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Changes of three COX isoforms expression after formalin induced inflammatory pain in brain and analgesic effects of different COX inhibitors

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ABSTRACT **AIM**: To compare the expression of three cyclooxygenase (COX) isoforms in the process of inflammatory pain and evaluate the analgesic effects of different protocols about usage of COX inhibitors on inflammatory pain. **METHODS**: Formalin was injected subplantarly to mice to induce inflammatory pain. The expression of COX-1, COX-2 and COX-3 was evaluated by radioimmunoassay and RT-PCR, respectively. For the analgesic effect assay, animals were divided into 5 groups including control, SC, NS, IN and NS+SC group. The former 4 groups received saline, SC-560 (300 $\mu g \cdot kg^{-1}$), NS-398 (150 $\mu g \cdot kg^{-1}$), and indomethacin (300 $\mu g \cdot kg^{-1}$), respectively. In the NS+SC group, animals received NS-398 during the first 1 month and SC-560 during the second month in the NS+SC group. **RESULTS**: The expression of COX-1 was higher at the late phase while that of COX-2 was higher at the early phase of inflammatory pain. The expression of COX-3 did not significantly change in the process of inflammatory pain. Additionally, behavioral assessment showed that using COX-2 inhibitors at the early phase followed by COX-1 inhibitors at the late phase could get better analgesic effect on inflammatory pain compared with single using COX-1 selective or COX-2 selective inhibitors. **CONCLUSION**: In brain, the expression of COX-2 increases rapidly in the inflammatory pain process while COX-1 expression does not increase till the late phase. Brain COX-3 is poorly involved in the inflammatory process. Combined use of COX-1 and COX-2 selective inhibitors may be a better protocol in inflammatory pain treatment.

KEY WORDS inflammatory pain; cyclooxygenase; COX inhibitor; radioimmunoassay; RT-PCR; hot plate

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Cyclooxygenase (COX) catalyzes the rate-limiting step of the prostanoid cascade. Arachidonic acid (AA) is converted to prostaglandin H_2 (PGH_2) by COX. PGH_2 is metabolized by different synthases into more biologically active products including the PGs (PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2) and thromboxane (TXA_2). Two distinct isoforms have been established for COX: COX-1 and COX-2. COX-1 displays the characteristics of a housekeeping gene. COX-1 is constitutively expressed in most tissues. The higher levels of that enzyme may be found in several specific tissues and cells, including endothelium, seminal vesicles, monocytes, and platelets. In contrast, COX-2 expression is markedly inducible in specialized cell types and is thought to be an inducible isoform. In particular tissues, COX-2 regulates specific physiological functions, such as the inflammatory process, ovulation, implantation, perinatal kidney development, ductus arteriosus remodeling, or ulcer healing. The activities of COX-1 and COX-2 are differentially inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs). For example, aspirin and indomethacin inhibit both enzymes, whereas NS-398 and SC-560 are selective inhibitors for COX-2 and COX-1, respectively.

NSAIDs have been used for treating inflammation for a long history. The COX-2 selective inhibitors are safer alternatives to the current NSAIDs in terms of gastrointestinal safety and are widely used in the treatment of the symptoms of osteoarthritis and the relief of acute pain. However, toxicological concerns regarding their renal and

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cardiovascular safety remain. However, it has been suspected that COX-2 selective inhibitors were not definitely preferable in inflammatory therapy. Although the level of PGs catalyzed by COX-2 is rapidly increased after inflammatory stimulation, the increase will not persist for a long time. Results of studies also showed that COX-2 may be beneficial to the healing of inflammation[1]. So blindly use of COX-2 inhibitors could be harmful. A continued need to develop more safe and effective strategy of COX inhibitor application fuels the ongoing investigations of COX and inflammatory pain.

Furthermore, a new isoform of COX has been identified by Chandrasekharan and his colleague recently and named COX-3[2]. It is thought to be a new target for pain therapy. Some proofs have been established for its role in anti-pyresis as well. However, its change in the process of inflammatory pain is still unknown.

We therefore observed the change of three COX isoforms in the process of inflammatory pain in brain and compared the analgesic effects of different protocol of COX inhibitor application.

