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Beneficial interaction of pycnogenol with indomethacin in rats

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Abstract. Cyclooxygenase 2 (COX-2) is responsible for the therapeutic effects of indomethacin, while inhibition of the COX-1 enzyme and oxidative stress are responsible for its gastro-toxic effects. It has been reported that pycnogenol increases the expression of COX-1, suppresses the expression rate of COX-2 and oxidative stress. Our aim in this study is to investigate the anti-inflammatory activities of indomethacin, pycnogenol, and their combination (PI) in rats and to examine their effects on stomach tissue. In the study, anti-inflammatory activity was investigated in carrageenan-induced inflammatory paw edema in albino Wistar male rats. Effects on stomach tissue were performed by applying the previous method. PI, indomethacin and pycnogenol were the best suppressors of carrageenan inflammation and oxidative stress in paw tissue, respectively. While the groups with the lowest COX-1 activity in paw tissue were IC, PIC and PC, respectively, PIC, IC and PC were the ones that best inhibited the increase in COX-2 activity. Pycnogenol inhibited the increase of malondialdehyde, the decrease of total glutathione and COX-1 in the stomach, and significantly suppressed the formation of indomethacin ulcers. Our experimental results showed that pycnogenol reduced the toxic effect of indomethacin on the stomach and increased anti-inflammatory activity. This beneficial interaction of pycnogenol and indomethacin suggests that PI will provide superior success in the treatment of inflammatory diseases.

Key words: Pycnogenol — Indomethacin — Ulcer — Rat — Anti-inflammatory activity

Introduction

Indomethacin, (1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid), is a nonsteroidal anti-inflammatory medicine with antipyretic and analgesic properties (Lucas 2016; Munjal and Allam 2020). Indomethacin is commonly used to treat pain and inflammation in diseases such as rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, bursitis, gout, postoperative pain, toothache, headache, and rheumatism (Nalamachu and Wortmann 2014; Munjal and Allam 2020). However, dose-related gastrointestinal, hepatic, renal, and cardiovascular side effects are observed when used. Especially, gastrointestinal ulcers cause termination of treatment (Lucas 2016). The therapeutic effects of indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are attributed to cyclooxygenase 2 (COX-2), while the side effects are attributed to cyclooxygenase 1 (COX-1) enzyme inhibition (Abdellatif et al. 2021). As is known, COX-1 gastrointestinal protection is a structural enzyme that is responsible for physiological functions like platelet aggregation, and vascular homeostasis (Suleyman

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et al. 2007). COX-2 is the inducible enzyme that is responsible for inflammatory reactions (Abdellatif et al. 2021). It is understood from the literature that, apart from COX-1 enzyme inhibition, reactive oxygen radicals (ROS) are one of the major components in the pathogenesis of indomethacin-induced gastric injury (Maziero et al. 2021). It has also been documented that indomethacin causes gastric tissue damage by interfering with enzymatic and non-enzymatic oxidant and antioxidant mechanisms (Suleyman et al. 2009, 2010). This information explains the significance to keep COX-1 and antioxidant levels within physiological limits in order to preserve gastric tissue integrity. Furthermore, it has been demonstrated that there is a positive correlation between COX-1 and antioxidant activity in terms of protective activity (Yapca et al. 2014).

Pycnogenol consists of a polyphenol concentrate derived from the bark of the French maritime pine (*Pinus pinaster Aiton*) (Rohdewald 2018). Pycnogenol has been reported to have various biological activities such as antioxidant and anti-inflammatory (Icel et al. 2018). Pycnogenol has also been reported to increase the expression of COX-1 and suppress the expression of COX-2 (Canali et al. 2009). All data obtained from the literature suggests that pycnogenol can reduce the toxic effect of indomethacin on the gastric tissue. It also suggests that pycnogenol may enhance anti-inflammatory effect of indomethacin. There was no data in the literature on the anti-inflammatory and gastric effects of indomethacin + pycnogenol combination (PI). The aim of our study is to examine the anti-inflammatory properties of pycnogenol, indomethacin, and PI in rats, as well as their effects on gastric tissue.

