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Rosmarinus Improved Skin Flap Survival Through mTOR Dependent Pathway

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Abstract

Introduction: Skin flap application in the clinical practice is restricted due to the ischemic damage and flap necrosis. Rosmarinus oil has been shown to improve a skin flap survival. In the present work we studied the role of mammalian target of rapamycin (mTOR) signaling on rosmarinus-induced flap protection. **Methods:** A flap surgery was performed on Sprauge-Dawley rats (8 cm in by 3 cm). A week before and a week after the surgery the flaps were treated with topical rosmarinus oil (twice per day). Rapamycin (m-TOR inhibitor) was administered 30 minutes before the flap surgery in rosmarinus-treated or not treated groups. A week after the surgery the malondialdehyde (MDA) contents, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities, expression of Bax, Bcl-2, mTOR and p-mTOR were measured in the flap tissue. **Results:** Topical application of the rosmarinus increased the flap survival ($p<0.05$), anti-oxidative enzyme activity (SOD, $p<0.05$) and anti-apoptotic protein Bcl-2 expression. Rosmarinus treatment decreased the flap MDA content, MPO activity, and pro-apoptotic protein Bax expression ($p<0.05$). Rosmarinus topical application did not change mTOR expression and phosphorylation in the flap tissue. Expression of p-mTOR in rosmarinus treated group was suppressed by rapamycin pre-treatment, which also abolished rosmarinus effects on the flap survival ($p<0.05$). **Conclusion:** These data suggested p-mTOR dependent mechanism in rosmarinus-induced flap survival.

Key words: Rosmarinus; Plant Oils; Ischemia; Reperfusion Injury; Surgical Flaps; Rats, Sprague Dawley; Graft Survival; Necrosis; Apoptosis

Introduction

An important option in the treatment of large damages to the skin tissue is the application of the random skin flaps (1). Regardless of their great potentials, skin flap applications are often limited due to the inadequate blood supply that leads to the partial or complete necrosis at distal parts of the flap (2). Ischemic damage to the flap tissue and subsequent necrosis can be augmented by clinical and pharmacological interventions that suppress oxidative tissue damage or increase tissue tolerance against injury (3, 4). There are several chains of molecular and cellular events which cause flap necrosis including the lack of the nutrients and ATP, oxidative stress, and inflammatory responses (5).

Rosemary (Rosmarinus officinalis L.) is a rich source of active antioxidant constituents such as phenolic diterpenes, flavonoids and phenolic acids. Previous studies on rosmarinus have demonstrated a wide range of beneficial properties including antimicrobial, antioxidant, anti-inflammatory, anti-diabetic, and anticancer effects (6). In two recent studies Ince et al., have shown that rosmarinus increases the skin flap survival by its anti-inflammatory, anti-oxidant and vasodilatory effects (7). In the present work we studied the effect of the oral rosmarinus intake for one week be-

fore flap elevation on decreasing apoptosis. In addition, we also evaluated the possible role of the mammalian target of rapamycin (mTOR) on rosmarinus-induced flap survival.

mTOR, as a central cell-growth regulator, integrates growth factor and metabolic signals to regulate cell survival (8). Pharmacological preconditioning of the tissue to increase its tolerance against ischemic injury has been shown to trigger particular patterns of protein expression including AMPK/mTOR pathway (AMP activated protein kinase and mTOR). Previous results have shown that mTOR phosphorylation can decrease ischemia-induced cellular damage. In accordance with the previous evidence, rosmarinus can activate mTOR/S6K and PI3kinase-akt-mTOR pathways to improve wound healing (9).

Material and Methods

Sprauge-Dawley rats (n=40) were divided into 6 groups (n=10). All protocols were approved by the Institutional Animal Care Committee of University accredited by the Ministry of Health and Medical Education. Skin flap surgery was performed by providing two incisions (8 cm) with 3cm distance on the dorsal surface of the rat and connecting them caudally by a third incision. A transparent foil paper of the same dimensions as the flap was placed between the flap and its donor bed. The whole procedure was done under general anaesthesia (50 mg/kg pentobarbital sodium; intraperitoneally). The percentage of the necrotic area was measured by measuring the total flap surface and necrotic area.

