NOTE *Clinical Pathology*

Relative potency of tyrosol in the treatment of endotoxin-induced uveitis in rats

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ABSTRACT. Tyrosol (Tyr) is a natural phenolic antioxidant with diverse biological activities. We compared the anti-inflammatory effects of intravenously administered Tyr versus prednisolone (PSL) in an endotoxin-induced uveitis (EIU) rat model. Intravenous administration of 100 mg/kg Tyr was performed 2 hr before, simultaneously and 2 hr after lipopolysaccharide (LPS) injection. Tyr treatment was associated with decreased inflammatory cell number, protein concentration, tumor necrosis factor (TNF)-α, PGE2 and NO levels in AqH and improvements in histopathologic evidence of EIU in ocular tissue at 24 hr after LPS injection. 100 mg/kg Tyr and 1 mg/kg PSL (administered on the same schedule as Tyr) had comparable anti-inflammatory effects. Taken together, Tyr may represent a promising therapeutic agent for the management of intraocular inflammatory diseases.

KEY WORDS: anti-inflammatory potency, endotoxin-induced uveitis, prednisolone, tyrosol

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The endotoxin-induced uveitis (EIU) animal model is based on the induction of acute anterior segment inflammation using lipopolysaccharide (LPS) injection [22]. This model has been used to investigate the pathogenesis of uveitis and evaluate the therapeutic effect of pharmacological agents [11, 14]. The EIU inflammatory response peaks at 24 hr in rats and is characterized by leukocyte infiltration and protein leakage into the anterior chamber and the breakdown of the blood–aqueous barrier (BAB) [5, 9]. Exposure to LPS stimulates the release of various inflammatory mediators, including tumor necrosis factor (TNF)-α, prostaglandin (PG)-E2 and nitric oxide (NO), all of which have been shown to contribute to EIU development [2, 3, 9, 12, 18].

Tyrosol (Tyr), a major component of olive oil, wine and plant extracts, has been shown to have numerous biological activities, including antioxidant and neuro- and cardioprotective properties [6, 10, 19, 23] and anti-inflammatory effects [4]. We previously found Tyr significantly suppresses the development of EIU in a dose-dependent manner (10–100 mg/kg) [25].

In the present study, we compared the efficacies of 100 mg/kg Tyr and 1 mg/kg prednisolone (PSL) in reducing intraocular inflammation in the EIU rat model.

Male Lewis rats (7 weeks, 170–180 g) were used in this study. Rats were randomly allocated to a control group, a LPS group, a LPS+Tyr group (Tyr group) and a LPS+PSL group (PSL group) in the same number. There were 29 rats for AqH collection from both eyes in each group; 8 of 29 rats were used for cell counting and protein concentration assay, and the other rats for measurement of inflammatory mediator levels. For histopathological examination, eight other rats were used in each group. Rats were anesthetized with isoflurane before the induction of EIU by subcutaneous injection of 200 μg LPS diluted in 0.2 m*l* sterilized saline. Two hr before, simultaneously and 2 hr after LPS injection, animals were intravenously administered 100 mg/kg Tyr, 2-(4-Hydroxyphenyl) ethanol (Sigma-Aldrich Co. LLC, St. Louis, MO, U.S.A.) diluted in 0.8 m*l* sterilized saline. The LPS and control groups were treated with 0.8 m*l* sterilized saline in the same manner as were the Tyr group. Intravenous administration of 1 mg/kg PSL (Sigma-Aldrich Co. LLC) was used as a positive control according to the same schedule as the Tyr group. All animals were handled and cared for in accordance with the Animal Care and Use committee of Kitasato University and in compliance with the ARVO statement for the USE of Animals in Ophthalmic and Vision Research. Rats were euthanized 24 hr after LPS injection, with aqueous humor (AqH) immediately collected from both eyes using a 30-gauge needle under a surgical microscope. Infiltrating cell numbers were counted with a hemocytometer (Bürker-Türk hemocytometer; Erma Inc., Tokyo, Japan) under an optical microscope by suspending aliquots of AqH samples in an equivalent volume of Türk's staining solution. Cell number per microliter values were obtained by averaging counts from four areas of the hemocytometer. The remaining AqH was immediately centrifuged at 2,500 rpm for 5 min at 4°C to obtain supernatants. Total AqH protein concentration was measured using bicinchoninic acid (BCA) protein assay kits (Pierce, Rockford, IL, U.S.A.).

Twenty-four hr after LPS injection, rats were euthanized, and both eyes were immediately enucleated under a surgical microscope. Eyes were fixed in 4% paraformaldehyde (Sigma-Aldrich Co. LLC) for 12 hr at 4°C before embedding in paraffin. Sagittal sections were cut at 3 μm thickness near the optic nerve head and stained with hematoxylin and eosin

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(H&E). Histopathologic evaluations of the anterior chamber, iris–ciliary body (ICB), vitreous and retina of EIU rats were scored from 0 to 3 using a light microscope, as we previously described [15]. Grades 0, 1, 2 and 3 represent no infiltrating cells, mild cell infiltration, moderate cell infiltration and severe cell infiltration in the ocular tissue, respectively.

