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Original article

# Isobolographic analysis of the cutaneous antinociceptive interaction between bupivacaine co-injected with serotonin in rats

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ABSTRACT

*Background:* The aim of this experiment was to investigate a long-lasting local anesthetic bupivacaine combined with serotonin at inducing cutaneous antinociception.
*Methods:* The skin antinociception, characterized by an inhibition of the cutaneous trunci muscle reflex (CTMR) following the pinprick on the dorsal skin of rats, was evaluated. The cutaneous antinociceptive effects of bupivacaine alone, serotonin alone, or bupivacaine co-injected with serotonin in a dose-dependent fashion were constructed, while the drug–drug interactions were evaluated by isobologram.
*Results:* Subcutaneous serotonin, as well as the local anesthetic bupivacaine provoked dose-related cutaneous antinociception. On an equipotent basis (50% effective dose [$ED_{50}$]), the relative potency was bupivacaine (0.43 [0.37–0.50] μmol) > serotonin (1.27 [1.15–1.40] μmol) ($p < 0.01$). At the equi-anesthetic doses ($ED_{75}$, $ED_{50}$ and $ED_{25}$), the duration of bupivacaine was similar to that of serotonin at producing cutaneous antinociceptive effects. Co-administration of bupivacaine and serotonin displayed a synergistic antinociception.
*Conclusions:* The preclinical data demonstrated that serotonin is less potent in eliciting cutaneous antinociceptive effects but has the similar duration of action, compared with bupivacaine. We also found a more significant depth of the sensory block with bupivacaine + serotonin than bupivacaine alone.


## Introduction

Local infiltration anesthesia by injecting a long-acting local anesthetic (e.g., bupivacaine) [1] is frequently used for minimally invasive surgery [2] or post-incisional pain management after the repair of inguinal hernias [3] owing to its relatively lack of side effects. Nevertheless, these methods are limited by the short duration of action at inducing analgesia or anesthesia [4]. For this reason, a local anesthetic containing adjuvant epinephrine (a vasoconstrictor) was co-injected to prolong the duration of local anesthesia [5,6]. However, it should be serious to restrict the dose usage of epinephrine for preventing adverse effects.

Interestingly, serotonin induced pulmonary vasoconstriction [7]. Using the characteristic of vasoconstriction, epinephrine can prolong the duration of local anesthesia [6,8]. Therefore, we proposed that the addition of serotonin to bupivacaine preparations may increase the depth of local anesthesia. The descriptive and analytical application of isobologram allows the experiment to present unambiguous and compact characteristics of the observed interaction [9]. Among the advantages of the isobolographic method is that it supplies a principle for estimating whether the biological response caused by the mixture of two agents is synergistic, antagonistic, or additive reactions [9]. Using isobologram, we showed that the addition of dopamine to propranolol preparation produced a synergistic cutaneous antinociception

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[10]. We aimed to investigate the isobolographic analysis of serotonin with bupivacaine in a rat model of skin infiltration anesthesia.

## Materials and methods

### Animals

Male Sprague-Dawley rats (210–260 g) were used in the present study. The experimental procedures were confirmed with the basis of International Association for the Study of Pain (IASP) ethical guidelines [11], and they were approved by the Institutional Animal Care and Use Committee of National Cheng-Kung University (Tainan, Taiwan). Animals were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and were kept under controlled environmental conditions: room temperature (22 °C) and 50% relative humidity with an established photoperiod of 12-h light/day (6:00 AM. to 6:00 P.M.). All animals had free access to food and tap water *ad libitum*.

### Drugs

Bupivacaine and serotonin were prepared in 0.9% NaCl (normal saline) before subcutaneous injection. Bupivacaine hydrochloride and serotonin creatinine sulfate monohydrate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

### Groups

Four experiments were planned (n = 8 for each dose of each group). In experiment 1, the dose-related curves of bupivacaine (0.15, 0.30, 0.45, 0.90 and 1.80 μmol) and serotonin (0.40, 0.80, 1.20, 2.40 and 4.80 μmol) were constructed to calculate the 50% effective dose ($ED_{50}$). In experiment 2, the area under the curves (AUCs), duration of action, and %MPE (percent of maximal possible effect) of bupivacaine at 1.80 μmol, serotonin at 4.80 μmol and vehicle (normal saline) were compared to each other on subcutaneous antinociception. In experiment 3, the isobolographic analysis was used to investigate the interaction of bupivacaine combined with serotonin as an infiltrative anesthetic. In experiment 4, to eliminate the possibility of systemic action of experimental agents from local skin subcutaneous antinociception, first group received intraperitoneal administration of co-administration of bupivacaine ($ED_{50}$) and serotonin ($ED_{50}$); second group underwent intraperitoneal administration of bupivacaine (1.80 μmol) or serotonin (4.80 μmol).

