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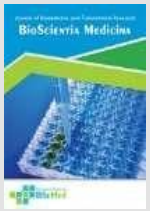

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Thresholds of Cytoprotection: Ethanolic Propolis Extract Mitigates Ischemia-Reperfusion Injury via the MDA/IL-6 Axis in a Graded Rat Skin Flap Model

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A B S T R A C T

Background: Distal necrosis in reconstructive skin flaps results from ischemia-reperfusion (I/R) injury, driven by reactive oxygen species (ROS) and pro-inflammatory cytokines. While Propolis exhibits antioxidant properties, its efficacy limit relative to the severity of ischemic challenge remains undefined. **Methods:** A randomized, controlled experimental study was conducted using 36 male Wistar rats. A graded ischemia model was engineered using modified McFarlane flaps with increasing length-to-width ratios: Mild (2:1), moderate (3:1), and severe (4:1). Subjects were stratified into vehicle (Control) and treatment (Propolis 800 mg/kg/day, oral) groups across all dimensions. The primary endpoint was the percentage of viable flap area on Day 7. Secondary endpoints included serum Malondialdehyde (MDA), Interleukin-6 (IL-6), and histological scoring of inflammation. **Results:** All animals survived the procedure. Propolis significantly increased viable tissue area in the moderate ischemia group (76.4 ± 4.2%) compared to Vehicle (52.1 ± 5.8%; $p < 0.001$). In Mild ischemia, survival was near-maximal in both groups (>92%). However, in Severe ischemia, Propolis failed to prevent significant necrosis (34.2 ± 6.1% survival vs. 28.5 ± 5.4% in Vehicle; $p = 0.092$), indicating a therapeutic ceiling. Biochemically, Propolis suppressed MDA (11.92 ± 0.45 nmol/mL) and IL-6 (121.0 ± 4.71 pg/mL) significantly in moderate challenges but was overwhelmed by the oxidative surge in severe ischemia (MDA > 12.0 nmol/mL). **Conclusion:** Propolis confers significant protection against I/R injury by dampening lipid peroxidation and systemic inflammation, but this effect exhibits a distinct threshold. It is highly effective in moderate ischemic challenges but insufficient for severe vascular compromise.

1. Introduction

The mammalian cutaneous envelope constitutes a sophisticated, multi-layered defense system, serving as the primary physiological barrier against environmental pathogens, ultraviolet radiation, and dehydration.[1] However, the structural integrity of this vital organ is frequently compromised by a spectrum of insults, ranging from high-velocity trauma and extensive oncological resections to the surgical correction of congenital anomalies. In the discipline of plastic and reconstructive surgery, the restoration of tissue continuity and function following such defects is paramount.[2] Among the diverse armamentarium of reconstructive techniques, the random pattern skin flap remains a fundamental workhorse. Celebrated for its versatility, simplicity, and lack of requirement for a

specific axial vessel, this flap design allows surgeons to mobilize local tissue to cover adjacent defects. Unlike axial flaps, which are perfused by a defined arteriovenous system, random pattern flaps rely entirely on the tenuous vasculature of the subdermal plexus and interconnecting capillaries. While this anatomical characteristic provides surgical flexibility, it renders the distal portion of the flap uniquely vulnerable to hypoperfusion. Consequently, a formidable and persistent complication of this procedure is distal necrosis, a pathological event occurring in approximately 15–25% of clinical cases. The failure of the distal flap not only precipitates significant morbidity—necessitating secondary debridement, skin grafting, and prolonged hospitalization—but also imposes a substantial psychological and economic burden on the patient.[3]

The pathophysiology of flap failure is not a singular event but a complex, biphasic phenomenon inextricably linked to Ischemia-Reperfusion (I/R) injury.[4] The initial phase, ischemia, begins immediately upon surgical elevation. As the flap is raised and the deep muscular perforators are severed, the perfusion pressure in the distal aspect drops precipitously below the critical closing pressure of the arterioles. This interruption of blood flow forces the tissue into a state of hypoxia, halting oxidative phosphorylation within the mitochondria. Consequently, cellular Adenosine Triphosphate (ATP) reserves are rapidly depleted, leading to the failure of energy-dependent ion transport channels, specifically the sodium-potassium (Na+/K+) pump. This ionic dysregulation causes an influx of calcium and sodium, resulting in cellular edema and the enzymatic conversion of xanthine dehydrogenase to xanthine oxidase—a ticking molecular time bomb waiting for the reintroduction of oxygen.