Materials and Methods

Experimental animals

A total of 54 albino Wistar male rats with a body weight changing between 265 and 277 grams were used in the experiment. It was obtained from Erzincan Binali Yildirim University Experimental Animals Application and Research Center. The animals were kept and fed at room temperature (22°C) in groups prior to the experiment. All the animal experiments were carried out in accordance with the National Guidelines for the Use and Care of Laboratory Animals and study was approved by Erzincan Binali Yildirim University animal ethics committee (date:11.11.2021, number: E-85748827-050.01.04-122466).

Chemicals

Indomethacin used in the experiment was supplied from Deva Holding (Turkey), pycnogenol from Solgar (USA), carrageenan from Sigma (Germany) and thiopental sodium from İ.E ULAGAY (Turkey).

Experiment procedure

Inflammatory paw edema test in rats

The anti-inflammatory effects of pycnogenol, indomethacin, and their combination in carrageenan-induced inflammatory paw edema were examined in these series of experiments (Albayrak et al. 2010). Five animal groups were used in this experiment: H, C, PC, IC and PIC (n = 6 *per* group). They were administered orally to the stomach by gavage: PC group, pycnogenol 40 mg/kg; IC group, indomethacin 25 mg/kg; PIC group, 40 mg/kg pycnogenol + 25 mg/kg indomethacin. The H (healthy) and C (carrageenan) groups received the same volume (0.5) of normal saline (0.9% NaCl) orally. One hour after the drugs and 0.9% NaCl were administered, 0.1 ml of 1% carrageenan was injected into the toe paws of all rats except H group. The reason we give drugs before carrageenan is because the carrageenan-induced inflammation process consists of an early and a late phase. The early phase (0–1 h) is associated with the release of inflammation mediators such as serotonin, bradykinin and histamine, and the late phase (after 1 h) is associated with prostaglandins (Mehrzadi et al. 2021). A plethysmometer was used to measure the foot volumes of the animals up to the knee joint prior to the carrageenan injection. Animals were trained for the measurement of paw volume. Before the experiment, the same procedure was applied 3 times on each animal to ensure adaptation to the procedure. Carrageenan-induced inflammatory paw volume increase (paw edema) was measured four times every hour. The reason we measure four times is that the late phase of carrageenan inflammation persists for four hours (Süleyman et al. 2004). The inflammatory paw volume increase (paw edema) caused by carrageenan was measured four times *per* hour. Following the completion of the measurements, all animals were dispatched under high-dose thiopental sodium (50 mg/kg) anesthesia, and the paw tissues were biochemically examined. All results obtained from the experiment were evaluated by comparing them across groups.

Ulcer tests in rats

To conduct the experiment, 40 mg/kg pycnogenol, 25 mg/kg indomethacin, 40 mg/kg pycnogenol + 25 mg/kg indomethacin, were orally administered to P (n = 6), I (n = 6), PI (n = 6) groups, respectively. H group (n = 6) received the same volume (0.5) of normal saline (0.9% NaCl) orally. Animals had been fasted 12 hours (drinking water is free). All experimental animals were killed under high-dose thiopental sodium (50 mg/kg) anesthesia six hours after

receiving drugs and 0.9% NaCl solution, and their stomachs were removed. The excised inner surface of the stomach was evaluated macroscopically. The width of the ulcer areas was determined using a magnifying glass and a millimeter paper. Afterwards, stomach tissues were biochemically examined.

Biochemical analysis

Determination of malondialdehyde (MDA)

The Cayman TBARS Measurement Kit (10009055, Cayman, Ann Arbor, MI, USA) is an easy-to-use, reproducible, and standardized tool for assessing lipid peroxidation in tissue homogenates. MDA-TBA that is formed by the reaction of MDA (μmol/g protein) and thiobarbutyric acid (TBA) under acidic conditions and at high temperatures (90–100°C) is calorimetrically measured at 530–540 nm.

Determination of glutathione (tGSH)

The Cayman GSH Measurement Kit (Item No: 703002, Cayman, Ann Arbor, MI, USA) employs an optimized enzymatic recycling method *via* glutathione reductase to quantify GSH (nmol/g protein). The sulfhydryl group of GSH reacts with 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) and a yellow 5-thio(2-nitrobenzoic) acid (TNB) is formed. GSTNB, a disulfide mixture of GSH and TNB produced concurrently, is reduced by glutathione reductase to recycle GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the sample, and the TNB absorbance value at 405–414 nm provides an accurate assessment of the GSH concentration in the sample.