1 MATERIALS AND METHODS

1.1 Animals and materials BALB/C mice obtained from the experimental animal center of fourth military medical university. Half of them were males and half of them were females. All of the animals weighed 18—22 g. Animals were housed in colonial stock following arrival. Food and water were available ad libitum. Temperature and humidity of the environment were controlled (23 ± 1 ℃ and 50% ±1.3 % of humidity) and the laboratory was maintained on a 12 h day/night cycle. All of the experiments were carried out during the light phase. All of the ethical manners for use of laboratory animals were considered carefully. All of the commercially available chemicals were analytical grade. NS-398, SC-560 and indomethacin were purchased from Sigma (USA).

1.2 Animal model Formalin induced pain model was used in this study. 50 μl of 5% formalin solution was injected subplantarly into the left hindpaw of the mice using a microsyringe 26-gauge needle. Time points were before injection, 1 h, 12 h, 1 d, 3 d, 7 d, 14 d, 30 d and 60 d after injection.

1.3 Total RNA extraction Animals were sacrificed at different time points. Brains were picked out and homogenated. Total RNA was extracted from the brain tissue using the TRIzol reagent (Invitrogen, UK) according to the manufacturer's instructions. RNA was measured using 260/280 UV spectrophotometry.

1.4 RT-PCR analysis First-strand cDNA was transcribed from 1 μg total RNA of mouse brain tissue with random primers by avian myeloblastosis virus reverse transcriptase (Takara, Japan). PCR was performed with primers specific for mouse GAPDH (5'-TGAACGGGAAGCTCACTGG-3' and 5'-TACAGCAACAGGGTGGTGGA-3', expected PCR product size, 307 bp), mouse COX-1 (5'-AGGAGATGGCTGCTGAGTTGG-3' and 5'-AATCTGACTTTCTGAGTTGCC-3'; expected PCR product size, 602 bp), mouse COX-2 (5'-GGGAAGCCTTCTCCAACC-3' and 5'-GAACCCAGGTCCTCGCTT-3', expected PCR product size, 293 bp), mouse COX-3 (5'-ATGAGTCGTGAGTCCGACCCCAGT-3' and 5'-TGTCGAGGCCAAAGCGGA-3', expected PCR product size, 290 bp). The samples were first denatured at 95 ℃ for 5 min, then it followed by 30 PCR cycles, the temperature profile was 95 ℃ for 30 sec, 60 ℃ for 30 sec, and 72 ℃ for 1 min. After the last cycle, an additional extension incubation of 7 min at 72 ℃ was performed. After amplification, PCR products (5 μl of each sample) were subjected to size separation by agarose gel (1%, Sigma) containing ethidium bromide. The bands were visualized by UV fluorescence. Densitometric analysis was performed by alpha imager. The percentage of IDV of COX mRNA to that of GAPDH mRNA (IDV%) is calculated.

1.5 Radioimmunological assay Animals were sacrificed at different time points. Brains were picked out and homogenated. Level of 6-keto-PGF$_{1\alpha}$ and PGE$_2$ in the supernatant were measured with RIA kit (Chemclin Biotech, Beijing, China) according to the manufacturer's instructions.

1.6 Group All animals were randomly divided into 5 groups: control group, NS group, SC group and IN group which received vehicle, NS-398, SC-560 and indomathacin, respectively at intervals of 1 d intragastrically. The NS+SC group received NS-398 during the first month and SC-560 during the second month. Doses of NS-398, SC-560 and indomethacin were 150, 300 and 300 $\mu g \cdot kg^{-1}$, respectively.

1.7 Behavior assessment Hot plate test were used for behavior assessment at different time points. For hot plate test, a metal hot plate was heated to a constant temperature. Behavioral measurements were taken at 55 ± 0.5 ℃. The temperature of the plate was monitored at all times. To confine the animals at a certain observation area, a colorless acrylic cylinder of 20 cm diameter was

placed on the hot plate. After each measurement, the plate was wiped with a damp cloth to remove traces of urine and faeces. Latency for the animal to lick its hind-paw was measured before injection and 1 h, 12 h, 1 d, 3 d, 7 d, 14 d, 30 d, 60 d after formalin injection.

1.8 Statistical analysis Data were presented as $\bar{x} \pm s$. Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by LSD multiple comparison test. $P < 0.05$ between the experimental groups were considered statistically significant.

