Therapeutic effects of traditional Chinese medicine formula Qianlie Tongli decoction on chronic prostatitis/chronic pelvic pain syndrome induced by peptide T2 in mice

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Abstract

Objectives: This study was undertaken to reveal therapeutic effects and the preliminary

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mechanism of Chinese medicine formula Qianlie Tongli decoction (QTD) in chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS).

Methods: A total of 50 male C57BL/6 mice were randomly divided into five groups. All groups except the control group were injected subcutaneously T2 peptide emulsion, which induced the CP/CPPS model. After the induction of CP/CPPS, the model group was given 0.9% NaCl by oral gavage while low-dose, medium-dose, and high-dose groups were treated with Chinese medicine formula. Micturition habits and pain behavior of mice were analyzed for each group. Hematoxylin and eosin staining were used to investigate prostate inflammation. The serum level of tumor necrosis factor-α (TNF-α) was measured by enzyme-linked immunosorbent assay (ELISA) kit.

Key findings: Chinese medicine formula significantly reduced the number of urine spots and improved pain response frequency in the medium-dose and high-dose group. The high-dose group showed reduced considerably inflammatory lesion and inflammatory cell infiltration than the low-dose and medium-dose groups. Serum levels of TNF-α in the high-dose group were significantly reduced compared with the model group.

Conclusions: The results demonstrated the therapeutic effects of Qianlie Tongli decoction in CP/CPPS mice by analyzing clinically relevant symptoms (urinary tract system, pelvic pain, and prostate inflammation), and preliminary explored the inflammatory-related treatment mechanisms by measuring TNF-α.

Keywords: Qianlie Tongli decoction; Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS); Anti-inflammation; Therapeutic effects; Immunization.

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1. Introduction

According to the National Institutes of Health (NIH), prostatitis has acute bacterial prostatitis (category I), chronic bacterial prostatitis (category II), chronic prostatitis/chronic pelvic pain syndrome (category III) and asymptomatic inflammatory prostatitis (category IV) [1]. NIH Category III Prostatitis is a highly extensive disease that affects men with a wide age range and severely affects the quality of life (QoL) [2]. Besides, population-based surveys have shown that CP/CPPS is as severe as Crohn's disease, diabetes, and heart failure [3]. As the most prevalent type of prostatitis, almost 90% to 95% of men have CP/CPPS with symptoms of chronic prostatitis [4]. CP/CPPS is mostly described by long-term, repeated pelvic pain or discomfort, lasting more than 3 months, with sexual dysfunction and variable urinary symptoms [5].

Presently, etiology and pathophysiology are not well-known. Many scholars believe that it might be related to pathogen infection, mental and psychological factors, neuroendocrine, immune function, and oxidative stress [6, 7]. Autoimmunity is a crucial factor in CP/CPPS, previous studies have shown that autoimmune prostatitis exists in human males, and immune disturbance leads to the loss of self-tolerance to prostatic antigens [8, 9].

Currently, there are no established treatments to alleviate symptoms for CP/CPPS. The therapeutic interventions for CP/CPPS mainly include alpha-blockers, antibiotics, pain medications, and multimodal therapy [10-12]. Weak evidence supports the use of α-blockers, pain medications, and a four to six weeks course of antibiotics for the treatment of CP/CPPS [13]. In China, for the treatment of numerous diseases, herbal has been widely used. Some studies provided evidence that herbal supplements could be effective in CP/CPPS [14].

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Herbal can improve the clinical symptoms, reverse some pathological changes, and restore the body's normal physiological function. For the treatment of CP/CPPS, there are several approaches in herbal, including alleviating pain and dispersing liver-qi, removing dampness, and clearing heat, and activating blood to dissolve stasis. Pro-inflammatory cytokines expedite CP/CPPS development, while anti-inflammatory cytokines alleviate the disease [15]. Numerous researchers have shown that herbal protects against cytokine production in atherosclerosis as the potential immunosuppressive agents [16]. Such diverse and flexible therapies in Chinese medicine uncover the advantages of herbal treatment for CP/CPPS.

