. *Life* 11:317, 2021.

15. **Champaneria MC, Workman AD, Gupta SC**. Sushruta: Father of plastic surgery. *Annals of Plastic Surgery* 73:2-7, 2014.

16. **Chase RA**. Skin and soft-tissue coverage - Retrospective. *Clinics in Plastic Surgery* 13:195-203, 1986.

17. **Cheng YT, Yang CC, Shyur LF**. Phytomedicine - Modulating oxidative stress and the tumor microenvironment for cancer therapy. *Pharmacological Research* 114:128-143, 2016.

18. **Cheuka PM, Mayoka G, Mutai P, Chibale K**. The role of natural products in drug discovery and development against neglected tropical diseases. *Molecules* 22:58, 2017.

19. **Chiba T, Ezaki O**. Dietary restriction suppresses inflammation and delays the onset of stroke in stroke-prone spontaneously hypertensive rats. *Biochemical and Biophysical Research Communications* 399:98-103, 2010.

20. **Cignarella F, Cantoni C, Ghezzi L, Salter A, Dorsett Y, Chen L, Phillips D, Weinstock GM, Fontana L, Cross AH, Zhou Y, Piccio L**. Intermittent fasting confers protection in CNS autoimmunity by altering the gut microbiota. *Cell Metabolism* 27:1222-1235, 2018.

21. **Clark AM**. Natural products as a resource for new drugs. *Pharmaceutical Research* 13:1133-1144, 1996.

22. **Delaney A, Diamantis S, Marks VJ**. Complications of tissue ischemia in dermatologic surgery. *Dermatologic Therapy* 24:551-557, 2011.

23. **Ekor M**. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology* 4:177, 2014.

24. **Eltzschig HK, Eckle T**. Ischemia and reperfusion-from mechanism to translation. *Nature Medicine* 17:1391-1401, 2011.

25. **Endrich B, Asaishi K, Götz A, Meßmer K**. Technical report - a new chamber technique for microvascular studies in unanesthetized hamsters. *Research in Experimental Medicine* 177:125-134, 1980.

26. **Fang F, Chung KC**. An evolutionary perspective on the history of flap reconstruction in the upper extremity. *Hand Clinics* 30:109-122, 2014.

27. **Faris MAIE, Kacimi S, Al-Kurd RA, Fararjeh MA, Bustanji YK, Mohammad MK, Salem ML.** Intermittent fasting during Ramadan attenuates proinflammatory cytokines and immune cells in healthy subjects. *Nutrition Research* 32:947-955, 2012.

28. **Fitzal F, DeLano FA, Young C, Schmid-Schönbein GW**. Early capillary no-reflow during low-flow reperfusion after hind limb ischemia in the rat. *Annals of Plastic Surgery* 49:170-180, 2002.

29. **Fitzhugh DJ, Shan S, Dewhirst MW, Hale LP**. Bromelain treatment decreases neutrophil migration to sites of inflammation. *Clinical Immunology* 128:66-74, 2008.

30. **Foroglou P, Karathanasis V, Demiri E, Koliakos G, Papadakis M**. Role of adipose-derived stromal cells in pedicle skin flap survival in experimental animal models. *World Journal of Stem Cells* 8:101-105, 2016.

31. **Freiman A, Shandalov Y, Rosenfeld D, Shor E, Ben-David D, Meretzki S, Levenberg S, Egozi D.** Engineering vascularized flaps using adipose-derived microvascular endothelial cells and mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine* 12:e130-e141, 2018.

32. **Gehmert S, Wenzel C, Loibl M, Brockhoff G, Huber M, Krutsch W, Nerlich M, Gosau M, Klein S, Schreml S, Prantl L, Gehmert S**. Adipose tissue-derived stem cell secreted IGF-1 protects myoblasts from the negative effect of myostatin. *BioMed Research International* 2014:129048, 2014.

33. **George BP, Chandran R, Abrahamse H**. Role of phytochemicals in cancer chemoprevention: Insights. *Antioxidants* 10:1455, 2021.

34. **Gillies HD, Millard DR**, The principles and art of plastic surgery. *Little, Brown & Company*, Boston, 1957.

35. **Gläser D, Hilberg T**. The influence of bromelain on platelet count and platelet activity in vitro. *Platelets* 17:37-41, 2006.

36. **Goodman T**. Grafts and flaps in plastic surgery: An overview. *AORN Journal* 48:650-655; 658-663, 1988.

37. **Grundmann F, Müller RU, Reppenhorst A, Hülswitt L, Späth MR, Kubacki T, Scherner M, Faust M, Becker I, Wahlers T, Schermer B, Benzing T, Burst V**. Preoperative short-term calorie restriction for prevention of acute kidney injury after cardiac surgery: A randomized, controlled, open-label, pilot trial. *Journal of the American Heart Association* 7:e008181, 2018.