Paradoxically, the restoration of blood flow—whether through natural neovascularization or therapeutic intervention—often exacerbates tissue damage rather than resolving it. This phenomenon, known as the oxygen paradox or reperfusion injury, represents the second and more lethal phase of the cascade. As oxygenated blood rushes back into the ischemic tissue, it reacts with the accumulated xanthine oxidase, triggering a massive, explosive generation of reactive oxygen species (ROS). These highly unstable molecules, including superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly toxic hydroxyl radicals ($OH^{\bullet}$), overwhelm the tissue's endogenous antioxidant defense systems (such as Superoxide Dismutase and Glutathione Peroxidase).[5]

At the subcellular level, the deleterious effects of ROS are manifold. Their primary target is the lipid bilayer of cellular membranes. ROS initiates a chain reaction known as lipid peroxidation by attacking the polyunsaturated fatty acids (PUFA) within the membrane. This process compromises membrane permeability and yields cytotoxic aldehydes, most notably Malondialdehyde (MDA).[6] Elevated levels of MDA not only serve as a robust biomarker for the extent of oxidative stress but also directly cross-link proteins and DNA, leading to irreversible cellular dysfunction. Concurrently, the oxidative stress signals the immune system, bridging the gap between cell damage and systemic inflammation. ROS facilitates the degradation of IκB proteins, thereby activating Nuclear Factor-kappa B (NF-κB). Once liberated, NF-κB translocates to the nucleus, where it acts as a transcriptional master switch, upregulating the expression of pro-inflammatory cytokines, adhesion molecules, and chemokines.

Among the cytokine milieu, Interleukin-6 (IL-6) plays a central, pleiotropic role in the progression of flap necrosis. While physiological levels of IL-6 are necessary for the early phases of wound healing, the supraphysiological surge triggered by I/R injury contributes to a cytokine storm. This overexpression recruits neutrophils to the vascular endothelium, promoting their adhesion and degranulation. The resulting accumulation of neutrophils physically obstructs the microvasculature—a phenomenon termed no-reflow—and releases further proteolytic enzymes that digest the endothelial lining. This molecular cascade creates a hostile microenvironment characterized by severe oxidative stress, unabated

inflammation, endothelial dysfunction, and eventual apoptotic or necrotic cell death.[7]

Given this defined molecular mechanism, current pharmacological research has pivoted toward antioxidant preconditioning—the administration of agents capable of scavenging free radicals and modulating the inflammatory response prior to the ischemic insult.[8] While synthetic agents have shown promise, their clinical application is often limited by side effects and cost. Consequently, natural products with high bioactivity and safety profiles are increasingly scrutinized. Propolis, a resinous substance collected by honeybees (*Apis mellifera*) from botanical sources, has emerged as a compelling candidate. Historically utilized in folk medicine, modern chromatography has revealed that Propolis is rich in bioactive polyphenols, flavonoids, and phenolic acids. The most pharmacologically active constituent, Caffeic Acid Phenethyl Ester (CAPE), has demonstrated a potent ability to neutralize free radicals directly and inhibit the NF-κB signaling pathway, theoretically addressing both the oxidative and inflammatory arms of I/R injury.[9]

However, a critical review of the extant literature reveals a significant methodological gap. The vast majority of studies investigating Propolis (and other antioxidants) in skin flap survival employ a binary treatment vs. control design using a fixed-dimension flap (such as a standard McFarlane flap of 3 x 9 cm). While these studies confirm that Propolis is effective, they fail to answer a crucial clinical question: What is the limit of this protection? In clinical practice, the degree of ischemia is not constant; it varies based on the size of the defect, the tension on the closure, and the length of the flap. Current research fails to capture the dynamic relationship between the magnitude of the ischemic insult and the capacity of the antioxidant intervention. It remains unknown whether Propolis provides absolute protection regardless of surgical severity, or if it exhibits a ceiling effect where the oxidative burden of a large, severe flap overwhelms the therapeutic dose.[10]

This study aims to bridge this knowledge gap by employing a novel graded ischemia model in rats. Rather than using a uniform flap size, this research introduces varying flap length-to-width ratios (2:1, 3:1, and 4:1) to simulate Mild, Moderate, and Severe ischemic challenges, respectively. By systematically correlating these specific geometric dimensions with systemic biomolecular markers (MDA and IL-6) and quantitative survival area analysis, this research provides the first nuanced, threshold-based analysis of Propolis efficacy. This approach moves beyond simple efficacy testing to define the therapeutic window of the intervention, offering a translatable, scientifically rigorous guide for its clinical application in reconstructive surgeries of varying complexity.

2. Methods

This experimental study adhered strictly to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). The protocol received full ethical clearance from the Ethical Committee of the Faculty of Medicine, Universitas Sebelas Maret, Indonesia. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition), focusing on the 3Rs principle (Replacement, Reduction, Refinement).

