African Journal of Biotechnology Vol. 9(34), pp. 5481-5487, 23 August, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.2012
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Quantification of substance P mRNA expression in the midbrain of ovariectomized migraine rats with SYBR green I real-time polymerase chain reaction

Ting-min Yu*, Gang Yao, Yu-hong Man and Xi-jing Mao

Department of Neurology, the Second Hospital of Jilin University, Changchun, Jilin 130041, China.

Accepted 8 July, 2010

This study was designed to develop a SYBR green I-based real-time polymerase chain reaction (RT-PCR) for quantitative detection of substance P (SP) mRNA in the midbrain of ovariectomized migraine rats and to evaluate the effects of estradiol on the mRNA expression of SP in order to shed light on the mechanisms underlying the pathogenesis of migraine and estrogen-conferred protection against migraine. 24 female rats were randomly assigned to the following groups: A: non-migraine controls; B: migraines; C, migraine rats receiving low estradiol; D, migraine rats receiving high estradiol. One week following ovariectomy, migraine was induced in groups B, C and D by nitroglycerin (i.p.). Behavior changes before and after migraine was examined. A SYBR green I-based RT-PCR assay was established to measure the absolute levels of SP mRNA in the midbrain. Behavioral changes in group D were significantly mitigated when compared with those in group B, whereas no marked behavioral changes were noted in groups C and B. In addition, mRNA copies of SP in group B were remarkably lower than group A, while the level of SP mRNA in both groups C and D was higher than group B, although no significance was reached (P > 0.05). SP mRNA expression decreased in the midbrain of migraine rats when compared with the non-migraine controls. High doses of estrogen partially restored SP expression in migraine rats and reduce migraine attack. Our study validated the SYBR green I-based RT-PCR technique for quantitative detection of SP mRNA.

Key words: Substance P, migraine, estrogen, midbrain, real-time quantitative polymerase chain reaction, rats.

INTRODUCTION

Migraine is a common neurological complaint with a higher incidence in women than in men, although the underlying mechanism of action remains poorly defined. Numerous studies have suggested a possible link between various neuropeptides such as substance P (SP), calcitonin generelated peptide and neurokinin A and migraine attack. Given the critical role of SP in sensory information transmission and pain modulation, intense effort has been made in attempt to unravel the precise mechanism of action of SP in migraine attack.

*Corresponding author. E-mail: yecolor@163.com. Tel: +86-431-88796298.

Abbreviations: RT-PCR, Real-time polymerase chain reaction; **SP,** substance P; **PAG,** periaqueductal gray; **Ct,** threshold cycles.

Multiple lines of evidence have shown that SP can trigger neurogenic inflammation, including vasodialtion, plasma extravasation and mast cell degranulation, thereby leading to hyperalgia. A study showed higher plasma SP levels in migraine patients compared with healthy individuals (Fusayasu et al., 2007). In addition, previous studies also demonstrated an abundant presence of SP-positive and SP receptor-positive neuronal cell bodies and dendrites in migraine-related brain areas such as periaqueductal gray (PAG) and raphe nuclei (Nakaya et al. 1994; Otsuka and Yoshioka, 1993). However, few studies have been conducted to directly address the quantification of SP mRNA in brain regions of migraine subjects.

Midbrain PAG is the main component of endogenous analgesic system involved in the autonomic regulation of pain, fear and anxiety. Raskin and colleagues found that the implantation of a stimulating electrode in PAG can induce a migraine-like headache (Raskin et al., 1987).

Lehner et al. (2004) showed high levels of pain-stimulated

c-fos expression in the PAG, further illustrating the concept that the PAG area is a critical region participating in the pain modulation. Migraine occurs more frequently in women than in men. Migraine attack in most female is associated with the menstrual cycle. Women are more prone to migraine attacks during the menstrual cycle, suggesting a role of low estrogen level in migraine and a possible benefit of estrogen supplementation in the management of migraine. An earlier study reported a reduction in the number of headache days per cycle following ethinyl estradiol treatment on days 1 to 21 and conjugated estradiol supplement on days 22 to 28 in women with menstrual-associated migraine (Calhoun, 2004).

As a widely accepted assessment for accurate and quantitative detection of nucleic acid molecules, real-time quantitative fluorescent polymerase chain reaction (PCR) has been widely used in various fields, including transgenic studies, gene expression profiling, drug efficacy evaluation and pathogen detection. Recently, great advances have been achieved in real-time PCR technique, among which, methods based on SYBR green I dye and TaqMan probes are most frequently applied.

