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Original Article

Aitongxiao improves pain symptoms of rats with cancer pain by reducing IL-1, TNF-α, and PGE2

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Abstract: Objective: To explore the mechanism of Aitongxiao in improving pain symptoms of rats with cancer pain. Methods: Walker 256 breast cancer cells were injected into the right tibial bone marrow cavity of normal female rats to establish a rat model of tibial cancer pain. The rats with successful model replication were randomly divided into normal group (NG), Hank solution group (HSG), cancer pain model group (CPMG), and Aitongxiao+cancer pain model group (ATX+CPMG). The pain response score, mechanical pain hindpaw withdrawal threshold, and latent heat pain of rats were evaluated, and the changes of serum IL-1β, TNF-α, PGE2 and blood cell counts of rats were detected. Results: Compared with the NG, the pain response score was increased, the mechanical pain hindpaw withdrawal threshold and latent heat pain were decreased, and IL-1β, TNF-α, and PGE2 were increased in CPMG. Compared with the CPMG, the pain response score was decreased, the mechanical pain hindpaw withdrawal threshold and latent heat pain were increased, and IL-1β, TNF-α, and PGE2 were decreased in ATX+CPMG. There was no significant change in blood cell count in each group. Conclusion: Aitongxiao can improve the pain symptoms of rats with tibial cancer pain. Its mechanism may be related to the reduction of IL-1β, TNF-α, and PGE2 levels.

Keywords: Aitongxiao, IL-1β, TNF-α, PGE2

Introduction

Pain originates from the protective mechanism of nociceptors on organisms [1]. Pain is divided into acute pain and chronic pain. Pain caused by rheumatoid arthritis, knee arthritis and cancer pain can be regarded as chronic pain. Cancer pain is a common symptom of cancer patients. Since pain is usually accompanied by an increase in inflammation, inflammatory reactions may play an important role in the progression of chronic pain [2-5]. Inflammatory mediators secreted by immune cells mediate the activity of nociceptors and pain sensitivity [1]. Tumor necrosis factor a (TNF-α) is an inflammatory mediator that not only mediates inflammatory progression, but also participates in the regulation of neuropathic pain. Some studies [6] have shown that TNF-α regulates the sensitivity of nociceptive neurons through potassium, sodium and calcium ion channels, thus possibly participating in the pain pathway. Alexander et al [7] found that interleukin-1β (IL-1β) not only induces inflammatory reaction, but also generates action potential by activating nociceptors. In addition, interleukin-17 can also promote neuropathic pain by inducing astrocyte proliferation and cytokines [8]. Besides cytokines, lipid medium prostaglandin E2 (PGE2) also plays a role in chronic neuropathic pain. Ma et al [9] believed that PGE2 can promote the release of nociceptive transmitters such as peptide substance P and CGRP. PGE2 can also inhibit neuronal activity through PGE2 receptor 4 on neuronal receptors [10].

Aitongxiao is a Chinese patent medicine, which may participate in regulating the proliferation and apoptosis of cancer cells. Wang et al [11] concluded that Aitongxiao inhibits proliferation of liver cancer cells and induces apoptosis by down-regulating Bcl-2 and survivin. Huang et al

Table 1. Pain response score of rats

Score	Behavioral performance
0	The foot soles of the hind legs were flat on the ground and there was no abnormal movement.
1	Both feet touched the ground, but the injection side was slightly spavined when walking.
2	The foot sole of the injection side touched the ground slightly and the center of body weight was obviously spavined when moving on the healthy side.
3	The foot sole of the injection side touched the ground slightly but did not dare to lift when walking with a heavy load.
4	The foot sole of the injection side was lifted without touching the ground.
5	Rats licked and bit or shook the foot sole of injection side.

[12] have suggested that Aitongxiao may induce apoptosis of hepatoma cells, block cell cycle and prevent exosomes release by inhibiting STAT3. Aitongxiao also has a significant effect on analgesia. Cong et al [13] concluded that Aitongxiao may alleviate the pain degree of patients with bone cancer pain clinically through cytokines such as IL-1β, IL-10 and TNF-α and interfere with constipation and nausea caused by morphine in the treatment of cancer pain.