Measurement of COX activity

We measured the COX activity (U/mg protein) in the gastric tissues of the experimental animals in this series of experiments using a COX activity assay kit (Item No. 760151, Cayman, Ann Arbor, MI, USA). Protein (catalog no: 704002) levels in supernatants obtained from tissue homogenates were measured. Gastric tissue was removed from gastric membranes and washed thoroughly with ice-cold Tris buffer, pH 7.4, containing 0.16 mg/ml of heparin, to remove any red blood cells and clots, then stored at −80°C until assayed. For each rat, a sample of gastric tissue was homogenized in 5 ml of cold buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA) *per* gram of tissue and centrifuged at 10000 × *g* for 15 min at 4°C. Supernatant was removed for assay and stored on ice. We then measured the protein concentration in the supernatant using the Bradford method (Bradford 1976). The COX activity assay kit measures the peroxidase activity of COX. This is assayed calorimetrically by monitoring the appearance of oxidized N, N, N', N'-tetramethyl-p-phenylenediamine at 590 nm. We measured COX-2 activity using the COX-1-specific inhibitor (Kulmacz and Lands 1983). Results for COX-1 and COX-2 activity are given as units *per* mg of protein. The activity of COX in the tissue was expressed as nmol/min/mg protein (U/mg protein).

Statistical analysis

All statistical analyses were performed using IBM SPSS 22 (IBM Corp. Released 2013. Armonk, NY). The results were presented as mean ± standard deviation (SD). Shapiro-Wilks test was used to assess the assumption of normality and Levene test for homogeneity of variances. Comparisons of normally distributed continuous variables between groups were performed using Student *t*-test, One Way Anova test or Welch Anova test. As *post hoc* tests, Games-Howell tests were used for COX-1 in stomach and paw tissue according to the homogeneity of variances, and Tukey HSD tests were used for other biochemical parameters. The changes in the time points of the groups in terms of paw edema were examined with the Two-way Repeated Measures Anova Analysis of Variance. Greenhouse-Geisser correction was used for the violation of sphericity. The statistical level of significance for all tests was considered to be 0.05.

Results

Carrageenan paw test

As a result of examining the variation of paw volume in the study groups over time, the group main effect was statistically significant ($F(3.20) = 406.769$; $p < 0.001$). All study groups differed from each other in a way that was statistically significant ($p < 0.001$). The main effect of time on paw volume values was also statistically significant ($F(2.93; 58.56) = 1849.12$; $p < 0.001$). When the time points were analyzed in pairs, the paw volume measurements between the 3rd and 4th hours were close to each other ($p = 0.397$) and the differences between all other time points were statistically significant ($p < 0.001$). Group and time interaction was also found statistically significant ($F(8.78; 58.56) = 242.75$; $p < 0.001$). When the interaction effect was examined, the IC group was different from the PC and PIC group in the first measurement ($p < 0.05$), but other groups were not statistically different from each other ($p > 0.05$). As shown in Figure 1 and Table 1, carrageenan injection caused inflammatory edema in the paw tissue of the study animals. While there was no statistically significant difference between the groups at the 1st h ($p > 0.05$), pycnogenol, indomethacin, and pycnogenol + indomethacin significantly inhibited the increase in carrageenan-related inflammatory

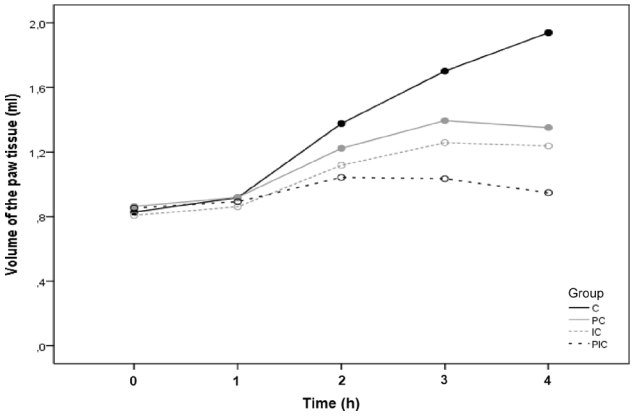

Figure 1. Paw volume measurements according to time in study groups. C, carrageenan group; PC, pycnogenol+carrageenan group; IC, indomethacin+carrageenan group; PIC, pycnogenol+indomethacin+carrageenan group.

paw edema at 2–4 h ($p < 0.001$) (Fig. 1). During this time, pycnogenol + indomethacin, indomethacin, and pycnogenol were the best inhibitors of carrageenan, resulting in paw edema reduction, respectively. Pycnogenol suppressed the carrageenan-induced inflammatory paw edema at a rate of 25.0%, 34.6%, 36.8% and 40.6%, respectively, in the 1st–4th hour, while indomethacin suppressed it at a rate of 37.5%, 43.6%, 48.3% and 61.3%, respectively. In the PIC group, this anti-inflammatory activity was 50.0%, 65.5%, 79.4% and 91.0%, respectively.

