Original Research Article

Antianxiety effect of Tong Luo Jiu Nao, traditional Chinese medicinal preparation, in rats

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A B S T R A C T

Tong Luo Jiu Nao (TLJN), a modern formula of traditional Chinese medicine, has proven to be clinically efficacious in several vascular cerebral diseases but its specific effect is not known. In the present study we investigated the acute and persisting effects of TLJN on anxiety model in Wistar male rats. TLJN was administered intragastrically during three successive days (Days 1–3) and then, the double TLJN dose was given on Day 7. For the evaluation of anxiety-related behavior of animals, we used the open field (OF) and elevated plus maze (EPM) paradigms. Testing in the OF was performed on Days 1, 2, 3, 4, 8, 14 and 22; in the EPM on the Day 23.

Two-way repeated-measures ANOVA revealed significant differences between control and TLJN treated animals in the open field model where TLJN induced increased exploratory activity, indicating reduced anxiety-like behavior. The effect persisted for several days after the treatment and was still present on Days 14 and 22. Reduced anxiety-like behavior was also observed on EPM 16 days after the last TLJN administration.

The results demonstrate behavioral effects of intragastric administered TLJN, which indicate reduced anxiety. Persistence of the induced behavioral changes suggests prolonged duration of action.

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Introduction

Traditional Chinese medicine (TCM) uses many approaches in therapy, which experience has shown to be very useful. The chemistry of naturally occurring compounds has been investigated for a long time in order to find compounds suitable for clinical treatments. The medicinal plants have traditionally been also used in several European countries for the treatment of many disorders and diseases (Patocka, 2003). One remedy prepared according to a TCM formula is Tong Luo Jiu Nao (TLJN), which is used in several clinical indications
(Hua et al., 2010; Li et al., 2012; Wang et al., 2012). TLJN is composed of active components of Fructus gardeniae and Radix notoginseng (Gardenia Jasminoides and Panax Notoginseng) (Hua et al., 2010) and has proven to be clinically efficacious in the treatment of ischemic cerebral stroke and dementia (Hua et al., 2010). This preparation protects brain blood vessel endothelial cells against ischemic attacks. TLJN has recently been patented and currently is investigated in preparation for future clinical studies (Liu et al., 2011).

It is assumed that brain microvascular endothelial cells secrete different active substances under different conditions which can influence the survival of neurons and so they can be suitable drugs targets for the treatment of brain diseases. It has been shown that intraperitoneal injection TLJN reduced ischemic damage to brain tissue and increased brain derived neuronal factor expression in rats (Alesheikh et al., 2011). Another study revealed reduction in beta-amyloid deposits after TLJN oral administration in the brains of mice, which served as a model of Alzheimer’s disease (Chen et al., 2006; He et al., 2013). Recent results showed the effect of TLJN on an animal model of Alzheimer’s disease that was evolved in rats by intracerebroventricular injection of beta-amyloid. The study demonstrated that intragastrically administered TLJN repaired the impaired structure of neurons and also corrected impairment of spontaneous alternation of rats in the Y maze and deterioration of learning and memory in the passive avoidance procedure (Liu et al., 2011).

We decided to investigate if TLJN may influence emotional functions that have not been explored so far. Accordingly, we examined the effects of the preparation on two measures of anxiety-related behavior: the open field model and the elevated plus maze. In order to explore both acute and persisting consequences of the TLJN treatment, we repeated testing in the open field over a period of three weeks and conducted elevated plus maze procedure at the end of behavioral experiments.

**Material and methods**

The study was reviewed and approved by the local Ethical Commission of the First Faculty of Medicine, Charles University in Prague and the treatment of animals was in accordance with the Declaration of Helsinki Guiding Principles on Care and Use of Animals (DHEW Publication, NHL 80-23). All effort was taken to minimize the number of animals used and their suffering.

**Animals**

Wistar male rats with starting body weight 250–280 g (VELAZ, Czech Republic) were used. Males are used to avoid the fluctuation of exploratory activity in dependence on the actual phase of estrous cycle in female rats. They were housed four or five per cages (42 cm × 26 cm × 25 cm, plexiglass) in a breeding room at constant temperature (21 ± 1 °C) with a 12L/12D schedule. The onset of the light phase was at 6:00 a.m. Rats were handled daily for 5 min by the same person for 10 days before the start of this experiment. Food and water were supplied ad libitum except during testing. The number of control treated animals was 10 per group.