Cancer pain is one of the links that cannot be ignored in the treatment of cancer patients, but there is no optimal analgesic strategy for pain relief. At the same time, there is still little research on whether Aitongxiao exerts analgesic effect through inflammatory mediators or cytokines. In this paper, the rat model of tibial cancer pain was established by subcutaneous injection of Walker 256 breast cancer cells into the right tibial bone marrow cavity to study whether Aitongxiao can improve the degree of rat cancer pain through IL-1α, TNF-α, and PGE2, so as to provide reliable basic experimental basis for clinical application and treatment of Aitongxiao.

Materials and methods

Rat model of tibial cancer pain

A total of 24 male SD rats (200-220 g) were selected. The rats were randomly divided into NG, HSG, CPMG and ATX+CPMG after being determined to have no obvious abnormal behavior, with 6 rats in each group. The study was approved by the hospital ethics committee. The operation process was strictly in accordance with the guidelines for care and use of experimental animals (published by NIH, revised in 1996, No. 85-23). The rats were fasted overnight and allowed to drink water freely before operation. After anesthesia with 2% sevoflurane, the tracheal intubation was used for ventilation, and the sevoflurane (1.5%~2%) was continuously introduced. In CPMG and ATX+CPMG, the rats lay on their backs on the operating table after anesthesia, and the left hind limb was removed to and carefully expose the tibia. Walker 256 cell was injected into the right tibia to establish a chronic tibial cancer pain model. After the injection, bone wax was used to seal the wound and antibiotic anti-infection treatment was performed. In ATX+CPMG, the Aitongxiao was externally applied to the rat's back, once a day for 7 days. In HSG, the rats were injected the same amount of Hank solution into the same part. In NG, the rats did not receive any treatment.

Pain assessment

After operation for 1 min, 30 min, 60 min and 24 h, the pain response, latent heat pain, and mechanical pain hindpaw withdrawal threshold of rats were observed respectively in each group. The pain response scores are shown in **Table 1** (refer to the studies by Shinoda et al [14]).

Time of latent heat pain

The plantar thermal tolerance of the rat's injured side limb was collected by thermal pain tester. The time taken by the rat from the start of irradiation to the lifting of the hind paw was called the time of latent heat pain, and the test was conducted once every 5 min, with a total of 3 times. For determination of mechanical pain hindpaw withdrawal threshold, a dynamic acupuncture pain measuring instrument was used to measure the minimum pressure value causing a withdrawal reaction of rats. The pressure value was called the mechanical pain hindpaw withdrawal threshold, and the test was conducted once every 5 min for a total of 3 times.

Table 2. Pain response score (Mean ± SEM, n=6)

	1 min	30 min	60 min	24 h	*F*	*P*
NG	0	0	0	0		
HSG	0.74±0.22	0.53±0.16	0	0		
CPMG	4.63±0.69	4.16±0.61	4.05±0.54	3.87±0.57	1.73	0.1940
ATX+model group*	4.56±0.59	2.62±0.12	1.83±0.48	1.39±0.52	54.66	<0.0001
t	11.52	13.25	14.67	11.28		
P	<0.0001	<0.0001	<0.0001	<0.0001		

*Note: ATX, Altongxiao.

Enzyme-linked immunosorbent assay

After 7 days of the experiment, the blood (1 mL) was drawn from rat abdominal aorta. The sample was placed in an anticoagulant tube, centrifuged at 3×10^3 g at low temperature for 30 min, and the supernatant was collected. The levels of IL-1α, TNF-α and PGE2 in the supernatant were detected by corresponding enzyme-linked immunosorbent assay kits (Abcam). All tests were strictly carried out according to the kit instructions.

Blood cell count

After 7 days of the experiment, the blood was drawn from the abdominal aorta of rats, and the red blood cell count, leukocyte count, platelet count and hemoglobin were counted by multiparameter blood cell analyzer.