Rosmarinus oil (Now® Foods; purchased from Nutrilife Srl, Italy) was applied in a dose

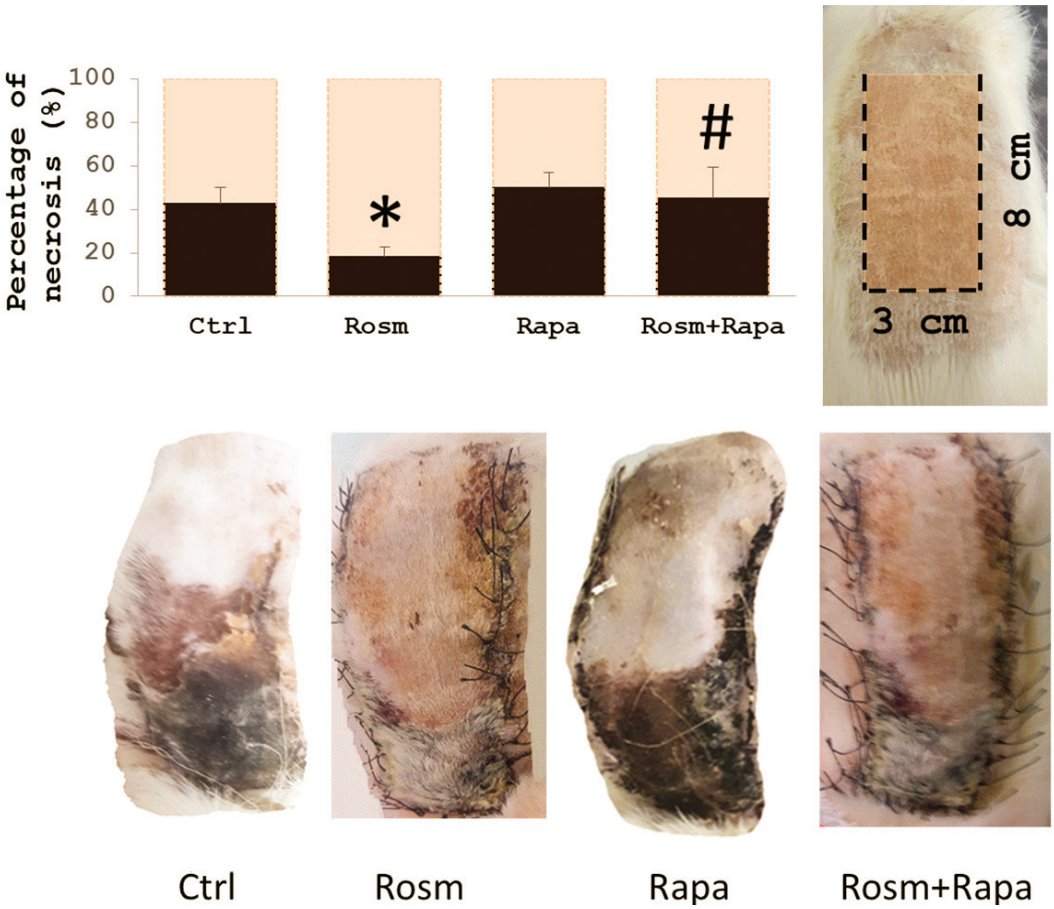

Figure 1. Percentage of the skin flap necrosis in different groups was calculated by dividing necrotic area (black bars) to whole flap area (orange fields). Data are shown as mean ± SDV. *P< 0.05 vs. control group. #p<0.05 when Rosm+Rapa group was compared with Rosmarinus group. Ctrl: control group with flap surgery, Rosm: Rosmarinus, Rapa: rapamycin.

of 0.5 ml per a flap twice a day for one week before and one week after surgery. In two other groups rapamycin (R0395 SIGMA) in a dose of 5 mg/kg was administered intraperitoneally in rosmarinus-treated or non-treated rats 30 minutes before flap surgery. The control group did not receive treatment.

