Investigation of mucus obtained from different fish species on the acute pain induced with scalpel incision in paw of rats

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Abstract: No comparative study could be found for the analgesic activity of mucuses from the Oncorhynchus mykiss (OM), Salvelinus fontinalis (SF), Salmo coruhensis (SC), Acipenser gueldenstaedtii (AG), and Acipenser baerii (AB) fish species in the literature. We aimed to investigate the effects of mucuses obtained from the abovementioned fish species on scalpel incision-induced pain in the rat paw and to examine the role of oxidant/antioxidant parameters and COX-2 gene expression in the analgesic activities. Animals were divided into groups: SIC (scalpel incision; SI), SIDS (SI+25 mg/kg diclofenac sodium), SOM (SI+25 mg/kg OM mucus), SFM (SI+25 mg/kg SF mucus), SCM (SI+25 mg/kg SC mucus), SAgM (SI+25 mg/kg AG mucus), SAbM (SI+25 mg/kg AB mucus), and HG (healthy). The paw pain thresholds were measured with a Basile algesimeter before and after diclofenac sodium (DS) or mucus administration, and then the rats were euthanized with thiopental sodium. Oxidant/antioxidant and COX-2 gene expression parameters were measured in paw tissues. OM, SC, AG, and AB fish mucuses could not decrease the SI-induced pain. However, SF fish mucus prevented this pain by 69% after the first hour and by 58.3% after the third hour. DS was shown to suppress pain more weakly than SF, preventing the pain by 62.1% and 50.0% after the first and third hours, respectively. SF mucus and DS significantly inhibited increase of COX-2 gene expression, while other fish mucuses could not. None of the fish mucuses except SF mucus in conjunction with DS could significantly inhibit the increase in oxidant parameters and decrease in antioxidants. SF fish mucus should be comparatively assessed in clinical practice for treatment of postoperative pain.

Key words: diclofenac sodium, gene expression, mucus, oxidant/antioxidants parameters, rat

Introduction

Pain has been revealed to be a sign of trauma and many diseases. One of the most common causes of pain is postoperative trauma [1]. It is know that current treatments for postoperative pain are insufficient and that moderate to severe pain is observed in about 75% of postoperative patients [2]. Postoperative pain that cannot be adequately suppressed is known to cause pulmonary, renal, and cardiac dysfunctions and chronic pain syndromes [3].

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Postoperative pain relief increases quality of life, decreases morbidity, and shortens hospitalization time [4]. For this purpose, narcotic and nonnarcotic analgesic drugs are used for the treatment of pain [5]. However, narcotic analgesics are addictive, cause tolerance, and have side effects restricting their use such as respiratory depression and constipation [6]. Nonsteroidal anti-inflammatory drugs (NSAIDs), which are nonnarcotic, cause serious adverse effects such as gastrointestinal ulcer, renal damage, inhibition of platelets, and hemorrhage [7]. For this reason, one of the most important purposes of developing medical science is discovery of new drugs that have less toxic effects and higher analgesic activity. Scientific studies conducted for this purpose have demonstrated the analgesic effect of mucus obtained from the Channa striatus, a species of fish [8]. There are also studies in the literature that have investigated use of fishery products in attenuation of postoperative pain and for contribution to wound healing [8]. However, except for some studies reporting a very small amount of data in the literature, there have not been any new studies investigating the analgesic activity of mucuses obtained from various fish species. The information from the literature suggests that mucuses of different fish species may have analgesic activity. The fish species used in this study included the Oncorhynchus mykiss, Salvelinus fontinalis, Salmo coruhensis, Acipenser gueldenstaedtii, and Acipenser baerii, and the fish were raised at the RTEU Fisheries research and Practise Center. As is widely known, analgesic activity can be induced in rats using formalin, carrageenan, and other methods [9–12]. The postoperative pain model used in this study was created with scalpel incision in the paw of rats [13]. Postoperative pain has been shown to result from tissue damage and accompanying inflammation [14]. The COX-2 enzyme is known to play a role in the mechanism of postoperative pain [15]. The activation of COX-2 causes production of free oxygen radicals, leading to serious oxidative tissue damage as well as inflammation [16]. We could not find a comparative study in the literature examining the effects of mucuses obtained from Oncorhynchus mykiss (OM), Salvelinus fontinalis (SF), Salmo coruhensis (SC), Acipenser gueldenstaedtii (AG), and Acipenser baerii (AB) fish species on pain induced by scalpel incision and oxidative damage. Therefore, the objective of our study was to investigate the effects of mucuses obtained from the abovementioned fish species on pain induced by scalpel incision in the paw of rats. In addition, we examined whether or not oxidant/antioxidant parameters and COX-2 gene expression have a role in analgesic activity.

