

## A Comparison Between the Effects of Estrogen and Soy Extract on Chronic Pain in Male Rats

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### Abstract

In addition to its effect on reproductive organs, estrogen exhibits complex effects on brain structure, function and behavior. In this regard, the influence of gonadal hormones especially estrogens on nociception has recently been accepted. Soybean is known as an important phytoestrogen containing plant that is used as an alternative for estrogen replacement therapy. In this study, the influences of soy extract and estrogen on formalin-induced nociception in male rats were examined. In the first experiment, the effect of soy extract added to drinking water (60 mg/kg per day) on the nociception was assessed and compared with a control group (with soy-free drinking water). There was a significant decrease in nociception in rats that received soy extract for two weeks. Furthermore, different doses of soy extract (50, 150 and 200 mg/kg) were injected subcutaneously 30 min before formalin test to assess its effect on acute and chronic pain perception. It was observed that different doses of soy extract had similar antinociceptive effects that were significantly different from saline injected control group. In other experiment, rats treated with a single dose of 150 µg of 17-β estradiol (s.c.) 48 h prior to the formalin test, to be compared with a control group just received estrogen vehicle (DMSO). According to our results Estradiol did not show any significant effect on pain suppression. These results suggest that the antinociceptive effect of soy extract on chronic pain is not a result of its phytoestrogenic activity, it might therefore be related to other substances within soy extract.

**Keywords:** Soy; Estrogen; Formalin test; Antinociception.

### Introduction

There are some evidences showing sex-dependent differences between males and females in pain perception (1). Estrogens are known as antinociceptive agents that modulate pain sensation (2). In addition, it has been reported that during pregnancy where there are high estrogen levels, pain threshold increases (3). Paradoxically, it has also been reported

that women are more likely to experience more severe and longer pain than men (4). Plant-derived phytoestrogens including soy products are molecules structurally and functionally similar to estradiol (5, 6). There are similarities in the structure, molecular weight and function between phytoestrogens and steroid estrogens (7). Soy has been identified as a novel dietary ingredient that significantly reduces hyperalgesia and neuropathic pain (8, 9). Soybean comprises of oil, carbohydrate, fatty acids (e.g. linoleic acid and palmitic acid), phospholipids (i.e. phosphatidyl choline, phosphatidyl ethanolamine

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**Table 1.** Effect of soy and estrogen on the second phase of formalin-induced pain.

Group	Dose	Pain score (Mean±SEM)	Percentage of inhibition
1-Control-a <i>n</i> =14	--	2.12±0.03	
2-Soy oral <i>n</i> =15	60 mg/kg/day	1.77±0.03	17% P<0.001
3-DMSO s.c. <i>n</i> =11	100 µl	1.98±0.03	
4-Estrogen s.c. <i>n</i> =8	150 µg	2±0.03	no inhibition P>0.05
5-Control-b Saline i.p. <i>n</i> =7	100 µl	1.93±0.04	
6-Soy i.p. <i>n</i> =7	50 mg/kg	1.87±0.03	12% P<0.001
7-Soy i.p. <i>n</i> =8	150 mg/kg	1.85±0.05	12% P<0.001
8-Soy i.p. <i>n</i> =7	200 mg/kg	1.83±0.05	12% P<0.001
9-Morphine sulfate <i>n</i> =6	15 mg/kg	1.73±0.03	13% P<0.001

All data represent means±S.E.M. Percentage of inhibition of soy and estrogen on nociception relative to their own control group is shown underneath of them. It is shown that rats which received oral soy had pain inhibition relative to their control group. *Control-a*: received soy-free drinking water and compared to orally soy-administrated rats. *Control-b*: received injective saline and compared to soy injected rats.

and phosphatidyl inositol) and minerals (calcium, iron and potassium). Isoflavones including diadzein and genistein are from phytoestrogens in soybean. Synthetic phytoestrogens resemble female hormones and have similar hormonal and non-hormonal effects (10). So far, there has not yet been simultaneous work to compare the phytoestrogen and estrogen influences on nociception. The aim of the present study was to investigate the effect of soy extract administration on formalin-induced nociception and figure out whether this effect is related to phytoestrogenic function of soy by direct comparison with 17-β estradiol administration in the same behavioral test.

