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Effects of Rapamycin on Reduction of Peridural Fibrosis: An Experimental Study

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Peridural fibrosis (PF) is a normal complication after lumbar surgery. It is a challenge for both surgeons and patients. Rapamycin (RPM), a novel antibiotic with anti-proliferative and immunosuppressive properties, has been shown to be effective in preventing uncontrolled scar proliferation diseases. The object of the present research was to investigate the effects of RPM on inhibiting PF *in vitro* and *in vivo*.

Material/Methods: *In vitro*, the fibroblasts collected and isolated from the rat tail skin were cultured with/without RPM and cell counting was performed. *In vivo*, the double-blinded study was conducted in 60 healthy Wistar rats divided randomly into 3 groups: 1) RPM treatment group; 2) Vehicle treatment group; 3) Control group. Rats underwent a L1-L2 level laminectomy with a satisfactory anesthetization. Four weeks post-operatively, the Rydell score, histological analysis, hydroxyproline content, vimentin expressional level, and inflammatory cytokines expressional levels were assessed.

Results: *In vitro*, RPM showed ability to prevent fibroblast proliferation. *In vivo*, the laminectomy was well tolerated by all rats, which were killed 4 weeks post-operatively. The Rydell score, histological evaluation, hydroxyproline content, vimentin expression level, and inflammatory activity showed the positive effect of RPM in preventing peridural adhesion, inhibiting fibrotic formation and collagen synthesis, and down-regulating inflammation.

Conclusions: In the present primary study, RPM showed good efficacy in preventing the proliferation of fibroblasts. RPM can prevent rat peridural adhesion through inhibiting collagen synthesis, fibroblasts proliferation, and inflammatory activity.

MeSH Keywords: **Fibroblasts • Laminectomy • Sirolimus • Tissue Adhesions**

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Background

Peridural fibrosis (PF) is a major challenge in spine surgery, with some patients having recurrent symptoms secondary to excessive formation of scar tissue, resulting in neurologic compression and in revision surgery when this scar needs to be mobilized for revision decompression. As first described in 1948 and a series of following-up reports, PF could be a contributing reason for leading failed back surgery syndrome (FBSS), with the main clinical characteristics of persistent back and/or leg pain [1–3]. PF remains a challenge for both patients and surgeons.

Multiple approaches for the prevention of PF have been used, including polytetrafluoroethylene membrane, antibiotic, immunosuppressant, ADCON-L, and some Chinese traditional medicines [4–8], and although some of them achieved good results in animals, no single treatment is widely accepted in clinical practice.

Rapamycin (RPM), also known as sirolimus, is a novel antibiotic with anti-proliferative and immunosuppressive properties [9]. RPM is widely used in coronary artery stents for its ability to decrease the rate of restenosis [10]. Recently, RPM has also been reported to be employed in treating uncontrolled scar proliferation diseases, such as keloid, hypertrophic scars, and intra-abdominal adhesion [11–13]. However, in the current literature, research investigating the effects of RPM in the prevention PF is unavailable.

The aim of this study was to investigate the effects of RPM in preventing the proliferation of fibroblasts, the formation of PF, and inflammation activity *in vitro* and *in vivo* by performing fibroblasts counting, Rydell assessment, histological analysis, measuring hydroxyproline content, assessing interleukin (IL)-6 and transforming growth factor (TGF)-β mRNA expressional levels.

Material and Methods

Animals and group

A total of 60 adult, healthy, Wistar rats (mean weight 250 g) were used in this study. The animals received care in accordance with the ethics standards laid down in the 1964 Declaration of Helsinki and its later amendments, and in compliance with the European Communities Council Directive (86/809/EEC) and with the principles of International Laboratory Animal Care. Rats were housed in the local laboratory with a 12-hour light-dark cycle, 18°C to 25°C room temperature, and free access to standard rat feed and clean water. All efforts were made to minimize the number of rats needed and their intra-operative suffering.

Rats were randomly divided into three groups according to their different treatments post-surgery (20 rats in each group): Group 1, RPM treatment group; Group 2, Vehicle treatment group (vehicle: 0.2% sodium carboxymethylcellulose and 0.25% polysorbate 80); and Group 3, Control group (laminectomy without intervention or treatment).

