Therapeutic anti-inflammatory effects of luteolin on endotoxin-induced uveitis in Lewis rats

Kazutaka KANAI1), Sho NAGATA1), Takuya HATTA1), Yuichi SUGIURA1), Kazuaki SATO1), Yohei YAMASHITA1), Yuya KIMURA1) and Naoyuki ITOH1)

1)Department of Small Animal Internal Medicine I, School of Veterinary Medicine, University of Kitasato, Towada, Aomori 034–8628, Japan

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ABSTRACT. The present study aimed to investigate the therapeutic efficacy of post-inflammatory treatment with luteolin on endotoxin-induced uveitis (EIU) in rats. Intraperitoneal injection of 10 mg/kg luteolin or 1 mg/kg prednisolone (Pred) at 4 hr post-lipopolysaccharide (LPS) injection (200 µg) was associated at 24 hr post-LPS injection with decreased clinical severity scores, number of inflammatory cells, protein levels and levels of tumor necrosis factor (TNF)-α, nitric oxide (NO) and prostaglandin (PG) E2 in the aqueous humor (AqH) and degrees of histological ocular tissue injury. The anti-inflammatory potency of luteolin was comparable to that of Pred. Luteolin exhibited robust efficacy in the treatment of EIU in rats, indicating its potential clinical utility in treating uveitis.

KEYWORDS: anti-inflammatory effect, endotoxin-induced uveitis, luteolin, prednisolone, therapeutic effect

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Luteolin is one of the most common flavonoids present in several dietary foods including olive oil, broccoli, celery, parsley, green pepper, herb, dandelion and Japanese honeysuckle [14]. The glycosidic form of luteolin is present in several dietary foods including olive oil, broccoli, celery, parsley, green pepper, herb, dandelion and Japanese honeysuckle [14]. The glycosidic form of luteolin is present in several plant species. Following oral consumption, glyco-

Endotoxin-induced uveitis (EIU) is an established and widely-used animal model of acute ocular inflammation [19]. This model has applications in evaluating the pathophysiology of acute ocular inflammation and investigating the potential efficacy of novel treatments [20]. EIU causes cellular infiltration and protein leakage into the aqueous humor (AqH) at 4 hr after lipopolysaccharide (LPS) systemic injection, with maximum injury developing at 18–24 hr after LPS injection [27]. LPS triggers the secretion of various inflammatory mediators, such as tumor necrosis factor-α (TNF-α) [24], prostaglandin E2 (PGE2) [3] and nitric oxide (NO) [15], which contribute to the pathophysiology of EIU. In most studies concerning EIU, ocular anti-inflammatory agents administered before or immediately after the injection of LPS have been effective in the prevention of ocular inflammation induced by LPS [6, 26, 29]. Therefore, therapeutic strategies, which are effective when administered after the onset of the inflammatory process, may have more translational relevance as therapies for uveitis treatment [21].

The purpose of the present study was to investigate the therapeutic efficacy of post-inflammatory treatment of luteo-

Male Lewis rats, aged 6 weeks and weighing 150–170 g, were used in this study. Animals were handled and cared in compliance with a protocol approved by the Animal Care and Use committee of Kitasato University, and with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. To evaluate the therapeutic efficacy of post-inflammatory treatment, animals were appropriately anesthetized using isoflurane, and EIU was induced by subcutaneous injection of LPS at two sites of 200 µg (100 µg for each site, LPS group) from Salmonella Typhimurium (Sigma-Aldrich, St. Louis, MO, U.S.A.) that was diluted in 0.1 m/ of phosphate buffered saline (PBS, pH 7.4) [25]. Animals that were not administered LPS subcutaneously received the same volume of pyrogen-free PBS (LPS (-) group). Mild anterior uveitis was observed in all rats at 4 hr after LPS injection using slit lamp, as evidenced by discrete dilation of the iridic and conjunctival vessels and cellular infiltration in the anterior chamber. Rats were intraperitoneally injected with 10 mg/kg luteolin, 3′,4′,5,7-tetrahydroxyflavone (Chroma Dex Inc., Irvine, CA, U.S.A.), in 0.8 m/ of PBS containing 0.1% dimethyl sulphoxide (DMSO, Sigma-Aldrich) in PBS. As a positive control, rats were intraperitoneally injected with 1 mg/kg prednisolone (Pred, Sigma-Aldrich) diluted in 0.8 m/ of PBS containing 0.1% DMSO. No adverse effects or toxicity associated with luteolin administration were
observed in the present study at 24 hr after LPS injection.