### Experimental

#### *Experimental procedure*

Adult male NMRI rats (195-220 g)

purchased from Razi Institute, Hesarak Iran, were used. Every group four rats were housed in one cage at temperature 21±2°C and 12 h light-dark cycling with standard chowder and water *ad libitum*. Rats were randomly divided in nine control and experimental groups as shown in Table 1. To determine the effect of estrogen on the nociception, rats were treated with a single dose of 150 µg 17 β-estradiol (Sigma UK) in 100 µl of DMSO injected s.c. 48 hours prior to formalin test and compared with a control group received 100 µl of vehicle (DMSO) (10). To determine the effect of orally administered soy extract, animals were treated with 60 mg/kg/day soy extract added to the drinking water for 2 weeks (11, 12) and the result was compared with a control group used (with soy-free drinking water). The average of the consumed drinking water was determined during one week in 10 rats and it was revealed

that each rat had approximately consumed 0.5 ml/g water per day. The drinking water with an overestimated volume of 10% was provided daily for every group of 4 rats in each cage. In other experimental groups rats treated with i.p. injection of soy extract, doses of 50, 150 and 200 mg/kg 25-30 min prior to the formalin test and were compared with saline injected control group. In positive control tests morphine sulfate (15 mg/kg; i.p.) dissolved in saline was used (13). Animals treated with 17 $\beta$ -estradiol, soy extract, morphine sulfate, DMSO or saline and those normal feeding control rats were assessed for nociception by formalin test.

#### *Preparation of plant extract*

The medicinal plant of soy was provided from the local market and was scientifically identified by the department of botany of Shaheed Beheshti University. To prepare the hydro alcoholic extract, 400 g of cleaned soy beans was crushed and mixed at ratio of 1 to 5 with methanol 70% and kept for 48 h at room temperature. During this time it was stirred several times and then the deposit was separated using paper filter. After filtration with it was maintained in a water bath at 65°C for 16 h to let the alcohol be evaporated from filtered solution to reach a final concentration of 25%.

#### *Formalin test*

Acute and chronic pain assessment was carried out via the formalin test protocol (14). All animals were habituated to the test chamber 30 min prior to the experiment. In all rats, 50  $\mu$ l of 2.5% formalin was subcutaneously injected into the plantar surface of hind paw. Following injection of formalin, the animals were immediately put in a chamber with a mirror angled at 45° positioned underneath the floor to allow an unobstructed view of the formalin injected paw by an observer. Observations continued for the next 60 min. The first 10 min was considered as the early phase and the period between 15 and 50-60 min as the second phase. The pain scoring was as follows: 0, normal weight bearing on the injected paw; 1, limping during locomotion or resting the paw lightly on the floor; 2, elevation of the injected paw; 3, licking or biting of the injected paw,

or grooming. Behavioral responses had been recorded every 15 sec. The average pain scores from every 3 min block in experimental groups were compared with similar blocks in control groups. The rats were not tested more than once and experiments were carried out between 09:00 a.m and 15:00 p.m.

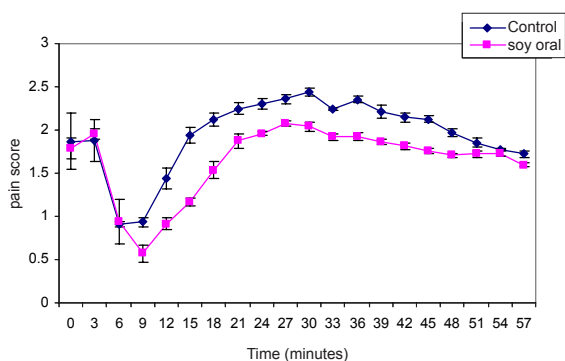
#### *Statistical analysis*

Data, were expressed as means $\pm$ S.E.M. Comparisons were carried out using one way analysis of variance (ANOVA) followed by post-hoc Tukey test and  $p < 0.05$  were considered as a significant difference.