Fibroblasts culture and rapamycin application *in vitro* and *in vivo*

Primary fibroblasts, collected and isolated from the rat tail skin, were cultured with a single-cell suspension method as previous study reported [14]. We used a culture solution including Dulbecco's modified medium (DMEM), 100 U/ml penicillin, 10% fetal bovine serum, and 100 μg/ml streptomycin. We cultured 4–6 passages of fibroblasts in 6-well plates and treated with 0.02 μg/ml RPM or saline (as a control). The number of fibroblasts was counted with a light microscope (Nikon) [12,15].

In vivo, rapamycin (Sigma) was dissolved in the vehicle containing 0.25% polysorbate 80 (Sigma) and 0.2% sodium carboxymethylcellulose (Sigma). Based on the previous literature, RPM was administered intraperitoneally post-surgery with a dose of 1.5 mg/kg/day for 28 days [13].

Surgical procedure

The rat laminectomy model was created as previously reported [4,15]. Sterile conditions and the basic micro-surgical tools were prepared before the operation. A general anesthetization was induced by 10% chloral hydrate (0.3 ml/100 g body weight). After the complete anesthetization, the rat was restrained in the prone position. The exposed skin was sterilized with the lower back fur around L1 and L2 level shaved. After that, L1-L2 total laminectomy was performed. Intra-operatively, close attention was paid to avoid traumatizing the nerve roots and the dura. The surgical sites were sutured after full hemostasis with saline.

Macroscopic evaluation of PF

Macroscopic evaluation was performed four weeks post-operatively. Five rats were randomly selected from each group. After satisfactory anesthetization, the surgical sites were reopened. The peridural adhesion evaluation was determined by assistants under double-blind trials based on the Rydell standard (Table 1) [3].

Histological analysis

Histological analysis was performed four weeks post-operatively. Five rats were randomly selected from each group. The whole L1-L2 vertebral column, including both peridural scar tissue and muscles, was harvested, and then the samples were

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Table 1. Rydell score system.

Score 0	Peridural scar tissue was not adherent to the dura mater
Score 1	Peridural scar tissue was adherent to the dura mater, but easily dissected
Score 2	Peridural scar tissue was adherent to the dura mater, and difficultly dissected without disrupting the dura matter
Score 3	Peridural scar tissue was firmly adherent to the dura mater, and could not be dissected

Table 2. Primer sequences for RTPCR.

Name	Sequence	Length
TGF-β1 forward	5'-GCCCTGCCCCTACATTTGG-3'	148bp
TGF-β1 reverse	5'-CTTGCGACCCACGTAGTAGACGAT-3'	
IL-6 forward	5'-ACCCCAACTTCCAATGCTCT 3'	131bp
IL-6 reverse	5'-TGCCGAGTAGACCTCATAGTGACC-3'	
GAPDH forward	5'-TCACCACCATGGAGAAGGC-3'	169bp
GAPDH reverse	5'-GCTAAGCAGTTGGTGGTGCA-3'	

fixed in 10% phosphate-buffered formaldehyde solution. The Cal-Ex II solution was employed for decalcification and dehydration for 3 days. Axial sections (5 µm) of the sample were made and were stained with hematoxylin-eosin (H&E).

With the aforementioned light microscope, the peridural adhesion was evaluated. To further evaluate the proliferative condition of fibroblasts, vimentin immunohistochemistry was performed with the monoclonal anti-vimentin antibody (Invitrogen) and the density of vimentin was evaluated. Specifically, both the fibroblasts and vimentin positive cells were counted in the three different visual fields, and the mean of the sample was calculated. Further analysis was performed with statistical package software.

Hydroxyproline content (HPC) detection

HPC evaluation was performed on the fourth week post-operatively. Five rats from each group were randomly selected. The wet scar tissue sample, weighing approximately 5 mg, was collected around the surgery site. The collected samples were rinsed, homogenized, centrifuged, and hydrolyzed. Then, 1 ml hydroxyproline developer (β-dimethylaminobenzaldehyde solution) was added to the standards and the samples. With a spectrophotometer, the absorbance at 550 nm was read. Then HPC per milligram of sample tissue was evaluated.

