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ABSTRACT

Bergamot essential oil (BEO) has proven wide evidence of pharmacological antinociceptive effectiveness both in nociceptive and in neuropathic pain models. The antinociceptive properties of BEO for inhalation have not been investigated. The purpose of this study is to evaluate the effects of the inhalation of BEO on formalin-induced nociceptive response in mice.

Male ddY-strain mice (Japan SLC, Hamamatsu, Japan) of 23–25 g of weight at the time the experiments underwent the formalin test. Twenty μl of formalin (2% in saline) were administered into the plantar surface of the mice hindpaw and the time of licking/biting was observed and recorded at intervals of 5 min. The device for BEO inhalatory delivery consisted in a filter paper disc soaked with known volume of BEO placed on the edge of the cage.

Inhalation of BEO exerted antinociceptive activity. In particular, it reduced the formalin-induced licking/biting behaviour in a manner that was dependent on the volume of BEO used in the device for its release and on the time of exposure to the phytocomplex.

The results support the use of BEO in aromatherapy for complementary management of chronic pain relief in a stepwise therapeutic programme.

1. Introduction

According to the Farmacopea Ufficiale Italiana (1991) bergamot essential oil (BEO) is obtained by cold pressing of the epicarp and, partly, of the mesocarp of the fresh fruit of bergamot (*Citrus bergamia* Risso et Poiteau). BEO comprises a volatile fraction (93–96% of total) containing monoterpene and sesquiterpene hydrocarbons (such as limonene) and oxygenated derivatives (such as linalool) and a non-volatile fraction (4–7% of total) containing waxes, polymethoxylated flavones, coumarins and psoralens such as bergapten (5-methoxypsoralen) and bergamottine (5-geranyloxypsoralen) [1, 2]. The most abundant compounds found in the volatile fraction are the monoterpene hydrocarbons limonene, γ-terpinene, and β-pinene, the monoterpene alcohol, linalool, and the monoterpene ester, linalyl acetate, which altogether constitute > 90% of the whole oil [3–5]. The nonvolatile residue is a natural odor fixative which influences the olfactory properties of the oil; however, it contains about 0.2% bergapten which is responsible for the phototoxicity of BEO [6, 7]. Therefore, a bergapten-free extract of the essence (BEO-BF) together with a natural essence deprived of the hydrocarbon fraction and of bergapten (BEO-HF/BF) are prepared by extractive industries for perfumery and cosmetic uses. Recently, this essential oil has been rigorously studied and some pharmacological activities of the utmost importance have been deciphered. In particular, strong evidence has been gathered for BEO to be endowed with analgesic activity, both in nociceptive and in neuropathic pain models (see [8]). In fact, intraplantar (i.pl.) BEO, or its components linalool and linalyl acetate, reduced the nociceptive response as assayed by the capsaicin test [9]. The latter antinociceptive effects were antagonized by the ipsilateral i.pl. injection of naloxone hydrochloride and by intraperitoneal (i.p.) naloxone methiodide, an

Abbreviations: BPSDs, Behavioural and psychological symptoms of dementia; BEO, Bergamot Essential Oil; BEO-BF, Bergapten-free extract of the essence; BEO-HF/BF, Essence deprived of the hydrocarbon fraction and of bergapten; i.p., Intraperitoneal; i.pl., Intraplantar; i.t., Intrathecal; PSNL, Partial Sciatic Nerve Ligation