## **Results**

Results of administration of soy extract and estrogen are shown in Table 1. Statistical comparison between the experimental groups (soy and estrogen) and control groups in the first 10 min indicate that neither soy nor estrogen has any effect on nociception in the whole first phase (early phase) of formalin-induced pain (Figure 1 and 2). An overall comparison by statistical analysis also indicates that oral soy administration has a significant inhibitory effect (17% pain reduction) in the second (chronic) phase (time interval 15-60 min) of formalin-induced nociception ( $P < 0.001$ ) (Figure 1 and Table 1). However, in some periods such as 51 to 57 min, oral soy administration did not cause any significant pain reduction. As shown in Table 1 and Figure 2, comparing to DMSO group, estrogen (150  $\mu$ g s.c.) had no effect on pain perception in the late phase (chronic phase) of formalin test ( $P > 0.05$ ).

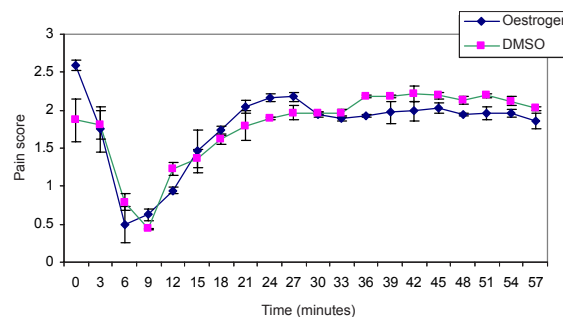
As demonstrated in Table 1, our results showed a significant reduction of pain perception (12% inhibition) in rats treated i.p. injection with different doses (50, 150, 200 mg/kg) of soy extract ( $P < 0.001$ ) relative to control group (treated with saline injection). Interestingly, this result is very close to our positive control group (morphine sulfate) that induced a 13% pain reduction ( $P < 0.001$ ). It also indicates that the differences of nociception among various doses of soy treatment are not significant. There was a similarity between oral and i.p. treatment of soy in analgesic effect.



**Figure 1.** Effect of oral soy (6.25% in pellet for 2 weeks) administration on pain scores in the first (early) and the second (late) phases of formalin-induced nociception in male rats (n=15). All data represent means±S.E.M. An overall marked antinociceptive effect was observed in the second phase (15-60 min) ( $P<0.001$ ).

### Discussion

The main finding of the present study is that under conditions of peripheral inflammation such as formalin injection into the plantar surface, soy appears to play an important role in pain relief in the second phase of formalin-evoked nociception. However, our finding shows that oral soy consumption does not result in alteration of formalin-induced acute phase nociception. The suppression of heat allodynia in a chronic neuropathic pain model in another report is in agreement with our result (9). In addition, an analgesic effect of soy-containing diet on cancer pain model as a neuropathic pain has been reported (15). Since there are different mechanisms for acute and chronic pain phases in formalin test, the discrepancy in responses in two phases might be resulted from different mechanisms. Acute pain induction is neurogenic and is mediated via the direct effect of formalin on the nerve endings going to central nervous system, and chronic pain is induced through peripheral pathway, resulted from inflammatory processes (16, 17). Experimental results demonstrated that substance P and bradykinin participate in the early phase, while histamine, serotonin, prostaglandins and nitric oxide are involved in the late phase of the formalin test (18). Blockade of tyrosine kinase activity and antioxidant activity are the known effects of isoflavones (19). Other reported activities of isoflavones, such as direct interaction with