### Methods

#### Animals

A total of 48 male albino Wistar rats weighing between 240–250 g were used in the experiment. All the rats were obtained from the Medical Experimental Application and Research Center of Recep Tayyip Erdogan University. Animals were kept in groups at normal room temperature (22°C), and relative humidity was maintained at approximately 50–60% under appropriate conditions in the Pharmacology Department Laboratory for 7 days before the experiment.

The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 27.04.2015; Meeting No.: 2015/36).

#### Chemical agents

Chemical agents used in the experiment included diclofenac sodium (Voltaren 50 mg Tablet) which was obtained from Novartis (Turkey), and thiopental sodium from İ.E ULAGAY (Turkey). At RTEU Fisheries Research and Practise Center, maintenance and feeding of several species for example, the rainbow trout, Oncorhynchus mykiss; brook trout, Salvelinus fontinalis; Black Sea trout, Salmo coruhensis (Syn=Salmo trutta labrax); sturgeon fish, Acipenser gueldenstaedtii; Siberian sturgeon, Acipenser baerii; Nile tilapia, Oreochromis niloticus; and goldfish, Carassius auratus, which have high economic value, is performed for student practice and study project purposes.

#### Experimental Groups

Experimental groups used in the scalpel incision pain test

The rats were divided into groups as follows: control, which only received a scalpel incision on the paw (SIC); scalpel incision + 25 mg/kg diclofenac sodium (SDS); scalpel incision + 25 mg/kg OM mucus (SOM); scalpel incision + 25 mg/kg SF mucus (SFM); scalpel incision + 25 mg/kg SC mucus (SCM); scalpel incision + 25 mg/kg AG mucus (SAGM); scalpel incision + 25 mg/kg AB mucus (SAbM); and healthy group (HG). The process of obtaining mucus from fish was performed in the laboratory setting under sterile conditions. The weights
listed above represent wet weights.

The reference study for the process of mucus extraction we used was performed by Jais et al. [17]. According to their study, prewashed fish were weighed and placed into the plastic bags (15 × 30 cm) containing an equal volume of distilled water at room temperature. Live fish were exposed to hypothermic stress to induce production of mucus and incubated at −20°C for 1 h. To collect mucus, a clean plastic spatula was applied to the fish by gently scratching their dorsolateral section. The collected mucus was centrifuged at 1,000 rpm for 30 min, and distilled water was used as a solvent. The supernatant containing the mucus was maintained as a stock supernatant (100% concentrated) and kept at +4°C until use.