**Drugs and experimental design**

In this study we used Tong Luo Jiu Nao (TLJN), a modern Chinese formula, based on traditional Chinese medicine (Hua et al., 2010; Liu et al., 2011). This is a multicomponent preparation whose active components were extracted from P. notoginseng and G. jasminoides. Based on clinical practice knowledge, the starting amount of herbs for extraction was 5.0 g and 8.5 g, respectively. TLJN was prepared according to the protocol of the National Medical Dictionary of China. We used HPLC to test the components and confirm the final concentration of active components in this oral solution. The final preparation TLJN contained 7.7 mg/ml of active compounds dissolved in water (Liu et al., 2011). HPLC shows the following concentrations: geniposide 4.95 mg/ml, geniposide acid 1.73 mg/ml and ginsenoside Rg1 1.02 mg/ml. For our behavioral study in rats, we used TLJN intragastrically in the volume of 3.0 ml/kg of water. Control animals received water in the same amount.

Design of the experiment is shown in Fig. 1. For the first three days rats received 3 ml/kg of either TLJN or water intragastrically between 8 and 9 a.m.; the behavioral tests were performed 1 h later and then on Day 4. Further, two doses separated by 3 h were given on Day 7, the open field test (OF) followed 24 h later (Day 8). Additional OF tests were performed on Days 14 and 22 without treatment. On Day 23 rats were tested on the elevated plus maze (EPM), also without treatment.

**Open field**

Behavior of rats was tested in a circular arena with the diameter of 150 cm, the walls being 50 cm high. The arena was divided into two concentric circles, the inner one defined by 110 cm diameter, the outer one by 20 cm wide area. Each rat was placed in the same position in the outer part of the arena and the following variables were scored for 5 min: total movement distance (m) (TMD), movement distance (m) in the central part (CMD). Behavioral parameters were recorded and subsequently analyzed by an automated activity monitoring system (AnyMaze, Stoelting, USA). Before testing the next rat, the arena was cleaned and dried. Behavioral testing was conducted between 08:00 and 12:00 h during the light phase in the experimental room which was separated from the breeding room. The experimental room was illuminated by two 60 W bulbs.

**Fig. 1** – Experimental schedule TLJN administration and behavioral testing. Design of the experiment using Tong Luo Jiu Nao (TLJN) or water in Wistar rats in the amount 3 ml/kg. Treatment was performed on Days 1, 2, 3 and open field (OF) test followed 60 min after intragastric treatment. OF test without treatment was performed on Days 4, 8, 14 and 22. On Day 23 elevated plus maze test (EPM) was performed.
Elevated plus-maze

Behavior of rats in the elevated plus maze (EPM) was tested in a device consisting of four arms. The opposite-facing open arms were 40 cm long and 10 cm wide with 0.2 mm edges; 40 cm high walls surrounded the opposite-facing closed arms. The central area measured 10 cm × 10 cm. The device was placed on the floor and the arms were elevated at a height of 50 cm. The experimental room was illuminated by two 60 W bulbs.

At the beginning of the test each rat was taken from its home cage and placed in the central area of the maze with the heads facing an open arm. The test lasted for 5 min. A four-paw criterion validated an entrance into an arm. The behavioral parameters, manually scored by two trained observers, were the following: time spent in the open arms, time spent in the closed arms, entries in the open arms and entries in the closed arms.

Statistical analyses

Data were analyzed by GraphPad PRISM, version 5.0, GraphPad Software 1994–2007 (www.graphpad.com). For the calculation of repeated measurements in the open field test, we used repeated-measures two-way ANOVA, using treatment and time as the factors. When overall data were significant we used Bonferroni post hoc test to compare means for individual days. Other data were analyzed with two-tailed t-test. The statistical significance was evaluated at the significance level 2α = 0.05.