2 RESULTS

2.1 RT-PCR The level of brain COX-1 mRNA expression was higher at the late phase after inflammation, while the level of COX-2 mRNA was higher at the early phase (Fig 1). IDV percents of COX-1 reached peak at the time point of 14 d to 60 d (Fig 2). IDV percents of COX-2 reached peak value at the time point of 1 d to 3 d and gradually decreased subsequently (Fig 3). The expression of brain COX-3 mRNA is comparatively lower than that of COX-1 and COX-2. Additionally, the level of COX-3 mRNA did not show significant change during the period of 60 d (Fig 4).

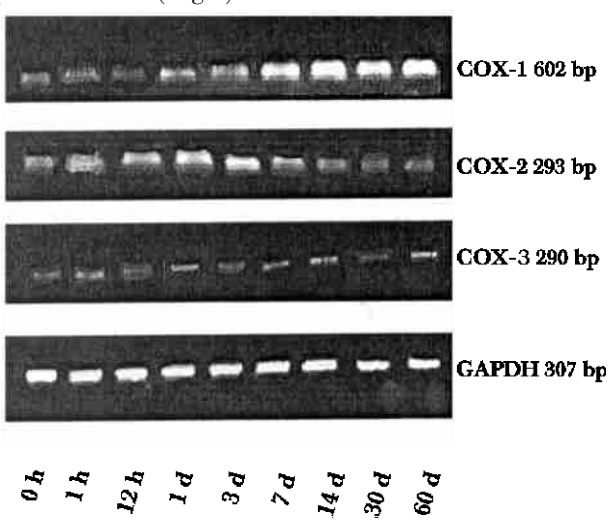

Fig 1 Electrophoresis assay of product of COX-1, COX-2, COX-3 and GAPDH RT-PCR

2.2 Radioimmunological assay The level of $PGF_{1\alpha}$ in brain tissue did not show significant change until 1 m. Overall, change of $PGF_{1\alpha}$ concentration during the process of inflammatory pain was moderate (Fig 5). Oppositely, change of PGE_2 after inflammatory stimulation is rapid and potent. Peak of PGE_2 concentration appeared at the time point of 1 d (Fig 6).

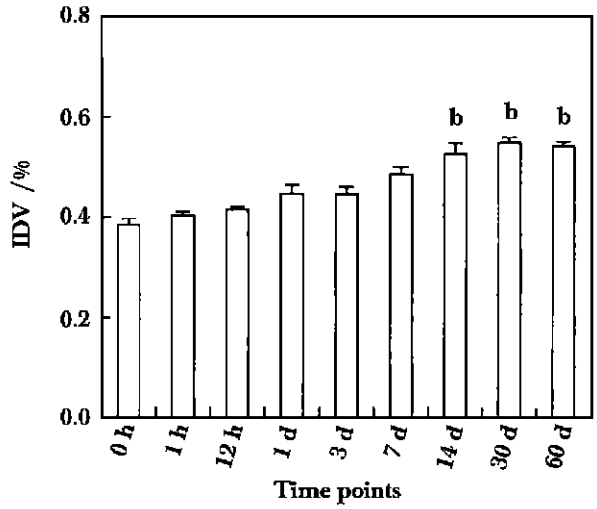

Fig 2 IDV percents of COX-1 mRNA at different time points after inflammatory stimulation ($\bar{x} \pm s$, $n = 6$)
Compared with the before injection time point, $^bP < 0.05$

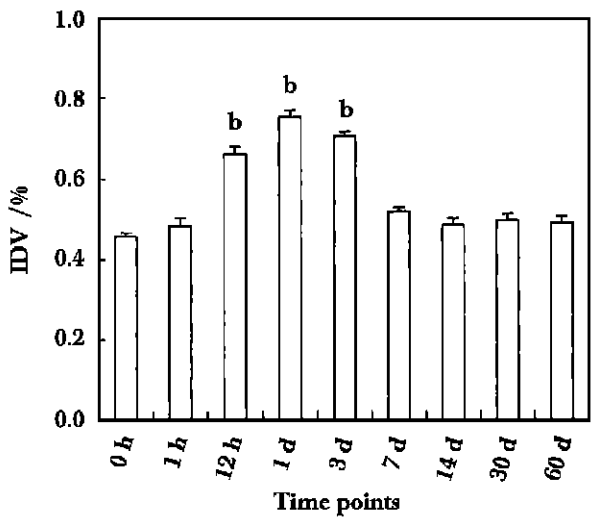

Fig 3 IDV percents of COX-2 mRNA at different time points after inflammatory stimulation ($\bar{x} \pm s$, $n = 6$)
Compared with the before injection time point, $^bP < 0.05$

