Original Paper

Physiological Responses and Gene Expression in Ultrasound-Guided Supraclavicular Brachial Plexus Block: a Comparative Study

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Key Words

Ultrasound-guided supraclavicular brachial plexus block • Midazolam • Neostigmine

Abstract

Background/Aims: Ultrasound-guided supraclavicular brachial plexus block (BPB) has come into wider use as a regional anesthetic during upper limb operations. This study assessed the neurological and hemodynamic changes and gene expression after co-administration of midazolam or neostigmine with bupivacaine during supraclavicular BPB. **Methods:** The study involved 90 adults divided into three groups: control (bupivacaine), midazolam (bupivacaine plus midazolam), and neostigmine (bupivacaine plus neostigmine). Blood samples were taken and interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) mRNA levels were measured by real-time PCR, and oxidative stress markers were identified. In addition to the hemodynamic variables, the onset and duration of sensory and motor blockades, duration of analgesia, pain scores, time of first request for an analgesic, and amounts of analgesics ingested were evaluated. **Results:** Compared with the control and neostigmine groups, the midazolam group experienced longer sensory and motor blockades, prolonged analgesia, lower pain scores at 12 h and 24 h, and lower need for postoperative analgesics. Moreover, the midazolam group exhibited lower oxidative stress markers with a higher fold change in IL-6 and TNF- α mRNA levels. **Conclusion:** Midazolam co-administered with bupivacaine provided better analgesic quality than did neostigmine with bupivacaine. This might be due to its superior antioxidant and anti-inflammatory effects.

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Introduction

Supraclavicular brachial plexus block (BPB) is an excellent substitute for general anesthesia during upper limb operations. It prevents the undesirable effects of using

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general anesthesia and upper airway instruments [1]. Moreover, it reduces the duration of hospitalization and costs, and provides complete muscle relaxation [1, 2]. The ultrasound-guided technique requires a lower anesthetic volume to provide an efficient block and decreases the risk associated with intravascular injections and trauma to the surrounding tissues [3].

Bupivacaine alone is now less frequently used as a local anesthetic because of its short duration of action [3]. Several adjuvants have been co-administered with bupivacaine, but the results have been either unconvincing or associated with untoward complications [4]. Several researchers have attempted to combine bupivacaine with other anesthetics such as ketamine [5], clonidine [6], midazolam [1], and neostigmine [7] with various success rates.

Midazolam, a water-soluble benzodiazepine, reduces pain perception [1] via postsynaptic gamma-aminobutyric acid (GABA_A) receptors, opioid receptors, and benzodiazepine receptors in the spinal cord. GABA_A receptor is a chloride ionophore that, when activated, decreases neural excitability by stabilizing the membrane potential near the resting potential [8]. Kohno et al. [9] have demonstrated the presence of GABA receptors within the brachial plexus and peripheral nerve trunks. Yamamoto et al. [10] have reported that these receptors enhance membrane translocation of alpha-7 nicotinic subtype acetylcholine receptors at presynaptic GABAergic neurons to allow endogenous acetylcholine to increase the release of presynaptic GABA within the rat neocortex.

Neostigmine, a reversible acetylcholinesterase inhibitor, prevents the breakdown of acetylcholine [7]. Lin et al. [11] reported that it enhances the release of nitric oxide, suppresses the expression of Fos, and activates muscarinic M2 receptors within the spinal cord.

This study aimed to estimate the neurological and hemodynamic changes and the antioxidant plus anti-inflammatory effects associated with adding either midazolam or neostigmine to bupivacaine during supraclavicular BPB.

Materials and Methods

Subjects

Inclusion criteria were unilateral upper limb operation except on the shoulder, age 18-70 years, weight 59-95 kg, and American Society of Anesthesiologist physical status I or II. Exclusion criteria were patient refusal; allergy to the drugs used; contraindications for regional block; use of drugs that may modify pain perception; upper limb peripheral neuropathy; cardiac, respiratory, hepatic, or renal disease; infection; incomplete block; and pregnancy. The protocol of this study was supported by the ethics committee of our institution. Written, informed consent was obtained from all patients.