The present study established a reliable and sensitive assay for quantifying the levels of SP mRNA in the midbrain of migraine rats via SYBR green I real-time PCR. In an attempt to shed some light on the underlying mechanisms of migraine development and estrogen-afforded protection against migraine development, this assay was later applied to assess the effects of estrogen administration on the SP mRNA expression in the midbrain of ovariectomized migraine rats.

MATERIALS AND METHODS

Reagents and equipment

Estradiol injection was obtained from General Pharmaceutical (Shanghai, China). Nitroglycerin was provided by Kangbao Biological Products (Shanxi, China). RNAiso Reagent, RNA PCR Kit (AMV) Ver.3.0, E.coli DH5α, pMD18-T Vector, restriction enzymes BamH I and Hind III and SYBR Premix Ex TaqTM were all purchased from TaKaRa (Dalian, China). An AxyPrep DNA Gel Extraction Kit and an AxyPrep Plasmid Miniprep Kit were obtained from Axygen Biosciences (USA). PCR was performed using GeneAmp PCR System2400 from PerkinElmer (Norwalk, USA). Centrifugation was performed using a Mikro 22R centrifuge (Hettich Zentrifugen, Germany). PCR products were separated using the Sub-Cell GT Agarose Gel Electrophoresis System (Bio-Rad, USA) with a BIO-RAD Power PAC 200/300 power supply (Bio-Rad, USA). The PCR products were later visualized with a UV-2401PC spectrophotometer (Shimadzu, Japan) and data were analyzed with Shimadzu UVPC software (version 3.5). The real-time quantitative PCR was performed by using ABI PRISM 7000.

Animals and treatment

Twenty-four adult healthy female Wistar rats provided by Experimental Animal Center of Basis Medical College of Jilin University weighing 210 ± 10 g, underwent ovariectomy after one week of habituation.

These ovariectomized rats were randomly divided into four equal groups: Group A, non-migraine controls; group B, migraine rats; group C, migraine rats receiving a low level of estradiol; group D, migraine rats receiving a high level of estradiol. Starting from the seventh day following ovariectomy, rats in groups A and B were given a daily intraperitoneal injection of 0.1 ml of peanut oil vehicle, while rats in groups C and D were intraperitoneally injected with 0.1 and 1.0 mg/kg of E2, respectively. Fourteen days after ovariectomy, rats in groups B, C and D were subcutaneously injected with 10 mg/kg of nitroglycerin to induce migraine, whereas group A was given equal volumes of peanut oil via subcutaneous injection. All procedures involving animal treatment were reviewed and approved by our institutes animal care and use committee.

Scoring of behavioral changes

Our previous studies found that the symptoms of migraine were most marked and clustered within the first five minutes following latency. Therefore, rats with 6 points during the first five minutes after latency were considered to be migraine-positive. The scoring of nitroglycerin-induced behavioral changes during the first five minutes after latency was performed as follows: Ten times of head scratching scored one point (each subsequent scratch scored 0.1 more point); two instances of turning scored one point (each subsequent turn added one more point); three occurrences of cage climbing scored one point (each subsequent climb added 0.1 point); one instance of forward and backward movement scored one point (each subsequent time added one more point); one instance of tail biting scored one point; and the presence of red ear scored one point.

RNA isolation and cDNA synthesis

Two hours after subcutaneous injection of nitroglycerin (peanut oil in group A), rats were anesthetized with 10% of chloral hydrate (0.3 ml/100 g) and sacrificed by cervical dislocation. Rat midbrains were then collected and stored immediately in liquid nitrogen at −70°C until detection. To isolate total RNA, 1 ml of RNAiso Reagen was added to 50 - 100 mg of midbrain tissues and the procedures for total RNA extraction were performed according to the instructions of the manufacturer. After gel electrophoresis, the purity and concentration of total extracted RNA was determined by absorbance at 260 and 280 nm. The A_{260}/A_{280} ratio of pure total RNA was expected to be in the range of 1.9 - 2.0.