38. **Gwozdzinski L, Bernasinska-Slomczewska J, Hikisz P, Wiktorowska-Owczarek A, Kowalczyk E, Pieniazek A**. The effect of diosmin, escin, and bromelain on human endothelial cells derived from the umbilical vein and the varicose vein - a preliminary study. *Biomedicines* 11:1702, 2023.

39. **Haidarali S, Patil C, Ojha S, Mohanraj R, Arya D, Goyal S**. Targeting apoptotic pathways in myocardial infarction: Attenuated by phytochemicals. *Cardiovascular & Hematological Agents in Medicinal Chemistry* 12:72-85, 2015.

40. **Hale LP, Greer PK, Sempowski GD**. Bromelain treatment alters leukocyte expression of cell surface molecules involved in cellular adhesion and activation. *Clinical Immunology* 104:183-190, 2002.

41. **Hanna AD, Lam A, Tham S, Dulhunty AF, Beard NA**. Adverse effects of doxorubicin and its metabolic product on cardiac RyR2 and SERCA2A. *Molecular Pharmacology* 86:438-449, 2014.

42. **Harder Y, Contaldo C, Klenk J, Banic A, Jakob SM, Erni D**. Improved skin flap survival after local heat preconditioning in pigs. *Journal of Surgical Research* 119:100-105, 2004a.

43. **Harder Y, Amon M, Erni D, Menger MD**. Evolution of ischemic tissue injury in a random pattern flap: A new mouse model using intravital microscopy. *Journal of Surgical Research* 121:197-205, 2004b.

44. **Harder Y, Schmauss D, Wettstein R, Egaña JT, Weiss F, Weinzierl A, Schuldt A, Machens HG, Menger MD, Rezaeian F**. Ischemic tissue injury in the dorsal skinfold chamber of the mouse: A skin flap model to investigate acute persistent ischemia. *Journal of Visualized Experiments* 2014:e51900, 2014.

45. **Hardiany NS, Karman AP, Calista ASP, Anindyanari BG, Rahardjo DE, Novira PR, Taufiq RR, Imtiyaz S, Antarianto RD**. The Effect of fasting on oxidative stress in the vital organs of New Zealand white rabbit. *Reports of Biochemistry and Molecular Biology* 11:190-199, 2022.

46. **He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB**. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through Flk-1/KDR activation of c-Src. *Journal of Biological Chemistry* 274:25130-25135, 1999.

47. **Hollenbeck ST, Senghaas A, Komatsu I, Zhang Y, Erdmann D, Klitzman B**. Tissue engraftment of hypoxic-preconditioned adipose-derived stem cells improves flap viability. *Wound Repair and Regeneration* 20:872-878, 2012.

48. **Il'yasova D, Fontana L, Bhapkar M, Pieper CF, Spasojevic I, Redman LM, Das SK, Huffman KM, Kraus WE.** Effects of 2 years of caloric restriction on oxidative status assessed by urinary F2-isoprostanes: The CALERIE 2 randomized clinical trial. *Aging Cell* 17:e12719, 2018.

49. **Insuan O, Janchai P, Thongchuai B, Chaiwongsa R, Khamchun S, Saoin S, Insuan W, Pothacharoen P, Apiwatanapiwat W, Boondaeng A, Vaithanomsat P**. Anti-inflammatory effect of pineapple rhizome bromelain through downregulation of the NF-κB-and MAPKs-signaling pathways in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. *Current Issues in Molecular Biology* 43:93-106, 2021.

50. **Ismaeel A, Greathouse KL, Newton N, Miserlis D, Papoutsi E, Smith RS, Eidson JL, Dawson DL, Milner CW, Widmer RJ, Bohannon WT, Koutakis P.** Phytochemicals as therapeutic interventions in peripheral artery disease. *Nutrients* 13:2143, 2021.

51. **Jongbloed F, De Bruin RWF, Pennings JLA, Payán-Gómez C, Van Den Engel S, Van Oostrom CT, De Bruin A, Hoeijmakers JHJ, Van Steeg H, IJzermans JNM, Dollé MET**. Preoperative fasting protects against renal ischemia-reperfusion injury in aged and overweight mice. *PLoS ONE* 9:e100853, 2014.

52. **Jongbloed F, de Bruin RWF, Klaassen RA, Beekhof P, van Steeg H, Dor FJMF, van der Harst E, Dollé MET, Ijzermans JNM**. Short-term preoperative calorie and protein restriction is feasible in healthy kidney donors and morbidly obese patients scheduled for surgery. *Nutrients* 8:306, 2016.

53. **Juhasz B, Thirunavukkarasu M, Pant R, Zhan L, Penumathsa SV, Secor ER, Srivastava S, Raychaudhuri U, Menon VP, Otani H, Thrall RS, Maulik N**. Bromelain induces cardioprotection against ischemia-reperfusion injury through Akt/FOXO pathway in rat myocardium. *American Journal of Physiology - Heart and Circulatory Physiology* 294:H1365-1370, 2008.

54. **Kalogeris T, Bao Y, Korthuis RJ**. Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology* 2:702-714, 2014.