**Experimental procedure**

A preoperative pain model in rats was created with a commonly used conventional method [13]. In this method, a 0.5 cm transverse subcutaneous incision was made with a scalpel in the upper part of the right paw of all the rats, except for those in the HG group (n=6). Only one paw per animal were subjected to scalpel incision. Then the cut edges of the skin were sutured using 5–0 silk suture. Twenty-four hours after the operation, the rats in the SIDS group (n=6) were administered 25 mg/kg diclofenac sodium directly to the stomach [18], while the SOM, SFm, SCm, SAgm, and SAbm groups were administered mucuses obtained from OM, SF, SC, AG, and AB fish at a dose of 25 mg/kg by oral route via oral gavage. Paw pain threshold was measured using a Basile algesimeter in all rat groups before administration and after the first and third hours after administration of diclofenac sodium (DS) and mucuses [19]. Rats were sacrificed with high-dose thiopental sodium anesthesia immediately after the measurement at more than three hours after administration, and paw tissues were removed. Malondialdehyde (MDA), myeloperoxidase (MPO), nitric oxide (NO), total reduced glutathione (tGSH), superoxide dismutase (SOD), and COX-2 gene expression parameters were measured in the extracted paw tissues. Analgesic activity was calculated by the method of Ince et al. [15]. All results obtained from the experiment were evaluated in comparison with the SIC group (n=6).

**Determination of COX-2 gene expression**

A mortar was used as a mechanical homogenizer to break up tissue. First, 200.0 µl of extract obtained from fragmented tissue was placed in a MagNA Pure Compact automatic RNA isolation device (Roche). Then, a 50.0 µl RNA sample was obtained through RNA isolation using a MagNA Pure Compact RNA Isolation Kit (Roche).

**Stage of cDNA synthesis**

The concentration of RNA obtained was measured. Based on the measured RNA concentration, the RNA was either diluted or undiluted to yield 15–20 ng of cDNA. Then, 10.0 µl of each calibrated sample, 2.0 µl of random primer, and 1.0 µl of distilled water from a Transcriptor First Strand cDNA Synthesis Kit were transferred into a 0.2 ml PCR tube. Denaturation was then conducted in a reverse-transcription PCR instrument at 65°C for 10 min. A mixture was then added to the denatured RNA to form cDNA. The quantities of the substances included in the mixture (from the Transcriptor First Strand cDNA Synthesis Kit) used for each sample were as follows: 4.0 µl of reaction buffer, 5.0 µl of RNase, 2.0 µl of deoxynucleotide mix, and 0.5 µl of reverse transcriptase. After the addition of 7.0 µl of the mixture to the denatured RNA, the tube was placed in a reverse-transcription PCR instrument and subjected to an appropriate PCR program.

**Gene expression analysis**

A pair of primers was designed to specifically detect mouse COX-2 mRNA. The sequences of these two primers were as follows: 5′-CAAGCAGTGGCAAGGCCTCA-3′ forward primer, and 5′-GGCACTTGCATTGATGGTGGCT-3′, reverse primer. Gene expression analysis was performed with real-time PCR reactions in a final volume of 5.0 µl cDNA, 8.0 µl distilled water, 10.0 µl LightCycler 480 Probes Master, and 2.0 µl primer-probe set. The following thermal cycling conditions were applied with a LightCycler 480 Instrument II: enzyme activation and denaturation at 95°C for 10 min, 45 cycles of amplification (95°C for 10 s, 60°C for 30 s), and signal detection at 72°C for 1 s with detection and cooling at 40°C for 30 s.

**Biochemical Procedures**

**Preparation of samples**

For determination of MPO in hindpaw, potassium phosphate buffer at pH=6 containing 0.5% HDTMAB (0.5% hexadecyl trimethyl ammonium bromide) for determination of MDA, 1.15% potassium chloride solution was completed to 2 ml for the other measurement in
phosphate buffer at pH=7.5 and homogenized in ice-cold environment. Samples were then centrifuged at 10,000 rpm at +4°C for 15 min. The supernatant fractions were used as the analysis samples.