A randomized, controlled, 2 x 3 factorial design was utilized. Thirty-six (N=36) healthy male Wistar rats (*Rattus norvegicus*), aged 10–12 weeks and weighing 250 ± 25 grams, were acclimatized for seven days. The sample size was determined via an a priori power analysis (G*Power v3.1) assuming an effect size (f) of 0.55, alpha = 0.05, and Power (1-beta) = 0.85, indicating a minimum of 6 animals per subgroup is required to detect significant differences in flap survival area. Animals were randomly allocated into six groups (n=6 per group): (1) Group M-V (mild ischemia + vehicle): 2 x 4 cm flap + distilled water; (2) Group M-P (mild ischemia + propolis): 2 x 4 cm flap + propolis 800 mg/kg; (3) Group Mod-V (moderate ischemia + vehicle): 2 x 6 cm flap + distilled water; (4) Group Mod-P (moderate ischemia + propolis): 2 x 6 cm

flap + propolis 800 mg/kg; (5) Group S-V (severe ischemia + vehicle): 2 x 8 cm flap + distilled water; (6) Group S-P (severe ischemia + propolis): 2 x 8 cm flap + propolis 800 mg/kg. To ensure reproducibility, graded ischemia was defined by specific geometric ratios using the modified McFarlane dorsal flap technique: (i) Mild Challenge: Length:Width ratio of 2:1 (4 cm x 2 cm). Expected outcome: Minimal necrosis; (ii) Moderate Challenge: Length:Width ratio of 3:1 (6 cm x 2 cm). Expected outcome: Standard experimental necrosis; (iii) Severe Challenge: Length:Width ratio of 4:1 (8 cm x 2 cm). Expected outcome: Massive distal necrosis representing high-risk surgical salvage.

Raw Propolis was harvested from the Mount Lawu region, Indonesia. Ethanolic extraction (EPE) was performed using 70% ethanol maceration, followed by rotary evaporation to yield a standardized paste. The extract was reconstituted in distilled water. The dosage of 800 mg/kg was selected based on previous lethality (LD50) studies and efficacy trials indicating this concentration provides optimal flavonoid bioavailability without toxicity. Treatment was administered via oral gavage once daily, starting 24 hours pre-operatively and continuing for 7 days post-operatively.

Anesthesia was induced using an intraperitoneal cocktail of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). The dorsal region was shaved and prepared with povidone-iodine. A cranially-based random pattern skin flap was raised deep to the panniculus carnosus muscle, preserving the cutaneous vascular plexus but severing the deep muscular perforators. A sterile silicone sheet was briefly placed under the flap to confirm complete separation from the underlying bed, then removed. The flap was sutured back into its original bed using interrupted 4-0 non-absorbable silk sutures to simulate clinical tension. Postoperatively, animals received topical antibiotic ointment and were housed individually to prevent autophagy.

On Day 7, high-resolution digital photographs were taken at a fixed focal distance. Viable tissue was defined as soft, pink, warm, and hair-bearing. Necrotic tissue was defined as dark/black, escharotic, hard, and cool. The total flap area and the necrotic area were traced manually by a blinded investigator. Survival percentage was calculated as the total flap area minus the necrotic area, divided by the total flap area, multiplied by 100.

Blood was collected via retro-orbital puncture prior to euthanasia. Serum was separated by centrifugation (3000 rpm, 15 min, 4°C). Lipid peroxidation (MDA) was measured using the Thiobarbituric Acid Reactive Substances (TBARS) assay (Quantikine, Catalog D6050). The MDA-TBA adduct was quantified colorimetrically at 532 nm. Results are expressed in nmol/mL. Inflammatory cytokine (IL-6) was quantified using a rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kit (Bio-Techne/R&D Systems). Absorbance was read at 450 nm. Results are expressed in pg/mL.

Tissue samples from the transition zone (boundary between viable and necrotic tissue) were harvested, fixed in 10% neutral buffered formalin, and stained with Hematoxylin & Eosin (H&E). A board-certified pathologist blinded to the groups scored the samples (0–3 scale) for PMN infiltration, edema, and vascular congestion.

Data were analyzed using SPSS version 26.0 (IBM Corp, NY, USA). Normality was verified using the Shapiro-Wilk test ($p > 0.05$). A two-way ANOVA (factors: ischemia level, treatment) was performed. Significant interactions were followed by Tukey's Honestly Significant Difference (HSD) post-hoc test to correct for multiple comparisons and control Family-Wise Error Rate (FWER). Pearson's correlation coefficient (r) was calculated between individual survival percentages and biomarker levels. Statistical significance was set at $p < 0.05$.