Qianlie Tongli decoction (QTD) is a Chinese medicine formula consisting of *Hedyotis diffusa,* *Epimedium brevicornu* Maxim*, Fritillaria thunbergii,* Cortex Phellodendri Chinensis (CPC)*,* and Earthworm (*Lumbricus terrestris*). These Chinese medicinal materials can be used individually or in combination. QTD has been used widely in the clinical setup in China for the treatment of various inflammatory conditions. Previous studies have revealed the anti-inflammatory effect of *Hedyotis diffusa, Epimedium brevicornu* Maxim*, Fritillaria thunbergii,* CPC*,* and Earthworm (*Lumbricus terrestris*) individually [17-21]. In this experiment, QTD takes "Bushen Tongluo" as the treatment principle, and *Epimedium* is the primary medicine to nourish the kidney and remove dampness in this formula. *Hedyotis diffusa* and *Fritillaria thunbergii* are the medicines for clearing heat and detoxifying. The adjuvant medicine-Earthworm can promote blood circulation and facilitate the effects of other medications. CPC and *Fritillaria thunbergii* used to disperse blood stasis and achieve the anti-inflammatory effect. Previously for the first time, we have evaluated the effects of QTD formula in an autoimmune prostatitis rat model [22]. In this study, we investigated the

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89 therapeutic effects of Chinese medicine formula QTD for CP/CPPS mice and explored the
90 preliminary mechanism.

91 **2. Method and materials**

92 The main reagent used in this experiment was T2 peptide with the sequence of CSEEM
93 RHRFR QLDTK LNDLKG amino acid from Transient receptor potential cation channel
94 subfamily M member 8 (TRPM8), it is an epitope of antigenic nature inducing experimental
95 autoimmune prostatitis (EAP) was purified and synthesized by Wuhan Buyers Biotechnology
96 Co., Ltd., China. Complete Freund's adjuvant (CFA) was obtained from the Sigma-Aldrich
97 Chemical Co. (St. Louis, MO, USA). Chinese medicine compounds included *Hedyotis diffusa*,
98 *Epimedium brevicornu* Maxim, *Fritillaria thunbergii*, CPC, and Earthworm (*Lumbricus*
99 *terrestris*), which were purchased from Nanjing Crane Age Pharmaceutical Service Co., Ltd.,
100 China (Batch #: Su Cai Zhunyin (2017) No. 019-022). The four voucher specimens were
101 preserved at the herbarium of China Pharmaceutical University, and the materials for formula
102 were authenticated by Prof. Dr. Zhang Chunfeng, College of Chinese Medicine, China
103 Pharmaceutical University [*Fritillaria thunbergii* (Liliaceae), CPU180910-1; *Epimedium*
104 *brevicornu* Maxim (Berberidaceae), CPU180910-2; *Hedyotis diffusa* (Rubiaceae),
105 CPU180910-3; Cortex Phellodendri Chinensis (Rutaceae), CPU180910-4; Earthworm
106 (*Lumbricus terrestris*), CPU180910-5].

107 ***2.1. Ethical Statement***

108 All procedures and experiments used in this study comply with guidelines of the local
109 institutional ethical committee of the China Pharmaceutical University (Jiangsu Province,
110 Nanjing, China) regarding care and welfare of animals under study (T/CPU, 235-2018,

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111 2018-09-09).

112 ***2.2. Animal model***

113 Fifty adult male 6-8 weeks old C57BL/6 mice weighing 18~22g were obtained from
114 Qinglong Mountain Animal Breeding Ground (Nanjing, China). These mice were kept and
115 fed under standard temperature and relative humidity with a 12-h light/dark cycle in an
116 animal room. The ethical committee approved animal handling and experimental procedures
117 of China Pharmaceutical University. These mice were randomized into five groups (n=10),
118 including the control, model, low-dose, medium-dose, and high-dose group.

119 ***2.3. Immunization***

120 T2 peptide was dissolved to 1mg/ml of reserves for a combination of an equal concentration
121 of CFA before immunization [23]. Except for the control group, the other groups were all
122 given a multipoint subcutaneous injection on days 0 and 14. Every time, mice were injected
123 with 200μl of a mixture subcutaneously with five injections separately in the back. For the
124 mice, the ultimate concentration of T2 peptide was 225μg/ml.