Results

Central movement distance (CMD) in the open field, which is indication of anxiety related behavior, is summarized in Fig. 2. Two-way ANOVA shows that TLJN treatment significantly increased CMD F(1,18) = 11.61) indicating decreased anxiety. There was also significant effect of time (F(6,108) = 17.60) and significant interaction is between treatment and time (F(6,108) = 3.89). Bonferroni post hoc test showed that significant differences between controls and TLJN group occurred on Days 14 and 22. Changes of total movement distance were similar to changes of CMD.

To further examine the persisting effect of TLJN, we investigated the behavior of rats in the elevated plus maze (EPM) on Day 23, i.e. 24 h after the last OF test. Group treated with TLJN spent more time in the open arms than the control group (t = 2.46) (Fig. 3A) and significantly less in the closed arms (t = 2.45) (Fig. 3B). The percent of time in the open arm was also significantly larger in TLJN treated group (Fig. 3C). Number of entries into the open and close arms did not differ between the control and treated groups.

Discussion

The present study demonstrated the ability of TLJN to influence the behavior of rats both in the open field and in the elevated plus maze. In the open field the drug increased central movement distance (CMD). The difference between treated and control animals reached significance on Days 14 and 22, in spite of the fact that TLJN increased CMD over the whole course of the experiment. The elevated plus maze test conducted on Day 23 showed that previous treatments of rats with TLJN significantly changed an important parameter – time spent in the open and closed arms. It has been suggested that the reluctance of rats to explore the open arms of the EPM is caused by fear of open spaces, rather than the novelty of the maze or its height. When compared to controls, TLJN group revealed a significant increase of time spent in the open arms (Fig. 3). The results indicate persisting consequences of the drug treatment on the investigated behaviors.

Both OF and EPM tests are widely used for assessing emotionality, or more specifically anxiety, in animals. The large illuminated arena of the open field is perceived by laboratory rodents as an aversive, frightening environment, and the behavior of a subject being placed in it reflects the reaction of the subject to that stressful event (Eilam et al., 1994; Ramos and Mormede, 1998). The increase in ambulation in the central field resulting from the effects of anxiety drugs like benzodiazepines is considered to indicate the attenuation of stress-induced inhibition of exploratory activity (Ramos and Mormede, 1998; Prut and Belzung, 2003; Hlinak et al., 2009). In the present experiments the interpretation of increased locomotion in the central field due to TLJN indicates an anxiolytic-like effect. Generally, in the open space rats and mice prefer to walk close to the walls that confer anxiety relieving body contact, so-called thigmotaxis as an index of anxiety (Eilam et al., 1994; Ramos and Mormede, 1998; Prut and Belzung, 2003). Consequently, the almost parallel pattern of changes in the total distance and distance traveled in the center observed in TLJN treated rats can be viewed as reflecting reduced anxiety level. However, the open field test also serves as a procedure to measure sedation or activation. The increased overall locomotion can therefore reflect also increased arousal and a strengthened drive to explore.

An anxiolytic-like effect of TLJN is further suggested by the results obtained in EPM. This procedure like the OF represents a fear-inducing experimental environment for rats and mice,

Fig. 2 – Central movement distance (CMD, meters) of Wistar rats tested in open field (OF) device: control group (white bars) and TLJN group (black and gray) treated on Days indicated on the schedule in Fig. 1. *Statistically significant using Bonferroni post hoc test.
in which they always will prefer the sites where the possible danger is minimal, specifically the closed arms (Pellow and File, 1986; Rodgers and Cole, 1994; Ruarte and Alvarez, 1999; Martinez et al., 2002). Anxiolytic compounds increase the percentage of time spent on open arms relative to time on open plus closed arms (Korte and De Boer, 2003). In the present experiment, TLJN treated rats spent more time than the controls in the open arms, which is a measure supporting the anxiety reducing effect of the preparation. Total arms entries did not differ between control and treated rats indicating that TLJN did not increase overall motor activity. Thus, the longer stay of rats in the open arms can be interpreted in terms of reduced anxiety (Pellow et al., 1985; Rodgers and Cole, 1994).

The present results confirmed the effectiveness of TLJN to modulate behavior after intragastric administration. In the present experiments we used naive, untreated animals and found changes in spontaneous unconditioned behaviors that indicate altered emotional functions. Although the increase in CMD was observed from the first open field testing, a significant CMD enhancement appeared later after the forth and last drug administration, i.e. in open field testing performed on days 14 and 22. This leads to a question, whether the slowly developing effect can be attributed to a chosen dose, or some kind of accumulating impact on the neuronal systems organizing the observed behavioral response.