Analysis of TGF-β1 and IL-6 expressions

The mRNA analyses of TGF-β1 and IL-6 were performed four weeks post-laminectomy. Five rats from each group were randomly selected. The scar tissue samples were collected as aforementioned approach from the laminectomy sites and the total RNA was extracted. The RNA (2 µg) was transcribed into cDNA employing AMV Reverse Transcriptase. Quantitative real-time PCR (RTPCR) was performed in the BioRad MYIQ2 (USA) (15). As shown in Table 2, according to the previous study, the primer is given [4,15]. GAPDH amplification was employed as an internal control.

Statistical analysis

The statistical analysis was conducted employing SPSS 13.0 statistical package software for Windows (USA). Data are expressed as mean ±SD. Both q-test and the single-factor analysis of variance (ANOVA) were applied to determine the significance of differences between independent samples. P values less than 0.05 were considered statistically significant.

Results

Ability of Rapamycin to prevent fibroblast proliferation *in vitro*

The potential ability of RPM to suppress the fibroblast proliferation is shown in Figure 1. The fibroblast counting showed a significant difference between the groups with/without RPM culture. Both the low-density and maturity levels of the fibroblast suggested that RPM can suppress fibroblast proliferation.

General macroscopic evaluation

The surgery was well performed on rats. None of rats died during the whole research process. None of the animals showed

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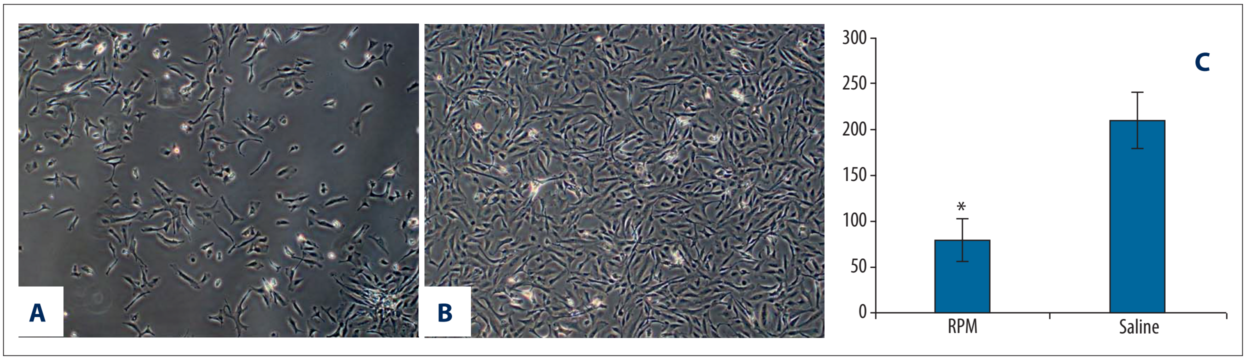

Figure 1. RPM's effect in preventing the proliferation of fibroblasts. Fibroblasts were cultured with (**A**) / without (**B**) RPM. The counts are given (**C**). * P<0.05 compared with the saline group.

Table 3. Peridural scar adhesion evaluations, according to the Rydell score.

Group	Score			
	0	1	2	3
RPM	4	1	0	0
Vehicle	0	0	0	5
Control	0	0	0	5

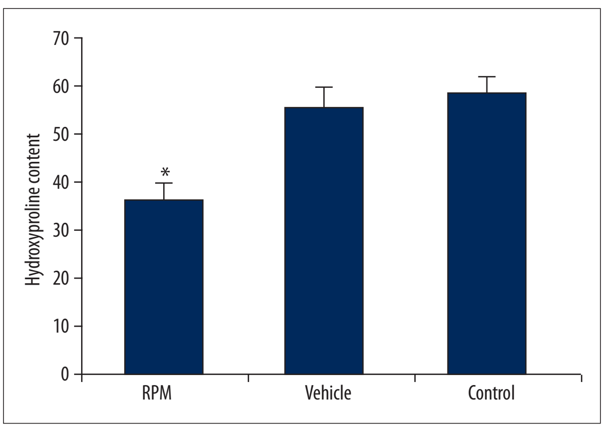

Figure 2. Hydroxyproline content evaluations among the three groups. The contents are given as micrograms per milligram (µg/mg). * P<0.05 compared with the control group.

any sign of wound infection, neurological deficit, or disturbance of wound healing.