Clinical manifestations of inflammation in the left eye of rats in each treated group at 24 hr after LPS injection were visualized and evaluated by three observers using slit lamp. There was no inter-observer variation in grading of clinical findings. Uveitis was scored from 0 to 4, as previously described [2]. Rats were subsequently euthanized, and the AqH was collected from both eyes immediately by anterior chamber puncture using a 30-gauge needle under a surgical microscope (Olympus, Tokyo, Japan). After determining the number of infiltrating cells and protein levels in the AqH, the samples were stored at −80°C until further use.

For counting the cells, the pooled AqH was suspended in an equal amount of Türk stain solution, and the cells were counted using a hemocytometer under light microscopy. The number of cells per field (in an equivalent of 0.1 µl) was obtained by averaging the counts from four fields per sample. Total protein concentrations in AqH samples were measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.).

The concentrations of NO, TNF-α and PGE2 in the AqH were measured using a total nitrite colorimetric assay (Oxis International, Portland, OR, U.S.A.), TNF-α and PGE2 ELISA kits (R&D systems, Minneapolis, MN, U.S.A.), respectively, according to the manufacturers’ instruction.

Rat eyes were enucleated at 24 hr after LPS injection and immediately stored in 4% paraformaldehyde in PBS for 24 hr at 4°C; the eyes were subsequently embedded in paraffin for histopathological examination. Paraffin-embedded sections were cut into 3 µm sections and stained with hematoxylin and eosin (HE). The anterior chamber, iris-ciliary body (ICB) and vitreous were observed in a blinded fashion under light microscopy. The number of infiltrating inflammatory cells surrounding ICB was counted in four sections per left eye, with the average of eight eyes used for each group. Histopathologic evaluation of inflammation was scored from 0 to 3, as previously described [11].

All data are expressed as mean ± standard deviation (SD). Parametric data were analyzed by variance (ANOVA), and the Tukey test was used for ad hoc comparisons between the two treatment groups. Non-parametric data were analyzed using the Kruskal-Wallis test, and the Newman-Keuls test was used for ad hoc comparisons between the two treatment groups. P-values of <0.05 were considered to be statistically significant.

First, we examined the therapeutic efficacy of luteolin. The mean clinical score in the LPS group was 3.5 ± 0.5 (n=6). Treatment of EIU rats with luteolin or Pred resulted in a significant (55% and 51%, respectively) decrease in clinical scores compared with the LPS group. Manual counting of infiltrating cells in the AqH revealed a significant (>25-fold) increase in the number of infiltrating cells in EIU rats, which significantly declined in luteolin and Pred-treated rats, (51% and 54%, respectively; n=6). Similarly, AqH protein levels in the LPS group were significantly increased (>25-fold) compared with those in the LPS (−) group; however, luteolin and Pred significantly (62%) ameliorated LPS-induced increases at AqH protein levels (Table 1).

Next, we determined AqH levels of TNF-α, NO and PGE2. Endotoxin-induced increases in inflammatory mediators in the AqH are a hallmark of uveitis. As shown in Table 2, increases at TNF-α, NO and PGE2 levels were observed in the LPS group. Luteolin and Pred significantly (P<0.05) inhibited LPS-induced inflammatory mediators in the AqH (n=5).

Furthermore, histopathological examination in the LPS group revealed severe LPS-induced cell infiltration in the anterior chamber and near the ICB. Decreases in the number of infiltrating cells were observed in rats treated with luteolin or Pred compared with that in the LPS group (Fig. 1A). The mean histopathological score in the LPS, luteolin and Pred groups was 2.8 ± 0.3, 1.5 ± 0.5 and 1.1 ± 0.3, respectively. Treatment of EIU rats with luteolin or Pred resulted in significantly decreased histopathological scores (53% and 60%, respectively) compared with the LPS group (n=8). No significant differences were present on the therapeutic anti-inflammatory efficacy between luteolin and Pred on the basis of histopathological scores or any AqH inflammatory parameters examined.