Biochemical results

MDA analysis results of the paw tissue

When the MDA data obtained from the study groups were evaluated, at least one group was statistically different ($F(4,25) = 608.608$; $p < 0.001$). As shown in Figure 2, MDA was increased in the C group compared to the H group. The groups in which this increase was most inhibited were PIC, IC and PC. All groups were statistically different from each other ($p < 0.05$).

tGSH analysis results of paw tissue

When evaluated in terms of tGSH results in the study groups, at least one group was statistically different from the others ($F(4,25) = 273.417$, $p < 0.001$). Carragenean treatment reduced tGSH. This decrease was most inhibited in the PIC, PC and IC groups, respectively. When the C, PC, IC groups were compared with H group, there was a statistical difference between them ($p < 0.001$), while the tGSH levels in the PIC group were at the level of the H group ($p = 0.538$).

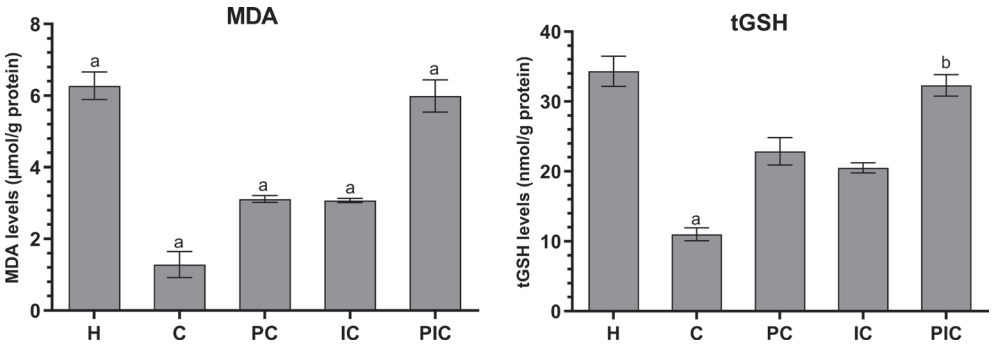

Figure 2. MDA and tGSH levels in the paw tissue of study groups. Data are mean ± SD ($n = 6$). [a] $p < 0.001$ *vs.* other groups; [b] $p = 0.538$ *vs.* H group. MDA, malondialdehyde; tGSH, total glutathione. H, healthy group; C, carrageenan group; PC, pycnogenol+carrageenan group; IC, indomethacin+carrageenan group; PIC, pycnogenol+indomethacin+carrageenan group.

Table 1. Paw volume measurement values and anti-inflammatory activity in study groups

Group	First measurement	Paw volume measurement values (ml)				Anti-inflammatory activity (%)			
		1st hour	2nd hour	3rd hour	4th hour	1st hour	2nd hour	3rd hour	4th hour
C	0.83 ± 0.02	0.91 ± 0.03	1.38 ± 0.04	1.70 ± 0.03	1.94 ± 0.03	–	–	–	–
PC	0.86 ± 0.02	0.92 ± 0.02	1.22 ± 0.06	1.41 ± 0.03	1.35 ± 0.02	25.0	36.8	36.8	55.9
IC	0.81 ± 0.03	0.86 ± 0.04	1.12 ± 0.03	1.26 ± 0.03	1.24 ± 0.02	37.5	43.6	48.3	61.3
PIC	0.85 ± 0.03	0.89 ± 0.04	1.04 ± 0.04	1.03 ± 0.03	0.95 ± 0.04	50.0	65.5	79.4	91.0

C, carrageenan group; PC, pycnogenol+carrageenan group; IC, indomethacin+carrageenan group; PIC, pycnogenol+indomethacin+carrageenan group.