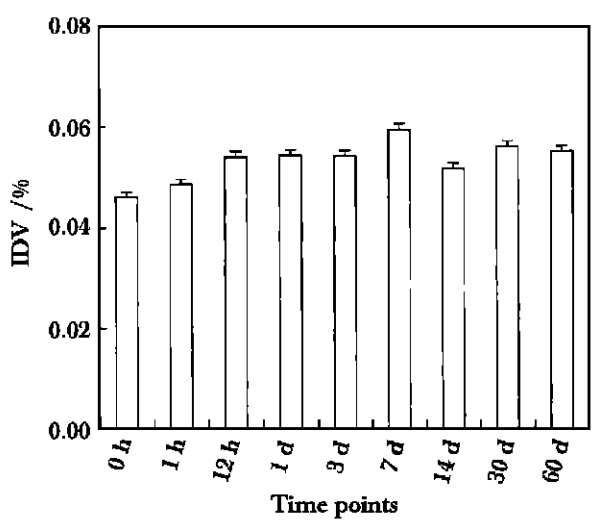

Fig 4 IDV percents of COX-3 mRNA at different time points after inflammatory stimulation ($\bar{x} \pm s$, $n = 6$)

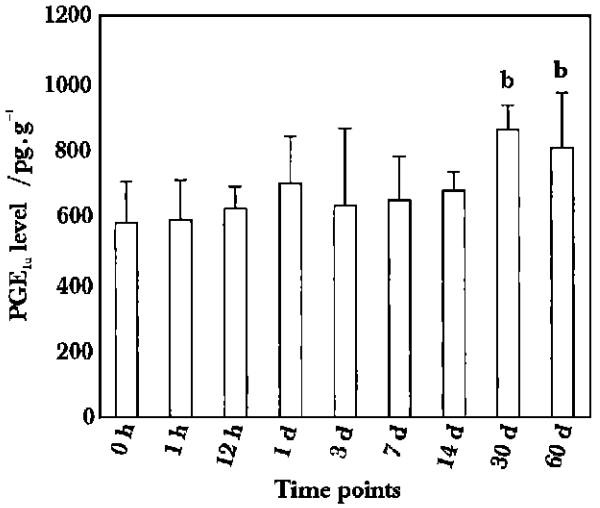

Fig 5 Concentration of $PGF_{1\alpha}$ at different time points after inflammatory stimulation($\bar{x}\pm s$, $n=6$)
Compared with the before injection time point, $^bP<0.05$

2.3 Behavioral assessment After formalin injection, all animals showed significant shorten of the reaction time. Compared with the control group, NS+SC group showed significant improvement of hyperalgesia. Moreover, the degree of improvement was gradually increased. Animals in the NS group got significant increase of reaction time at the early time and the extent increased gradually until 7 d. Oppositively, animals in the SC group did not show significant potent effect on reaction time. Effect observed in the IN group was not as potent as that in the NS+SC group, but it stronger than that in the SC group (Fig 7).

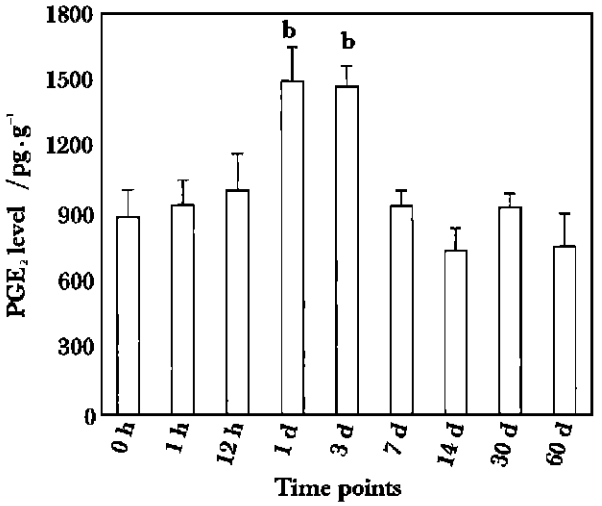

Fig 6 Concentration of PGE_2 at different time points after inflammatory stimulation($\bar{x}\pm s$, $n=6$)
Compared with the before injection time point, $^bP<0.05$