This was a prospective double-blind study; neither the patients nor operators were informed of which drugs were used in each case. Another anesthetist was responsible for allocating the patients to the study groups and loading the drugs for each block. A total of 90 patients took part in the study (30 patients in each group). Patients were allocated randomly by the computer-generated block method into the following groups.

1. Control group: Ultrasound-guided supraclavicular BPB with 30 mL 0.5% bupivacaine.
2. Midazolam group: Ultrasound-guided supraclavicular BPB with 28 mL bupivacaine 0.5% plus 2 mL midazolam (50 µg/kg body weight).
3. Neostigmine group: Ultrasound-guided supraclavicular BPB with 28 mL bupivacaine 0.5% plus 2 mL neostigmine (200 µg).

Supraclavicular block

After aseptic preparation of the area, the supraclavicular block was performed by the anesthetist using SonoAce 6 ultrasound machine with a high-frequency linear probe. The patient head was slightly raised and rotated opposite to the surgery site. Anesthetist stood at the patient's head with ultrasound machine. A superficial linear probe put in supraclavicular fossa, the supraclavicular artery was identified

as a hypoechoic pulsating structure above first rib which visualized as hyperechoic line. The plexus usually appeared as a set of hypoechoic rounded structures (grapes bunch) reclined superior and dorso-lateral to subclavian artery.

Hemodynamic variables

Heart rate (HR) and mean arterial blood pressure (MABP) measurements were recorded pre-operatively (baseline level), intra-operatively (these variables were recorded at 5 min, 10 min, 15 min, 30 min, 60 min, and at the end of surgery), and again postoperatively at 30 min and 60 min.

Neurological assessment

The following parameters were monitored:

Sensory block onset is defined as the time taken between administration of the anesthetic drug(s) and complete block. Sensory block was monitored by pinprick from C4 to T1 dermatomes and scored as follows: 0 = normal sensation and 1 = loss of tactile sensation (anesthesia).

Motor block onset is defined as the time taken between the administration of the anesthetic drug(s) and total muscle power loss. Motor block score: 0 = normal motor function with full flexion and extension of elbow, wrist, and fingers; 1 = decreased muscle strength but able to move the fingers only; and 2 = total motor block with an inability to move the fingers.

Sensory block duration is the time taken between the beginning of total sensory block and the appearance of pain.

Motor block duration is the time from total motor block to the return of full muscle power.

Analgesia duration is the time taken between successful block and the subject sensing pain that requires an analgesic.

First analgesic request is how long it takes the patient to ask for the first postoperative analgesic.

Pain management was assessed by evaluating pain on a visual analog scale (VAS) where zero represents no pain and 10 the worst possible pain. Significant pain is described as a pain level score of 4 requiring analgesia (15 mg/kg of paracetamol every 6 h). In cases of VAS ≥ 6 an additional analgesic was given in the form of 1 $\times$ 25- μ g fentanyl transdermal patch/h. VAS was measured at 0 min, 30 min, 60 min, 2 h, 4 h, 12 h, and 24 h postoperatively.

Laboratory

Samples (3 mL) of blood were saved in heparinized tubes immediately before the analgesics were administered (baseline), and at 2 h, 4 h, and 24 h thereafter. Blood was immediately centrifuged at 4 °C at 3,000 $\times$ g for 10 min, divided into aliquots, and stored at -80°C until the enzyme-linked immunosorbent assays for the measurement of 8-Epi-prostaglandin F2 alpha (8-Epi-PGF_{2 α}) and superoxide dismutase (SOD) according to the instructions supplied with each kit. Lipid peroxidation product malondialdehyde (MDA) was measured using spectrophotometry [12]. Quantitative real time-polymerase chain reaction (qRT-PCR) was utilized to estimate interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) mRNA levels, as described below in the RNA extraction subsection. All PCR reagents were from Applied Biosystems (Foster City, CA, USA). Subsequently, the cDNA was amplified with the SYBR Green I PCR Master Kit (Fermentas) using the Step One instrument and examined using RT²SYBR® (Precision PLUS Master Mix premixed with SYBR green). Extracted RNA was transformed to cDNA. Primer Express software was used for PCR amplification. Each reaction had 50 cycles and was performed in triplicate, as per the manufacturer's recommendation.