125 ***2.4. Preparation and administration of QTD***

126 The composition of TCM formula QTD is as follows: *Hedyotis diffusa* (60g), *Epimedium*
127 *brevicornu* Maxim (60g), *Fritillaria thunbergii* (60g), CPC (60g), and Earthworm (60g)
128 (Table 1). Based on the traditional Chinese herbal decoction method, these five agents were
129 mixed and kept for 1 hour in the arenaceous pot with eight volumes of cold water. Then the
130 mixture was boiled for 40min and filtered. The first filtered solution was then reserved, and
131 formula materials were again mixed in six volumes of cold water, boiled for 40 min and
132 filtered. The second filtered solution was then reserved, and then in four volumes of cold

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133 water, the formula materials were mixed again, boiled for 40 min and filtered. The three filter
134 liquors were combined and concentrated 0.5g/ml, 0.75g/ml, and 1.0 g/ml for the low, medium,
135 and high-dose groups, respectively. After the development of the CP/CPPS model on day 28,
136 based on previous methodology [24, 25], each mouse in the low-dose, medium-dose, and
137 high-dose group received 0.15ml/10g daily by oral gavage between the day 29 and 56.

Scientific name	**Latin binomial**	**Chinese name**	**Part used**	**Weight(g)**	**Voucher #**
Fritillaria thunbergii	*Fritillaria thunbergii*	Zhebeimu	Bulb	60g	CPU180910-1
Epimedium brevicornu Maxim	*Epimedii Folium*	Yinyanghuo	Leaf	60g	CPU180910-2
Hedyotis diffusa	Oldenlandia diffusa	Baihuasheshecao	Whole plant	60g	CPU180910-3
Cortex Phellodendri Chinensis (CPC)	Phellodendri Chinensis	Huangbo	Bark	60g	CPU180910-4
Lumbricus terrestris (Earthworm)	Lumbricidae terrestris	Dilong	Whole Body	60g	CPU180910-5
Total amount				300g	

138 **Table 1.** The composition of TCM formula QTD.

139 ***2.5. Voiding Behavior Analysis***

140 Changes of urine spots were analyzed by voiding the spot assay (VSA) test [26].
141 Experimental mice were placed on the filter paper in a cage individually. After one hour, total
142 filter papers were collected, and the images of urine spots were taken under the ultraviolet

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light. Then the urine spots of mice were evaluated by the Fiji version of ImageJ software. The number of urine spots represents the urine frequencies, and the spots with size ≥ 6.6mm^2 were calculated. Blinded observers collected data.

2.6. Pain Threshold Assessment

Mice were tested on days 28 and 56 by using von Frey filaments (vFF) applied to the abdomen and the plantar region of the hind paw. Referred hyperalgesia and tactile allodynia were tested. The technique was done in stainless steel chambers. Ten fibers with forces of 0.04, 0.07, 0.16, 0.40, 0.60, 1.00, 1.40, 2.00, 4.00 and 6.00 of consistently increasing weights were exerted to pelvic regions, and withdrawal responses of mice were evaluated. Each filament was applied for 1~2s with a 5s interval for a total of 10 times, and in ascending order of force, the hairs were tested. Immediate licking or scratching of the area of filament stimulation, sharp retraction of the abdomen, and jumping of mice were measured as a positive response to fiber stimulation. Response frequency was considered as the mean percentage of positive responses.

2.7. Histopathology

Prostate tissues from mice were soaked in 10% PFA for 24-48 hours. The fixed tissues were dehydrated in different solvents and fixed in paraffin. After that, the samples were sliced into sections of 5μm, which were stained with H&E staining and examined under a microscope. The severity of prostate tissue inflammation was assessed by using four-point scores in a random double-blind method. Grade 0 means no inflammation, and the grade 1 means part of acinar epithelial cell detachment and slight focal infiltration, the grade 2 means most of the acinar epithelial cell detachment, epithelial cell necrosis, moderate focal and mild diffuse

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infiltration and the grade 3 means most of the acinar epithelial cell detachment, epithelial cell necrosis and severe diffuse infiltrate [27].