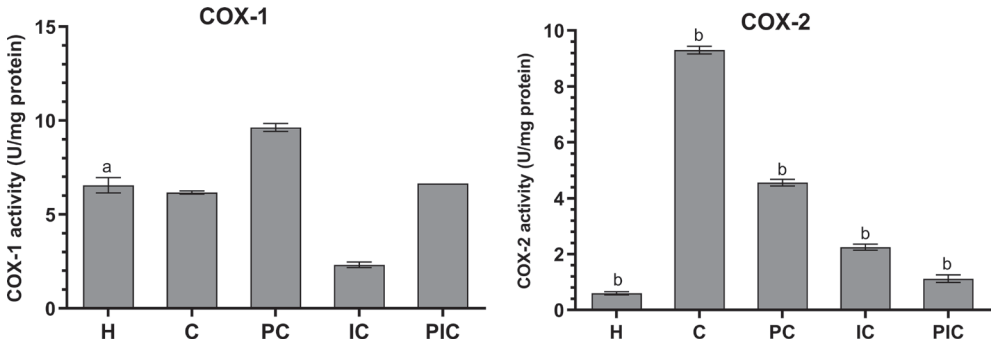

Figure 3. COX-1 and COX-2 levels in the paw tissue of study groups. Data are mean ± SD (n = 6). [a] $p > 0.05$ *vs.* C and PIC groups; [b] $p < 0.001$ *vs.* other groups. COX-1, cyclooxygenase-1; COX-2, cyclooxygenase -2. For more abbreviations, see Figure 2.

The effects of indomethacin alone and pignegenon alone in preventing tGSH decrease were the same ($p = 0.999$) (Fig. 2).

COX-1 and COX-2 analysis results of paw tissue

When the animal groups were compared in terms of COX-1 ($F(4,11.689) = 1150.455$, $p < 0.001$) and COX-2 ($F(4,25) = 5716.760$, $p < 0.001$) values in paw tissue, at least one group was different from the others. As indicated in Figure 3, there was no significant difference in COX-1 activity in the paw tissues of carrageenan-injected animals compared to H group ($p = 0.290$). However, COX-1 was found to be higher in the paw tissue of the PC group compared to H group ($p < 0.001$). While COX-1 activity was significantly reduced in I group ($p < 0.001$), COX-1 activity in the PIC group was nearly equal to that of the H group ($p = 0.329$). Carrageenan increased COX-2 levels in animal paw tissue. The best suppressors of the carrageenan-related COX-2 increase were IPC, indomethacin, and pycnogeneol, respectively.

Macroscopic findings of gastric tissue

Gastric tissues in the H and P groups were normal (Fig. 4A, B). However, marked hyperemia was observed on the gastric surface of animals treated with indomethacin alone, and foci of damage (ulcer) in various numbers and diameters were detected throughout the gastric tissue. The ulcers consisted of round-oval and irregular mucosal defects of varying depth, scattered over the entire gastric surface. The borders of the ulcers were visible (Fig. 4C). The gastric tissue of PI group was seen healthier than in I group (Fig. 4D). As shown in Table 2, no ulcer areas were found in the gastric tissue of healthy animals given pycnogenol alone. However, the mean ulcer area in the stomach tissue in the I group was 52 ± 2.5 mm^2, and the ulcer area in the stomach tissue of the PI group was 0.5 ± 0.5 mm^2. Furthermore, in addition, the mean number of ulcers in the stomach tissue of the animals in the I group was 22.0 ± 2.6, and the mean number of ulcers in the PI group was 0.8 ± 1.12. In terms of both ulcer area and ulcer number, the PI group data were statistically significantly lower than in the I group ($t(10) = 18.143$, $p < 0.001$).

Table 2. The effects of pycnogenol, indomethacin and their combination on gastric tissue

Group	Ulcer area (mm^2)	p	Number of ulcers	p
H	–	–	–	
P	–	–	–	–
I	52.0 ± 2.5	<0.001*	22.0 ± 2.6	<0.001*
PI	0.5 ± 0.5	–	0.8 ± 1.2	–

H, healthy group; P, pycnogenol group; I, indomethacin group; PI, pycnogenol+indomethacin group. Statistical significance was determined by Student's t-test. Data are presented as mean value ± SD; * $p < 0.001$ *vs.* PI group.