**Figure 2.** Effect of s.c. injection of  $17\beta$ -estradiol (150  $\mu$ g s.c.) on formalin-induced nociception in male rats (n=8). All data represent means±S.E.M. There is no significant difference between control (DMSO) and treated (estrogen) groups in both phases.

several intracellular enzymes, are also candidate contributors to the inhibition of inflammatory response (20). Furthermore, it is reported that genistein inhibits monocyte adhesion to TNF-activated endothelial cells and also inhibits platelet aggregation and release of pro-inflammatory cytokines (20, 21). Therefore, the inhibitory effect of soy on nociceptive response in the late phase of formalin test suggests that this effect might be due to its peripheral action and it is possibly associated with the increases in the activities of antioxidant enzymes and also related to the blockade of some of inflammatory mediators such as histamine, serotonin, and prostaglandins.

It has been reported that Sabra strain rats fed with soy have reduced heat hyperalgesia (8). However, no reduction of heat hyperalgesia (induced by Hargreave device) in Wistar rats after nerve injury, fed with soy has been observed (8, 12). Shir and colleagues in 2001 used Hargreave device to induce noxious heat stimulation and acute pain which is different from their study in 1998 (using laser) and our method in the first phase of formalin test. The acute phase is resulted from nociceptors' activation; the chronic phase is more reflective of inflammatory/injury-induced central and peripheral sensitization. Hyperalgesia responses in second phase would be more comparable to nerve injury results than acute nociceptive responses (1<sup>st</sup> phase). Therefore, the types of noxious stimuli (formalin vs. Hargreave device or laser) or rat strain (NMRA vs. Sabra

or Wistar) are the probable reasons for different results.

Notably, it has been shown that soy-derived isoflavones such as active aglycones (daidzein and genistein) and inactive glucosides (mainly daidzin and genistin) did not alter pain threshold (22). Furthermore, no significant differences in pain threshold were detected between the phytoestrogen-treated and control groups tested with hot plate device (23). Therefore, our observation is in agreement with these previous reports about acute pain, where the antinociceptive effect of soy was not observed against allodynia and hyperalgesia.

Likewise, i.p. injection of different concentrations of soy has significantly reduced chronic pain scores in rats. The pain suppression was not significantly different among three used concentrations. Shir and colleagues (12) have mentioned that low and high concentrations of phytoestrogens do not induce sensitivity reduction in neuropathic pain. Therefore, those doses used by us are possibly in mid ranges that do not show different functions. Lower and higher doses should be used to confirm the hypothesis. Furthermore, the non-specific function of different doses of soy in this experiment could be another reason to justify this subject. Since the analgesic effect of soy administration in both oral and s.c. injection showed similarity, one can conclude that possibly the effective ingredient of soy in nociception can pass through gastrointestinal tract. Regarding the mechanism of second phase of formalin-induced nociception which is related to the release of inflammatory mediators such as prostaglandin and substance p (13), probably the antinociceptive effect of soy is mediated via its modulatory effect on inflammatory mediators.

Our study showed that estrogen has no inhibitory effect on pain perception in acute and chronic phases of formalin test, while we have used the same dose that was administrated by others (10). There are paradoxical reports concerning estrogen involvement in pain perception. For instance, it has been shown that estrogen administration increases the proportion of heat sensitive fibers that are also mechanosensitive (25). However, the reduction of sensitivity to noxious stimuli during

pregnancy which is accompanied by elevated gonadal hormone level in women and female rats is also reported (26). There is evidence that the modulatory effect of estrogen is sex-dependent, and in most cases estrogen has excitatory and inhibitory effects on second messenger pathways in females and males respectively. In females, estrogen can activate second messengers including PKA, PKC and PLC and increase intracellular calcium in different tissues such as DRG neurons via a non-genomic mechanism (27, 28). Conversely, estrogen can inhibit PKA and PKC activation in the male rat (29, 30). It is likely that estrogen effect on nociception is related to many parameters such as gender, the type of pain, animal strain and other gradients that accompany estrogen.