2.8. Enzyme-linked immunosorbent assay (ELISA)

To examine the anti-inflammatory effects of QTD, the TNF-α level in the serum was identified by ELISA. The blood plasma obtained from the heart was put on room temperature for 2 hours; it was then centrifuged at 3000 rpm for 10 min. After that, the supernatant was collected and kept at -80°C. The content of TNF-α was calculated by using the ELISA kit (Elabscience Biotechnology Co., Ltd., China) according to the manufacturer's protocol.

2.9. Statistical analysis

The statistical differences between control, model, and treatment groups were analyzed by analysis of variance (ANOVA) analysis and expressed as mean, standard deviation (±SD). In all the analyzes, $p<0.05$, $p<0.01$, $p<0.001$ was considered statistically significant.

3. Results

3.1. Voiding behavior analysis

As shown in (**Fig. 1a)**, urine spots were collected on filter paper, and a VSA test was used to analyze the voiding behaviors. Several urine spots on day 28 were significantly higher as compared to the day -1 and day 56 **(Fig. 1b-d)**. After day 56, the number of urine spots was significantly higher in the model group as compared to the control, the low-dose, medium-dose, and high-dose group **(Fig. 1e)**.

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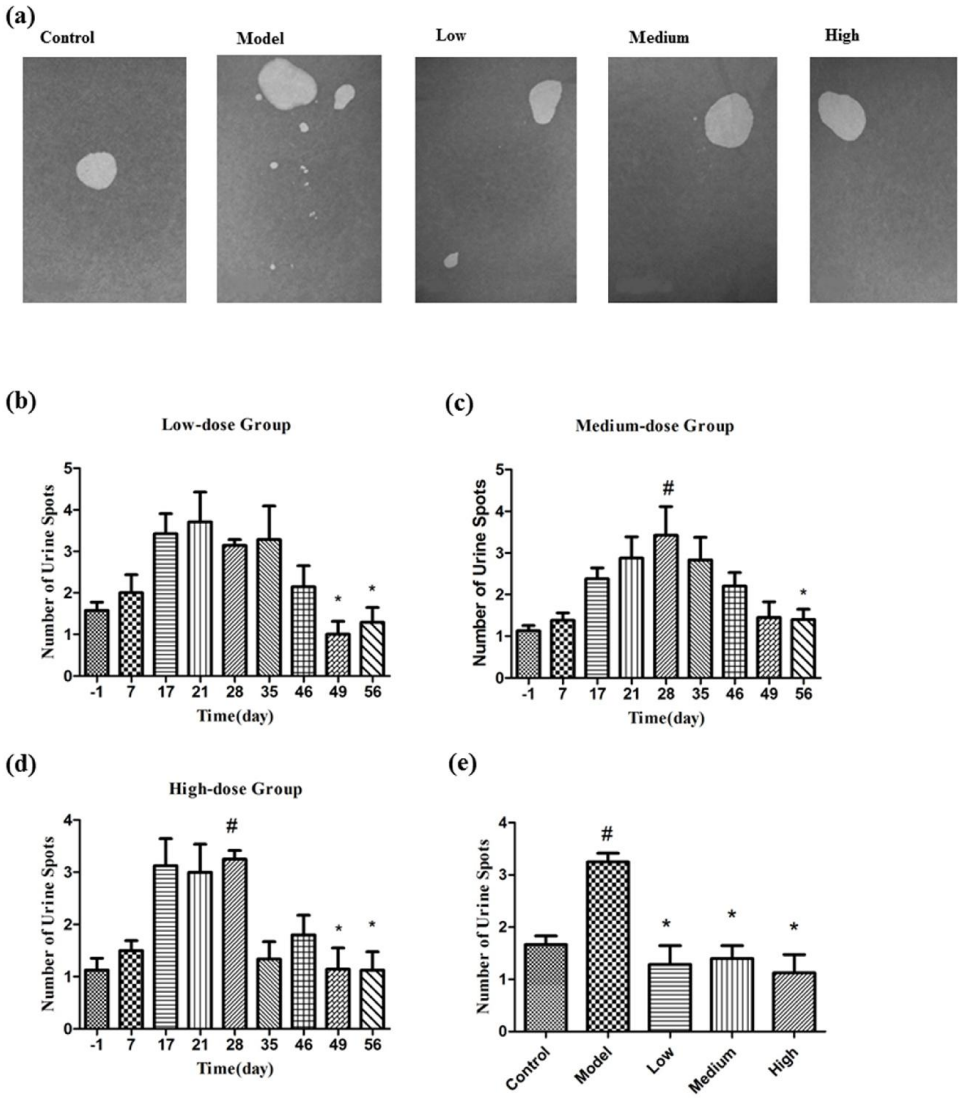