Seven days after flap surgery the necrotic areas were calculated based on the scoring of the length of necrotic area according to the photographs and the percentage of the necrotic area in each flap was calculated. The skin flap samples were collected and preserved for biochemical analysis after sacrificing the animals with an intra-cardiac administration of ketamine (150 mg/kg).

MDA contents and MPO and SOD activities in the flap

The flap tissue was homogenized in 50 mM Tris–ethylenediaminetetraaceticacid (EDTA) buffer (pH 7.0, 4 °C), then centrifuged at 14000×g for 15 min (4 °C). The obtained supernatant was used to determine the levels of MDA contents and MPO and SOD activities. The tissue malondialdehyde (MDA) level, as an index of lipid peroxidation, was determined in homogenized solutions using the Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, Canada) according to the manufacturer's guide. The superoxide dismutase (SOD) activity was determined using the xanthine oxidase method in accordance to the manufacturer's protocols (Nanjing Jiancheng Biology Institution, Nanjing, China). The MPO activity (Sigma-Aldrich) of the supernatant was determined based on H_2O_2-dependent oxidation of odianizidine 2HCl with spectrophotometer (1unit per minute at 460 nm). The obtained values were expressed as U.mg protein^{-1}.

Bax, Bcl-2, mTOR and p-mTOR expressions in the skin flaps

A bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) was used to deter-

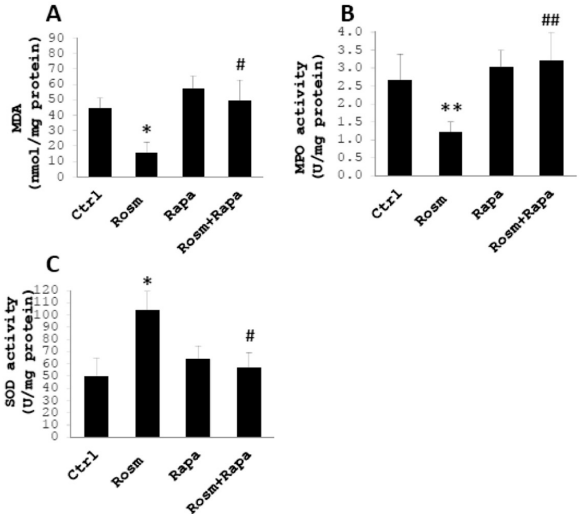

Figure 2. MDA contents and MPO and SOD activities in different groups. A) MDA contents as an index of lipid peroxidation, B) SOD activity as an index of the anti-oxidative activity, and C) MPO activity as an index of the inflammatory reactions. Data are shown as mean ± SDV. *P < 0.05 vs. control group. #p<0.05 when Rosm+Rapa group was compared with Rosmarinus group. Ctrl: control group with flap surgery, Rosm: Rosmarinus, Rapa: rapamycin.