Later, the cDNA synthesis was performed in a 20 µl of reaction system comprising 300 ng of RNA, 4 µl of $MgCl_2$, 2 µl of 10×RT buffer, 2 µl of dNTP mixture (10 mM each), 0.5 µl of RNase inhibitor, 1 µl of AMV reverse transcriptase, 1 µl of random 9 mers and RNase Free dH_2O. The total RNA was reverse transcribed at 30°C for 10 min, 42°C for 30 min, denatured at 99°C for 5 min and quenched at 5°C for 5 min.

Preparation of SP standard plasmids

The SP-specific primers were synthesized as follows: forward, 5'-TGGCGGTCTTTTTTCTCGTT-3' and reverse, 5'-GCATTGCCTCC TTGATTTGG-3 (Tang et al., 2007). A product size of 114 bp was expected to be generated after PCR amplification. The PCR program was initiated at 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 54°C for 50 s, 72°C for 60 s and a final step of 72°C for 10 min. The amplified PCR products were separated on a 2% agarose gel and recovered thereafter. The recovered PCR products were then ligated into pMD-18T vectors, which were later transformed into competent *Escherichia Coli* DH5α cells. After selection for ampicillin-resistant clones, the positive plasmids were extracted and

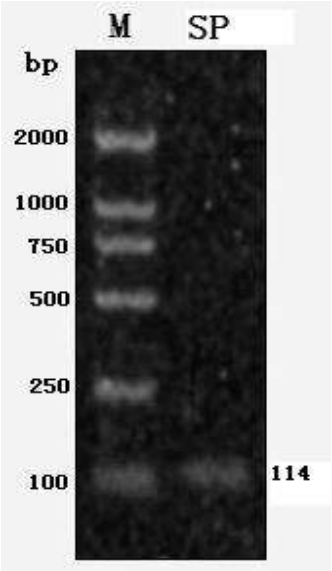

Figure 1. Agarose gel electrophoresis of PCR amplification products of SP. The band at 114 bp indicates the SP target gene.

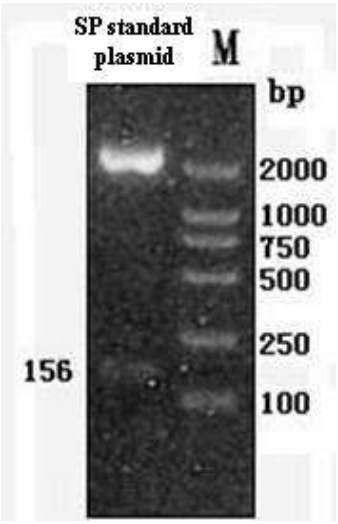

Figure 2. SP gene standard plasmids double digested by restriction enzymes Hind III/BamH I. The band at 156 bp shows the SP recombinant plasmid.

identified by restriction enzymes Hind III and BamH I. The full cDNA sequence of SP was later confirmed to be correct by sequencing analysis. Subsequently, OD_{260} values of the extracted plasmids were assayed at a wavelength of 260 nm.

SYBR green I real-time PCR

SYBR green I real-time PCR was performed according to the manufacture's protocol in a 20 µl of reaction system comprising of 10 µl of SYBR Premix Ex Taq^{TM}, 0.4 µl of forward and reverse primers each (10 uM), 0.4 µl of ROX reference dye, 2.0 µl of cDNA sample and 6.8 µl of dH_2O. To generate a standard curve, the recombinant standard plasmids were serially diluted with sterilized water to 1.6 × 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 10^3, 10^2, 10^1 and 10^0 copies/µl and were subjected to PCR amplification (in triplicates). The program for PCR amplification was initiated at 94°C for two minutes, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 54°C for 50 s and extension at 72°C for 60 s. Fluorescent signals were detected at the end of annealing in each PCR cycle. The threshold of fluorescence detection was set at the number of threshold cycles (Ct) corresponding to the inflection point of the fluorescence curve from the baseline to the exponential growth phase. The melting analysis was performed during the PCR reaction using the following program: 95°C for 15 s, 60°C for 20 s and 95°C for 15 s.