MDA analysis results of gastric tissue

When the MDA data obtained from the study groups were evaluated, at least one group was statistically differ-

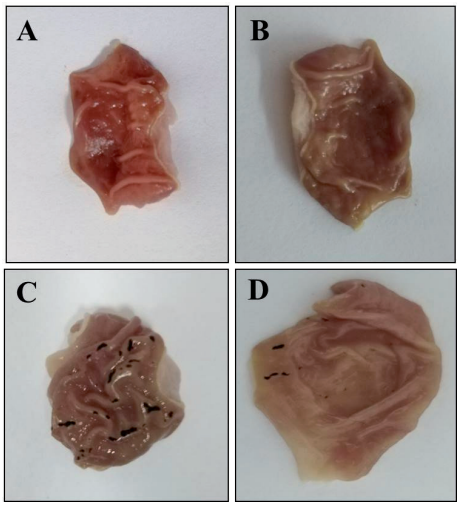

Figure 4. Gastric tissues of H group (**A**), P group (**B**), I group (**C**) and PI group (**D**). The ulcer area was evaluated in mm^2 using millimetric paper. H, healthy group; P, pycnogenol group; I, indomethacin group; PI, pycnogenol+indomethacin group.

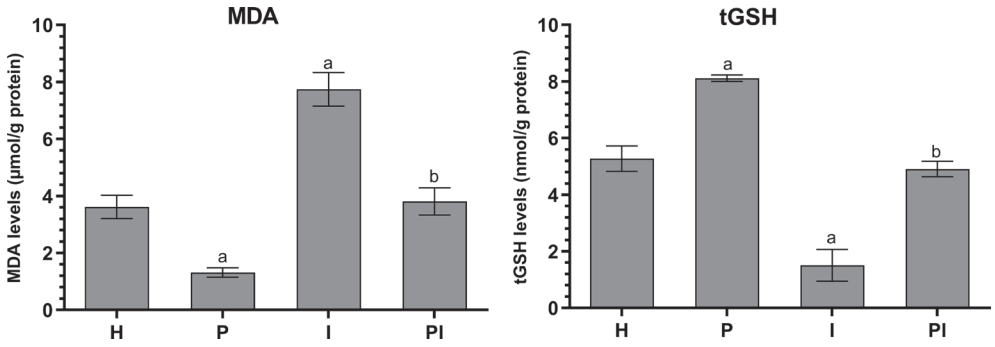

Figure 5. MDA and tGSH levels in the gastric tissue of study groups. Data are mean ± SD (n = 6). [a] $p < 0.001$ *vs.* other groups; [b] $p > 0.05$ *vs.* H group. MDA, malondialdehyde. tGSH, total glutathione. For more abbreviations, see Figure 4.

ent (F(3,20) = 220.269, $p < 0.001$). The amount of MDA in the gastric tissue of animals orally given pycnogenol alone (P group) decreased compared to the H group ($p < 0.001$) but increased in I groupwhen compared to HC group ($p < 0.001$). MDA levels in the PI group were similar to those in the H group ($p = 0.868$) (Fig. 5).

tGSH analysis results of gastric tissue

When evaluated in terms of tGSH results in the study groups, at least one group was statistically different from the others (F(3,20) = 292.467, $p < 0.001$). When compared to the control group, pycnogenol alone increased tGSH in gastric tissue ($p < 0.001$). However, indomethacin alone resulted in a significant decrease in tGSH in gastric tissue ($p < 0.001$). PI group had no significant difference on the tGSH level in the gastric tissue when compared with H group ($p = 0.381$) (Fig. 5).

COX-1 and COX-2 analysis results of gastric tissue

When the animal groups were compared in terms of COX-1 (F(3,8.65) = 264.381, $p < 0.001$) and COX-2 (F(3,20) = 775.131, $p < 0.001$) values in gastric tissue, at least one group was different from the others. When pycnogenol was administered alone, COX-1 activity in gastric tissue increased when compared to the healthy group ($p < 0.001$). On the other hand, indomethacin significantly decreased COX-1 activity in gastric tissue ($p < 0.001$). However, the PI group had no significant difference on COX-1 activity in gastric tissue when compared with HC group ($p = 0.095$). The treatments that best inhibited COX-2 activity in gastric tissue were pycnogenol + indomethacin, indomethacin, and pycnogenol, respectively (Fig. 6).