55. **Karlsen M, Hovden AO, Vogelsang P, Tysnes BB, Appel S**. Bromelain treatment leads to maturation of monocyte-derived dendritic cells but cannot replace PGE 2 in a cocktail of IL-1β, IL-6, TNF-α and PGE 2. *Scandinavian Journal of Immunology* 74:135-143, 2011.

56. **Kasemsuk T, Vivithanaporn P, Unchern S**. Anti-inflammatory effects of bromelain in lps-induced human U937 macrophages. *Chiang Mai Journal of Science* 45:299-307, 2018.

57. **Kip P, Trocha KM, Tao M, O'leary JJ, Ruske J, Giulietti JM, Trevino-Villareal JH, MacArthur MR, Bolze A, Burak MF, Patterson S, Ho KJ, Carmody RN, Guzman RJ, Mitchell JR, Ozaki CK**. Insights from a short-term protein-calorie restriction exploratory trial in elective carotid endarterectomy patients. *Vascular and Endovascular Surgery* 53:470-476, 2019.

58. **Kip P, Sluiter TJ, Moore JK, Hart A, Ruske J, O'leary J, Jung J, Tao M, Macarthur MR, Heindel P, de Jong A, de Vries MR, Burak MF, Mitchell SJ, Mitchell JR, Ozaki CK**. Short-term pre-operative protein caloric restriction in elective vascular surgery patients: A randomized clinical trial. *Nutrients* 13:4024, 2021.

59. **Kondo M, Shibata R, Miura R, Shimano M, Kondo K, Li P, Ohashi T, Kihara S, Maeda N, Walsh K, Ouchi N, Murohara T**. Caloric restriction stimulates revascularization in response to ischemia via adiponectin-mediated activation of endothelial nitric-oxide synthase. *Journal of Biological Chemistry* 284:1718-1724, 2009.

60. **Küntscher M V., Hartmann B, Germann G**. Remote ischemic preconditioning of flaps: A review. *Microsurgery* 25:346-352, 2005.

61. **Laschke MW, Kleer S, Scheuer C, Schuler S, Garcia P, Eglin D, Alini M, Menger MD**. Vascularisation of porous scaffolds is improved by incorporation of adipose tissue-derived microvascular fragments. *European Cells and Materials* 24:266-277, 2012.

62. **Laschke MW, Menger MD**. Adipose tissue-derived microvascular fragments: Natural vascularization units for regenerative medicine. *Trends in Biotechnology* 33:442-448, 2015.

63. **Laschke MW, Menger MD**. The dorsal skinfold chamber: A versatile tool for preclinical research in tissue engineering and regenerative medicine. *European Cells and Materials* 32:202-215, 2016.

64. **Laschke MW, Karschnia P, Scheuer C, Heß A, Metzger W, Menger MD**. Effects of cryopreservation on adipose tissue-derived microvascular fragments. *Journal of Tissue Engineering and Regenerative Medicine* 12:1020-1030, 2018.

65. **Laschke MW, Später T, Menger MD**. Microvascular fragments: More than just natural vascularization units. *Trends in Biotechnology* 39:24-33, 2021.

66. **Laschke MW, Menger MD.** Microvascular fragments in microcirculation research and regenerative medicine. *Tissue Engineering Part B: Reviews* 28:1109-1120, 2022.

67. **Lee DW, Jeon YR, Cho EJ, Kang JH, Lew DH**. Optimal administration routes for adipose-derived stem cells therapy in ischaemic flaps. *Journal of Tissue Engineering and Regenerative Medicine* 8:596-603, 2014.

68. **Lee JH, Lee JT, Park HR, Kim JB**. The potential use of bromelain as a natural oral medicine having anticarcinogenic activities. *Food Science and Nutrition* 7:1656-1667, 2019.

69. **Lee SH.** Human adipose-derived stem cells' paracrine factors in conditioned medium can enhance porcine oocyte maturation and subsequent embryo development. *International Journal of Molecular Sciences* 22:579, 2021.

70. **Li J, Bao G, ALyafeai E, Ding J, Li S, Sheng S, Shen Z, Jia Z, Lin C, Zhang C, Lou Z, Xu H, Gao W, Zhou K**. Betulinic acid enhances the viability of random-pattern skin flaps by activating autophagy. *Frontiers in Pharmacology* 10:1017, 2019.

71. **Liu RH**. Health-promoting components of fruits and vegetables in the diet. *Advances in Nutrition* 4:384S-392S, 2013.

72. **Lucas JB**. The physiology and biomechanics of skin flaps. *Facial Plastic Surgery Clinics of North America* 25:303-311, 2017.

73. **Madeo F, Carmona-Gutierrez D, Hofer SJ, Kroemer G**. Caloric restriction mimetics against age-associated disease: Targets, mechanisms, and therapeutic potential. *Cell Metabolism* 29:592-610, 2019.

74. **Malinowski B, Zalewska K, Węsierska A, Sokołowska MM, Socha M, Liczner G, Pawlak-Osińska K, Wiciński M.** Intermittent fasting in cardiovascular disorders - an overview. *Nutrients* 11:673, 2019.