Statistics and analysis

SPSS20.0 (IBM) was used for statistical comparison of data. GraphPad Prism 8.0 was used for data mapping. Excel analysis tools was used to simulate standard curves. The measurement data were expressed by Mean ± SEM. The NG, HSG, CPMG and ATX+CPMG were tested by K-S and conformed to normal distribution, and one-way analysis of variance was used. Repeated analysis of variance was used to compare the differences in different periods in the group. The confidence interval was 95%. The difference was statistically significant if $P<0.05$.

Results

Aitongxiao relieved the degree of pain in rats

In this study, the pain response score was used as the basis for evaluating rats with tibial cancer pain treated by Aitongxiao. If the pain response score was decreased statistically, the pain state of rats was improved. **Table 2** showed the pain response scores of each group in different periods. Compared with the NG in the same period, the pain response score increased in CPMG. Compared with the simultaneous CPMG, the pain response score of the ATX+CPMG was significantly reduced in the 30 min-24 h period after operation. Compared with that after operation for 1 min, the pain response score of the ATX+CPMG was significantly reduced within 30 min to 24 h. The above results indicated that Aitongxiao reduced the degree of pain in rats.

Table 3 showed that after operation for 1 min, the CPMG and ATX+CPMG had significantly lower latent heat pain and obvious heat sensitivity compared with the NG. Within 1 min-24 h after operation, there was no statistical difference in latent heat pain between NG and HSG in different periods. Compared with that after operation for 1 min, there was no obvious change in the latent heat pain in CPMG at the 30 min-24 h, and the ltime of atent heat pain increased to 15.62±2.16 s in ATX+CPMG at the 30 min-24 h. After operation for 30 min-24 h, the time of latent heat pain in ATX+CPMG increased statistically compared with the simultaneous CPMG.

Table 4 showed that after operation for 1 min, the CPMG and ATX+CPMG had significantly lower mechanical pain hindpaw withdrawal threshold compared with the NG. Within 1 min-24 h after operation, there was no statistical difference in mechanical pain hindpaw withdrawal threshold between NG and HSG in different periods. Compared with 1 min after operation, there was no obvious change in the mechanical pain hindpaw withdrawal threshold in CPMG at the 30 min-24 h, and the mechanical pain hindpaw withdrawal threshold incre-

Table 3. Time of latent heat pain (s, Mean ± SEM, n=6)

	1 min	30 min	60 min	24 h	F	*P*
NG	18.26±2.36	17.25±2.12	18.19±2.02	18.64±2.31	0.43	0.7338
HSG	17.89±2.11	17.95±2.03	18.65±2.74	18.36±2.18	0.15	0.9296
CPMG	11.63±1.89	12.35±1.97	12.95±2.16	13.47±2.57	0.81	0.5061
ATX+model group*	11.56±2.07	13.69±2.14	14.89±2.32	15.62±2.16	3.99	0.0221
F	18.82	10.36	8.22	6.74		
P	<0.0001	<0.0001	<0.0001	0.0025		

*Note: ATX, Altongxiao.

Table 4. Mechanical pain hindpaw withdrawal threshold (g, Mean ± SEM, n=6)

	1 min	30 min	60 min	24 h	F	*P*
NG	37.63±3.25	36.11±3.98	35.87±3.17	33.98±3.65	0.65	0.3798
HSG	37.83±3.49	36.94±3.62	36.12±3.29	35.74±3.94	0.41	0.7775
CPMG	14.69±3.26	15.25±3.12	16.22±3.08	16.68±3.95	0.43	0.7325
ATX+model group*	14.51±3.59	17.68±3.22	19.57±3.25	22.39±3.74	5.50	0.0064
F	92.53	66.17	65.15	34.60		
P	<0.0001	<0.0001	<0.0001	<0.0001		

*Note: ATX, Altongxiao.