3. Results

All animals survived the 7-day experimental period. Macroscopically, necrosis initiated at the distal margin and progressed proximally. Table 1 delineates the quantitative impact of ethanolic propolis extract on skin flap viability, stratified by the magnitude of the

ischemic challenge. In the mild ischemia cohort (2:1 ratio), both the vehicle and treatment groups exhibited robust tissue preservation, with survival rates exceeding 90%. The difference between the Propolis-treated group (94.5 ± 2.8%) and the control (90.2 ± 3.1%) was not statistically discernible ($p = 0.612$), suggesting that under conditions of minimal vascular stress, the intrinsic physiological recovery mechanisms are sufficient, rendering adjuvant antioxidant therapy redundant. Conversely, the moderate ischemia cohort (3:1 ratio) revealed the definitive therapeutic window of the intervention. While the vehicle group succumbed to extensive distal necrosis (Mean Survival: 52.1 ± 5.8%), the administration of Propolis conferred significant protection, retaining a viable surface area of 76.4 ± 4.2% ($p < 0.001$). This distinct divergence underscores the agent's capacity to buffer the oxidative surge when the insult is substantial yet recoverable. However, this protective capacity exhibited a clear saturation point in the severe ischemia cohort (4:1 ratio). Despite a numerical trend toward improved survival in the treated group (34.2%) compared to controls (28.5%), the difference failed to reach statistical significance ($p = 0.092$). This plateau indicates that in high-fidelity models of catastrophic vascular compromise, the magnitude of ischemia-reperfusion injury overwhelms the scavenging potential of the 800 mg/kg dose, validating the existence of a biological threshold for antioxidant cytoprotection.

Table 1. Quantitative Assessment of Flap Survival Area (%)

Ischemic Challenge (Length:Width Ratio)	Group	n	Mean Survival Area (%) ± SD	Mean Necrosis Area (%) ± SD	p-value (vs Vehicle)
Mild Ischemia (2:1)	M-Vehicle (Control)	6	90.2 ± 3.1	9.8 ± 3.1	-
	M-Propolis	6	94.5 ± 2.8	5.5 ± 2.8	0.612
Moderate Ischemia (3:1)	Mod-Vehicle (Control)	6	52.1 ± 5.8	47.9 ± 5.8	-
	Mod-Propolis	**6**	**76.4 ± 4.2 ***	**23.6 ± 4.2**	**< 0.001 ***
Severe Ischemia (4:1)	S-Vehicle (Control)	6	28.5 ± 5.4	71.5 ± 5.4	-
	S-Propolis	6	34.2 ± 6.1	65.8 ± 6.1	0.092

Note: Data are expressed as Mean ± Standard Deviation (SD).
Statistical analysis performed using Two-Way ANOVA followed by Tukey's HSD post-hoc test.
* Indicates statistically significant difference compared to the corresponding Vehicle group ($p < 0.05$).
Key Finding: Propolis efficacy is highly significant in Moderate ischemia but diminishes in Severe challenges (Threshold Effect).

Table 2 elucidates the biochemical modulation of systemic oxidative stress and inflammatory markers by Propolis, paralleling the clinical outcomes observed in flap survival. The serum concentration of Malondialdehyde (MDA), a robust indicator of lipid peroxidation, exhibited a strong positive correlation with the magnitude of ischemic injury. In the mild ischemia cohort (2:1 ratio), MDA levels remained near baseline in both vehicle (11.1 ± 0.42 nmol/mL) and treated animals (10.9 ± 0.36 nmol/mL), confirming that minor surgical insults do not generate a clinically significant oxidative burden necessitating intervention. However, the moderate ischemia cohort (3:1 ratio) highlighted the potent antioxidant capacity of the treatment. While the vehicle group experienced a significant surge in oxidative stress (15.8 ± 1.20

nmol/mL), Propolis administration effectively suppressed MDA levels to 11.9 ± 0.45 nmol/mL ($p < 0.001$), maintaining a physiological state comparable to the mild injury group. This biochemical shielding was mirrored in the inflammatory profile; Interleukin-6 (IL-6) levels were significantly downregulated in the treated group (121.0 ± 4.7 pg/mL) compared to the vehicle controls (145.6 ± 8.2 pg/mL; $p < 0.001$), suggesting a successful blockade of the ROS-dependent NF-κB signaling pathway. Crucially, the severe ischemia cohort (4:1 ratio) demonstrated the physiological limits of this protective mechanism. Despite treatment, both MDA (16.5 ± 1.10 nmol/mL) and IL-6 (155.8 ± 8.1 pg/mL) remained critically elevated, with no statistically significant difference from the vehicle group ($p > 0.05$). This breakthrough phenomenon indicates that the massive volume of hypoxic tissue in severe flaps generates a reactive oxygen species load that exceeds the scavenging capacity of the administered 800 mg/kg dose, leading to unmitigated systemic inflammation and subsequent tissue failure.