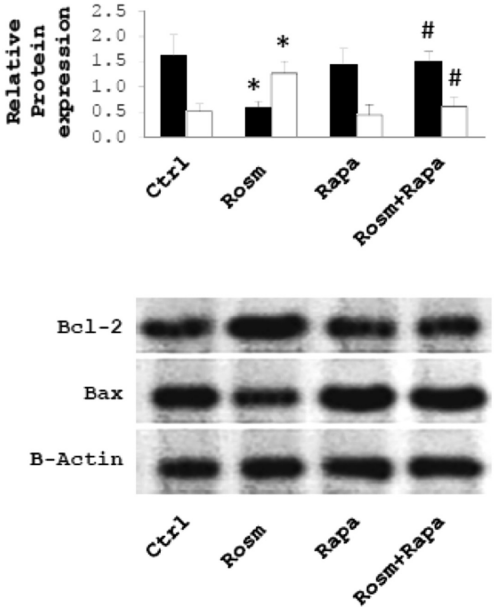

Figure 3. Relative expression of the Bax or Bcl-2 proteins to the β-actin expression in the same sample. Lower panel shows a representative sample from each group. Data are shown as mean±SDV. *P < 0.05 vs. control group. # P < 0.05 when Rosm+Rapa group was compared with Rosmarinus group. Ctrl: control flap surgery, Rosm: Rosmarinus, Rapa: rapamycin.

mine the protein concentrations in the tissue extracts (–80°C). The samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 7% gel, and incubated for 1.5 h with rabbit polyclonal anti-rat Bcl-2 (ab59348), anti-rat Bax (ab53154), anti-rat b-actin antibodies (ab8227), anti-rat mTOR (ab2732), and anti-phospho-mTOR Ser2481 (Merck, Germany). The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (rabbit IgG secondary antibody; H&L-Pre-Adsorbed; Abcam), and the protein bands were visualized with an enhanced chemiluminescence system (10).

Statistical analysis

All analyses were performed in SPSS (version 17) and data were presented as Means ± standard deviation. One-way analysis of variance (ANOVA) followed by and Post-Hoc analysis (Tukey test) was used for comparisons between the groups. The level of statistical significance was accepted as $p < 0.05$.

Results

Seven days after flap surgery the percentage of the necrotic area in the non-treated control group was 42.53 ± 7.28 % of the total flap surface. Rosmarinus treatment decreased percentage of the necrotic are to 18.07 ± 4.79 ($p<0.05$; Figure 1). Rapamycin administration before flap surgery did not change necrosis (49.9 ± 6.89). Rapamycin administration in rosmarinus treated group increased the flap necrosis (45.2 ± 13.93 vs. rosmarinus treated group, $p<0.05$; Figure 1).

Skin flap MDA contents and SOD and MPO activities

MDA contents in the skin flap tissue of the control group was 44.01 ± 7.1 nmol/mg protein that was decreased by rosmarinus topical application for one week before surgery and a week after surgery (15.62 ± 6.95, $p<0.05$; Figure 2 A). Rapamycin by itself did not change the MDA contents but it increased the MDA levels in the rosmarinus treated flaps (49.2 ± 13.56, $p<0.05$ vs. the rosmarinus group; Figure 2 A).

The MPO activity, as an index of the neutrophil infiltration, was decreased by the rosmarinus topical application ($p<0.05$ vs. control group; Figure 2 C). This effect was inhibited by rapamycin administration 30 min before surgery ($p<0.05$ vs. rosmarinus group, Figure 2 C). Rapamycin by itself did not change the MPO activity.