These information suggest that whole plant with different concentrations of various phytoestrogens is suitable for pain relief and besides estrogen other substances exist in soy that causes its effect. The main ingredient in soy because of which it is involved in nociception is not known well. The most studied candidates are phytoestrogens that inhibit protein kinase C (31). Activation of PKC is involved in development of neuropathic pain (31). However, phytoestrogens showed estrogenic role in some aspects of analgesic activity (8) and further investigation with different doses of estrogen in both male and female with measuring the estrogen plasma level could be helpful.

### **Conclusion**

The present study showed that soy consumption (either oral or i.p. injection) in male rats has a clear inhibitory effect on pain suppression in chronic phase. However, neither oral administration nor i.p. injection of soy could change nociception in early phase of formalin-induced pain. These findings suggest that soy is possibly involved in anti-inflammatory mechanism in pain relief. Nonetheless, estrogen did not exert any antinociceptive effect in both acute and chronic pain in our experiment. Taken together, the hypotheses that antinociceptive effect of soy is resulted from soy protein or estrogen or different concentrations of isoflavones are left to be more investigated.

## References

- (1) Berkley KJ. Sex differences in pain. *Behav. Brain Sci.* (1997) 20: 371-380
- (2) Liu NJ and Gintzler AR. Prolonged ovarian sex steroid treatment of male rats produces antinociception: identification of sex-based divergent analgesic mechanisms. *Pain* (2000) 85: 273-281
- (3) Dawson-Basoa M and Gintzler AR. Nociceptin (Orphanin FQ) abolishes gestational and ovarian sex steroid-induced antinociception and induces hyperalgesia. *Brain Res.* (1997)750: 48-52
- (4) Unruh AM. Gender variations in clinical pain experience. *Pain* (1996) 65: 123-167
- (5) Adlercreutz H. Epidemiology of phytoestrogens. *Clin. Endocrinol. Metab.* (1998) 12: 605-623
- (6) Lephart ED, West TW, Weber KS, Rhees RW, Setchell KD, Adlercreutz H and Lund TD. Neurobehavioral effects of dietary soy phytoestrogens. *Neurotoxicol. Teratol.* (2002) 24: 5-16
- (7) Shutt DA and Cox RI. Steroid and phyto-estrogen binding to sheep uterine receptors *in vitro*. *J. Endocrinol.* (1972) 52: 299-310
- (8) Shir Y, Ratner A, Raja SN, Campbell JN and Seltzer Z. Neuropathic pain following partial nerve injury in rats is suppressed by dietary soy. *Neurosci. Lett.* (1998) 240: 73-76
- (9) Shir Y, Sheth R, Campbell JN, Raja SN and Seltzer Z. Soy-containing diet suppresses chronic neuropathic sensory disorders in rats. *Anesth. Analg.* (2001) 92: 1029-1034
- (10) Kuba T, Wu HB, Nazrian A, Festa ED, Barr GA, Jenab S, Inturrisi CE and Quinones-Jenab V. Estradiol and progesterone differentially regulate formalin-induced nociception in ovariectomized female rats. *Hormones and behaviors* (2006) 49: 441-9
- (11) Perez-Martin M, Salazar V, Castillo C, Ariznavarreta C, Azcoitia I, Garcia-Segura LM and Tresguerres JA. Estradiol and soy extract increase the production of new cells in the dentate gyrus of old rats. *Exp. Gerontol.* (2005) 40: 450-453
- (12) Shir Y, Campbell JN, Raja SN and Seltzer Z. The correlation between dietary soy phytoestrogens and neuropathic pain behavior in rats after partial denervation. *Anesth. Analg.* (2002) 94: 421-6