As show in Table 3, based on the Rydell standard, the grades of peridural scar adhesion in different groups are given. In the laminectomy sites of both Vehicle and Control groups, severe peridural scar adhesions were observed. Dissection of the peridural scar would lead to risk of tearing the dura mater or nerve root injury and serious bleeding. In the RPM group, soft or weak peridural scar adhesion was observed around the laminectomy sites and the peridural scar can be easily dissected without serious bleeding.

Hydroxyproline content (HPC)

Figure 2 shows the HPC level of scar tissue from each group. Compared with the Vehicle group and Control group, the RPM group showed a significant reduction (P=0.002, P=0.001, separately). Compared with that of the Control group, the HPC level in the Vehicle group showed no significant difference (P=0.301).

Histological analysis

The histological results showed that there was little adhesion in the laminectomy sites of RPM rats and little or loose peridural scarring (Figure 3A) but in the operative sites of the Vehicle group and the Control group there were severe adhesions to the dura mater and nerve root caused by dense peridural scar tissue (Figure 3B, 3C).

As shown in Figure 3, the fibroblasts density evaluation showed less density in the RPM group (63.44±22.13) compared with the Vehicle (90.72±31.49, P<0.001) and the Control group (99.81±35.27, P<0.001) but there was no significant difference between the Vehicle group and the Control group (P=0.471).

As shown in the representative sections in Figure 4, the vimentin cells immunohistochemistry result showed lower vimentin density in the RPM group (13.92±19.16) versus the Vehicle (36.91±27.47, P<0.001) and Control group (37.39±25.41, P<0.001) but there was no significant difference between the Vehicle group and the Control group (P=0.388).

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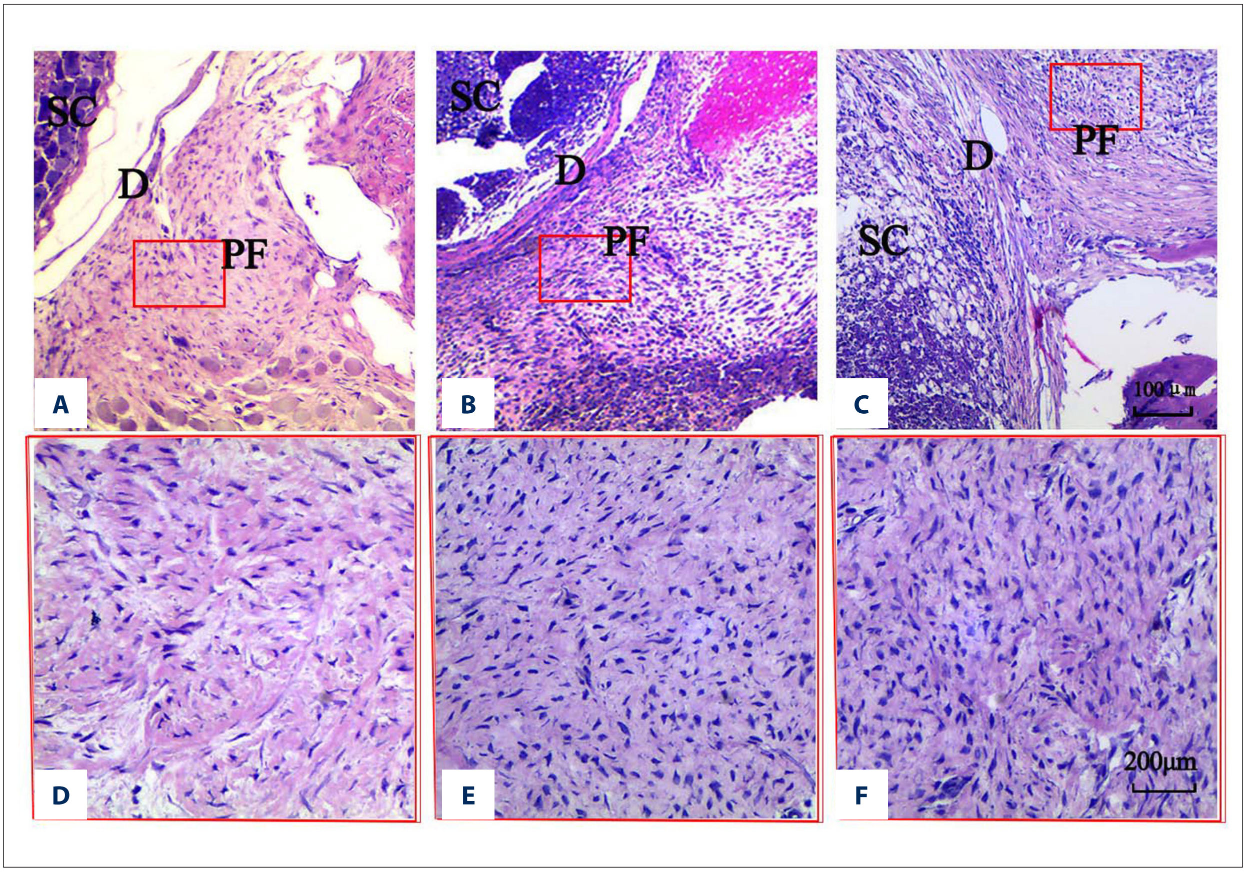