TNF-α and PGE2 levels in AqH were measured using commercially available enzyme-linked immunosorbent assay kits (R&D Systems Inc., Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Total NO levels in AqH were measured using colorimetric nitric oxide assay kits (Oxford Biochemical Research Inc., Oxford, MI, U.S.A.) in accordance with the manufacturer's instructions.

All results are expressed as means ± SD. Parametric data were compared by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Non-parametric data were compared using the Kruskal–Wallis test followed by the Newman–Keuls *post-hoc* test for multiple comparisons. *P*-values <0.05 were considered statistically significant.

The mean infiltrating cell number and protein concentration in AqH samples collected 24 hr after LPS injection were $25.6 \pm 2.4 \times 10^5$ cells/m*l* and 24.9 ± 2.6 mg/m*l*, respectively (mean ± SD, n=8). Rats treated with 100 mg/kg Tyr and 1 mg/kg PSL had significantly lower numbers of infiltrating inflammatory cells (Tyr: $8.3 \pm 2.8 \times 10^5$ cells/m*l*, $P < 0.001$; and PSL: $8.4 \pm 3.1 \times 10^5$ cells/m*l*, $P < 0.01$) and AqH protein concentrations (Tyr: 14.6 ± 2.4 mg/m*l*, $P < 0.001$; and PSL: 15.1 ± 2.4 mg/m*l*, $P < 0.001$) compared with the LPS group (Table 1). No infiltrating cells were observed in the AqH of rats that did not receive LPS injection (control group), with a mean AqH protein concentration of 1.4 ± 0.3 mg/m*l*. Severe inflammatory cell infiltration was observed in the anterior chamber and adjacent to the ICB in LPS-injected rats. The mean histopathologic score in the LPS group was 2.875 (n=8). In Tyr- and PSL-treated rats, the degree of inflammation was significantly reduced, with similar histopathological findings (Fig. 1A and 1B). Increased levels of TNF-α, PGE2 and NO were detected in the AqH of LPS-injected rats (n=7); however, treatment with Tyr and PSL significantly ameliorated these increases (Table 2). No significant differences in any measured parameters were observed between the Tyr and PSL groups.

Table 1. Effects of Tyr and PSL on infiltrating cell number and protein concentration in AqH

Group	Infiltrating cells ($\times 10^5$ cells/m*l*)	Protein concentration (mg/m*l*)
Control	0	1.4 ± 0.3
LPS	25.6 ± 2.4	24.9 ± 2.6
Tyr	8.3 ± 2.8 [a)]	14.6 ± 2.4 [a)]
PSL	8.4 ± 3.1 [a)]	15.1 ± 2.4 [a)]

Data represent the mean ± SD (n=8). a) $P<0.001$ significantly different from the LPS group.

In this study, Tyr significantly attenuated the intraocular inflammatory response in EIU rats at 24 hr after LPS injection, with significant decreases in cellular infiltration and protein leakage into the AqH and histopathologic improvements in the characteristic manifestations of EIU. The anti-inflammatory effects of Tyr may be associated with suppression of TNF-α, PGE2 and NO production in the anterior segment. A similar trend was observed in PSL-treated