Statistical analysis

All data were analyzed by the Statistical Analysis System (SAS) software. The results of behavioral scoring were expressed as mean ± standard derivation ($\bar{X}$ ± S). Paired t tests were used to compare differences between the various groups. Due to the wide variations of SP mRNA copies among different groups, the results of SP mRNA were presented as median ± interquartile range (M ± Q). A rank sum test was conducted to statistically comparing mRNA copy differences between groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Behavioral observation

In accordance with the scoring criteria for nitroglycerin-induced migraine model (those having 6 points within the first five minutes after latency were considered to have migraine), the success rate of migraine induction achieved 100% in all of our animal groups. The behavioral scores of groups A, B, C and D were 1.60 ± 0.53, 28.78 ± 2.51, 25.70 ± 2.32 and 23.25 ±3 .35, respectively. Compared with the control group, significant differences regarding the behavioral scores of groups B, C and D were noted ($P < 0.01$). In contrast, no marked difference of behavioral scores was observed between groups C and B ($P > 0.05$), whereas a remarkable discrepancy of behavioral scores was found between groups D and B ($P < 0.05$).

Preparation of SP plasmid standards

A 114-bp amplification fragment of SP gene was generated by using a conventional PCR method and separated on a 2% agarose gel (Figure 1). The length of the pMD-18T vector was 2692 bp; thus the resultant recombinant vector was 2806 bp in length. After double digestion by restriction enzymes Hind III and BamH I, the recombinant vector yielded two fragments corresponding to two DNA bands at 2650 and 156 bp, respectively (Figure 2), which was consistent with the expected product lengths. DNA sequencing results of the cloned DNA provided by Takara (Dalian, China) revealed that the cloned fragment had

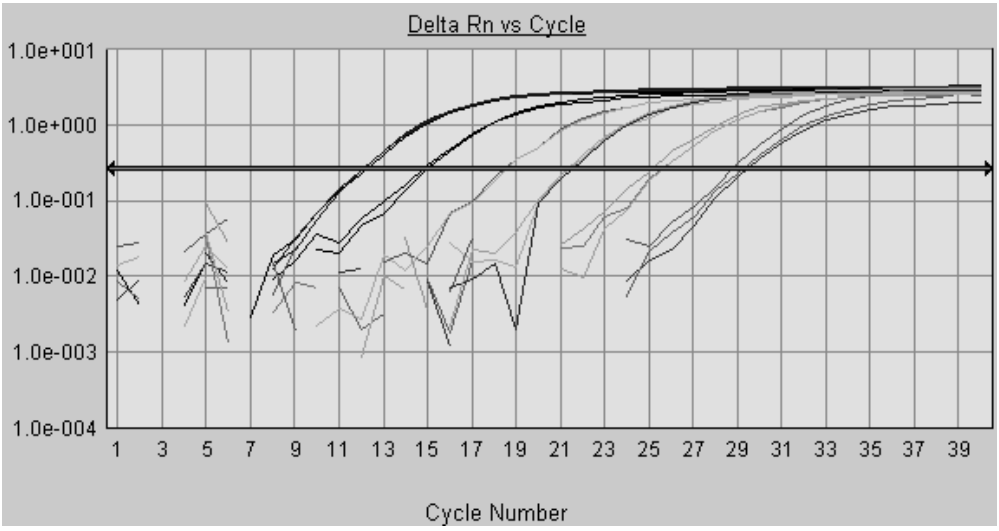

Figure 3. Amplification curves of serial dilutions of SP gene standard plasmids. The copy number of SP gene template varied from 1.6×10^9copies/μl in the leftmost to 1.6×10^4copies/μl in the rightmost of the amplification curves.