**Malondialdehyde (MDA) analysis**

MDA measurement was based on the method used by Ohkawa et al. [20]. This method is based on spectrophotometric measurement of the absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA at a high temperature (95°C) at a wavelength of 532 nm. Homogenates were centrifuged at 5,000 g for 20 min, and the supernatants were used for determination of the MDA amount. For this purpose, 250 µl homogenates, 100 µl 8% sodium dodecyl sulfate (SDS), 750 µl 20% acetic acid, 750 µl 0.08% TBA, and 50% µl pure water were pipetted and vortexed. After this, the mixture was incubated at 100°C for 60 min, 2.5 ml n-butanol was added to it, and spectrophotometric measurement was performed. The amount of red color that formed was read at 532 nm using 3 ml cuvettes, and the amount of MDA was determined with the standard chart created with an MDA stock solution that had previously been prepared by taking account of dilution coefficients.

**Determination of myeloperoxidase (MPO) activity**

For determination of MPO in the paw tissue homogenates, potassium phosphate buffer (pH=6) containing 0.5% HDTMAB (0.5% hexadecyltrimethylammonium bromide) was prepared. The homogenates were then centrifuged at 10,000 rpm at +4°C for 15 min. The supernatant fractions were used as the analysis samples. In determination of MPO enzyme activity, oxidation reaction with MPO mediated H2O2 containing 4-aminantipyrine/phenol solution was used as substrate [21].

**Nitric oxide (NO) analysis**

Nitric oxide levels were measured using the Griess reaction, which is based on a two-step process. In the first step, nitrate is converted into nitrite by nitrate reductase. In the second step, nitrite reacts with the Griess reagent. At the end of this reaction, a deep purple azo compound forms. The absorbance of this azo compound was measured photometrically at a wavelength of 540 nm. This azo chromophore can be used to accurately determine nitrite concentrations as a marker of NO [22, 23].

**Total glutathione (tGSH) analysis**

The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications [24]. Each sample was weighed and homogenized in 2 ml of 50 mmol/l Tris–HCl buffer containing 20 mmol/l EDTA and 0.2 mmol/l sucrose (pH 7.5). The homogenate was immediately precipitated with 0.1 ml of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4,200 rpm for 40 min at 4°C. The supernatant was used to determine the GSH level. For this purpose, 1,500 µl of measurement buffer (200 mmol/l Tris–HCl buffer containing 0.2 mmol/l EDTA [pH 7.5]), 500 µl supernatant, 100 µl DTNB (10 mmol/l), and 7,900 µl methanol were added to a tube, vortexed, and incubated for 30 min at 37°C. 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was used as a chromogen, and it formed a yellow-colored complex with sulfhydryl groups. The absorbance was measured at 412 nm using a spectrophotometer (DU 500, Beckman Coulter, Inc., Brea, CA, USA). The standard curve was obtained by using reduced glutathione.

**Superoxide dismutase (SOD) analysis**

Measurements were performed according to the method of Sun et al. [25]. When xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT, and a purple-colored formazan dye is produced. Each sample was weighed and homogenized in 2 ml of 20 mmol/l phosphate buffer containing 10 mmol/l EDTA (pH 7.8). It was then centrifuged at 6,000 rpm for 10 min, and the brilliant supernatant was used as the assay sample. Next, 2,450 µl measurement mixture (0.3 mmol/l xanthine, 0.6 mmol/l EDTA, 150 µmol/l NBT, 0.4 mol/l Na2CO3, 1 g/l bovine serum albumin), 500 µl supernatant, and 50 µl xanthine oxidase (167 U/l) were combined and vortexed. This mixture was then incubated for 10 min. Formazan was produced at the end of the reaction. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least O2− radical that reacts with NBT occurs.

**Statistical analysis**

Data from the experiments were expressed as the mean ± standard deviation (× ±/− SD). Significance of differences between groups was defined using one-way ANOVA followed by Fisher’s post hoc LSD (least significant
Effects of Fish Mucus on the Acute Pain in Rats

All statistical analyses were performed with the PASW Statistics for Windows, Version 18.0 statistical software, and \( P < 0.05 \) values were considered statistically significant.