75. **Martinez-Lopez N, Tarabra E, Toledo M, Garcia-Macia M, Sahu S, Coletto L, Batista-Gonzalez A, Barzilai N, Pessin JE, Schwartz GJ, Kersten S, Singh R**. System-wide benefits of intermeal fasting by autophagy. *Cell Metabolism* 26:856-871, 2017.

76. **Maurer HR**. Bromelain: Biochemistry, pharmacology and medical use. *Cellular and Molecular Life Sciences* 58:1234-1245, 2001.

77. **McGregor IA, Morgan G**. Axial and random pattern flaps. *British Journal of Plastic Surgery* 26:202-213, 1973.

78. **Menger MD, Laschke MW, Vollmar B**. Viewing the microcirculation through the window: Some twenty years experience with the hamster dorsal skinfold chamber. *European Surgical Research* 34:83-91, 2002.

79. **Menger MD, Laschke MW, Amon M, Schramm R, Thorlacius H, Rücker M, Vollmar B**. Experimental models to study microcirculatory dysfunction in muscle ischemia-reperfusion and osteomyocutaneous flap transfer. *Langenbeck's Archives of Surgery* 388:281-290, 2003.

80. **Metzig C, Grabowska E, Eckert K, Rehse K, Maurer HR**. Bromelain proteases reduce human platelet aggregation in vitro, adhesion to bovine endothelial cells and thrombus formation in rat vessels in vivo. *In Vivo* 13:7-12, 1999.

81. **Michalsen A, Li C.** Fasting therapy for treating and preventing disease - current state of evidence. *Forschende Komplementarmedizin* 20:444-453, 2013.

82. **Milton SH**. Pedicled skin-flaps: The fallacy of the length : width ratio. *British Journal of Surgery* 57:502-508, 1970.

83. **Mitchell JR, Verweij M, Brand K, van de Ven M, Goemaere N, van den Engel S, Chu T, Forrer F, Müller C, de Jong M, van IJcken W, IJzermans JNM, Hoeijmakers JHJ, de Bruin RWF**. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell* 9:40-53, 2010.

84. **Mitchell JR, Beckman JA, Nguyen LL, Ozaki CK**. Reducing elective vascular surgery perioperative risk with brief preoperative dietary restriction. *Surgery* 153:594-598, 2013.

85. **Nakai M, Ribeiro R V., Stevens BR, Gill P, Muralitharan RR, Yiallourou S, Muir J, Carrington M, Head GA, Kaye DM, Marques FZ**. Essential hypertension is associated with changes in gut microbial metabolic pathways: A multisite analysis of ambulatory blood pressure. *Hypertension* 78:804-815, 2021.

86. **Nakano M, Nakajima Y, Kudo S, Tsuchida Y, Nakamura H, Fukuda O**. Effect of autotransplantation of microvessel fragments on experimental random-pattern flaps in the rat. *European Surgical Research* 30:149-160, 1998.

87. **Neri M, Fineschi V, Paolo M, Pomara C, Riezzo I, Turillazzi E, Cerretani D**. Cardiac oxidative stress and inflammatory cytokines response after myocardial infarction. *Current Vascular Pharmacology* 13:26-36, 2015.

88. **Neumayer C, Fügl A, Nanobashvili J, Blumer R, Punz A, Gruber H, Polterauer P, Huk I**. Combined enzymatic and antioxidative treatment reduces ischemia-reperfusion injury in rabbit skeletal muscle. *Journal of Surgical Research* 133:150-158, 2006.

89. **Patil RR, Guhagarkar SA, Devarajan PV**. Engineered nanocarriers of doxorubicin: A current update. *Critical Reviews in Therapeutic Drug Carrier Systems* 25:1-61, 2008.

90. **Pavan R, Jain S, Shraddha, Kumar A**. Properties and therapeutic application of bromelain: A review. *Biotechnology Research International* 2012:976203, 2012.

91. **Pei Y, Zhang L, Mao X, Liu Z, Cui W, Sun X, Zhang Y**. Biomaterial scaffolds for improving vascularization during skin flap regeneration. *Chinese Journal of Plastic and Reconstructive Surgery* 2:109-119, 2020.

92. **Pekas EJ, Shin J, Headid RJ, Son WM, Layec G, Yadav SK, Scott SD, Park SY.** Combined anthocyanins and bromelain supplement improves endothelial function and skeletal muscle oxygenation status in adults: A double-blind placebo-controlled randomised crossover clinical trial. *British Journal of Nutrition* 125:161-171, 2021.

93. **Prasanna PL, Renu K, Valsala Gopalakrishnan A**. New molecular and biochemical insights of doxorubicin-induced hepatotoxicity. *Life Sciences* 250:117599, 2020.

94. **Pribaz JJ, Fine NA**. Prelamination: Defining the prefabricated flap - a case report and review. *Microsurgery* 15:618-623, 1994.