Figure 3. H&E staining for peridural adhesion at the laminectomy sites treated with RPM (**A**), vehicle (**B**), and nothing (**C**). The fibroblasts condition at the laminectomy sites treated with RPM (**D**), vehicle (**E**), and nothing (**F**). a: Loose peridural scar tissue without adherence to the spinal cord was observed in the RPM group. b, c: Dense scar tissue adherent to the spinal cord was seen in the vehicle and control groups. D: dural, SC: spinal cord, PF: peridural fibrosis.

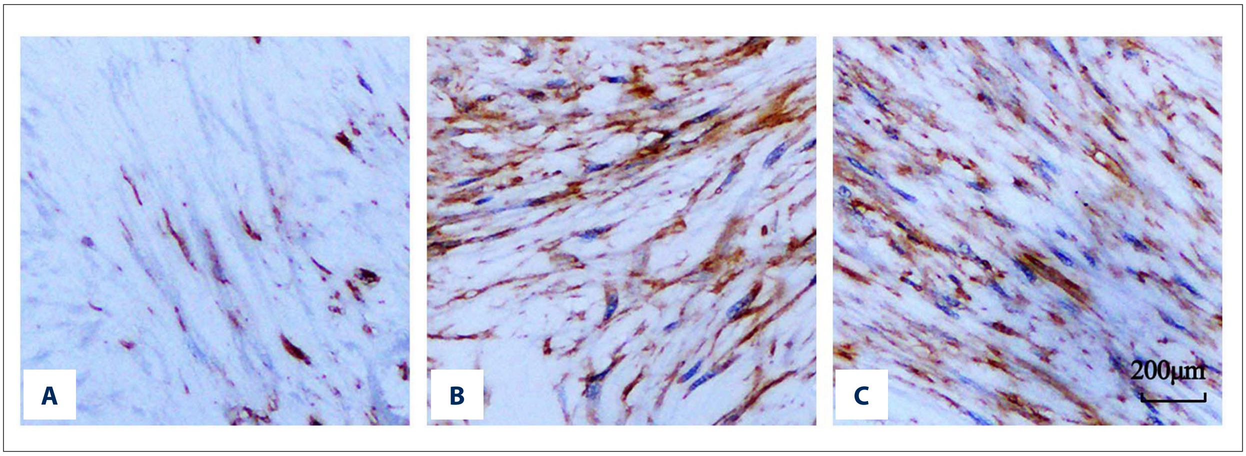

Figure 4. Vimentin-positive cells expressional levels in post-operative scar tissue in different groups: RPM (**A**), vehicle (**B**), and control (**C**).

Effect of RPM on TGF-β1 and IL-6

The results of mRNA expression levels of TGF-β1 and IL-6 are shown in Figure 5. The expression levels of TGF-β1 and IL-6 in the RPM group were significantly lower than in the Control group (P=0.002) and Vehicle group (P=0.018) but the expression levels between the Control group and the Vehicle group showed no significant difference (P=0.377).