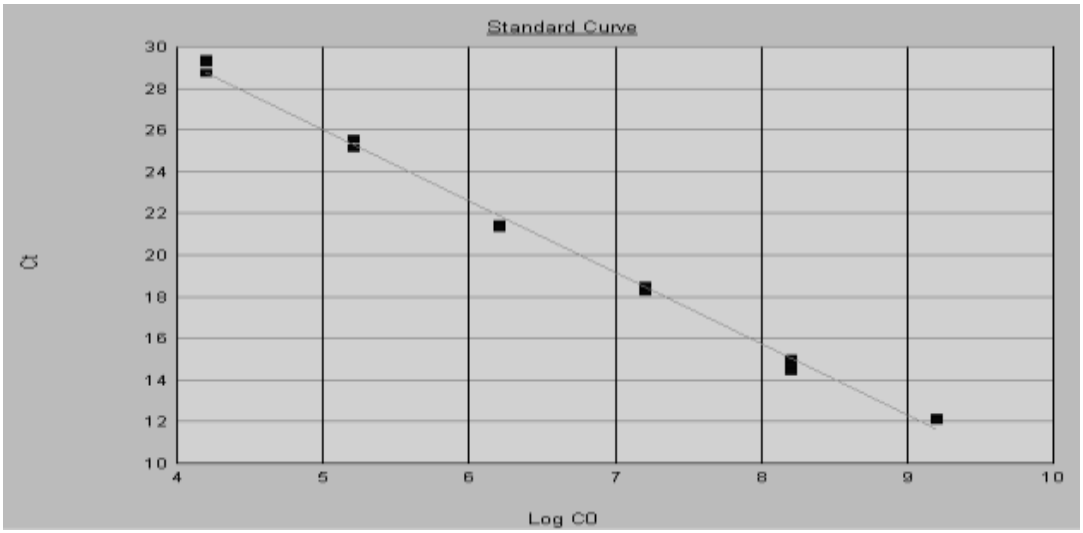

Figure 4. The standard curve of SP gene plasmid standards used in real-time fluorescent quantitative PCR (Ct = -3.428Log(x) + 43.18, r = 0.995).

100% sequence homology with the target SP gene sequence.

Amplification curves and linear detection range

The PCR standard curve was plotted with the standard SP plasmids. The linear range of the standard curve covered six orders of magnitude. The maximum detection limit was 1.6×10^9 copies/μl and the minimal detection limit was 1.6×10^4 copies/μl (Figure 3). The regression equation of the standard curve was Ct = -3.428Log(x) + 43.18，r = 0.995 (Figure 4).

Homogeneity of amplified products

The melting curve analysis shows that the peaks of the PCR products derived from serially diluted SP gene plasmid standards were uniformly situated at 82°C with a sharp peak, indicating the high homogeneity of the obtained PCR products (Figure 5).

Expression of SP mRNA in the midbrain of rats with or without migraine

Based on the standard curve of serially diluted SP plasmid

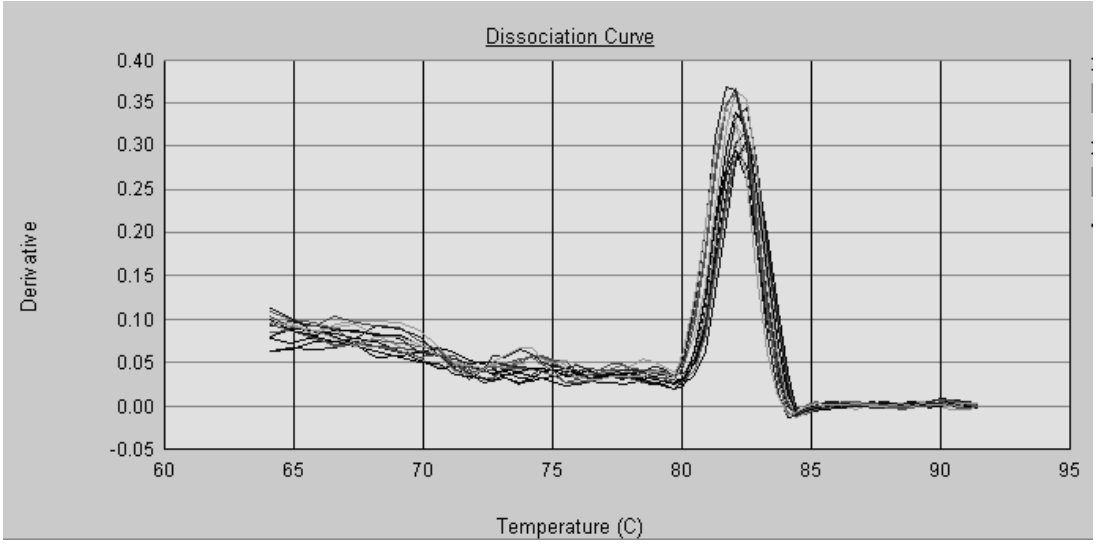

Figure 5. Melting curve analysis of the PCR products amplified with serially diluted SP gene plasmid standards. The peaks of the PCR products were uniformly situated at 82°C with a sharp peak, indicating high homogeneity of the obtained PCR products.