95. **Puhr-Westerheide D, Schink SJ, Fabritius M, Mittmann L, Hessenauer MET, Pircher J, Zuchtriegel G, Uhl B, Holzer M, Massberg S, Krombach F, Reichel CA**. Neutrophils promote venular thrombosis by shaping the rheological environment for platelet aggregation. *Scientific Reports* 9:15932, 2019.

96. **Qian Q, He W, Liu D, Yin J, Ye L, Chen P, Xu D, Liu J, Li Y, Zeng G, Li M, Wu Z, Zhang Y, Wang X, DiSanto ME, Zhang X**. M2a macrophage can rescue proliferation and gene expression of benign prostate hyperplasia epithelial and stroma cells from insulin-like growth factor 1 knockdown. *Prostate* 81:530-542, 2021.

97. **Raffaghello L, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, Longo VD**. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proceedings of the National Academy of Sciences of the United States of America* 105:8215-8220, 2008.

98. **Rathnavelu V, Alitheen NB, Sohila S, Kanagesan S, Ramesh R**. Potential role of bromelain in clinical and therapeutic applications (Review). *Biomedical Reports* 5:283-288, 2016.

99. **Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, Romashkan S, Williamson DA, Meydani SN, Villareal DT, Smith SR, Stein RI, Scott TM, Stewart TM, Saltzman E, Klein S, Bhapkar M, Martin CK, Gilhooly CH, Holloszy JO, Hadley EC, Roberts SB**. A 2-year randomized controlled trial of human caloric restriction: Feasibility and effects on predictors of health span and longevity. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* 70:1097-1104, 2015.

100. **Redman LM, Smith SR, Burton JH, Martin CK, Il'yasova D, Ravussin E**. Metabolic slowing and reduced oxidative damage with sustained caloric restriction support the rate of living and oxidative damage theories of aging. *Cell Metabolism* 27:805-815, 2018.

101. **Reinisch JF**. The pathophysiology of skin flap circulation: The delay phenomenon. *Plastic and Reconstructive Surgery* 54:585-598, 1974.

102. **Rezaeian F, Wettstein R, Scheuer C, Schenck TL, Egaña JT, Machens HG, Menger MD, Harder Y**. Long-term preconditioning with erythropoietin reduces ischemia-induced skin necrosis. *Microcirculation* 20:693-702, 2013.

103. **Richards EM, Li J, Stevens BR, Pepine CJ, Raizada MK.** Gut microbiome and neuroinflammation in hypertension. *Circulation Research* 130:401-417, 2022.

104. **Richards RR, Seaber A V., Urbaniak JR**. Chemically induced vasospasm: The effect of ischemia, vessel occlusion, and adrenergic blockade. *Plastic and Reconstructive Surgery* 75:238-244, 1985.

105. **Rinninella E, Cintoni M, Raoul P, Ianiro G, Laterza L, Lopetuso LR, Ponziani FR, Gasbarrini A, Mele MC**. Gut microbiota during dietary restrictions: New insights in non-communicable diseases. *Microorganisms* 8:1140, 2020.

106. **Robertson LT, Mitchell JR**. Benefits of short-term dietary restriction in mammals. *Experimental Gerontology* 48:1043-1048, 2013.

107. **Romano B, Fasolino I, Pagano E, Capasso R, Pace S, de Rosa G, Milic N, Orlando P, Izzo AA, Borrelli F**. The chemopreventive action of bromelain, from pineapple stem (Ananas comosus L.), on colon carcinogenesis is related to antiproliferative and proapoptotic effects. *Molecular Nutrition and Food Research* 58:457-465, 2014.

108. **Schmauss D, Weinzierl A, Weiss F, Egaña JTT, Rezaeian F, Hopfner U, Schmauss V, Machens HG, Harder Y**. Long-term pre- and postconditioning with low doses of erythropoietin protects critically perfused musculocutaneous tissue from necrosis. *Journal of Plastic, Reconstructive and Aesthetic Surgery* 72:590-599, 2019.

109. **Shepherd BR, Chen HYS, Smith CM, Gruionu G, Williams SK, Hoying JB**. Rapid perfusion and network remodeling in a microvascular construct after implantation. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24:898-904, 2004.

110. **Shoba E, Lakra R, Kiran MS, Korrapati PS**. Fabrication of core-shell nanofibers for controlled delivery of bromelain and salvianolic acid B for skin regeneration in wound therapeutics. *Biomedical Materials* 12:035005, 2017.

111. **Shoba E, Lakra R, Kiran MS, Korrapati PS.** 3 D nano bilayered spatially and functionally graded scaffold impregnated bromelain conjugated magnesium doped hydroxyapatite nanoparticle for periodontal regeneration. *Journal of the Mechanical Behavior of Biomedical Materials* 109:103822, 2020.

112. **Siddiqui J, Singh A, Chagtoo M, Singh N, Godbole M, Chakravarti B.** Phytochemicals for breast cancer therapy: Current status and future implications. *Current Cancer Drug Targets* 15:116-135, 2015.