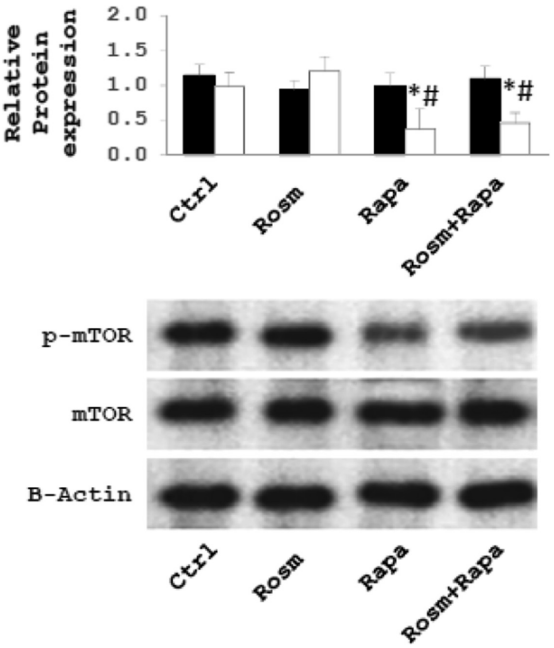

Figure 4. mTOR and p-mTOR proteins expression in the flap tissue. Relative expression of the mTOR or p-mTOR to β-actin was calculated and presented as mean ±SDV. Lower panel shows a representative sample from each group. *P<0.05 vs. control group. # $p < 0.05$ when Rosm+Rapa group was compared with Rosmarinus group. Ctrl: control flap surgery, Rosm: Rosmarinus, Rapa: rapamycin.

The topical application of rosmarinus increased the SOD activity in the flap tissue (103.67 ± 15.66 vs. 49.16 ± 15.56 U/mg protein in the control group, $p<0.05$; Figure 2 B). Pretreatment with rapamycin decreased the SOD activity in the rosmarinus treated groups (56.36 ± 12.82, $p<0.05$; Figure 2 B).

Bax and Bcl-2 expression

The topical application of rosmarinus decreased the expression of the pro-apoptotic protein Bax (0.59 ± 0.12 vs. 1.6 ± 0.42 in the control group, $p<0.05$) and increased the anti-apoptotic protein Bcl-2 expression (1.27 ± 0.23 vs. 0.51 ± 0.14 in the control group, $p<0.01$; Figure 3). Bax and Bcl-2 ex-

pressions did not change by rapamycin administration. However, rapamycin in the rosmarinus treated groups increased Bax expression (1.5 ± 0.19, $p<0.05$ vs. rosmarinus group), and decreased Bcl-2 expression (0.61 ± 0.18, $p<0.05$ vs. the rosmarinus group; Figure 3).

Expression mTOR and phosphorylated mTOR (p-mTOR) in skin flaps

The topical application of rosmarinus for one week before and one week after flap surgery did not change mTOR and p-mTOR expressions in the flap tissue (Figure 4). Rapamycin decreased phosphorylated mTOR (p-mTOR) expression (0.38 ± 0.28 vs. 0.98 ± 0.19 in the control group, $p<0.05$). Rapamycin also decreased p-mTOR expression in the rosmarinus treated flaps (0.46 ± 0.14, $p<0.05$ vs. 1.21 ± 0.2 in rosmarinus group; Figure 4).

Discussion

The rosmarinus topical application for one week before and one week after flap surgery attenuated distal flap necrosis, decreased flap MDA contents, MPO activity, Bax protein expression and increased SOD activity and Bcl-2 protein expression. Anti-necrotic, anti-apoptotic and anti-oxidative effects of the topical rosmarinus are all reversed by blocking mTOR phosphorylation and suggested the involvement of a p-mTOR dependent mechanism in protective effects of rosmarinus.

Several pharmacological agents have been tried to decrease ischemia reperfusion injury in different organs. Protective effects of the rosmarinus topical application, oral administration or intraperitoneal injection on improving the skin flap survival after ischemic injury have been already proved in works by Ince et al. (7). They have shown that rosmarinus treatment increases the blood vessel diameter and therefore the blood supply into the flap tissue. Rosmarinus has also been shown to protect against ischemia reperfusion injury in other organs including the brain, lung and kidney (11). In addition, rosmarinus has been applied on diabetic wounds and radiation-induced tissue damage which confirmed its favorable effects on wound healing (12). In the present work we have shown that rosmarinus suppresses oxidative stress in the flap tissue that is reflected by decreased lipid peroxidation, neutrophil infiltration and increased anti-oxidative enzyme activity.