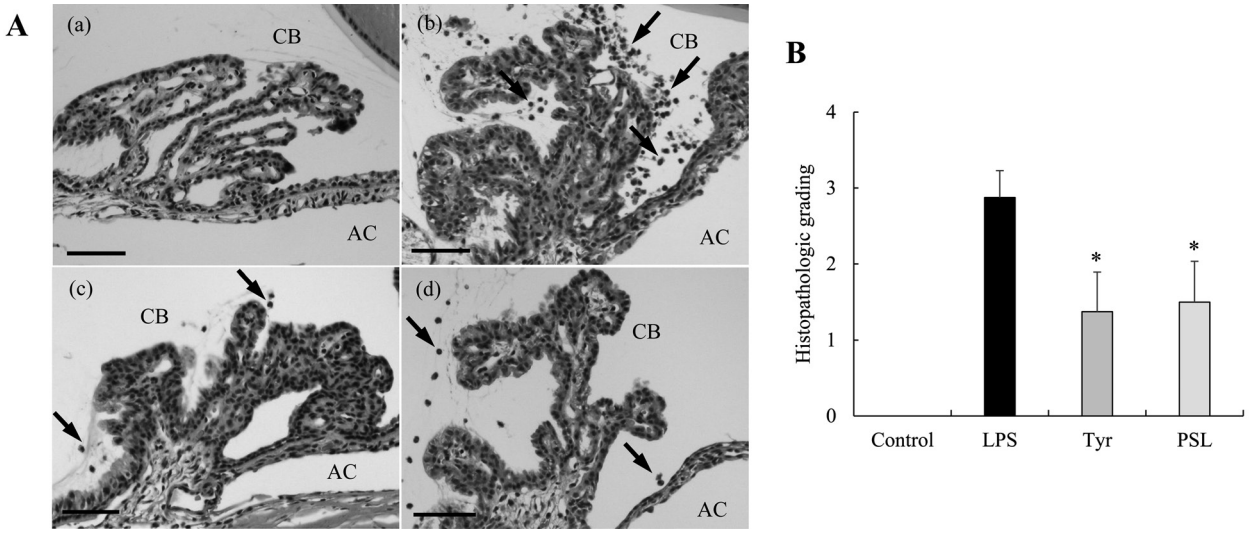

Fig. 1. Effects of Tyr and PSL on histopathological appearance of EIU. A: histopathological changes in the anterior segment of eyes at 24 hr after LPS injection. No inflammation was observed in the anterior segment of rats that did not receive LPS injection (a). Severe inflammatory cell infiltration was observed in untreated EIU rats (b). Rats treated with 100 mg/kg Tyr (c) and 1 mg/kg PSL (d) demonstrated significantly reduced anterior segment inflammation. H&E staining. Bars, 25 μm. AC, anterior chamber. CB, ciliary body. Arrows indicate inflammatory cells. B: effect of 100 mg/kg Tyr and 1 mg/kg PSL on histopathological EIU scores. Scores presented as the mean ± SD (n=8). *P <0.001 for comparisons with the LPS group.

Table. 2. Effects of Tyr and PSL on TNF-α, PGE2 and NO levels in AqH

Group	TNF-α (*pg*/m*l*)	PGE2 (*pg*/m*l*)	NO (*μ*M)
Control	ND	ND	7.9 ± 2.3
LPS	195.2 ± 30.6	1,088.4 ± 301.2	222.6 ± 48.1
Tyr	111.2 ± 35.1 [a)]	298.8 ± 146.1 [a)]	117.1 ± 22.7 [a)]
PSL	106.9 ± 22.5 [a)]	308.0 ± 157.5 [a)]	114.0 ± 19.1 [a)]

Data represent the mean ± SD (n=7). a) $P<0.001$ significantly different from the LPS group. ND=no detected.

rats. At present, corticosteroids are the front-line therapy for uveitis; however, long-time exposure to these drugs is associated with significant adverse effects, including intraocular pressure increase, cataract formation and increased susceptibility to infection [10]. Therefore, further studies facilitating the development of safe and effective therapies for uveitis are required. The results of recent studies have indicated that natural plant products and antioxidants may prevent ocular inflammation in experimental animals [13, 16, 21]. Tyr is a natural phenolic antioxidant that has been shown to have efficacy in preventing various disorders, such as inflammation, cancer, obesity, hyperglycemia, and neurodegenerative and cardiovascular diseases [1, 4, 6, 7, 10, 20, 23]. Tyr has very low acute toxicity, with a reported LD_{50} of 2,700 and 1,700 mg/kg in mice following intragastric and intraperitoneal injections, respectively, and 7,079 mg/kg in rats following intragastric administration. No toxicity was reported in a study of chronic intragastric administration of Tyr at a dose of 200 mg/kg for 3 months in male rats and 10 mg/kg in dogs [24]. We administered three consecutive doses of 100 mg/kg Tyr intravenously in rats, resulting in a total dose of 300 mg/kg Tyr. At the doses, no side effects or toxicity associated with Tyr administration was observed at 24 hr after LPS injection or at autopsy in the present study. Intravenously administered Tyr is rapidly eliminated from rat organs, with plasma concentrations reported to have a half-life of approximately 70 min [8]. In rats, Tyr-derived metabolites are rapidly distributed to major organs and tissues, predominantly the liver and kidney, within 1 hr of oral administration of 200 mg/kg Tyr and excreted via the kidney within 4 hr [17]. Thus, intravenous administration of Tyr at a dose of 100 mg/kg three times every 2 hr may maintain higher plasma concentrations of Tyr compared to a single dose of 300 mg/kg. These consecutive doses of 100 mg/kg Tyr were found to have a significant suppressive effect on EIU, with comparable efficacy to 1 mg/kg PSL, without any side effects in the present study.

Overall, the results of the present study demonstrate that Tyr treatment suppresses the inflammatory process of EIU. The present findings indicate that Tyr may present an attractive therapeutic alternative to currently available anti-inflammatory drugs for the management of intraocular inflammatory diseases.

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