113. **Soñanez-Organis JG, Vázquez-Medina JP, Crocker DE, Ortiz RM.** Prolonged fasting activates hypoxia inducible factors-1α, -2α and -3α in a tissue-specific manner in northern elephant seal pups. *Gene* 526:155-163, 2013.

114. **Später T, Menger MM, Nickels RM, Menger MD, Laschke MW**. Macrophages promote network formation and maturation of transplanted adipose tissue–derived microvascular fragments. *Journal of Tissue Engineering* 11:2041731420911816, 2020.

115. **Stone R, Rathbone CR**. Microvascular fragment transplantation improves rat dorsal skin flap survival. *Plastic and Reconstructive Surgery - Global Open* 4:e1140, 2016.

116. **Takase MT, Hashimoto I, Nakanishi H, Nakaya Y, Harada H, Sedo H, Kanda I**. Sarpogrelate hydrochloride, an antagonist of 5-hydroxytryptamine receptor, improves skin flap survival and suppresses thrombus formation in injured microvessels of rabbits. *Journal of Reconstructive Microsurgery* 22:59-65, 2006.

117. **Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT**. PAMPs and DAMPs: Signal 0s that spur autophagy and immunity. *Immunological Reviews* 249:158-175, 2012.

118. **Taussig SJ, Batkin S**. Bromelain, the enzyme complex of pineapple (Ananas comosus) and its clinical application. An update. *Journal of Ethnopharmacology* 22:191-203, 1988.

119. **Thangapazham RL, Sharad S, Maheshwari RK**. Phytochemicals in wound healing. *Advances in Wound Care* 5:230-241, 2016.

120. **Tinkum KL, Stemler KM, White LS, Loza AJ, Jeter-Jones S, Michalski BM, Kuzmicki C, Pless R, Stappenbeck TS, Piwnica-Worms D, Piwnica-Worms H**. Fasting protects mice from lethal DNA damage by promoting small intestinal epithelial stem cell survival. *Proceedings of the National Academy of Sciences of the United States of America* 112:E7148-E7154, 2015.

121. **Tobalem M, Wettstein R, Pittet-Cuénod B, Vigato E, Machens HG, Lohmeyer JA, Rezaeian F, Harder Y**. Local shockwave-induced capillary recruitment improves survival of musculocutaneous flaps. *Journal of Surgical Research* 184:1196-1204, 2013.

122. **Uysal AC, Mizuno H, Tobita M, Ogawa R, Hyakusoku H**. The effect of adipose-derived stem cells on ischemia-reperfusion injury: Immunohistochemical and ultrastructural evaluation. *Plastic and Reconstructive Surgery* 124:804-815, 2009.

123. **Vogt PM, Boorboor P, Vaske B, Topsakal E, Schneider M, Muehlberger T**. Significant angiogenic potential is present in the microenvironment of muscle flaps in humans. *Journal of Reconstructive Microsurgery* 21:517-523, 2005.

124. **Wahab MSA, Zaini MH, Ali AA, Sahudin S, Mehat MZ, Hamid HA, Mustaffa MF, Othman N, Maniam S.** The use of herbal and dietary supplement among community-dwelling elderly in a suburban town of Malaysia. *BMC Complementary Medicine and Therapies* 21:110, 2021.

125. **Walsh ME, Shi Y, Van Remmen H**. The effects of dietary restriction on oxidative stress in rodents. *Free Radical Biology and Medicine* 66:88-99, 2014.

126. **Wang WZ, Baynosa RC, Zamboni WA**. Update on ischemia-reperfusion injury for the plastic surgeon: 2011. *Plastic and Reconstructive Surgery* 128:658e-692e, 2011.

127. **Weinzierl A, Ampofo E, Menger MD, Laschke MW**. Tissue-protective mechanisms of bioactive phytochemicals in flap surgery. *Frontiers in Pharmacology* 13:864351, 2022.

128. **Yao ST**. Microvascular transplantation of prefabricated free thigh flap. *Plastic and Reconstructive Surgery* 69:568, 1982.

129. **Zahir KS, Syed SA, Zink JR, Restifo RJ, Thomson JG**. Ischemic preconditioning improves the survival of skin and myocutaneous flaps in a rat model. *Plastic and Reconstructive Surgery* 102:140-150, 1998.

130. **Zhang YJ, Gan RY, Li S, Zhou Y, Li AN, Xu DP, Li H Bin, Kitts DD.** Antioxidant phytochemicals for the prevention and treatment of chronic diseases. *Molecules* 20:21138-21156, 2015.

131. **Zheng X, Wang S, Jia W.** Calorie restriction and its impact on gut microbial composition and global metabolism. *Frontiers of Medicine* 12:643-644, 2018.

132. **Zhu C, Song K, Shen Z, Quan Y, Tan B, Luo W, Wu S, Tang K, Yang Z, Wang X**. Roseburia intestinalis inhibits interleukin-17 excretion and promotes regulatory T-cells differentiation in colitis. *Molecular Medicine Reports* 17:7567-7574, 2018.