- (13) Khalili M, Atyabi M. Antinociceptive effects of oral and intraperitoneal administration of alcoholic *Datura stramonium* seeds extract in male rats. *Iranian J. Pharmaceut. Res.* (2004) 4: 231-236
- (14) Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* (1977) 4: 161-174
- (15) Zhao C, Wacnik PW, Tall JM, Johns DC, Wilcox GL, Meyer RA and Raja SN. Analgesic effects of a soy-containing diet in three murine bone cancer pain models. *J. Pain* (2004) 5: 104-110
- (16) Hunskaar S and Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* (1987) 30: 103-114
- (17) Shibata M, Ohkubo T, Takahashi H and Inoki R. Modified formalin test: characteristic biphasic pain response. *Pain* (1989) 38: 347-352
- (18) Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. *Pain* (1992) 51: 5-17
- (19) Kim H, Peterson TG and Barnes S. Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor beta signaling pathways. *Am. J. Clin. Nutr.* (1998) 68: 1418S-1425S
- (20) Chacko BK, Chandler RT, Mundhekar A, Khoo N, Pruitt HM, Kucik DF, Parks DA, Kevil CG, Barnes S and Patel RP. Revealing anti-inflammatory mechanisms of soy isoflavones by flow: modulation of leukocyte-endothelial cell interactions. *Am. J. Physiol. Heart Circ. Physiol.* (2005) 289(2):908-15
- (21) Gottstein N, Ewins BA, Eccleston C, Hubbard GP, Kavanagh IC, Minihane AM, Weinberg PD and Rimbach G. Effect of genistein and daidzein on platelet aggregation and monocyte and endothelial function. *Br. J. Nutr.* (2003) 89: 607-616
- (22) Bu L, Setchell KD and Lephart ED. Influences of dietary soy isoflavones on metabolism but not nociception and stress hormone responses in ovariectomized female rats. *Reprod. Biol. Endocrinol.* (2005) 3: 58
- (23) Lephart ED, Galindo E and Bu LH. Stress (hypothalamic-pituitary-adrenal axis) and pain response in male rats exposed lifelong to high vs. low phytoestrogen diets. *Neurosci. Lett.* (2003) 342: 65-68
- (24) McRoberts JA, Li J, Ennes HS and Mayer EA. Sex-dependent differences in the activity and modulation of *N*-methyl-d-aspartic acid receptors in rat dorsal root ganglia neurons. *Neuroscience* (2007) 148: 1015-20
- (25) Liu B, Eisenach JC and Tong C. Chronic estrogen sensitizes a subset of mechanosensitive afferents innervating the uterine cervix. *J. Neurophysiol.* (2005) 93: 2167-2173
- (26) Dawson-Basoa M and Gintzler AR. Involvement of spinal cord delta opiate receptors in the antinociception of gestation and its hormonal simulation. *Brain Res.* (1997) 757: 37-42
- (27) Stefano GB, Prevot V, Beauvillain JC, Fimiani C, Welters I, Cadet P, Breton C, Pestel J, Salzet M and Bilfinger TV. Estradiol coupling to human monocyte nitric oxide release is dependent on intracellular calcium transients: evidence for an estrogen surface receptor. *J. Immunol.* (1999) 163: 3758-3763
- (28) Qiu J, Bosch MA, Tobias SC, Grandy DK, Scanlan TS, Ronnekleiv OK and Kelly MJ. Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. *J. Neurosci.* (2003) 23: 9529-9540
- (29) Dina OA, Aley KO, Isenberg W, Messing RO and Levine JD. Sex hormones regulate the contribution of PKCepsilon and PKA signalling in inflammatory pain

- in the rat. *Eur. J. Neurosci.* (2001) 13: 2227-2233
- (30) Joseph EK and Levine JD. Sexual dimorphism for protein kinase c epsilon signaling in a rat model of vincristine-induced painful peripheral neuropathy. *Neuroscience* (2003) 119: 831-838
- (31) Malmberg AB, Chen C, Tonegawa S and Basbaum

AI. Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. *Science* (1997) 278: 279-283

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