RNA extraction

RNA extraction was achieved with Rnase extraction kit (according to manufacturer's directions). The Ct values of four genes relative to the concentrations of housekeeping gene RNAs were calculated. Briefly, RNA expression was determined using Precision PLUS Master Mix premixed with SYBR green, and 12.8 mL (8 $\times$ 1.6 mL) samples were prepared for reverse transcription using the Precision NanoScript 2 Reverse Transcription kit.

Reverse transcription

One-microgram samples of total RNA were reverse transcribed using the Applied Biosystems Taqman RT-Kit. IL-6 and TNF- α gene expression levels were assessed with qRT-PCR. The primer

sequences used were: IL-6, forward primer (5'-`GGTACATCCTCGACGGCATCT-3`) and reverse primer (5'-GTGCCTCTTTGCTGCTTTTCAC-3`); TNF- $\alpha$ , forward primer (5'-GCG ACG TGG AAC TGG CAG AAG-3`) and reverse primer (5'-GGT ACA ACC CAT CGG CTG GCA-3`) [14]. Each sample was run in triplicate in a reaction volume of 20 mL for 50 cycles using standard RT-PCR cycling conditions. All samples were normalized to a relative standard curve and the amounts of gene transcripts were normalized to the housekeeping genes. The reactions were performed by a thermal cycler (MJ Research, Inc., MA, USA). The amplification conditions were: 30 cycles of template denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, then polymerization at 72 °C for 2 min. However, annealing for TNF- α was performed at 58 °C for 90 s and extension was at 72 °C for 140 s. All data were normalized by the mean critical threshold (Ct) values of the GAPDH housekeeping gene [13], and are reported as fold changes in expression [forward primer (5'-TCG TGG AAG GAC TCATGA C-3`) and reverse primer (5'-CCA TCA CGC CAC AGT TT-3`)].

Statistics

Data were analyzed by the Statistical Package for the Social Sciences, version 21 (SPSS, Chicago, Illinois, USA). Data are presented as the mean $\pm$ standard deviation (SD) or number. The Shapiro-Wilk test was used to detect normal distribution. Categorical data were examined by the Chi-squared test. Continuous variables were examined by analysis of variance or the Kruskal-Wallis test. Data at multiple time points were analyzed by repeated measures analysis of variance with Tukey's post-hoc test. Differences between the groups were regarded as statistically significant when the *P* value was less than 0.05.

Results

Table 1 presents the demographic data and characteristics of the participants. No significant intergroup differences were found among the patients' characteristics.

Fig. 1 shows the motor and sensory blockades onset times. Compared with the

Table 1. Demographic and clinical characteristics of the groups studied. Values are presented as the mean $\pm$ standard deviation or number and were analyzed by one way analysis of variance followed by Bonferroni or chi-squared tests. ASA, American Society of Anesthesiologists; BMI, body mass index

	Control group (n = 30)	Midazolam group (n = 30)	Neostigmine group (n = 30)
Age (years)	33.1 (11)	37.1 (10.6)	33.1 (9)
Men/women	26/4	27/3	24/6
Height (cm)	175.9 (5.2)	174 (7.5)	178.5 (6.8)
Weight (kg)	55.9 (6.1)	51.3 (5.8)	53.1 (5.2)
BMI (kg/m ²)	18.1 (2.4)	17.1 (2.7)	17.1 (1.9)
Duration of surgery (min)	103.3 (17.4)	106.8 (21.5)	104.6 (23.2)
Type of surgery			
Bone/soft tissue	18/12	14/16	20/10
ASA grade			
I/II	24/6	26/4	28/2

Fig. 1. Onset times of motor and sensory blockades. Data are presented as the mean $\pm$ standard deviation, n = 30 subjects per group. Kruskal-Wallis test was used to examine

the data. ** *P* < 0.01 and *** *P* < 0.001, significantly different compared with the control group.