133. **Zimna A, Kurpisz M**. Hypoxia-inducible factor-1 in physiological and pathophysiological angiogenesis: Applications and therapies. *BioMed Research International* 2015:549412, 2015.

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9. PUBLICATIONS

9.1. ORIGINAL ARTICLES

1. Harder Y, Schmauss D, Wettstein R, Egaña JT, Weiss F, **Weinzierl A**, Schuldt A, Machens HG, Menger MD, Rezaeian F. Ischemic tissue injury in the dorsal skinfold chamber of the mouse: A skin flap model to investigate acute persistent ischemia. *Journal of Visualized Experiments* 17:e51900, 2014.

2. Schmauss D, Megerle K, **Weinzierl A**, Agua K, Cerny M, Schmauss V, Lohmeyer JA, Machens HG, Erne H. Microsurgeons do better - tactile training might prevent the age-dependent decline of the sensibility of the hand. *Journal des Peripheren Nerven Systems* 20:392-396, 2015.

3. Schmauss D, **Weinzierl A**, Weiss F, Egaña JT, Rezaeian F, Hopfner U, Schmauss V, Machens HG, Harder Y. Long-term pre- and postconditioning with low doses of erythropoietin protects critically perfused musculocutaneous tissue from necrosis. *Journal of Plastic Reconstructive and Aesthetic Surgery* 72:590-599, 2019.

4. Schmauss D, **Weinzierl A**, Harder Y. [Local Heat preconditioning for the prevention of ischemic complications in plastic surgery.] *Journal für Ästhetische Chirurgie* 12:182-183, 2019.

5. **Weinzierl A**, Harder Y, Schmauss D, Ampofo E, Menger MD, Laschke MW. Improved vascularization and survival of white compared to brown adipose tissue grafts in the dorsal skinfold chamber. *Biomedicines* 10:23, 2021.

6. **Weinzierl A**, Harder Y, Schmauss D, Menger MD, Laschke MW. Boosting tissue vascularization: Nanofat as a potential source of functional microvessel segments. *Frontiers in Bioengineering and Biotechnology* 10:820835, 2022.

7. Weiss F, Agua K, **Weinzierl A**, Schuldt A, Egana JT, Schlitter AM, Steiger K, Machens HG, Harder Y, Schmauss D. A modified burn comb model with a new dorsal frame that allows for local treatment in partial-thickness burns in rats. *Journal of Burn Care Research* 43:1329-1336, 2022.

8. Wrublewsky S, Speer T, Nalbach L, Boewe AS, Pack M, Alansary D, Roma LP, Hoffmann MDA, Schmitt BM, **Weinzierl A**, Menger MD, Laschke MW, Ampofo E. Targeting pancreatic islet NLRP3 improves islet graft revascularization. *Diabetes* 71:1706-1720, 2022.

9. **Weinzierl A**, Harder Y, Schmauss D, Menger MD, Laschke MW. Bromelain protects critically perfused musculocutaneous flap tissue from necrosis. *Biomedicines* 10:1449, 2022.

10. Wrublewsky S, **Weinzierl A**, Hornung I, Prates-Roma L, Menger MD, Laschke MW, Ampofo E. Co-transplantation of pancreatic islets and microvascular fragments effectively restores normoglycemia in diabetic mice. *NPJ Regenerative Medicine* 7:67, 2022.

11. Schmauss D, Pöhlmann S, **Weinzierl A**, Schmauss V, Moog P, Germann G, Bickert B, Megerle K. Relevance of the scaphoid shift test for the investigation of scapholunate ligament injuries. *Journal of Clinical Medicine* 11:6322, 2022.

12. **Weinzierl A,** Harder Y, Menger MD, Laschke MW. Perioperative intermittent fasting protects ischemic musculocutaneous flap tissue from necrosis. *Plastic and Reconstructive Surgery* 151:1030-1041, 2023

13. **Weinzierl A,** Harder Y, Schmauss D, Menger MD, Laschke MW. Microvascular fragments protect ischemic musculocutaneous flap tissue from necrosis by improving nutritive tissue perfusion and suppressing apoptosis. *Biomedicines* 11:1454, 2023.

14. **Weinzierl A**, Barbon C, Gousopoulos E, von Reibnitz D, Giovanoli P, Grünherz L, Lindenblatt N. Benefits of robotic-assisted lymphatic microsurgery in deep anatomical planes. *Journal of Plastic Reconstructive and Aesthetic Surgery Open* 37:145-154, 2023.

15. **Weinzierl A**, Coerper M, Harder Y, Menger MD, Laschke MW. Caloric restriction: A novel conditioning strategy to improve the survival of ischemically challenged musculocutaneous random pattern flaps. *Nutrients* 15:4076, 2023.

16. Limido E, **Weinzierl A**, Ampofo E, Harder Y, Menger MD, Laschke MW. Nanofat accelerates and improves the vascularization, lymphatic drainage and healing of full-thickness murine skin wounds. *International Journal of Molecular Sciences* 25:851, 2024.