185

186 **Fig.1a.** Analysis of voiding behavior using ImageJ software. Urine spots with size ≥ 6.6mm^2

187 were considered. **b, c, d, e.** Several urine spots. (b) *means p<0.05 compared with day 21

188 (c) $^{\#}$means p<0.05 compared with day -1, *means p<0.05 compared with day 28 (d) $^{\#}$means

189 p<0.05 compared with day -1, *means p<0.05 compared with day 28 (e) *means p<0.05

190 compared with model group, $^{\#}$means p<0.05 compared with control group.

191 ***3.2. Pain behavior analysis***

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On day 28 of CP/CPPS induction and before the treatment with QTD, pain response frequency was recorded for all groups. On day 28th, maximum pain response frequency was observed in the model, low-dose, medium-dose, and the high-dose group **(Fig. 2a)** as compared to the control group. After the induction of CP/CPPS, the treatment groups received the treatment for 28 days, and the follow-up pain threshold on day 56 was compared to the day 28 pain threshold. On day 56, no significant difference was observed in the model and low-dose group, though the medium and high dose groups showed minimum pain response frequencies **(Fig. 2b)** as compared to day 28.

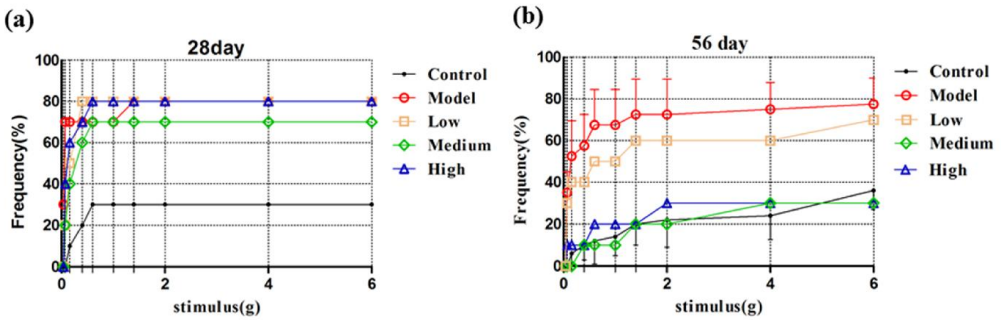

Fig.2. Pain withdrawal response frequencies in the model, low-dose, medium-dose, high-dose, and control group of mice after application of forces.

3.3. Histopathology

After Hematoxylin and eosin staining of the prostates, the model group appeared to have more severe inflammation than the control group. The low-dose group showed a large number of inflammatory infiltrations, while only scattered inflammatory infiltrates were seen in the medium-dose group. However, after the treatment, no significant inflammation and infiltration of inflammatory cells in a high-dose group were observed **(Fig. 3a)**. The

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inflammation score was most notable in the model group (p<0.001) compared to the control group. The low-dose, medium-dose, and high-dose group showed a significant difference (p<0.001) as compared to the model group. Compared to the control group, the low-dose and medium-dose groups were significantly different (p<0.05). The high-dose group showed no significant difference as compared to the control group. **(Fig. 3b)**.