Discussion

The anti-inflammatory effects of pycnogenol, indomethacin, and IPC in carrageenan-induced inflammatory paw edema in rats were investigated in the first series of this study (Albayrak et al. 2010). Paw tissues were also biochemically examined. The carrageenan inflammation model is well-known for determining anti-inflammatory activity (Suleyman et al. 2008; Tanas et al. 2010; Salman et al. 2011). The inflammatory process induced by carrageenan consists of an early and a late phase. The release of inflammation mediators such as serotonin, bradykinin, and histamine is associated with the early phase (0–1 h), and prostaglandins are associated with the late phase (after 1 h) (Mehrzadi et al. 2021). According to our findings, pycnogenol, indomethacin, and IPC significantly reduced carrageenan-induced inflammatory paw edema in both the early and late stages. Furthermore, pycnogenol, indomethacin, and PI carrageenan were discovered to be more effective in the second phase of inflammation than in the first. Previous research has also demonstrated that indomethacin has

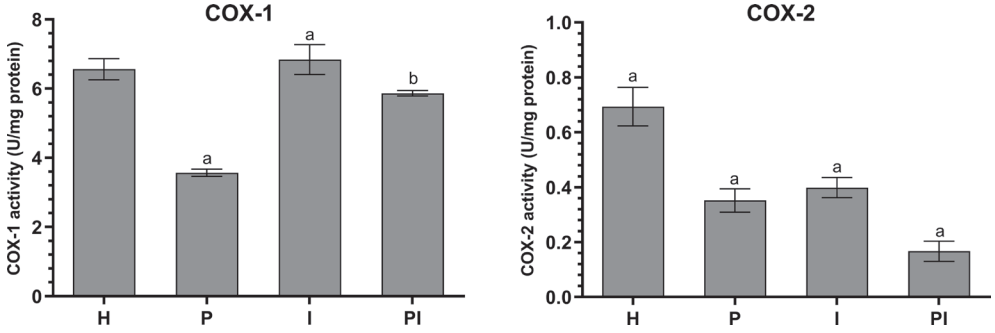

Figure 6. COX-1 and COX-2 levels in the gastric tissue of study groups. Data are mean ± SD (n = 6). [a] $p < 0.001$ *vs.*other groups; [b] $p = 0.095$ *vs.* H group. COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2. For more abbreviations, see Figure 4.

anti-inflammatory effects in inflammation models induced by carrageenan, histamine, and serotonin (Süleyman et al. 1999). Indomethacin is also known to inhibit the increase in prostaglandins that causes the late stage of carrageenan inflammation in paw tissue (Houshmand et al. 2016). There have been no studies that investigate the direct effect of pycnogenol on histamine, bradykinin, and prostaglandin release in inflamed tissue; however, it has been reported that it reduces histamine release from mast cells due to oxidative stress (Sharma et al. 2003). As previously stated, pycnogenol inhibits COX-2 expression (Canali et al. 2009). This suggests that pycnogenol may have indirectly suppressed prostaglandin synthesis. The most pronounced anti-inflammatory effects of indomethacin, pycnogenol, and PI were observed at the fourth hour of carrageenan inflammation in our study. These findings suggest that the drugs work by inhibiting histamine, bradykinin, and prostaglandin synthesis.

In this study, which is consistent with the literature, PI, which best reduced carrageenan paw edema, significantly reduced MDA increase in inflamed tissue more than pycnogenol and indomethacin. ROS, as previously stated, causes lipid peroxidation (LPO) and MDA production from lipids. As a result, MDA is recognized as a dependable oxidant marker of oxidative stress-induced LPO (Ghonimi et al. 2021). According to a study that backs up these findings, inflammation and oxidative stress are processes that are linked (Mitrea et al. 2020). Haddadi et al. (2020) emphasized that indomethacin, which has an anti-inflammatory effect, prevents the increase in the amount of MDA in paw tissue when combined with carrageenan. Ince et al. (2009) discovered that pycnogenol reduced carrageenan-induced inflammation. According to the literature, pycnogenol protects brain tissue from inflammatory and oxidative damage by preventing the increase in MDA levels caused by ischemia-reperfusion in rats (Ozoner et al. 2019).