In the present study, mild anterior uveitis was observed in all rats at 4 hr after LPS injection. Treatment with luteolin at 4 hr after LPS injection resulted in a significant decrease at 24 hr after LPS injection in clinical uveitis scores, values of LPS-induced cellular infiltration, AqH protein and inflammatory mediators, and histopathologic scores. Overall, the anti-inflammatory effect of luteolin may be derived through inhibition of release of inflammatory mediators. To note, these anti-inflammatory effects were achieved even when luteolin was administered at 4 hr after LPS injection, indicating that luteolin has therapeutic efficacy after the onset of EIU in rats. In addition, the anti-inflammatory potency of 10 mg/kg luteolin was comparable to that of 1 mg/kg Pred.

### Table 1. Therapeutic effects of luteolin on clinical scoring, cellular infiltration and protein levels in the aqueous humor at 24 hr post-LPS injection

| Group     | Clinical scoring | Infiltrating cells (>10⁶ cells/ml) | Protein levels (mg/ml) |
|-----------|-----------------|---------------------------------|------------------------|
| LPS (−)   | 0               | 0                               | 0.8 ± 0.2              |
| LPS       | 3.5 ± 0.5       | 28.2 ± 5.3                      | 27.8 ± 3.0             |
| Luteolin  | 1.6 ± 0.50      | 14.4 ± 2.5                      | 17.4 ± 2.70            |
| Pred      | 1.8 ± 0.49      | 15.3 ± 3.4                      | 17.3 ± 3.20            |

Each value represents the mean ± SD (n=6). a) P<0.001, compared with LPS group.

### Table 2. Therapeutic effects of luteolin on TNF-α, NO and PGE2 levels in the aqueous humor at 24 hr post-LPS injection

| Group     | TNF-α (pg/ml) | NO (µM) | PGE2 (pg/ml) |
|-----------|---------------|---------|--------------|
| LPS (−)   | ND            | 6.8 ± 2.1 | ND           |
| LPS       | 294.6 ± 76.8  | 231.1 ± 33.9 | 2,159.7 ± 366.4 |
| Luteolin  | 160.9 ± 31.6   | 138.8 ± 20.7 | 1,359.8 ± 441.5 |
| Pred      | 164.3 ± 44.9   | 142.3 ± 16.6 | 1,316.9 ± 393.5 |

Each value represents the mean ± SD (n=5). a) P<0.05, b) P<0.01 and c) P<0.001, compared with LPS group. ND=not detected.
Current treatments for uveitis include topical, periocular and systemic administration of corticosteroids [23]. However, such therapies are limited by potentially serious adverse effects, such as decreased resistance to infection, decreased corneal wound healing, increased intraocular pressure, induction of glaucoma and cataracts [4]. Therefore, safer and long-lasting alternative treatments are awaited. Many alternative approaches, including anti-cytokine therapies, such as anti-TNF [13] and antioxidants [28], have been studied in experimental animals for the suppression of ocular inflammation.

Luteolin has been in use in traditional medical practice for the treatment of a variety of disorders [1]. Luteolin has various molecular targets, including transcription factors, cytokines, enzymes, apoptosis and growth factors [5,17]. Many researchers have reported that the administration of luteolin attenuates inflammation in experimental animal models of inflammatory disorders [8,9,16]. In collaboration with these studies, we recently demonstrated preventive anti-inflammatory effects of luteolin in EIU in a dose-dependent manner and comparable anti-inflammatory potency of 10 mg/kg luteolin to 1 mg/kg Pred [10]. Intraperitoneal and oral LD50 of luteolin in rats is calculated 411 mg/kg and >5000 mg/kg, respectively [7]. However, the safety profile of luteolin in human remains unclear [14]. To our knowledge, only one report regarding the pharmacokinetic differences of luteolin between humans and rats or dogs has been published [12], and particularly, the pharmacokinetic data of luteolin in dogs are scarce. Therefore, further studies are required to evaluate the clinical use of luteolin as an anti-inflammatory agent in dogs and human. Accordingly, we plan to investigate the additive effect of luteolin and corticosteroid in EIU rats.

In summary, to the best of our knowledge, this study is the first approach to demonstrate the efficacy of luteolin treatment in ocular inflammation after the onset of EIU. The results of the present study indicate that luteolin has the potential efficacy in the treatment of uveitis.

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