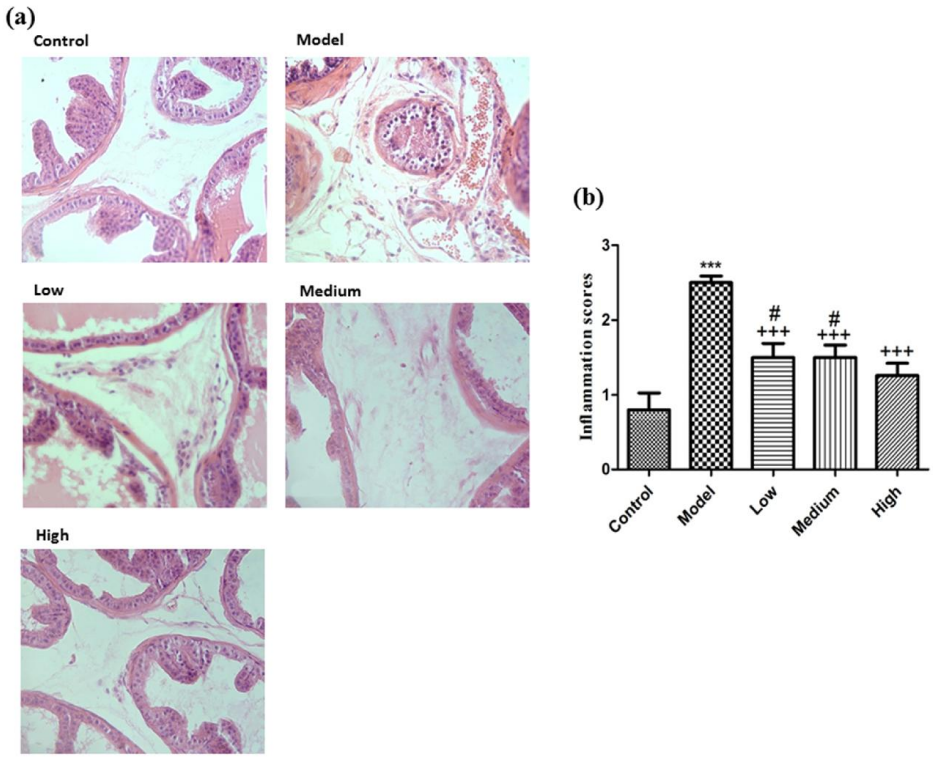

Fig. 3a. Observation of histopathology of the prostate tissue from C57BL/6 mice in the different groups (resolution: 400X). The model group appeared to have the most severe inflammatory lesion and the most extensive infiltration of inflammatory cells. The low-dose group showed a large number of inflammatory infiltrations. The medium-dose group displayed scattered inflammatory infiltrates. No significant inflammation and infiltration of inflammatory cells were observed in the high-dose group. The control group showed no

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inflammation. **b.** Mean inflammation scores of each group. ***The most significant difference from the control group at $p<0.001$. $^{+++}$The most notable difference from the model group at $p<0.001$. $^{\#}$Significant difference from the control group at $p<0.05$.

3.4. Serum levels of TNF-a

To detect the TNF-α level in the serum ELISA was used. As shown in **Fig. 4** and **Table 2**, the serum level of TNF-α in the model group was significantly higher than the control group ($p<0.05$). The low-dose and medium-dose groups showed no significant difference as compared to the control group ($p>0.05$). The TNF-α levels in the high-dose group also showed no significant difference from the control group ($p>0.05$). The high-dose group showed the most significant difference ($p<0.01$) as compared to the model group. When compared to the model group, the low-dose and medium-dose group showed a significant difference ($p<0.05$).

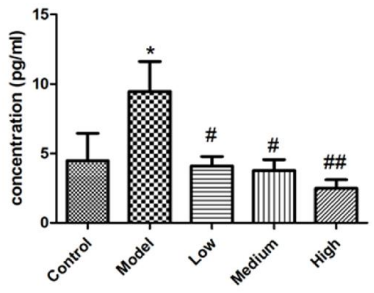

Fig. 4 Evaluation of the expression levels of TNF-α in serum. The ELISA kit detected levels of TNF-α in blood serum. The model group showed increased levels as compared to the control group ($^{*}p<0.05$). The medium-dose group and the low-dose group showed a significant difference ($^{\#}p<0.05$) as compared to the model group. The high-dose group displayed the most significant differences ($^{\#\#}p<0.01$) as compared to the model group.