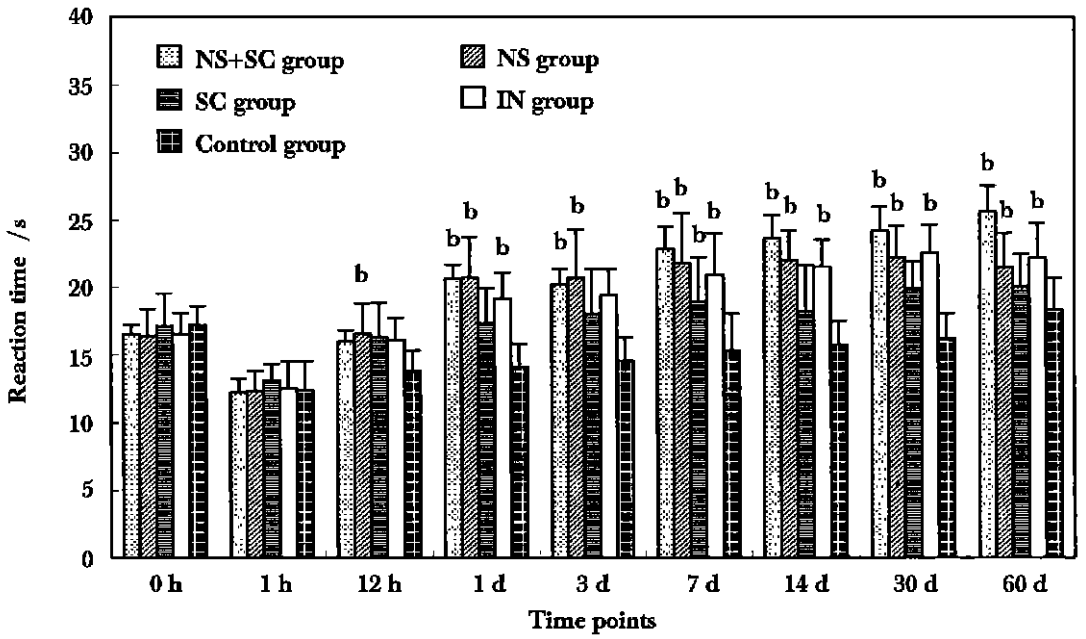

Fig 7 Analgesic effects of different COX inhibitors application on inflammatory pain($\bar{x}\pm s$, $n=6$)
Compared with the before injection time point, $^bP<0.05$

3 DISCUSSION

Over thirty years ago, the mechanisms of action of aspirin-like drugs or non-steroidal anti-inflammatory drugs (NSAIDs) was proposed through their inhibition of prostaglandin biosynthesis via the enzyme COX. 20 years after the initial discovery, it was discovered that there are at least two COX isoforms, COX-1 and COX-2. Almost all available non-specific NSAIDs block both COX isoforms, which can decrease the amounts of prostaglandins formed by COX-1 and COX-2. Furthermore, recent researches suggest that the possibility of a third COX isoform with the

cognomen of "COX-3"[2].

New classes of selective COX-2 inhibiting medications have entered the worldwide market based on our increased understanding of COX inhibition. As well as benefiting for the arthritic patients, these specific inhibitors of COX-2 may demonstrate new therapeutic potential, slowing down tumor growth[3,4], delaying the birth process[5], and impeding the degenerative changes associated with Alzheimer's disease[6,7].