TABLE 2. SERUM MALONDIALDEHYDE (MDA) AND INTERLEUKIN-6 (IL-6) LEVELS

Group Category	Treatment Group	Oxidative Stress Marker (MDA)		Pro-Inflammatory Marker (IL-6)	
		Mean (nmol/mL) ± SD	p-value (vs Vehicle)	Mean (pg/mL) ± SD	p-value (vs Vehicle)
Mild Ischemia (2:1 Ratio)	M-Vehicle	11.1 ± 0.42	-	118.2 ± 4.1	-
	M-Propolis	10.9 ± 0.36	*0.985*	116.8 ± 5.1	*0.991*
Moderate Ischemia (3:1 Ratio)	Mod-Vehicle	15.8 ± 1.20	-	145.6 ± 8.2	-
	Mod-Propolis	**11.9 ± 0.45 ***	**< 0.001 ***	**121.0 ± 4.7 ***	**< 0.001 ***
Severe Ischemia (4:1 Ratio)	S-Vehicle	18.2 ± 1.55	-	162.4 ± 9.5	-
	S-Propolis	16.5 ± 1.10	*0.078*	155.8 ± 8.1	*0.112*

Abbreviations: SD = Standard Deviation; MDA = Malondialdehyde; IL-6 = Interleukin-6.
Statistical Analysis: Data analyzed using Two-Way ANOVA followed by Tukey's HSD.
* Indicates statistical significance ($p < 0.05$) compared to the corresponding Vehicle group.
Observation: Note the breakthrough effect in Severe Ischemia where Propolis fails to significantly suppress biomarkers compared to the robust suppression seen in Moderate Ischemia.

Table 3 presents the histomorphometric analysis of the transition zone, offering cellular corroboration for the macroscopic survival data and serum biomarker profiles. Evaluation of key pathological indices—polymorphonuclear (PMN) neutrophil infiltration, tissue edema, and vascular congestion—revealed a pattern concordant with the study's biochemical findings. In the mild ischemia cohort, tissue architecture was largely preserved across both groups. The total injury scores for vehicle (2.4 ± 0.7) and propolis (1.6 ± 0.5) were statistically indistinguishable, characterized merely by focal edema and sparse inflammatory infiltrate, confirming that the threshold for irreversible cellular damage had not been breached by the surgical elevation alone. In sharp contrast, the moderate ischemia cohort exposed the pivotal protective mechanism of the intervention. The vehicle group exhibited signs of acute tissue failure, including diffuse hemorrhagic edema and dense neutrophilic cuffing around the microvasculature (Mean Score: 8.1 ± 1.1). However, propolis administration significantly attenuated these pathological changes (Mean Score: 3.6 ± 1.0; $p < 0.01$). The marked reduction in PMN infiltration in this group provides the structural basis

for the suppressed serum IL-6 levels observed earlier, suggesting that propolis effectively limits the recruitment of cytotoxic leukocytes to the ischemic penumbra, thereby preserving the microcirculation.

Nevertheless, the severe ischemia cohort demonstrated the physiological ceiling of this protection. Histological sections from both the vehicle and propolis-treated groups were dominated by extensive coagulative necrosis, loss of nuclear staining (karyolysis), and widespread thrombosis. The lack of a significant difference in total injury scores (8.8 vs 8.1) indicates that in the face of profound vascular compromise, the inflammatory cascade proceeds to irreversible tissue destruction regardless of antioxidant supplementation. Thus, the histopathology confirms that propolis acts as a potent modulator of inflammation in salvageable tissue but cannot reverse the fundamental necrosis dictated by severe hypoxia.

TABLE 3. HISTOPATHOLOGICAL EVALUATION OF TISSUE INJURY (TRANSITION ZONE)

Experimental Group	Semi-Quantitative Score (Mean ± SD)			Total Injury Score (Max 9)	p-value (vs Vehicle)	Qualitative Microscopic Description
	PMN Infiltration (0-3)	Tissue Edema (0-3)	Vascular Congestion (0-3)			
M-Vehicle	0.8 ± 0.2	0.9 ± 0.3	0.7 ± 0.2	2.4 ± 0.7	-	*Minor interstitial edema; intact epidermis; minimal inflammatory infiltrate.*
M-Propolis	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	1.6 ± 0.5	0.082	*Normal dermal architecture; sparse neutrophils; healthy collagen bundles.*
Mod-Vehicle	2.6 ± 0.4	2.8 ± 0.4	2.7 ± 0.3	8.1 ± 1.1	-	*Severe neutrophil accumulation; widespread separation of collagen fibers (edema); hemorrhage.*
Mod-Propolis	1.1 ± 0.3	1.3 ± 0.4	1.2 ± 0.3	3.6 ± 1.0	< 0.01 *	*Markedly reduced inflammation; preserved vascular integrity; minimal congestion.*
S-Vehicle	2.9 ± 0.1	2.9 ± 0.2	3.0 ± 0.0	8.8 ± 0.3	-	*Massive coagulative necrosis; loss of nuclear staining; extensive thrombosis.*
S-Propolis	2.7 ± 0.3	2.6 ± 0.4	2.8 ± 0.3	8.1 ± 1.0	0.145	*Diffuse necrosis persisting; heavy leukocyte infiltration similar to control.*

Scoring System (0-3 Scale):

0 = None/Normal | 1 = Mild (Focal) | 2 = Moderate (Multifocal) | 3 = Severe (Diffuse/Extensive)

PMN: Polymorphonuclear Neutrophils. Data presented as Mean ± Standard Deviation (n=6).
* Indicates statistically significant improvement compared to Vehicle group (Two-Way ANOVA, Tukey HSD).
Note: Histological preservation in the Moderate-Propolis group corroborates the reduction in IL-6 levels.