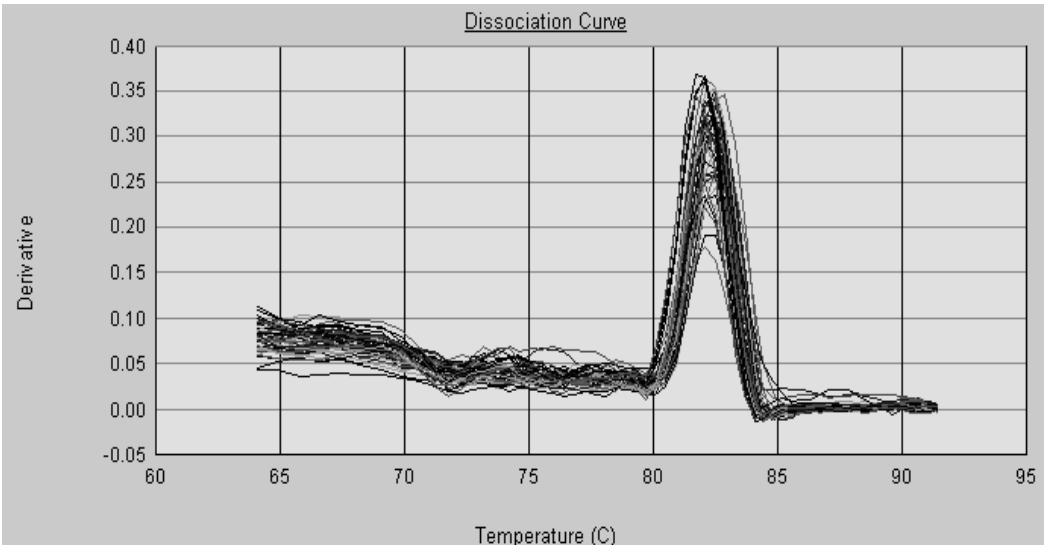

Figure 6. Melting curve analysis of amplified SP products in four groups. The peaks of the melting curves of PCR products amplified with SP templates in all four groups were also uniformly localized at 82°C, showing high homogeneity of PCR products.

standards and real-time quantitative PCR, the initial content of SP DNA templates in various groups was calculated. The peaks of the melting curves of PCR products amplified with SP templates in all four groups were also uniformly localized at 82°C, showing a high homogeneity of PCR products (Figure 6). The SP mRNA copies ($\times 10^5$) in every 300 ng of total RNA in groups A, B, C and D were 2.08 ± 1.70, 0.50 ± 0.79, 1.19 ± 0.26 and 0.94 ± 0.84, respectively. The mRNA copies of SP in the midbrain of migraine rats were significantly lower than those of non-migraine controls ($P < 0.05$), whereas the mRNA copies of SP in the midbrain of migraine rats treated with either low or high levels of estradiol were higher than those of migraine rats without estradiol intervention but not to a significant level ($P > 0.05$) (Table 1).

DISCUSSION

In 1931, Von and Gaddum discovered a substance named substance P in the intestine and brain of the horse

Table 1. Comparison of SP mRNA expression levels among various groups ($\bar{X}$ ±S).

Group	No.	RNA copies/300ng (10^5)
Control (group A)	6	2.08±1.70
Migraine (group B)	6	0.50±0.79a
Low-dose estradiol (group C)	6	1.19±0.26
High-dose estradiol (group D)	6	0.94±0.84

a: Significant difference ($P < 0.05$) when compared with control group.

that could cause contraction of smooth intestinal muscle, vasodilation and reduction of blood pressure (Von and Gaddum, 1931). With continuous progress being made toward a better understanding of pain, the roles of SP in the sensory information transmission and pain modulation have attracted increasing attention. Currently, it is generally believed that SP in the peripheral tissues transmits pain messages to the central nervous system where SP acts mainly as an analgesic.

Han and colleagues showed that SP could induce the release of enkephalin from the PAG and thus may function as an analgesic (Zhou et al., 1985). A study found that SP was capable of modulating the function of PAG by affecting changes of inward and outward currents in the neurons localized in PAG (Drew et al., 2005). In addition, another study indicated that increased release of SP in PAG can boost the analgesic effects of opioid substances (Rosén et al., 2004).