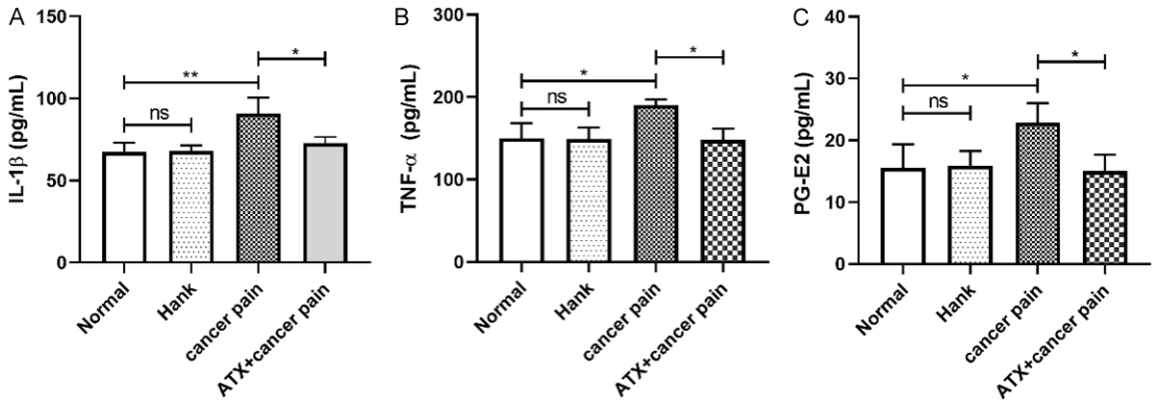

Figure 1. Aitongxiao down-regulated serum IL-1β, serum TNF-α and serum PGE2. A: Levels of serum IL-1β in each group. B: Levels of serum TNF-α in each group. C: Levels of serum PGE2 in each group. ns, P>0.05, *P<0.05, **P<0.01.

ased to 23.39±3.74 g in ATX+CPMG at the 30 min-24 h. After operation for 30 min-24 h, the mechanical pain hindpaw withdrawal threshold in ATX+CPMG increased statistically compared with the simultaneous CPMG.

Aitongxiao down-regulated serum IL-1β, TNF-α, and PGE2 in rats with cancer pain

After 7 days of the experiment, the blood was drawn from the abdominal aorta of rats, and the levels of IL-1β, TNF-α, and PGE2 in the supernatant were detected by corresponding kits (Abcam). **Figure 1** showed that compared with the NG, there was no significant difference in IL-1β, TNF-α, and PGE2 in HSG, while IL-1β, TNF-α, and PGE2 in CpMG were significantly increased. Compared with the CPMG, IL-1β, TNF-α and PGE2-2 were significantly reduced in ATX+CPMG. The above results showed that Aitongxiao could down-regulate serum IL-1β, TNF-α and PGE2 in rats with cancer pain.

Aitongxiao did not affect blood cell count

After 7 days of the experiment, the blood was drawn from the abdominal aorta of rats, and the red blood cell count, leukocyte count, plate-

Table 5. Blood cell count (Mean ± SEM, n=6)

	RBC* (10^{12} cells/L)	WBC* (10^9 cells/L)	PLT* (10^9 cells/L)	Hb* (g/L)
NG	7.13±0.86	4.05±0.71	981.25±65.32	149.87±13.42
HSG	7.54±0.78	3.79±0.64	899.67±71.36	157.78±14.84
CPMG	8.16±0.74	4.62±0.73	946.23±67.41	155.39±17.21
ATX+model group*	7.76±0.89	4.13±0.61	916.27±74.59	143.42±15.36
F	1.65	1.59	1.59	1.05
P	0.2095	0.2238	0.2239	0.3920

*Note: RBC, red blood cell count; WBC, leukocyte count; PLT, platelet count; Hb, hemoglobin.

let count and hemoglobin were counted by multiparameter blood cell analyzer. There was no statistical difference in red blood cell count, leukocyte count, platelet count, and hemoglobin level among the groups (**Table 5**). The above results showed that Aitongxiao had no obvious effect on blood cells.