Fig. 2. Durations of motor and sensory blockades and analgesia. Data are presented as the mean $\pm$ standard deviation; n = 30 subjects per group. Kruskal-Wallis test was used to examine the data. * *P* < 0.05 and *** *P* < 0.001, significantly different compared with the control group.

control group, the midazolam and neostigmine groups had significantly faster onsets of motor block (7.60 ± 0.87 min vs. 5.02 ± 0.53 min, $P < 0.001$, and 4.90 ± 0.75 min, $P < 0.001$, respectively) and sensory block (6.99 ± 0.63 min vs. 4.86 ± 0.53 min, $P < 0.001$, and 4.38 ± 0.69 min, $P < 0.001$, respectively). There was no difference between the midazolam and neostigmine groups.

The durations of motor block, sensory block, and analgesia are shown in Fig. 2. Compared with the control group, the midazolam and neostigmine groups showed significantly longer durations of motor block (261.10 ± 34.66 min vs. 508.50 ± 62.09 min, $P < 0.001$, and 412.00 ± 43.72 min, $P < 0.05$, respectively) but only the midazolam group experienced longer sensory blocks (186.50 ± 36.31 min versus 458.50 ± 58.87 min, $P < 0.001$). Similarly, analgesia was significantly longer in the midazolam group compared with the control group (469.00 ± 49.30 min vs. 199.00 ± 32.91 min, $P < 0.001$); however, there was no difference between the midazolam and neostigmine groups.

Fig. 3 shows the VAS pain scores at different time points. The highest and most significant VAS pain scores in the control group were found at 2 h, 4 h, 12 h, and 24 h postoperatively, while the midazolam group had lower VAS scores than did the neostigmine group ($P < 0.05$ at 12 h and 24 h postoperatively).

The first requests for an analgesic were significantly later in the midazolam and neostigmine groups than in the control group (9.56 ± 2.24 h, $P < 0.001$ and 7.65 ± 1.53 h, $P < 0.05$ versus 6.05 ± 1.17 h, respectively; Fig. 4). The amount of paracetamol consumed in the 24 h following surgery was significantly lower in the midazolam group than in the control and neostigmine groups (1.96 ± 0.35 g vs. 2.91 ± 0.34 g, $P < 0.001$, and 2.49 ± 0.64 g, $P < 0.01$, respectively).

Fig. 3. Visual analogue scale. Values are presented as the mean $\pm$ standard deviation; $n = 30$ subjects per group. Values were analyzed by repeated measures analysis of variance. Differences from the baseline level of every group were examined by Mann-Whitney test. *** $P < 0.001$, significantly different from baseline; ††† $P < 0.001$, significantly different from the control group; and ‡ $P < 0.05$ and ‡‡‡ $P < 0.001$, significantly different from the neostigmine group.

Fig. 4. Time of the first request for an analgesic (A) and total amount of paracetamol (B). Data are presented as the mean $\pm$ standard deviation, $n = 30$ subjects in every group. Kruskal-Wallis test was performed to examine the data. * $P < 0.05$ and *** $P < 0.001$, significantly different from the control group; and †† $P < 0.01$, significantly different from the neostigmine group.

Fig. 5. Heart rates (A) and MABPs (B) of the participants. Data are presented as the mean $\pm$ standard deviation, $n = 30$ subjects per group. Data were examined by repeated measures analysis of variance. MABP, mean arterial blood pressure.

Fig. 5 shows the hemodynamic parameters. There were no significant intergroup differences of HR and MABP levels.

Fig. 6 shows the plasma 8-Epi-PGF_{2α}, MDA and SOD concentrations. The concentrations of 8-Epi-PGF_{2α}, MDA, and SOD were not statistically different at either baseline or 24 h following anesthetic drug injection among the study groups. However, at 2 h and 4 h, the plasma 8-Epi-PGF_{2α} concentrations increased significantly in all groups compared with the baseline concentrations ($P < 0.001$ for each group), with significant elevations in the control group compared with the midazolam and neostigmine groups (272.40 ± 28.46 pg/mL vs. 221.50 ± 14.74 pg/mL, $P < 0.001$, and 247.10 ± 22.33 pg/mL, $P < 0.05$, respectively) and (386.60 ± 26.06 pg/mL vs. 336.70 ± 42.51 pg/mL, $P < 0.001$, and 361.90 ± 32.62 pg/mL, $P < 0.05$, respectively), with a more significant decline in the midazolam group than in the neostigmine group ($P < 0.05$ at every time point).