Furthermore, it has been proven that differential expression of SP exists in various regions of the central nervous system when pain occurs. The number of SP-positive neurons was increased in the nucleus trigeminalis caudalis, while it was decreased in lumbar dorsal horns in a nitroglycerin-induced pain model (Greco et al., 2008). The current study shows that SP mRNA expression in the midbrain region of migraine rats was dramatically lower than that of non-migraine controls ($P < 0.05$), suggesting that the expression of SP gene decreases with the development of pain. The reduction of SP expression in the midbrain region may attenuate the analgesic effects of SP, which may contribute to the persistence of pain. However, the underlying cause for the reduction of SP gene expression in the midbrain region of nitroglycerin-induced migraine rats remains to be characterized. Nevertheless, since little is known about the pathogenesis and influencing factors of migraine, further study is warranted to better delineate the role of SP in migraine attacks.

The nitroglycerin-induced migraine animal model is a well-accepted model for migraine headache (Tassorelli et al., 2003). The behavioral and histopathological changes of successfully induced migraine rats (red ear, head scratching and cage climbing) bear some resemblance to the manifestations of migraine attack in humans. As a result, this model is commonly used in studies focusing on pathogenesis, pathophysiological and molecular basis of migraine. Behavior-based evaluation can be used to assess whether the nitroglycerin-induced migraine has been successfully established in experimental animals. A study suggested that the behavior-based assessment of nitroglycerin-induced migraine animal model could be utilized to evaluate the efficacy of therapeutics tailored for migraine headache (Fu et al., 2005). Hence, based on such behavioral criteria, the current study achieved fully successful model construction.

The prevalence of migraine headache is much higher in women than in men (Stewart et al., 1992). In addition, most of the migraine attacks in women seem to be closely correlated with their menstrual cycles, during which migraine attack develops more frequently than during intermenstrual periods, suggesting a possible involvement of estrogen in the development of migraine in women. A study indicated that a supplement of estrogen during menstruation could control and prevent the onset of migraine (Labbé et al., 1997). In this study, the estrogen level of the ovariectomized rats was kept at relatively constant levels by intraperitoneal administration of estradiol. Later, a migraine model was induced in these rats in order to observe the effects of different concentrations of estrogen on the behaviors and SP mRNA expression. Significant behavioral differences were noted between groups D and B, indicating that high levels of estrogen might offer some protection against the risk of migraine. This finding is consistent with the fact that the symptoms of migraine headache are alleviated in pregnancy during which estrogen usually reaches a higher level. Furthermore, SP mRNA copies in the midbrain region of groups C and D showed a trend of increase compared with group B, albeit, non-significant ($P > 0.05$). One of the previous studies demonstrated that estrogen is capable of stimulating the expression of SP receptors (Villablanca and Hanley, 1997). Nevertheless, this current study depicted that estradiol supplementation triggered a slight but insignificant increase in the expression of SP mRNA in the midbrain region of ovariectomized rats with migraine. These seemingly conflicting data with regards to the regulatory roles of estrogen on SP expression may suggest involvement of multiple processes in estrogen-conferred protection against migraine.

Conventional RT-PCR assay has been extensively used in the detection of mRNA transcription. Nevertheless, the inherent non-quantitative nature and poor reproducibility of

RT-PCR have greatly limited the application of this technique and the proper interpretation of its detection results. In recent years, real-time quantitative PCR has emerged as a highly promising method for mRNA quantification. Real-time fluorescence quantitative PCR can detect PCR products during the PCR reaction in a real-time manner and quantify the amounts of PCR products generated during the exponential phase of PCR reaction by the introduction of standard curves. Therefore, fluorescent signals emitted during the whole PCR amplification can be monitored in real-time and continuously analyzed in the real-time quantitative PCR. Along with the progression of PCR reaction, changes in fluorescent signals can be plotted. The number of amplification cycles needed to reach the preset threshold for fluorescent signal detection in each reaction tube is referred to as the Ct value. The Ct value of each DNA template is linearly correlated to the logarithm of its initial template copies. The higher the initial copy number, the smaller the Ct value. A standard curve can be drawn using serially diluted standard plasmids with known initial template copies in the real-time PCR. Once the Ct value of the sample of interest is obtained, the initial copies of this sample can be calculated from this standard curve (Ginziger, 2002). This study obtained the Ct value by a series of 10-fold dilution of standard plasmids and the correlation coefficient of the standard curve achieved was as high as 0.995. Therefore, the initial DNA template contents in various samples were calculated accurately based on the regression equation. Because the non-specific products and primer dimers originated from the nonspecific binding between SYBR green I and double DNA strands, similar amplification curves may be generated during the PCR amplification. Consequently, the inclusion of melting curve analysis is of importance in the PCR reaction. Results from our current study proved that PCR products from different groups have a uniform melting temperature and a high specificity.