**Results**

Table 1 shows that mucuses from the OM, SC, AG, and AB fish species could not decrease the pain induced by scalpel incision. However, SF fish mucus prevented this pain by 69% after the first hour (\( P < 0.0001 \)) and suppressed it by 58.3% after the third hour (\( P < 0.001 \)). DS suppressed the pain more weakly than SF after the first and third hours, suppressing pain by 62.1 (\( P < 0.001 \)) and 50% (\( P < 0.01 \)), respectively. The pain thresholds of SiC, Som, SFm, SCm, Sagm, Sabm, and SidS groups were decreased by 29 ± 3.7 g, 26 ± 7.8 g, 9 ± 2.2 g, 28 ± 6.4 g, 30 ± 7.4 g, 11 ± 2.0 g after the first hour and by 36 ± 4.9 g, 33 ± 7.3 g, 15 ± 3.7 g, 37 ± 7.2 g, 34 ± 7.8 g, 35 ± 8.1 g, and 18 ± 2.8 g after the third hour, respectively.

**Results of COX-2 gene expression**

COX-2 gene expression significantly increased in the groups subjected to scalpel incision in the paw compared with the healthy group. SF mucus and DS significantly inhibited the increase in COX-2 gene expression. However, the mucuses of the OM, SC, AG, and AB fish failed to inhibit the increase in COX-2 gene expression (Fig. 1.)

**Biochemical findings**

The results of biochemical analyses showed that the mucuses of the OM, SC, AG, and AB fish could not prevent the increase in MDA resulting from scalpel incision in the paw, but SF mucus and DS inhibited MDA almost the same rate (Fig. 2). SF mucus and DS also significantly inhibited the increase in MPO activity resulting from incision, whereas the mucuses of the other fish could not (Fig. 3). In addition, the increase in amount of NO caused by scalpel incision in the paw significantly decreased in only the SF mucus and DS groups (Fig. 4). The levels of GSH and SOD antioxidant parameters were found to decrease in parallel with the increase in oxidant activities in the tissue subjected to incision. While SF mucus more significantly inhibited the decrease in tGSH...
and SOD compared with DS, the effects of the mucuses from the OM, SC, AG, and AB fish species on tGSH and SOD were not statistically significant (Figs. 5 and 6).