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Group	TNF-α (pg/ml) mean ±SD
Control	2.59±1.39
Model	9.46±6.81*
Low dose	4.10±1.53#
Medium dose	3.77±1.91#
High dose	2.47±1.53##

Table 2. TNF-α level in serum (means ±SD). *p<0.05 means, compared with the control group. #p<0.05 indicates, comparison with the model group. ##p<0.01 means, comparison with the model group.

4. Discussion

CP/CPPS severely weakens the psychological and physical health of men. The etiology of this syndrome is not fully understood, and by western medicine, its treatment is frequently unsuccessful [28]. Traditional Chinese medicine is paying increasing attention to academic circles. Many scholars verified that TCM plays an essential role in reducing the risk of chronic disease [29]. Compared to the specific drug, the advantage of TCM therapeutics for CP/CPPS is “multiple components, multiple targets” to a complex disease[30]. CP/CPPS belongs to “Gonorrhea,” “Stranguria,” and “Turbid Semen.” The theories of TCM state that there are four kinds of common syndromes; downward flow syndrome of dampness-heat, gan-qi stagnation syndrome, blood-stasis syndrome, and deficiency of kidney-yang syndrome, which influence the treatment options [31-33]. The symptoms of damp heat and blood stasis are the most prevalent clinical syndrome, Wang Z's meta-analysis and literature review

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demonstrate that TCM ranks highest in terms of improvement of CP/CPPS associated with damp-heat and blood-stasis syndromes [30].

Qianlie Tongli decoction is composed of *Hedyotis diffusa*, *Epimedium brevicornu* Maxim, *Fritillaria thunbergii*, CPC, and Earthworm (*Lumbricus terrestris)*. QTD can clear heat, remove dampness, and promote blood circulation. Nankang tablets are included in the 《Chinese Pharmacopoeia》, *Epimedium* is the main drug, and adjuvants include CPC and *Hedyotis diffusa*. Icariin is the quality control indicator, Ni Yan's study [34] found that berberine and oleanolic acid also as indicators. Xiang Dong's study [35] analyzed the determination of icariine in Qianlietong tablets by HPLC; *Epimedium* is the main medicine in Qianlietong tablets; the result suggests the icariine is the main active ingredient. Xie Xueyuan's study [36] analyzed the determination of berberine in Qianlietong granules by HPLC, CPC is the main medicine in Qianlietong granules, the negative control doesn't contain CPC, the result suggests the berberine is the main active ingredient. 《Compendium of Materia Medica》 and 《Shennong Materia Medica》 recorded that earthworm has the functions of activating meridians, activating blood circulation and removing stasis. Shuxuetong's main component is the earthworm, which can significantly improve the symptoms of CP/CPPS [37]. Our lab found *Fritillaria thunbergii* can improve CP/CPPS mice symptoms [18], so it as an adjunct. Fritillaria is insoluble in water, even if it may be major active ingredients. So, we presumed that QTD' major ingredients were berberine, icariine, and oleanolic acid. Due to a Chinese herbal formula has many active ingredients, which need further research.

Numerous studies have shown that immune-related effects of TCM are associated with