Conclusion

In conclusion, our current study successfully established a SYBR green I-based real-time PCR assay for quantitative detection of SP mRNA. The standard curve constructed, exhibits a high linear correlation, sensitivity and reproducibility, thereby ensuring more accurate and reliable interpretation of the study results. This is supported by the application of this technique to quantify the expression of SP mRNA in midbrain of migraine rats where a decreased SP mRNA expression was found. These data suggest that the aberrant expression of SP gene may be implicated in the development of migraine. Although, estrogen intervention showed a trend to restore SP mRNA expression in ovariectomized migraine rats, this effect failed to reach significance.

Further studies are warranted to better elucidate the underlying mechanisms of down regulated expression of SP in migraine rats, as well as the association between migraine attack and estrogen levels. Research on the relationship between SP, migraine attack and estrogen should offer new guidance for the development of therapeutic strategies against migraine headache.

REFERENCES

Calhoun AH (2004). A novel specific prophylaxis for menstrual associated migraine. South Med. J. 97(9): 819-822.

Drew GM, Mitchell VA, Vaughan CW (2005). Postsynaptic actions of substance P on rat periaqueductal grey neurons *in vitro*. Neuropharmacology, 49(5): 587-595.

Fu XJ, Song XX, Zhou YH (2005). Evaluation of behavior and symptom of nitroglycerin induced hyperalgesia in model rats of migraine. Chin. J. Neuromed. 4(5): 449-451.

Fusayasu E, Kowa H, Takeshima T, Nakaso K, Nakashima K (2007). Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. Pain, 128(3): 209-214.

Ginziger DG (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exp. Hematol. 30(6): 503-512.

Greco R, Tassorelli C, Sandrini G, Di Bella P, Buscone S, Nappi G (2008). Role of calcitonin gene-related peptide and substance P in different models of pain. Cephalalgia, 28(2): 114-126.

Labbé EE, Murphy L, O'Brien C (1997). Psychosocial factors and prediction of headaches in college adults. Headache, 37(1): 1-5.

Lehner M, Taracha E, Skórzewska A, Wistowska A, Zienowicz M, Maciejak, P, Szyndler J, Bidziński A, Ptaźnik A (2004). Sensitivity to pain and c-Fos expression in brain structures in rats. Neurosci. Lett. 370(1): 74-79.

Nakaya Y, Kaneko T, Shigemoto R, Nakanishi S, Mizuno N (1994). Immunohistoche-mical localization of substance P receptor in the central nervous system of the adult rat. J. Comparative Neurol. 347(2): 249-274.

Otsuka M, Yoshioka K (1993). Neurotransmitter functions of mammalian tachvkinins. Physiol. Rev. 73(2): 229-308.

Raskin NH, Hosobuchi Y, Lamb S (1987). Headache may arise from perturbation of brain. Headache, 27(8): 416-420.

Rosén A, Zhang YX, Lund I, Lundeberg T, Yu LC (2004). Substance P microinjected into the periaqueductal gray matter induces antinociception and is released following morphine administration. Brain Res. 1001(1-2): 87-94.

Stewart WF, Lipton RB, Celentano DD, Reed ML (1992). Prevalence of migraine headache in the United States: relation to age, income, race, and other sociodemographic factors. JAMA, 267(1): 64-69.

Tang W, Song B, Zhou ZS, Lu GS (2007). Intrathecal administration of resiniferatoxin produces analgesia against prostatodynia in rats. Chin. Med. J. 120(18): 1616-1621.

Tassorelli C, Greco R, Wang D, Sandrini M, Sandrini G, Nappi G (2003). Nitroglycerin induces hyperalgesia in rats-a time-course study. Eur. J. Pharmacol. 464(2-3): 159-162.

Villablanca AC, Hanley MR (1997). 17beta-estradiol stimulates substance P receptor gene expression. Mol. Cell. Endocrinol. 135(2): 109-117.

Von EUS, Gaddum JH (1931). An unidentified depressor substance in certain tissue extracts. J. Physiol. 72(1): 74-87.

Zhou ZF, Xie GX, Han JS (1985). Release of enkephalin in periaqueductal gray stimulated by substance P can relieve pain in rabbit. Chin. Sci. Bull. 30: 69-73.