4. Discussion

The present study delineates the molecular efficacy and, crucially, the physiological limits of ethanolic propolis extract (EPE) in mitigating the complex pathology of ischemia-reperfusion (I/R) injury. By engineering a robust 2 x 3 factorial design, this research successfully disentangled the specific effects of the pharmacological intervention from the variable magnitude of the surgical insult. This methodological nuance addresses a persistent ambiguity in the literature, where binary treatment versus control models have historically failed to capture the dynamic range of ischemic stress encountered in clinical practice. The pivotal finding of this investigation is the identification of a distinct threshold effect.[11] Our data demonstrates that propolis, administered at a dosage of 800 mg/kg, functions as a potent physiological buffer capable of maintaining homeostasis in the face of mild-to-moderate ischemic challenges (up to a 3:1 length-to-width ratio). However, this protective capacity is finite; it is overwhelmed by the catastrophic reactive oxygen species (ROS) load and inflammatory cascade generated during severe (4:1) ischemia. This finding fundamentally shifts the understanding of

herbal cytoprotection from a concept of absolute efficacy to one of dose-dependent and capacity-limited support.[12]

The primary driver of distal necrosis in random pattern skin flaps is the unchecked propagation of free radicals. Following the restoration of blood flow to ischemic tissue, the sudden influx of molecular oxygen interacts with accumulated hypoxanthine and xanthine oxidase, precipitating a respiratory burst. Malondialdehyde (MDA), a toxic aldehyde generated during the decomposition of polyunsaturated fatty acids in cellular membranes, serves as a highly sensitive proxy for the extent of this lipid peroxidation.[13] Our results indicate a linear escalation of serum MDA levels concomitant with the increasing dimensions of the flap, thereby validating the graded ischemia model as a reliable platform for testing antioxidant resilience. In the context of moderate ischemia, the administration of propolis yielded a statistically profound reduction in systemic oxidative stress (11.9 nmol/mL in treated animals versus 15.8 nmol/mL in controls). This suggests that the bioactive constituents of propolis—specifically flavonoids like caffeic acid phenethyl ester (CAPE), galangin, and quercetin—effectively intercepted superoxide anions and hydroxyl radicals before they could initiate the self-propagating chain reaction of membrane destruction. Mechanistically, these phenolic compounds possess hydroxyl groups that donate hydrogen atoms to unstable radicals, stabilizing them while becoming relatively stable phenoxy radicals themselves, thus terminating the oxidative chain.

However, the failure of propolis to significantly attenuate MDA levels in the severe ischemia group ($P > 0.05$) illuminates the phenomenon of antioxidant saturation. We hypothesize that in the 4:1 flap model, the sheer volume of hypoxic tissue creates a ROS storm upon reperfusion that is kinetically faster than the scavenging rate of the exogenous antioxidant. In this scenario, the rate of radical generation exceeds the stoichiometric capacity of the circulating flavonoids. Furthermore, severe ischemia likely induces the irreversible opening of the mitochondrial permeability transition pore (mPTP), causing a collapse of the mitochondrial membrane potential. Once this metabolic tipping point is breached, the cell ceases to function as a viable unit, and no amount of exogenous antioxidant can reverse the structural disintegration. This aligns with the point of no return hypothesis, suggesting that standard antioxidant therapies become futile once mitochondrial integrity is fundamentally compromised by prolonged, severe hypoxia.[14]

While oxidative stress initiates the injury, it is the subsequent inflammatory response that propagates tissue death into the viable penumbra (Figure 1). Ischemia acts as a sterile inflammatory trigger. The stabilization of hypoxia-inducible factor 1-alpha (HIF-1α) in low-oxygen environments leads to the activation of the nuclear factor-kappa B (NF-κB) signaling pathway. NF-κB serves as the master transcriptional regulator of inflammation, driving the expression of cytokines, including Interleukin-6 (IL-6). Our study established a strong positive correlation ($r = 0.78$) between serum IL-6 levels and the percentage of macroscopic necrosis, confirming IL-6 as a critical mediator of flap failure. In the moderate ischemia cohort, propolis treatment effectively uncoupled this axis, maintaining IL-6 levels near the baseline observed in mild injury. The molecular rationale for this effect is likely attributed to the specific inhibitory action of CAPE on the IκB kinase (IKK) complex.[15] Under normal conditions, NF-κB is sequestered in the cytoplasm by the inhibitor protein IκB-alpha. Reactive oxygen species and inflammatory signals trigger the phosphorylation and degradation of IκB-alpha, allowing NF-κB to translocate to the nucleus. Propolis appears to inhibit this phosphorylation step, effectively locking NF-κB in the cytoplasm and silencing the gene expression of pro-inflammatory cytokines.