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antagonist acting at the peripheral opioid receptors; morphine-induced antinociception after i.p. and intrathecal (i.t.) injections was markedly enhanced by the combined injection of i.pl. BEO or linalool, its main oxygenated monoterpene [9]. Interestingly, for i.pl. injection BEO or linalool reduced partial sciatic nerve ligation (PSNL)-induced neuropathic pain symptoms in mice and inhibition of spinal ERK phosphorylation seems to be involved in this anti-allodynic effect [10]. These same Authors [9] have shown that BEO or linalool modulate morphine-induced anti-allodynic effect under neuropathic pain, a condition known to be resistant to opioid treatment (see [11]). More recently, for i.pl. injection BEO and linalool have been reported to reduce behavioural signs of formalin-induced nociception in a dose-dependent manner. The formalin test is characterized by formalin-induced biphasic nocifensive behaviour of licking/biting, with the early nociceptive phase being followed by a late, second, phase that involves peripheral inflammation and central sensitization (see [12]). Due to the lack of relevant information about the effects of the inhalation of BEO on nocifensive behaviour, the purpose of this study was to investigate the antinociceptive action of BEO *via* the inhalatory route of administration. Indeed, according to the literature, BEO inhalation was found to produce anxiolytic-like behaviour [13], but there are no data available about its effect on nociception and this may be relevant to the use of BEO in aromatherapy.

2. Materials and methods

2.1. Animals

For the study, male ddY-strain mice (Japan SLC, Hamamatsu, Japan) of 23–25 g of weight at the time of these experiments were used. Mice were individually housed in a colony maintained in a controlled environment (12 h light/dark cycle, room temperature 23 °C, 50–60% relative humidity), with food and water *ad libitum*. All of the experiments were performed in agreement with the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals and approved by the Committee of Animal Care and Use of Tohoku Medical and Pharmaceutical University, for minimizing animal suffering and to use only the number of animals necessary to produce reliable results.

2.2. Experimental protocol for BEO administration via inhalatory route

The habituation of mice was carried out in a transparent cage (22.0 cm × 15.0 cm × 12.5 cm), that also served as observation chamber. Another plexiglass cage was turned upside down and placed over the first cage, in order to avoid any leaks of BEO. A filter paper dry disc (control) or soaked with different volumes of BEO according to the experiment (100, 200, 400, 800 µl) was applied on the edge of the cage 5 min before placing the mice in the observation chamber, so that it was saturated with BEO. Mice were divided into three experimental groups (post-inhalation, pre-inhalation and double-inhalation). In the post-inhalation group the inhalation of BEO was carried out immediately after the i.pl. injection of formalin and for the whole duration of the formalin test. In the pre-inhalation group the inhalation of BEO was carried out as pre-treatment for 1 h, during the mice habituation, at the end of which BEO-releasing filter paper was removed and the formalin test was performed. In the double-inhalation group the inhalation of BEO was carried out both as pre-treatment for 1 h during the habituation and immediately after formalin administration for the whole duration of the formalin test, in order to assess the total effects of these two different options of delivery of BEO.

2.3. Formalin test

After 1 h of habituation 20 µl of formalin (2% in saline) were i.pl. administered to the mice, using a microsyringe with 26-gauge needle. The time of licking/biting was recorded with a handheld stop-watch at intervals of 5 min: during the early phase, beginning immediately after formalin administration and lasting for 10 min (0–10 min), and during the late phase, starting 10 min after formalin injection and lasting for 20 min (10–30 min).

2.4. BEO composition

BEO was obtained from “Capua Company1880 S.r.l.,” Campo Calabro, Reggio Calabria (Italy). According to chromatographic analysis provided in the certificate of analysis, this batch of BEO contains: D-limonene (39.60%), linalyl acetate (31.09%), linalool (9.55%).

2.5. Statistical analysis

The results are presented as mean ± s.e.m. duration (seconds) of nociceptive response and evaluated statistically for differences by ANOVA followed by Bonferroni's test and considered significant when $p < 0.05$.

3. Results

3.1. Effect of BEO inhalation as post-treatment on formalin test evoked licking/biting

In the post-inhalation group (see treatment scheme in Fig. 1 a) the filter paper disc, applied on the edge of the cage, was soaked with 200, 400 or 800 µl of BEO and the mice were subjected to BEO inhalation from the time of formalin injection for the following 30 min, during which the formalin test was carried out. Under these experimental conditions, BEO did not show significant effects on the early phase (0–10 min) (Fig. 2). However, administration of 400 and 800 µl of BEO significantly reduced the time of licking/biting in the late phase (10–30 min) in a dose-dependent manner (Fig. 2).