Similarly, at 2 h, the MDA concentrations increased significantly in the control and neostigmine groups compared with the baseline concentrations ($P < 0.001$ for each). At 4 h, plasma MDA concentrations increased significantly in each of the three groups ($P < 0.001$ for each). Compared with that in the control group, the concentration of MDA diminished significantly in the midazolam and neostigmine groups (8.69 ± 1.20 nmol/L vs. 4.83 ± 0.98 nmol/L, $P < 0.001$, and 6.75 ± 0.82 nmol/L, $P < 0.05$, respectively), and (11.01 ± 1.70 nmol/L vs. 6.94 ± 0.83 nmol/L, $P < 0.001$, and 8.98 ± 1.51 nmol/L, $P < 0.05$, respectively), with a more significant reduction in the midazolam group than in the neostigmine group ($P < 0.05$ at every time point).

In contrast, at 2 h and 4 h, the plasma SOD concentration declined significantly in the control ($P < 0.001$ in each time point) and neostigmine ($P < 0.01$ and $P < 0.001$, respectively) groups compared with the baseline concentrations, while they were no different from the

Fig. 6. Plasma concentrations of (A) 8-Epi-PGF_{2α}, (B) MDA, and (C) SOD among the groups. Data are presented as the mean $\pm$ standard deviation, $n = 30$ subjects per group. Data were examined by repeated measures analysis of variance. Differences from baseline in each group were examined by Mann-Whitney test. *** $P < 0.001$, significantly different from the baseline level. † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$, significantly different from the control group; ‡ $P < 0.05$ and ‡‡ $P < 0.01$, significantly different from the neostigmine group. 8-Epi-PGF_{2α}, 8-Epi-prostaglandin F₂ alpha; MDA, malondialdehyde; and SOD, superoxide dismutase.

Fig. 7. Real-time PCR products of (A) IL-6 and (B) TNF-alpha in Ct values relative to housekeeping gene RNAs. Data are presented as the mean $\pm$ standard deviation, $n = 30$ subjects per group. Data were examined by repeated measures analysis of variance. Differences from baseline in each group were examined by Mann-Whitney test. *** $P < 0.001$, significantly different from baseline level. † $P < 0.05$, significantly different from the control group. IL-6, interleukin-6; TNF- α , tumor necrosis factor-alpha.

baseline concentration at 2 h and had decreased significantly at 4 h ($P < 0.001$) in the midazolam group. Compared with the control group, the reduction in SOD concentration was less significant in the midazolam and neostigmine groups (286.50 ± 35.65 U/L vs. 406.60 ± 19.03 U/L, $P < 0.001$, and 346.80 ± 42.59 U/L, $P < 0.01$, respectively) and (204.80 ± 26.06 U/L vs. 329.00 ± 32.64 U/L, $P < 0.001$, and 269.00 ± 44.83 U/L, $P < 0.01$, respectively), with a significant decline in the midazolam group compared with the neostigmine group ($P < 0.01$ at every time point).

Fig. 7 demonstrates the IL-6 and TNF- α RT-PCR Ct values relative to housekeeping gene RNAs. IL-6 and TNF- α gene concentrations rose significantly compared with baseline concentrations ($P < 0.001$ for every parameter at each time point) in the three groups but there was no difference among the groups at 0 h, 4 h, and 24 h following the administration of the anesthetic drugs. At 2 h, the fold change in IL-6 was higher in the midazolam group ($P < 0.05$ for every parameter) compared with the control group.

Discussion

Many additives are used to improve the quality of analgesics, but the results are either uncertain or associated with undesirable adverse effects [1]. This study revealed that the addition of midazolam to bupivacaine during ultrasound-guided supraclavicular BPB provided better neurological and analgesic qualities than the addition of neostigmine. These effects might be attributable to the greater anti-oxidant and anti-inflammatory effects of midazolam.

Analgesic quality is improved by a quick onset time plus prolonged sensory and motor blockades [14]. The present study concurred with the data of Kamali et al. [15] who found more prolonged sensory blockade in their midazolam- group than in the control group. In contrast, Kumar et al. [16] found that neostigmine provided more prolonged sensory and motor blockades and a longer period of analgesia than midazolam.