Discussion

In this study, the analgesic activities of the mucuses obtained from the OM, SF, SC, AG, and AB fish species were investigated in a pain model created with scalpel incision in the rat paw. In addition, we examined whether or not oxidant/antioxidant parameters and COX-2 gene expression have a role in hyperalgesia and analgesic activities. In a previous study, the pain threshold was demonstrated to statistically decrease in the rat paw that underwent scalpel incision compared with the normal value [13]. In our study, the hindpaw pain threshold of the animals that underwent scalpel incision was found to be lower than the value before incision, which is consistent with the literature. It has been suggested that mucuses from the OM, SC, AG, and AB fishes have analgesic activities, but it was found that they could not prevent a decrease in pain threshold. However, SF fish mucus prevented pain more significantly than DS after the first and third hours. As mentioned above, mucus isolated from some fish species showed an analgesic effect [26]. In order to highlight the analgesic effect of the mucuses used in our study and the degree of effect, diclofenac was used as positive control, and the evaluation was performed with a dose of 25 mg/kg. This dose is the most effective dose of diclofenac [18]. For this reason, in order to evaluate the gravimetric effect of the mucuses, the same dose as used for diclofenac was preferred for the mucuses in the experimental design. The antinociceptive effect of mucus isolated from the Channa striatus is mainly derived from its lipid (oleic acid, linoleic acid) and amino acid content [26]. In our study, the antinociceptive activity of SF, the analgesic activity of which was also found to be significant, was thought to be derived from its content, as mentioned above. In a previous study, it was found that pretreatment of a Channa striatus (haruan) extract with distilled water resulted in a significant antinociceptive effect that was concentration dependent [27]. However, there was no significant effect between the concentrations of 25% and 50%. Extracts with 50% and 100% were pretreated with 10% concentration α-amylase and lipase and 0.1% concentration protease, respectively, and the results showed that α-amylase and protease pretreatment resulted in concentration-dependent activity, whereas this activity was not remarkable compared with the positive control pretreated with distilled water. The extract pretreated with lipase similarly showed significant activity, but interestingly it was not concentration dependent. The finding revealing no antinociceptive effect of α-amylase and protease suggests that the bioactive content is neither a carbohydrate nor a protein [27]. There is a limited un-
understanding of the contents of mucus of SF fishes in the current literature. Although this previous study showed the analgesic activity of mucus from the Channa striatus, the mechanism of this effect was not explored. There are also no studies that have used the same SF fish as use and explained the analgesic activity of SF mucus or found any novel contents in the mucus responsible for its analgesic activity. Postoperative pain is defined as an acute pain accompanied by an inflammatory process [28]. An increase of COX-2 enzyme activity is responsible for postoperative pain [29]. The COX-2 enzyme has been reported to play a role in induction of inflammation by increasing production of proinflammatory prostaglandins from arachidonic acid [7]. In another study, COX-2 was reported to be involved in the production of prostaglandin induced by proinflammatory agents and to increase pain sensitivity in the peripheral tissues [30]. It is known that the analgesic, anti-inflammatory,
and antipyretic effects of DS, which we compared with SF in the present experiment, are the result of inhibition of COX-2 [31]. Mucus from SF also inhibited COX-2 gene expression, which increased in rat paw tissue due to the incision. The present study also found that the amount of MDA, which is an oxidant parameter, was significantly increased in the groups subjected to scalpel incision in the paw compared with the healthy group. Because, the activation of COX-2 may lead to inflammation as well as production of free oxygen radicals that may cause tissue damage [16, 32]. Furthermore, the amount of MDA has been reported to be elevated both in inflammatory and noninflammatory pains; this suggests that oxidative stress has a role in pain pathogenesis [33]. Ilker et al. stated the importance of oxidative stress in the pathogenesis of incision-related postoperative pain and reported that MPO gene expression increased in damaged tissue that underwent incision; in addition, they demonstrated that metamizol, which significantly inhibited the increase of MPO gene expression, had a more potent analgesic effect than paracetamol, which could not inhibit MPO expression [15]. SF mucus and DS inhibited increase of MPO activity in the paw as a result of incision, and this suggests that inhibition of the increase of MPO activity is one of the significant analgesic activity mechanisms of SF.

In the present study, the amount of NO was found to be significantly higher in the tissue from the groups subjected to scalpel incision than in the healthy group. There is an increasing volume of evidence about the contribution of NO to hyperalgesia, which occurs as a result of various stimuli [34]. The role of NO in peripheric and central nociception is rather complex [35]. Many studies have recognized that NO plays a role in perception and has an important function in modulation of pain [35, 36]. This data from the literature supports our experimental results.

The present study also found that the tGSH and SOD levels were low in the rat paw tissue with high levels of MDA, MPO, and NO. While this results in a change in the oxidant/antioxidant balance in favor of oxidants in the paw tissue subjected to scalpel incision, the balance in the paw tissue treated with SF and DS changed in favor of antioxidants. TGHSH, which is one of the endogenous antioxidant parameters, reacts with free radicals and converts them into harmless products. By this mechanism, tGSH protects cells against oxidative damage that may be caused by free radicals [37]. SOD is known to be an endogenous antioxidant with decreased activity in damaged tissue [38].

In conclusion, scalpel incision caused a decrease in the pain threshold, reflecting hyperalgesia in the rat paw. The OM, SC, AG, and AB fish mucus could not inhibit the decrease in this pain threshold caused by scalpel incision in a paw of the rats. However, SF fish mucus appeared to inhibit the decrease in pain threshold better than DS and to provide a more significant analgesic effect. These promising results show that SF fish mucus can be adapted for use in clinical practice for treatment of postoperative pain.

Conflict of interest

The authors declare that they have no conflicts of interest.

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