3.2. Effect of BEO inhalation as pre-treatment on licking/biting

Mice were subjected to the inhalation of BEO (filter paper disc soaked with 200, 400 or 800 µl) for the whole habituation period of 1 h (see treatment scheme in Fig. 1 b) but not during the formalin test.

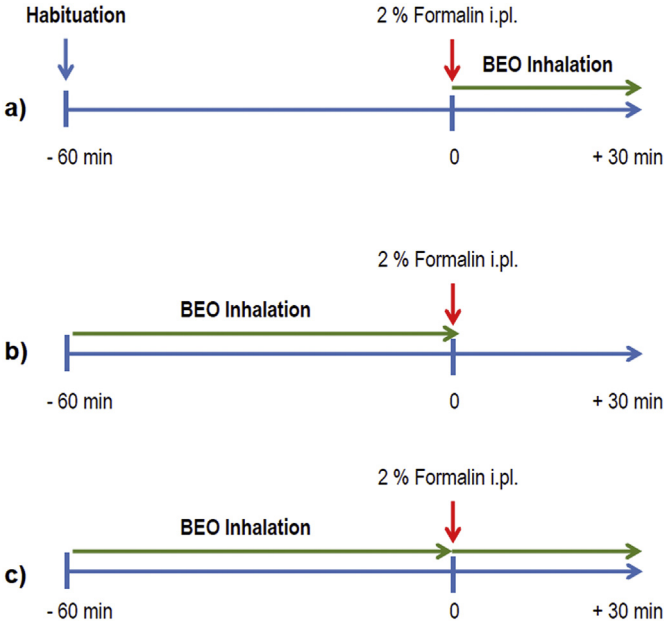

Fig. 1. BEO inhalation scheme. Schematic representation of the administration scheme of BEO as: a) post-inhalation, b) pre-inhalation and c) double-inhalation in relation to formalin intraplantar (i.pl.) administration.

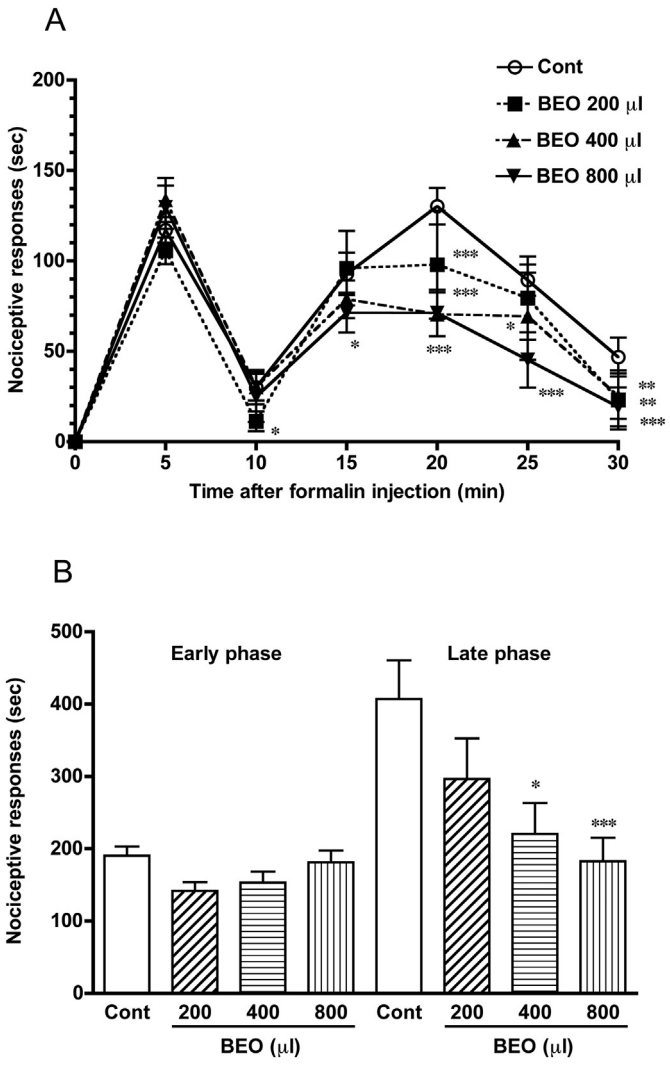

Fig. 2. Effect of BEO inhalation on the time of licking/biting in the post-inhalation group. Time of licking/biting is represented as mean ± s.e.m. duration expressed in seconds (sec). Statistical significance was by ANOVA followed by Bonferroni's test (n = 8 mice per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* control (Cont).