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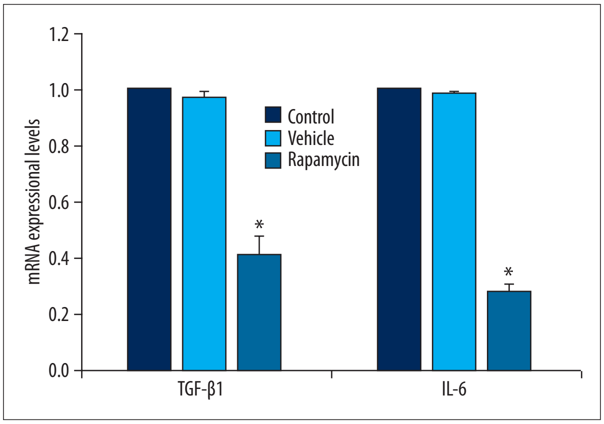

Figure 5. The relative inflammatory cytokines expression levels in peridural scar tissue. * $P<0.05$ compared with the control group.

Discussion

According to previous reports the clinical rate of FBSS for post-lumbar laminectomy patients is 8–40% [16]. Based on our own experiences and previous reports, up to 25% of FBSS patients have PF [17,18].

It was reported that revision surgery was effective in treating PF. However, the complications, such as nerve root injury, dural tears, wound infection, and peridural bleeding, cannot be ignored. It has been gradually accepted that the success rate of reoperation is poor [19]. Among the various kinds of agents and materials studied to inhibit PF post-laminectomy, mitomycin C (MMC) was reported to be effective in reducing collagen synthesis experimentally [20,21]. Although the effects of MMC in preventing collagen synthesis and scar formation were proved in the aforementioned studies, due to the toxicity of MMC, some researchers doubted that MMC would lead to an increased rate of infection [22]. A recent study provided clear evidence that all-trans retinoic acid (ATRA) prevents PF through the NF-κB signaling pathway [15] and another study showed the ability of azithromycin to prevent PF [7]. In the present study we found a similar effect of RPM in prevention of PF, as reflected by Rydell score and the anti-inflammatory and anti-fibrotic abilities of RPM also were as good as ATRA and azithromycin. Additionally, from the results of the HPC evaluation, RPM was able to reduce collagen deposition as effectively as MMC and ATRA [15,23]. Thus, in the present primary research shows the abilities of RPM to prevent fibroblasts proliferation, reduce peridural adhesion, inhibit collagen synthesis, and curtail inflammatory activity.

The pathophysiological mechanism of PF is still unclear, but it is accepted that inflammatory activity plays an important role in promoting PF formation [24]. In the present research, the down-regulation of IL-6 and TGF-β suggest the efficacy of RMP in preventing inflammation. The latest research suggests that RPM is able to attenuate inflammation and fibrosis through blocking the mTOR signaling pathway [25]. As previously reported, the mTOR signaling pathway plays a key role in regulating the activation of myofibroblasts and macrophages [26,27]. Thus, the results of the present study data and previous reports demonstrate some if not all of the possible ways in which RPM prevents PF.

Our extensive literature review suggests it is unlikely that any single approach will sufficiently inhibit PF. Many new approaches have recently been reported, such as the application of cross-linked high-molecular-weight hyaluronic acid, hyperbaric oxygen treatment, and hybrid chitosan membranes [17,28–30]. Thus, we hypothesized that PF could be significantly prevented with the combined application of RPM with these new approaches.

To the best of our knowledge, the present research is the first primary study investigating RPM's effect on preventing PF in laminectomy rats. However, we did not investigate different doses, safe concentration, and long-term effects of RPM in the present study and future research is needed before clinical trials can be conducted.

Conclusions

Our *in vitro* and *in vivo* data suggest that RPM has good efficacy in preventing the proliferation of fibroblasts and that RPM can prevent rat peridural adhesion through inhibiting collagen synthesis, fibroblasts proliferation, and inflammatory activity. More research evaluating different doses, safe concentration, and long-term effects should be performed.

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