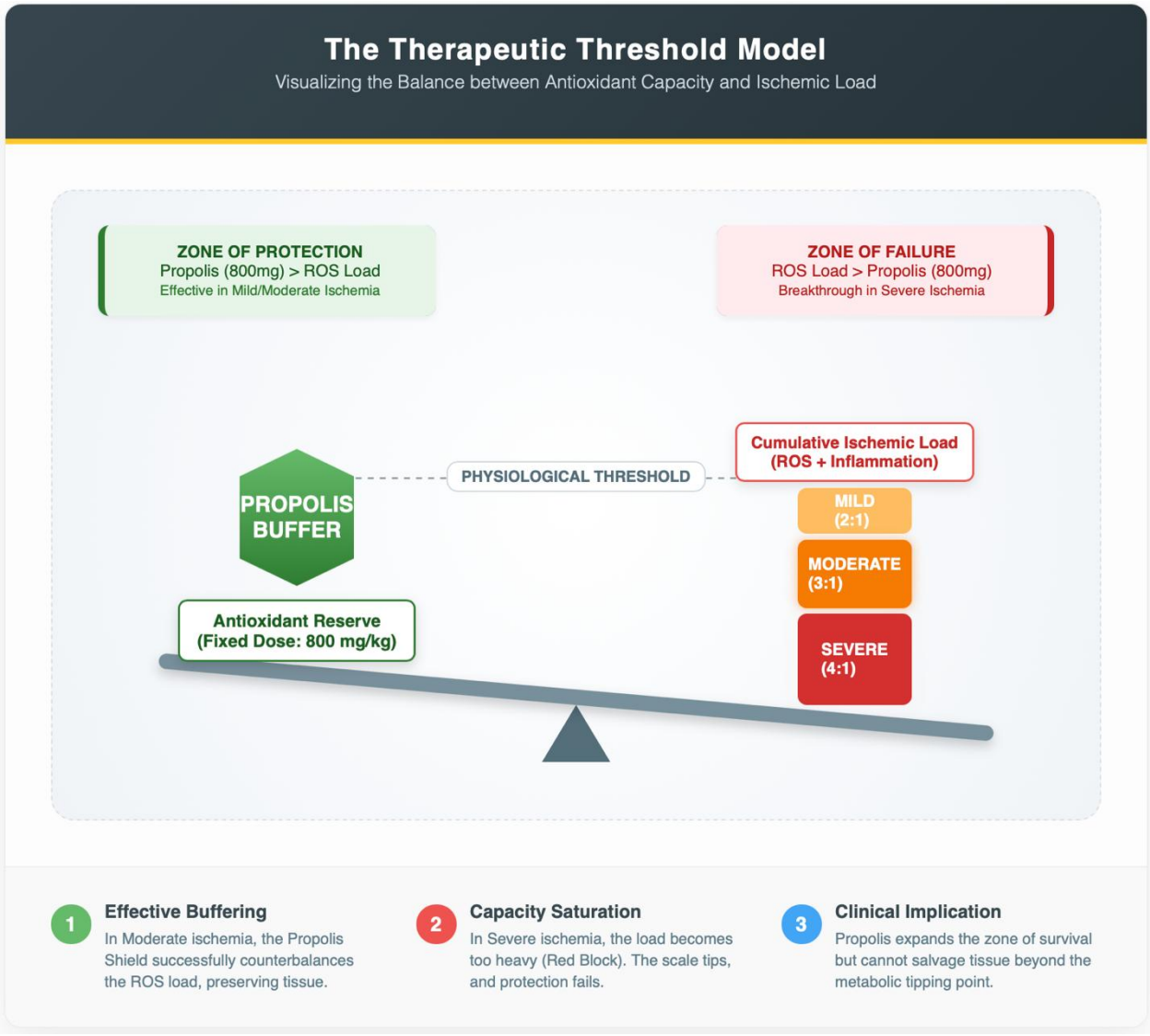

Figure 1. The therapeutic threshold model.