Postoperative pain, a physiological reaction to tissue injury, can result in unpleasant physiological and metabolic effects as well as adverse emotional experiences if it is inadequately managed [15, 17]. The following study revealed that the addition of midazolam to bupivacaine resulted in a greater reduction in postoperative pain and the need for fewer analgesics than did the addition of neostigmine. Similarly, Kamali et al. [15] found that co-administration of midazolam and lidocaine during spinal anesthesia decreased the severity of postoperative pain and postoperative analgesic consumption to a greater degree than co-administration of neostigmine and lidocaine. In contrast, Kumar et al. revealed that the time points of the first postoperative analgesics were not considerably different between the bupivacaine-neostigmine and bupivacaine-midazolam groups [16].

This study showed that the addition of either midazolam or neostigmine to bupivacaine provoked no intra- or postoperative changes in HR or MABP. These results agree with previous studies that have reported stable HR and MABP measurements throughout the surgical procedure when bupivacaine is co-administered with either midazolam [14] or neostigmine [16].

Surgical stress can decrease antioxidant activities in the body and oxidative stress can lead to elevations in the levels of free radicals [18]. Thus, the use of an anesthetic drug with antioxidant effects to lessen oxidative stress is crucial and of significant clinical value [19]. Lipid peroxidation, the principal mechanism of cell damage mediated via free radicals, can reduce membrane fluidity along with permeability and cause denaturation of cell membrane proteins [20]. The arachidonic acid peroxidation product 8-epi-PGF_{2 α} is an often used as an oxidative stress marker [21]. MDA is a lipid peroxidation product often used as an oxidative stress indicator. SOD is the main free radical scavenger concerned with the elimination of superoxide anion [17]. This study revealed that co-administration of midazolam with bupivacaine exhibited a more potent antioxidant effect than the co-administration of neostigmine with bupivacaine by decreasing the plasma levels of 8-Epi-PGF_{2 α} and MDA and

enhancing those of SOD. In line with the present study, Kang et al. reported that midazolam exerts antioxidant activity whereas neostigmine has no such effect [19]. In contrast, Bondok et al. demonstrated in an experimental acute liver failure model that neostigmine could have a protective effect by reducing MDA and increasing SOD levels in the liver [22]. This suggests that postoperative pain may in part result from oxidative stress. Kim et al. reported that antioxidants reduce the hyperalgesia of neuropathic pain. In addition [23], Eisenberg et al. showed that salivary and serum antioxidants, in addition to serum MDA concentrations, are raised in subjects with complex regional pain syndrome and spinal neurons are sensitized [24].

It has been implied that anesthesia might reduce inflammatory responses by repressing the release of cytokines [25]. The present study revealed that the co-administration of midazolam and bupivacaine reduced inflammation, which was identified by higher fold changes in the expression of IL-6 mRNA. In contrast, Bondok et al. [22] reported that neostigmine reduces the expression of TNF- α and increases the expression of IL-10 in rat liver. Unfortunately, the results of this study cannot be compared with those of Bondok et al. [22] because in that study, neostigmine was the only drug investigated. Our results suggested that midazolam may reduce inflammatory responses; however, further studies are needed.

Several studies have suggested that proinflammatory cytokines facilitate the development and continuation of persistent pain syndrome. TNF- α has been proposed to play a substantial role in the pathogenesis of pain [26, 27]. Furthermore, IL-6 provokes muscle and joint hyperalgesia [28] and the development of injury-mediated hyperalgesia [29]. These findings suggest that postoperative pain could partly result from inflammatory reactions.

In conclusion, this study demonstrated that midazolam co-administered with bupivacaine provided superior analgesic quality than did neostigmine by prolonging the duration of sensory and motor blockades and postoperative analgesia alongside reducing the need for postoperative pain relief. In addition, these beneficial effects were associated with hemodynamic stability. This might be explained by midazolam's better antioxidant and anti-inflammatory effects. Therefore, this drug could be considered an option for enhancing the effects of analgesics.

Disclosure Statement

The authors declare no conflicts of interest. The researchers are responsible for the content and writing of the paper.

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