The purported effective components of the TLJN preparation, geniposide, ginsenoside Rg1 and genposidic acid are molecules that are not expected to penetrate the blood brain barrier due to their size. To explain the observed improvement of ischemic brain alterations or alleviation of behavioral changes in Alzheimer’s disease model after systemic application of TLJN, several authors have suggested that TLJN stimulates microvascular endothelial cells to produce so far not recognized neuroprotective factors, which are transported to the brain (Hua et al., 2010; Alesheikh et al., 2011; Liu et al., 2011). However, some direct penetration of TLJN to the brain cannot be excluded, and the circumventricular organs have to be taken into consideration (Partridge, 1986; Zdarova-Karaseva et al., 2010). For example, molecules of several neuropeptides, such as oxytocin, vasopressin or galanin, influence emotional functions after systemic application (both in humans and in animal behavioral models) (Klenerova et al., 2009, 2010, 2011), and some possibilities for the penetration of these molecules have been suggested (Begley, 1994; Broadwell et al., 1998; Smith and Ferguson, 2012).

The anxiolytic effect of TLJN may be mediated by increasing expression of the brain-derived neurotrophic factor (BDNF) (Alesheikh et al., 2011) since it has been recently shown (Weidner et al., 2014) that transgenic mice overexpressing BDNF show anxiolytic activity in the elevated plus-maze.

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**Fig. 3 – Behavior of Wistar rats tested in the elevated plus maze (EPM) on Day 23. CO = controls, TLJN = TLJN treated.** Columns indicate mean values ± S.E.M. *Statistically significant as compared with controls.
These mice also develop spontaneous seizures and it is possible that administration of TLJN to humans may result in increased risk of seizures. Level of BDNF is known to be up-regulated in the epilepsy and has been linked with polymorphisms in BDNF (Papaleo et al., 2011). The overexpression of BDNF was found to be transgenic mice with developing of spontaneous seizures. However, clinical studies with TLJN so far did not mention the higher susceptibility to seizures.

Pharmacokinetically, TLJN was metabolized very fast in rats. The plasma concentration of two components of TLJN, geniposide and gensenoside Rg1, achieved the maximum level within 45 min after intragastric administration. Neither is detectable after 24 h (Zhou et al., 2008). In brain of Sprague-Dawley rats, the components were detectable by RP-HPLC 60 min after intragastric administration. In heart, 10 min after treatment, the component can be detected and maximum concentration was achieved after 60 min of treatment (Zhou et al., 2008, 2009). Due to the fast metabolism of TLJN components, we can with relatively high probability assume that the two presumably active compounds disappeared from the plasma during the 15 days period without treatment. Although we do not know precisely how rapidly components of TLJN are metabolized within the brain, the results showed lasting consequences of drug administration. Thus, we propose that some comparatively long-lasting changes have occurred in neural systems associated with these behaviors.

In conclusion, intragastric administration of TLJN produced behavioral changes that persisted after the initial treatment. The data obtained in the two tests, namely the increase in exploratory activity in the central part of open field, as well as prolonged time spent in the open arms of the elevated plus maze, suggest reduced anxiety level. The results extend the knowledge about the actions of the TLJN preparation, derived from traditional Chinese medicine, with an interesting feature that may contribute to its therapeutic profile.

Conflict of interest

None.

Authors' contributions

VK designed the study and the data analysis strategy, participated in the interpretation of data and elaboration of the manuscript. SH carried out data collection from behavioral studies, performed the statistical analysis, drew the figures and wrote the manuscript. QH prepared the TLJN preparation, participated in the design of the study, interpretation of data and review of the manuscript. XJL participated in the preparation of TLJN and review of the manuscript. All authors read and approved the final manuscript.

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References


Papaleo, F., Silverman, J.L., Aney, J., Tian, Q., Barkan, C.L., Chadman, K.K., Crawley, J.N., 2011. Working memory...
deficits, increased anxiety-like traits, and seizure susceptibility in BDNF overexpressing mice. Learn. Mem. 18, 534–544.