The lack of a significant anti-inflammatory effect in the Severe group, despite the same dosage, suggests the activation of redundant, non-canonical pathways. In massive necrosis, dying cells release danger-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1) protein and heat shock proteins. These DAMPs bind to toll-like receptors (specifically TLR4) on macrophages and endothelial cells, triggering an overwhelming inflammatory response that may bypass the specific checkpoint inhibited by CAPE.[16] This cytokine storm recruits massive waves of neutrophils, whose physical accumulation plugs the microvasculature (the no-reflow phenomenon), rendering the anti-inflammatory properties of propolis insufficient to halt the cascade.

A potential methodological critique of this study is the reliance on systemic (serum) biomarkers rather than localized tissue homogenates. It acts as a limitation, as serum levels are subject to dilution by the total blood volume.[17] However, the data reveal a compelling narrative regarding the systemic consequences of local injury. The strong inverse correlation observed between flap survival area and serum MDA ($r = -0.84$) suggests that in significant

ischemic events (Moderate and Severe), the local oxidative stress is substantial enough to cause a systemic spillover.

This spillover is likely facilitated by the widespread endothelial dysfunction and increased vascular permeability induced by cytokines like IL-6.[18] As the endothelial barrier breakdown occurs in the flap, locally generated aldehydes and cytokines leak into the systemic circulation. The strong correlation validates the use of serum MDA and IL-6 not just as research tools, but potentially as minimally invasive clinical markers. In a human clinical setting, monitoring these serum markers could offer surgeons a liquid biopsy to assess the physiological status of a buried or monitoring-difficult flap before external signs of necrosis become irreversible, corroborating the diagnostic utility suggested by a previous study. The identification of a therapeutic threshold has profound implications for the translation of propolis into reconstructive surgery. The all-or-nothing paradigm of drug efficacy is insufficient; instead, surgeons must view cytoprotective agents as tools to expand the zone of survival or the ischemic penumbra.[19]

Our findings imply that propolis is an excellent adjuvant therapy for standard flaps or those with marginal perfusion risks—situations corresponding to the moderate challenge in our model. In these cases, the tissue is threatened but viable, and the buffering capacity of propolis can tip the balance away from apoptosis and toward survival, resulting in a clinically significant gain in tissue area (from 52% to 76% survival in our study). Conversely, the data warns against viewing Propolis as a magic bullet for technically flawed, excessively large, or severely compromised flaps. In the severe group, where the metabolic demand vastly outstripped the vascular supply (4:1 ratio), Propolis could not prevent necrosis. This suggests that pharmacological intervention cannot compensate for poor surgical design or fundamental vascular inadequacy. Therefore, Propolis should be integrated into perioperative protocols as a means to optimize the resilience of viable tissue, potentially allowing for slightly more aggressive flap dimensions or providing a safety net for patients with comorbidities (such as diabetes and smoking) that impair microcirculation, provided the surgical fundamentals are sound.[20]

5. Conclusion

The present investigation provides compelling, mechanistic evidence that the oral administration of ethanolic propolis extract (800 mg/kg) significantly enhances random pattern skin flap survival by simultaneously dampening the MDA-mediated oxidative cascade and the IL-6-driven inflammatory response. The study moves beyond the binary assessment of efficacy to establish a nuanced, biologically grounded model of cytoprotective limits. We have identified a distinct therapeutic window: the efficacy of propolis is maximal in mild-to-moderate ischemic challenges, where it successfully preserves mitochondrial function and endothelial integrity. However, this protective effect diminishes in the face of severe physiological stress, where the magnitude of the reperfusion injury overwhelms the antioxidant capacity of the administered dose. This threshold effect is a critical contribution to the field, clarifying why natural antioxidants may show variable results in clinical trials where injury severity is not standardized. Based on these findings, we advocate for the inclusion of standardized Propolis extracts in perioperative pharmacological protocols for reconstructive surgery, particularly for patients undergoing procedures with high risks of marginal necrosis. However, clinicians must recognize the limits of this therapy; it is a vital support for the ischemic penumbra, not a substitute for adequate perfusion. To overcome the threshold identified in this study, future research should focus on three key trajectories: (1) Dose-escalation and kinetics: Investigating whether higher doses (such as 1200 mg/kg) or more frequent dosing schedules can elevate the saturation point in severe ischemia without inducing toxicity; (2) Targeted delivery systems: Developing nanotechnology-based carriers (liposomes or hydrogels) for localized,

transdermal delivery of CAPE directly to the flap tissue. This would achieve higher local tissue concentrations than oral administration, potentially overcoming the spillover dilution and providing a stronger localized oxidative shield; (3) Combination Therapies: Exploring the synergistic potential of combining Propolis with agents that target the mitochondrial permeability transition pore (such as cyclosporine A) or vasodilators (such as nitroglycerin) to attack the pathology of I/R injury from multiple physiological angles simultaneously. By defining the limits of current interventions, this study lays the groundwork for the next generation of targeted, optimized therapies in plastic and reconstructive sciences.

6. References

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