Previous studies have demonstrated that rosmarinus extract can suppress Hydrogen peroxide (H_2O_2)-induced cellular apoptosis by downregulating Bax and caspase-3 and caspase-9 proteins and upregulation of the Bcl-2 (13). Parallel to its anti-oxidative effects, rosmarinus in the present work decreased apoptotic markers in the flap tissue and prevented from apoptotic cell death. This effect can be related to its vasodilatory effects, which decrease metabolic damage to the mitochondria and therefore decreases the expression of the apoptotic proteins (14).

mTOR integrates different cues, such as oxygen and nutrient levels and energy availability, to regulate cell growth and survival (15). In accordance with the previous study, the increased phosphorylation of mTOR and downstream signaling molecules (S6K1 and 4EBP1) protected the mouse embryonic stem cells (mESCs) against hypoxia-induced apoptosis. An effect that was reversed by mTOR inhibitor rapamycin. It has been shown that rosmarinus treatment can induce mTOR/S6K and PI3kinase-akt-mTOR signaling to improve wound healing (10). In tissue ischemia reperfusion models, mTOR phosphorylation decreased tissue and cellular damage. In the present work rosmarinus treatment did not change the mTOR expression and phosphorylation; however, its protective effect was blocked by suppressing mTOR phosphorylation that suggested mTOR-dependent mechanism for rosmarinus-induced flap survival.

In conclusion, the rosmarinus topical application to the flap tissue decreases necrosis, oxidative damage and apoptosis through mTOR phosphorylation. Even though our data have not shown a direct effect of rosmarinus on mTOR expression and phosphorylation, it seems that mTOR phosphorylation is a key element interacting with signaling pathways involved in the rosmarinus-induced flap survival.

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Ruzmarinom poboljšano preživljavanje režnja kože delovanjem na mehanizme zavisne od mTOR

Sažetak

Uvod. Primena kožnog režnja u kliničkoj praksi je ograničena zbog ishemijskog oštećenja i nekroze režnja. Dokazano je da ulje ruzmarina poboljšava preživljavanje kožnog režnja. U ovom radu proučavali smo ulogu signaliziranja ciljnog molekula za rapamicin kod sisara (*mammalian target of rapamycin* (mTOR)) u zaštiti režnja pomoću ruzmarina. **Metode.** Operacija režnjem je izvedena na *Sprauge-Dawley* pacovima (8 cm sa 3 cm). Nedelju dana pre i nedelju dana posle operacije, režnjevi su tretirani topikalno uljem ruzmarina (dva puta dnevno). Rapamicin (m-TOR inhibitor) je primenjen 30 minuta pre operacije režnjem u grupama koje su tretirane i koje nisu tretirane ruzmarinom. Nedelju dana posle operacije izmeren je sadržaj malondialdehida (MDA), aktivnosti mijeloperoksidaze (MPO) i superoksidaze dismutaze (SOD), ekspresija Bax, Bcl-2, mTOR I p-mTOR u tikvu režnja. **Rezultati.** Topikalna primena ruzmarina povećava preživljavanje režnja ($p < 0,05$), antioksidativnu aktivnost enzima (SOD, $p < 0,05$) i ekspresiju antiapoptotičkog proteina Bax ($p < 0,05$). Tretman ruzmarinom je smanjio sadržaj malondialdehida u režnju, aktivnost mijeloperoksidaze i ekspresiju proapoptotičkog proteina Bax ($p < 0,05$). Topikalna primena ruzmarina nije promenila mTOR ekspresiju i fosforilaciju u tkivu režnja. Ekspresija p-mTOR-a u grupi tretiranoj ruzmarinom je suprimirana prethodnim tretmanom rapamicinom, koji je takođe poništio efekte ruzmarina na preživljavanje režnja ($p < 0,05$). **Zaključak.** Ti podaci su ukazivali na mehanizme zavisne od p-mTOR-a u preživljavanju režnja izazvanom ruzmarinom.

Ključne reči: Ruzmarin; Biljna ulja; Ishemija; Reperfuzione povrede; Hirurški režnjevi; Sprague Dawley pacovi; Preživljavanje grafta; Nekroza; Apoptoza

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