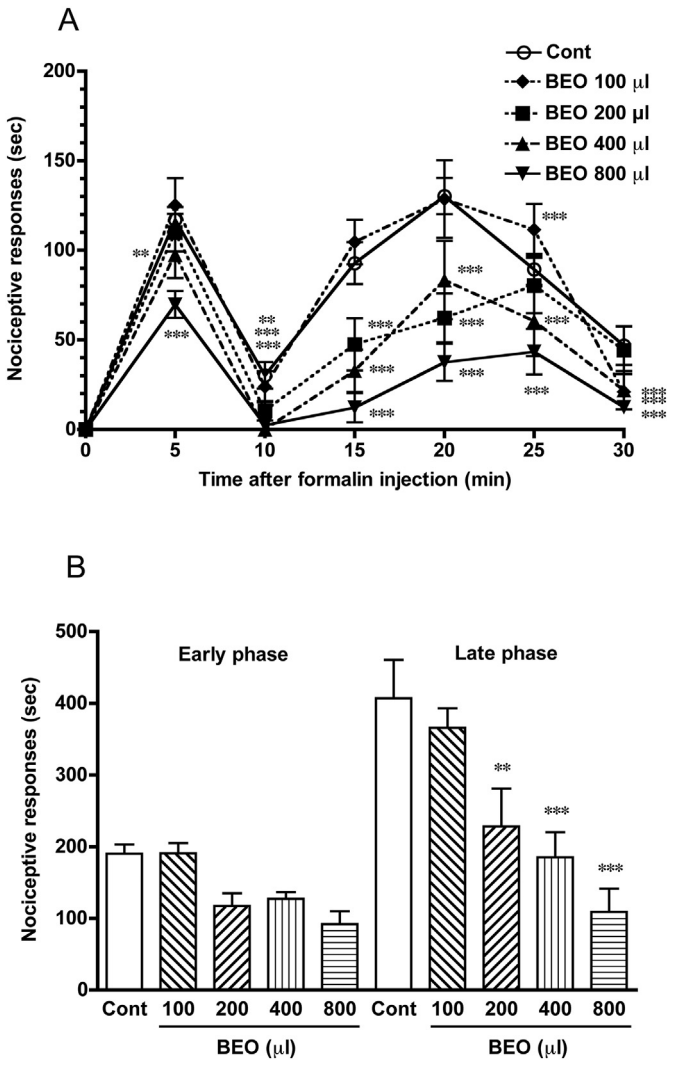

Fig. 3. Effect of BEO inhalation as pre-treatment (pre-inhalation group) on the time of licking/biting. Time of licking/biting is represented as mean ± s.e.m. duration expressed in seconds (sec). Statistical significance was by ANOVA followed by Bonferroni's test (n = 8 mice per group). *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control (Cont).

Under these experimental conditions, the effects of BEO were dose-dependent and even the smaller volume, which had previously resulted ineffective in the post-inhalation group, resulted effective (Fig. 3).

3.3. Effect of the inhalation of BEO on licking/biting after formalin administration (post-inhalation group) as compared with the effect reported in the pre-inhalation and the double-inhalation groups

Comparison of the effects of the inhalation of BEO (800 µl) on the time of licking/biting, recorded at 5 min intervals, in the 3 experimental groups (post-, pre- and double-inhalation groups) unraveled that, while in the early phase BEO was efficacious in a statistically significant manner only at 5 min and if administered as pre-treatment (pre-inhalation group) or both as pre-treatment and after formalin injection (double-inhalation group) ($p < 0,001$; Fig. 4 a), in the late phase it resulted effective also when administered just immediately after formalin injection (post-inhalation group) (Fig. 4 a and b). However, BEO administered in pre-inhalation and in double-inhalation scheme resulted more effective than when administered after formalin injection (post-inhalation group).