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276 cytokine regulation [38]. Inflammation may contribute to CP/CPPS. Many types of research
277 have shown that several pro-inflammatory cytokines recruit activated immune and
278 inflammatory cells to the sites of lesions, thus increasing and maintaining the inflammatory
279 condition [39]. *Hedyotis diffusa* is a slender, annual plant that widely spread in the Asian
280 country, which has been used to treat inflammation, and urethral infection [40]. And it could
281 protect the renal damage by down-regulating the levels of TNF-α, IL-1β, IL-6, and
282 up-regulating the level of IL-10[41]. *Epimedium brevicornu* Maxim also shown an
283 anti-inflammatory effect in LPS-induced peritonitis by inhibiting the production of TNF-α,
284 IL-1β, and IL-6 [17]. Icariin is a major bioactive monomer, and studies have shown its
285 potential effect to treat immune and inflammatory diseases [42]. Berberine is an alkaloid
286 derivative extracted from a variety of plants, and Liu X'experiment reveals its
287 immunomodulatory effects in an autoimmune myocarditis rat model [43]. All these herbal
288 products had a synergic anti-inflammation effect.
289 In particular, the TNF-a level was usually high in the expressed prostatic secretions (EPS)
290 from men with CP/CPPS [44]. TNF-α is mainly derived from monocytes and macrophages.
291 The study found $CD4^+$ T cells and macrophages are key factors in the development of
292 CP/CPPS[45]. Increased inflammasome may be a possible mechanism of CP/CPPS, and
293 inhibiting the inflammasome-related pathway may be a new therapeutic approach [46]. Some
294 studies revealed that inflammasome might be a target for pain therapy [47]. The essential
295 problem of CP/CPPS is congestion, collateral blockage, and inflammatory cells infiltration.
296 All these herbals play a vital role in reducing inflammation and activate blood circulation of
297 chronic diseases.

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298 Our results also showed the therapeutic role of QTD in ameliorating urinary tract symptoms,
299 relieve pain, and reduce inflammation in CP/CPPS therapies. H&E staining of prostate tissue
300 has demonstrated that the high-dose group of QTD had a most significant anti-inflammatory
301 effect on CP/CPPS. TNF-α is a well-known pro-inflammatory cytokine. In the CP/CPPS
302 model group, the expression levels of TNF-α were higher than the control group. After
303 treatment, the TNF-a levels decreased significantly and were similar to the control group,
304 which demonstrated that QTD plays an important role in the immunomodulatory of the
305 CP/CPPS mice model. Here, we used Chinese medicines to treatment CP/CPPS, and its
306 possible mechanism of action is to suppress the immune response by reducing the release of
307 TNF-α.
308 CP/CPPS prostate involvement pain may be related to the axon reflex of the dorsal root
309 ganglion and cause neurogenic inflammation. TNF-α is involved in pain perception during
310 antigen-induced neurogenic inflammation [48]. Increased levels of TNF-α may also cause
311 neuronal damage. By upregulating the voltage-gated channels of dorsal root ganglion neurons,
312 uninjured neurons are involved in neuropathic pain [49]. The pain threshold was significantly
313 lower in the model group, which may be local cytokine production or local
314 neuroinflammation caused by autoimmunity. After 4 weeks of the intervention of QTD, the
315 pain in the mice was significantly relieved, this may be related to the level of cytokine TNF-α.
316 We only analyzed serum levels of TNF-α, which may be the principal limitation of this study.
317 In subsequent research, we will analyze more cytokines in the serum and prostatic tissue.
318 The current treatment for CP/CPPS is mainly using a combination of antibiotics, α-blockers,
319 and anti-inflammatory drugs. The improvement in symptoms and response to treatment is

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very variable; for CP/CPPS, management will likely progress toward symptom-specific rather than a generic, 'one strategy fits all' treatment [50]. However, the TCM formula has multiple ingredients; it is a more comprehensive treatment for multiple symptomatic diseases. Based on the UPOINT system [51], the QTD has unique advantages in urinary symptoms, organ function, tenderness of muscles, and regulate psychology.

In this study, we revealed that Qianlie Tongli's decoction could reduce the number of urine spots, improve the pain threshold, and reduce inflammatory lesion and inflammatory cells, and decreases the TNF-α level in the serum. However, we additionally expect that Qianlie Tongli's decoction may be useful for all kinds of prostatitis diseases.

5. Conclusion

CP/CPPS is an enigma for many researchers. Although its treatment is not yet absolutely understood, traditional Chinese medicines have a significant effect on the medication for CP/CPPS. This Chinese medicine formula (QTD) provides a new therapeutic approach to CP/CPPS. However, to conclude the long-term effectiveness of this treatment, more clinical study is needed to explore the practice of traditional Chinese medicine in the treatment of CP/CPPS.

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Conflict of interest

All authors declare no conflict of interest.

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