Oxidative tissue damage occurs when the oxidant antioxidant balance shifts in favor of oxidants (Kisaoglu et al. 2013). As can be seen from our experimental results, the amount of MDA in the carrageenan group is high while the amount of tGSH is low. GSH is a tripeptide made up of glutamate, cysteine, and glycine that is essential for antioxidant defense. GSH detoxifies through interactions with reactive nitrogen species, hydroxyl radicals, hypochlorous acid, and other reactive species (Bjørklund et al. 2020). PIC, which reduced inflammatory paw edema the best, prevented tGSH store reduction with carrageenan more effectively than pycnogenol and indomethacin. In a recent experimental study, Zammel et al. (2021) discovered that the amount of MDA was higher in carrageenan-inflamed paw tissue, while the amount of GSH was lower than in the healthy group. On the contrary, MDA was measured to be low and GSH was high in the ethylene group treated with indomethacin (Mitrea et al. 2020). There have been no studies on the effect of pycnogenol on the amount of GSH in carrageenan-induced inflamed paw tissue; however, it is known that it prevents the decrease in the amount of tGSH in brain tissue in areas of inflammation caused by ischemia-reperfusion (Ozoner et al. 2019).

Previous research found that the severity of inflammation caused by carrageenan was linked to an increase in COX-2 activity, while the anti-inflammatory effect was linked to a decrease in COX-2 activity (Albayrak et al. 2010). As previously stated, COX-2 enzyme inhibition is responsible for indomethacin's anti-inflammatory effect, while COX-1 enzyme inhibition is responsible for its side effects (Abdellatif et al. 2021). Pycnogenol is also known to have an anti-inflammatory (Icel et al. 2018) and inhibitory effect on COX-2 expression (Canali et al. 2009). In our study, COX-2 activity was found to be significantly lower in the PIC group than pycnogenol and indomethacin, which had a stronger antioxidant effect. There are statements in the literature that support our experimental results that COX-2 is a source of ROS and that inhibiting its activity can reduce oxidative stress (Ahmed et al. 2014).

The effects of pycnogenol, indomethacin, and their combination on gastric tissue were investigated in the second series of our study. There were no macroscopic symptoms in the gastric tissue of the study animals given pycnogeneol alone. However, significant hyperemia and ulcer foci were found in the gastric tissue of the study animals given indomethacin alone. In previous studies, it has been reported that irregular mucosal defects of different shapes and diameters develop in the gastric tissue of study animals given indomethacin (Polat et al. 2011). It has been reported in the literature that indomethacin causes gastric damage by inhibiting the production of protective factors such as bicarbonate, mucus, antioxidants, and COX-1, thereby increasing acid secretion and oxidant production (Suleyman et al. 2010). According to recent research, maintaining high antioxidant and COX-1 levels is beneficial in the treatment of indomethacin-associated gastric ulcers (Kuadkaew et al. 2021). As can be seen from the results of our tests, no damage was found in the gastric tissue with the highest COX-1 level. On the other hand, the gastric tissue of the indomethacin group with the lowest COX-1 level showed significant damage. As a result, significant inflammation developed in the paw tissue of carrageenan-treated animals. Pycnogenol suppressed carrageenan-induced inflammation nearly as well as indomethacin. Pycnogenol and indomethacin combination has been shown to be the most effective at suppressing carrageenan inflammation. The results of our biochemical experiments revealed that the severity of inflammation was directly proportional to the increase in oxidants and COX-2 and the decrease in antioxidants. Furthermore, indomethacin caused a damage in the gastric tissue of animals. Pycnogenol reduced the gastric tissue damage caused

by indomethacin. In the indomethacin group, there was significant gastric tissue damage due to high oxidant levels, low antioxidant levels, and COX-1 levels. On the contrary, no damage was found in the gastric tissue of the healthy and pycnogenol groups, which had low oxidant levels, high antioxidant levels, and low COX-1 levels. Furthermore, pycnogenol has a gastroprotective effect by preventing indomethacin-induced increases in oxidant levels in gastric tissue as well as decreases in antioxidant and COX-1 levels. According to our findings, pycnogenol reduced the toxic effect of indomethacin on the gastric tissue while increasing its anti-inflammatory activity. This advantageous interaction of pycnogenol and indomethacin suggests that combination of pycnogenol and indomethacin will be more effective in the treatment of inflammatory diseases.

Author contribution statement. BE and HS conceived and designed research. BS, RM, MG and CE conducted experiments. BE and YKA analyzed data. BE and HS wrote the manuscript. BE, BS, RM, CE, SB, MG, SH and HS revised the manuscript critically for important intellectual content. All authors read and approved the manuscript.

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