4. Discussion

BEO is often used in aromatherapy (see [13]), a specialized form of phytotherapy recorded for thousand years, used nowadays in industrialized countries as complementary medicine to control symptoms of anxiety, depression, pain and sleep disorders, among others. At variance with the psychological responses, most reliant on the individual experience, pharmacological mechanisms for inhalatory aromatherapy stem from components of the phytocomplex entering the body *via* the bloodstream by absorption through the lungs or olfactory mucosa [14]. In line with the latter concept, our data demonstrate that BEO is endowed with antinociceptive effects when administered *via* the inhalatory route in mice. In fact, under our present experimental conditions, a paper filter disc soaked with different volumes of BEO and applied at the edge of a plexiglass cage allowed the effects of inhalation of BEO to be studied in the formalin-induced nocifensive response in ddY-strain mice. Exposure to BEO *via* inhalation occurred according to three different treatment schedules (Fig. 1): the post-inhalation group of mice was exposed to BEO immediately after formalin injection, the pre-inhalation group was exposed for the whole habituation, but not during the formalin test, and the double-inhalation group was exposed

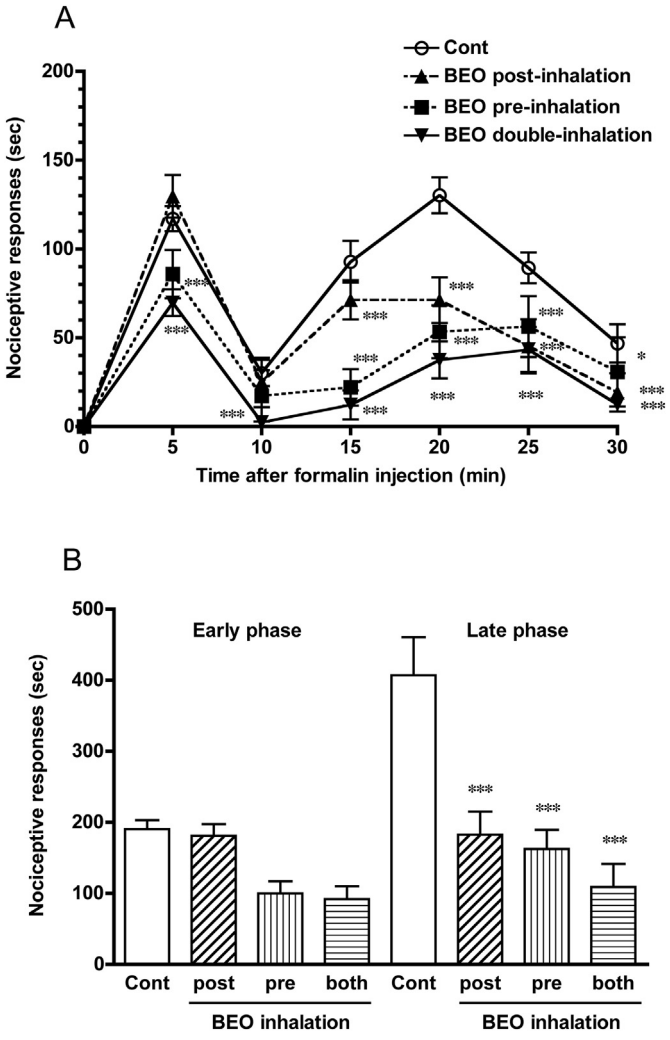

Fig. 4. Effect of the inhalation of BEO (800 μl) after formalin administration (post-inhalation group) compared with its inhalation as pre-treatment (pre-inhalation group) and with its inhalation during habituation and for the whole duration of the formalin test (double inhalation group) on the duration of licking/biting. Time of licking/biting is represented as mean ± s.e.m. duration expressed in seconds (sec). Statistical significance was by ANOVA followed by Bonferroni's test (n = 8 mice per group). *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control (Cont).