17. **Weinzierl A**, Coerper M, Harder Y, Menger MD, Laschke MW. Short-term periodic fasting reduces ischemia-induced necrosis in musculocutaneous flap tissue. *Biomedicines* 12:690, 2024.

18. Von Reibnitz D, **Weinzierl A**, Barbon C, Gutschow CA, Giovanoli P, Grünherz L, Lindenblatt N. 100 anastomoses: a two-year single-center experience with robotic-assisted micro- and supermicrosurgery for lymphatic reconstruction. *Journal of Robotic Surgery* 18:164, 2024.

19. **Weinzierl A**, Grünherz L, Puippe GD, Gnannt R, von Reibnitz D, Giovanoli P, Vetter D, Möhrlen U, Wildgruber M, Müller A, Pieper CC, Gutschow CA, Lindenblatt N. Microsurgical central lymphatic reconstruction-the role of thoracic duct lymphovenous anastomoses at different anatomical levels. *Frontiers in Surgery* 11:1415010, 2024.

20. Limido E, **Weinzierl A**, Ampofo E, Harder Y, Menger MD, Laschke MW. Short-term cryoprotectant-free cryopreservation at -20°C does not affect the viability and regenerative capacity of nanofat. *Frontiers in Bioengineering and Biotechnology* 12:1427232, 2024.

9.2. REVIEW ARTICLES

1. Schmauss D, **Weinzierl A**, Schmauss V, Harder Y. Common rodent flap models in experimental surgery. *European Surgical Research* 59:255-264, 2018.

2. **Weinzierl A**, Ampofo E, Menger MD, Laschke MW. Tissue-protective mechanisms of bioactive phytochemicals in flap surgery. *Frontiers in Pharmacology* 13:864351, 2022.

3. **Weinzierl A**, Schmauss D, Brucato D, Harder Y. Implant-based breast reconstruction after mastectomy, from the subpectoral to the prepectoral approach: An evidence-based change of mind? *Journal of Clinical Medicine* 11:3079, 2022.

4. **Weinzierl A**, Schmauss D, Harder Y. [The significance of oncoplastic breast reconstruction after tumorectomy in surgical breast cancer therapy]. *Handchirurgie Mikrochirurgie Plastische Chirurgie* 54:305-313, 2022.

5. **Weinzierl A**, Schmauss D, Harder Y. [The value of synthetic and biologic meshes in implant-based breast reconstruction]. *Handchirurgie Mikrochirurgie Plastische Chirurgie* 54:269-278, 2022.

6. Limido E, **Weinzierl A**, Harder Y, Menger MD, Laschke MW. Fatter Is better: Boosting the vascularization of adipose tissue grafts. *Tissue Engineering Part B Reviews*. 29:605-622, 2023.

7. Brucato D, Ülgür II, Alberti A, **Weinzierl A**, Harder Y. Complications associated with facial autologous fat grafting for aesthetic purposes: A systematic review of the literature. *Plastic and Reconstructive Surgery Global Open* 12:e5538, 2024.

8. Bonomi F, Limido E, **Weinzierl A**, Harder Y, Menger MD, Laschke MW. Preconditioning Strategies for improving the outcome of fat grafting. *Tissue Engineering Part B Reviews* online ahead of print, 2024.

9.3. CASE REPORTS

1. Lo Presti G, **Weinzierl A**, Feuerlein K. [Cetuximab Multi Re-Challenge for Adenocarcinoma of the Sigmoid]. *Schweizer Krebsbulletin* 41:81-83, 2021

2. Grünherz L, **Weinzierl A**, Puippe GD, von Reibnitz D, Barbon C, Schneider MA, Giovanoli P, Gutschow CA, Lindenblatt N. First-in-human use of a microsurgical robotic system for central lymphatic reconstruction. *Plastic and Reconstructive Surgery Global Open.* 11:e5484, 2023.

3. Breckwoldt T, Niggemann P, Grünherz L, **Weinzierl A**, Lindenblatt N. Arm lymphedema after vascularized lymph node harvest following Covid-19 vaccination. *Case Reports in Plastic Surgery and Hand Surgery* 11:2342332, 2024.

10. SCHOLARSHIPS AND AWARDS

10/2010 - 04/2017 Max Weber Program Bavaria - Scholarship for gifted students

03/2018 *"Best Abstract in the context of a doctoral thesis"* at the 8th Research Day, University della Svizzera Italiana, Lugano, Switzerland

05/2023 European Research Council „Best Paper Award" for the paper *"Boosting tissue vascularization: Nanofat as a potential source of functional microvessel segments"*

09/2023 Swiss Plastic Surgery Scientific Award 2023 for the project *"Dietary conditioning strategies for increasing tissue survival in critically perfused musculocutaneous flaps"*

05/2024 European Research Council „Best Paper Award" second place for the paper *"Perioperative intermittent fasting protects ischemic musculocutaneous flap tissue from necrosis."*

11. Curriculum Vitae

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.