Discussion

Pain is a common clinical feature of patients with advanced cancer, so it is very necessary how to relieve the pain degree of cancer patients [15, 16]. Cancer not only causes pain, but also seriously affects the quality of life and recovery of patients. Inflammatory mediators and cytokines are closely related to chronic cancer pain. Inflammation may mediate the generation and transmission of cancer pain by affecting the normal metabolism of neurons. Therefore, this study was designed to evaluate the analgesic mechanism of Aitongxiao by inflammatory mediators and cytokines.

Chronic pain is accompanied by an increase in PGE2 and inflammatory mediators and induces the development of pain and hyperalgesia [5, 9, 17]. The results revealed that the rats showed obvious pain symptoms (increased pain sensitivity) after injection with Walker 256 breast cancer cells, accompanied by up-regulation of IL-1β, TNF-α, and PGE2. Nociceptors have many receptors on their terminals. When normal cells or tissues are damaged, immune cells secrete cytokines and lipid media to improve cell damage. Cytokines bind to receptor on surface, causing intracellular signal transmission of receptor, thus affecting receptor pain sensitivity or ion channel [1]. Therefore, tibial pain caused by cancer cells is accompanied by up-regulation of IL-1β, TNF-α, and PGE2.

After Aitongxiao treatment in rats with cancer pain, it was found that the pain score of rats was decreased, the time of latent heat pain was shortened, the threshold of mechanical pain was increased, and IL-1β, TNF-α, and PGE2 were decreased after Aitongxiao treatment for 30 min. Altongxiao uses Paniculate Swallowwort, Gecko, Olibanum, Myrrh, Eupolyphaga Seu Steleophaga, Scolopendra, Scorpio, Realgar, Toad Venom, Artificial Musk and Borneol as raw materials, and it has certain analgesic and tumor inhibiting effects. Borneol may inhibit neuronal inflammation and TRP channels through NF-κB pathway [18, 19]. Toad venom can regulate the central nervous system through a sodium/potassium pump [20]. Olibanum and myrrh may inhibit the degree of pain in neuropathic mice through the TRPV1 channel [21]. Musk combined with Borneol exerts neuron protection [22]. Realgar can inhibit the activity of dopamine neurons and microglia [23]. The above research has shown that the raw materials of Aitongxiao can improve neuronal inflammation and regulate neuronal channels. Therefore, the rats with chronic pain showed a down-regulation of IL-1β, TNF-α, and PGE2 and pain relief after being treated with Aitongxiao. There was no significant change in the blood cell count, indicating that Aitongxiao had no significant effect on blood cells. Blood cell counts (such as red blood cell count, leukocyte count, platelet count, and hemoglobin) are associated with cancer, and abnormal blood counts in vivo indicate the possibility of cancer. Therefore, this study was designed to detect the blood cell count during the tumor experiment in vivo. Since Aitongxiao seemed to have no significant effect on blood cell count, this suggested that Aitongxiao may have no effect on blood cells.

The rat model of tibial cancer pain was established using Walker 256 breast cancer cells and treated with Aitongxiao. After comparing the serum inflammatory factors, blood cell

count, and degree of pain of rats in different groups, it was found that Aitongxiao significantly reduced serum IL-1β, TNF-α, and PGE2 related to pain, and reduced pain and pain sensitivity in rats. However, there are limitations to the study. The mechanism by which Aitongxiao improves pain through IL-1β, TNF-α, and PGE2 remains unclear. Previous studies have shown that inflammatory factors and PGE2 are involved in regulating the potential changes and neurotransmitters of nociceptors, so the relationship between Aitongxiao and nociceptors will be discussed in future studies. Pain is divided into chronic pain and acute pain. The mechanism by which Aitongxiao participates in acute pain also needs to be further studied.

To sum up, we conclude that Aitongxiao can improve the degree of pain in rats by lowering serum IL-1β, TNF-α, and PGE2 through a chronic pain model in this study. Therefore, Aitongxiao may reduce the activity of nociceptors by inhibiting the excessive secretion of inflammatory mediators and cytokines, thus reducing the release of nociceptive neurotransmitters from afferent nerves.

Disclosure of conflict of interest

None.

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