### Subcutaneous injection of drugs

Subcutaneous injection was executed as described previously [12,13]. In the beginning the hair of animals on the dorsal region of the thoracolumbar area (10 cm × 6 cm) was shaved mechanically before starting an injection session. Then, a total volume of 0.6 mL agent was subcutaneously administrated using a 30-gauge needle at the dorsal site of the thoracolumbar area of the unanesthetized animal. For consistency, an experienced investigator, who was blind to the treatment group, was responsible for every neurobehavioral assessment. After subcutaneous injection, a wheal (a circular elevation of the skin) approximately 20 mm in diameter appeared, and it was marked using ink.

### Neurobehavioral estimation

Local dorsal skin pinpricks elicited the cutaneous trunci muscle reflex (CTMR) in rats. The CTMR, characterized *via* the reflex of skin movement over the back of a rat, was provoked *via* twitches of the lateral thoracispinal muscle [14,15]. When we see a CTMR to a pinprick probed on the contralateral side or outside the wheal, six pinpricks with a frequency of 2 Hz were applied inside the wheal. This cutaneous analgesic effect according to the block of the CTMR was graded on a scale of 0 to 6 (the number to which the animal failed to react a CTMR) [16,17]. To provoke a standardized nociceptive pinprick (19 ± 1 g), a von Frey hair (No.15; Somedic Sales AB, Stockholm, Sweden) affixing an 18-gauge needle was used. The complete lack of six CTMR following six pinpricks was recorded as complete sensory block (100% PE; 100% of possible effect), while the maximum block in the time course of nociceptive/sensory block was defined as the %MPE (maximal possible effect).

### The AUCs, duration of action, and $ED_{50}$

A computer software, Kinetica version 2.0.1 (InnaPhase Corporation, Philadelphia, PA), was employed to calculate the AUCs [18,19]. The drug's duration (full recovery time) was defined as the time interval from injection of the tested drugs (i.e., time = 0) to complete recovery (no antinociception) [20,21]. To obtain the $ED_{50}$s, the dose-response curves of bupivacaine and serotonin were constructed, and these two curves were then fitted using the computer-derived SAS NLIN Procedures (SAS Institute Inc., Carey, NC) [10,22]. The $ED_{50}$, defined as the dose that provoked 50% block of the CTMR, was obtained. Moreover, the $ED_{75}$ and $ED_{25}$ of agents were computed through the same computer-derived curve-fitting (SAS NLIN analysis) [23,24]. Complete block time (duration of complete blockade) was defined as the time interval from the first time point of 100% block to the last time point up to 100% block.

### Isobologram methods

We used the isobolographic analysis (version 1.27, Pharm Tools Pro, McCary Group, Wynnewood, PA) for evaluating the drug–drug interactions [9,25]. After constructing the dose–response curve of combined drugs (i.e., serotonin co-injected with bupivacaine at a ratio of $ED_{50}$ *vs.* $ED_{50}$) under the equipotent doses, the experimental data were calculated according to a standard procedure [9]. The dose ranges administered for the combination were $1/8(ED_{50} + ED_{50})$, $1/6(ED_{50} + ED_{50})$, $1/4(ED_{50} + ED_{50})$, and $1/2(ED_{50} + ED_{50})$. An isobologram was employed for assessing the drug–drug interactions based on Tallarida [9,26]. We investigated the difference between an experimental value of $ED_{50}$ (taken from a dose–related curve of the mixture of two drugs) and a theoretical value of $ED_{50}$ (taken from the theoretical additive line *via* computer simulation) [27,28]. On the X and Y axes, the theoretical additive line was plotted using the $ED_{50}$ of bupivacaine and serotonin, respectively. The $ED_{50}$ of combined drugs was then constructed against the theoretical additive line.

### Statistical analyses

A statistical software, SPSS for Windows (version 17.0, SPSS, Inc, Chicago, IL, USA), was used, while $p$ values of $<0.05$ were considered statistically significant. Either the Student's $t$-test or one-way or two-way analysis of variance (ANOVA) followed by pairwise Tukey's honest significance difference (HSD) test was used to analyze the experimental data. Experimental values are expressed as mean ± SEM or the $ED_{50}$ values with 95% confidence interval (95% CI).