to BEO both during habituation and immediately after formalin injection, in order to evaluate the total effect of either inhalation times. The results demonstrate that, when administered immediately after formalin, BEO reduced in a dose-dependent and statistically significant manner the nocifensive behaviour in the late phase. When administered as pre-treatment, during the habituation of the mice, BEO was dose-dependently effective; interestingly, the lower volume used, inactive in the post-treatment (post-inhalation group), here resulted active. Finally, BEO resulted effective in the late phase when administered immediately after formalin and this is at variance with the effect on the early phase that has been observed only at 5 min, after pre-treatment administration or if administered both as pre-treatment and after formalin. Nevertheless, the administration of BEO carried out as pre-treatment and, both as pre-treatment and immediately after formalin for the whole duration of the formalin test, remained more effective than BEO given immediately after formalin injection. These results demonstrate that BEO is endowed with consistent and reproducible antinociceptive properties when administered *via* the inhalatory route. It has been previously reported that in the formalin test i.pl. linalool recapitulates the antinociception of BEO (given i.pl.) and that opioid receptor antagonists (*e.g.* naloxone hydrochloride and methiodide, the latter being unable to cross the blood brain barrier) could attenuate BEO- or linalool-induced antinociception, suggesting that the peripheral opioid system takes part in the analgesic activity of the essential oil [12]. Indeed, for systemic administration BEO interferes with exocytotic and membrane transporter-mediated release of glutamate in the rat hippocampus and this effect is lost when the monoterpene hydrocarbon-free fraction of the essential oil is used [15]. Most recently, BEO has been shown to enhance basal and induced autophagy [16], a mechanism whose derangement has been implicated in pain sensitization [17]. On the other hand, linalool inhibited the licking/biting response induced by i.t. injection of proinflammatory cytokines, *e.g.* IL-1β or TNF-α [18]. Furthermore, it has been reported that linalool could produce antinociception through interactions with opioid, muscarinic M2 or adenosine A1 receptors, or by modulating nitric oxide (NO) synthesis [19–22]. There is also evidence to suggest that linalool may modulate glutamatergic neurotransmission *via* NMDA receptors [9, 23, 24]. Accordingly, it is conceivable that antinociceptive properties of systemic administration of BEO and linalool may involve additional, central, mechanisms to the demonstrated activation of the opioid system in the periphery [8, 9], [11]. Altogether, our findings could support the development of aromatherapy clinical trials for the inhalatory administration of BEO for the treatment of chronic pain and of age-related pathological conditions often associated with chronic pain (see osteoarthritis, post-diabetic and post-herpetic neuropathic pain and behavioural and psychological symptoms of dementia, BPSDs). Indeed, the data gathered so far demonstrating a strong analgesic effectiveness of BEO do support aromatherapy with BEO for the control of chronic pain and a reduced use of drugs like opioids, bearing serious side effects. The latter are very often prescribed despite the lack of strong evidence for their efficacy in chronic non-cancer pain [25]. Some 40–60% among care homes demented patients suffer from BPSDs [26] and these are still not adequately controlled through atypical antipsychotics; in fact, BPSDs are strongly associated with pain states [27] and atypical antipsychotics are devoid of analgesic effects. At variance with the latter concept, aromatherapy with BEO, known to be endowed with analgesic activity, may be a promising management option for the control of BPSDs. Quite importantly, aromatherapy for inhalation might have its effects on pain in the absence of any psychological perception of the fragrance and this is of primary importance, since most demented people may be anosmic because of the early loss of olfactory neurons [28]. Recently, the inhalatory administration of BEO in clinical setting was found to exert an effect on mental health, assessed through the Positive and Negative Affect Scale, improving mood states [29]. Furthermore, the recent finding that the anxiolytic-like/relaxant effects of BEO are devoid of sedation, in contrast with benzodiazepines [30], strengthens the usefulness of aromatherapeutic treatment with BEO in the elderly. Therefore, additional studies are needed for a deeper knowledge of the effects of BEO given *via* inhalation, both from a basic and clinical perspective.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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