Traditionally, the use of COX inhibitors in treatment of patients with inflammatory pain was apt to COX-2 selective inhibitors. A number of COX-2 selective inhibitors have been developed. However, with the development of researches, the role of COX-1 and COX-2 was heralded to be more complex. Though COX-2 was considered to be an important inducer in the inflammatory process, recently it was also considered to be an important role for inflammatory recovery. As for inflammatory pain, it was proposed that both COX-1 and COX-2 were involved in the pain process. In this study, the change of both COX-1 and COX-2 was observed. Both direct and indirect methods were used and both them showed the same results. Compared with COX-1, the level of COX-2 in the brain was higher during the whole process. Furthermore, COX-1 was found to be higher at the late phase of inflammatory pain, while COX-2 was found to be higher at the early phase. This may lead to the indication that the former isoform was involved in the sustenance of inflammatory pain but the latter one was involved in the pain origination. So dealing with COX-1 or COX-2 singly may not be enough for the pain treatment. Meanwhile, COX inhibitors of low selectivity lead to more side effects. So the optimal protocol of using COX inhibitors in treatment of patients of inflammatory pain maybe using different COX selective inhibitors at different time. Based on this suggestion, the second part of this study was designed. In this part, animals were divided into five groups to compare the effects of different protocols on inflammatory pain. Results showed that protocol of best effect was the combined use of both COX-1 and COX-2 selective inhibitors. Detailedly, COX-1 selective inhibitor was used at the late phase and COX-2 selective inhibitor was used at the early phase. Single use of COX-1 inhibitor showed the least effect. At the early phase, non-selective inhibitor showed less effects than COX-2 selective inhibitor. But at the endpoint of 2 mon, effects of the former was found to be better. So inhibition of COX-2 at the late phase was not beneficial for inflammation recovery.

Unlike COX-1 and COX-2, COX-3 did not show significant change after formalin injection. COX-3 is a splice variant of COX-1. The structure of COX-3 mRNA retains intron-1 and a signal peptide compared with that of COX-1. The retention of intron-1 could alter the active site of enzyme. As a result, there are some differences among the three isoforms. COX-3 was proved to be a promising target for pain control[2,8,9]. However, our study indicated that inflammatory stimulation could not lead to a significant change of brain COX-3 expression. That means the involvement of brain COX-3 in the inflammatory process could be poor. There is still no investigation on the expression of peripheral COX-3. As peripheral mechanism is important for the development of inflammatory pain, poor involvement of COX-3 in the process can not lead to the conclusion that COX-3 is less inflammatory. Further studies are important to get more evidences.

The findings of this study show that among the three isoforms, the expression of brain COX-1 was higher at the late phase while the expression of COX-2 was higher at the early phase of inflammatory pain. Moreover, the expression of brain COX-3 did not significantly change in the process of inflammatory pain. To our knowledge, this is the first time that COX-3 expression is evaluated in the inflammatory process. Additionally, pharmacological analysis shows that using COX-2 inhibitors at the early phase subsequently with COX-1 inhibitors at the late phase could get better analgesic effect on inflammatory pain compared with simply using COX-1 or COX-2 selective inhibitors.

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福尔马林所致炎性痛后脑内三种 COX 亚型的变化及不同选择性 COX 抑制剂的镇痛效应比较

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摘要　目的: 比较炎性痛后三种环氧合酶(cyclooxygenase, COX)亚型的表达变化, 以及选择性 COX 抑制剂不同应用方式对炎性痛的镇痛效应。方法: 小鼠足底注射福尔马林诱导炎性痛。用放射免疫分析及 RT-PCR 分别评估脑 COX-1、COX-2 及 COX-3 在福尔马林注射前、注射后 1、12 h、1、3、7、14、30、60 d 的变化。在镇痛效应的比较中, 动物被分成 5 组: 对照组、SC 组、NS 组、IN 组及 NS＋SC 组。前 4 组分别灌胃生理盐水、SC-560、NS-398 和 indomethacin。NS＋SC 组在前一个月接受 NS-398, 后一个月接受 SC-560。测定各组动物在福尔马林注射前、注射后 1、12 h、1、3、7、14、30、60 d 的热痛阈。结果: COX-2 的表达在炎性痛后 12 h 到 3 d 升高显著, 而 COX-1 的表达在 2 周到 2 月升高显著。在整个观察时限内 COX-3 的表达无明显变化。与其他组相比, NS＋SC 组动物的热痛阈在整个炎性痛过程中均明显提高。结论: 炎性痛后早期 COX-2 升高而晚期 COX-1 升高。COX-3 变化不明显。COX-1 抑制剂和 COX-2 抑制剂的结合使用比单纯使用其中一种能取得更好的镇痛效果。

关键词　炎性痛; 环氧合酶; 环氧合酶抑制剂; 放射免疫分析; RT-PCR; 热板试验; 小鼠