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Comparison of neurotoxicity of dexmedetomidine as an adjuvant in brachial plexus block in rats of different age

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ABSTRACT

Dexmedetomidine is a local anesthetic adjuvant that exerts neuroprotective effects in addition to its sedative and analgesic properties. However, it is not clear whether dexmedetomidine causes any neurotoxicity in neonates. We injected dexmedetomidine alone or in combination with ropivacaine to induce brachial plexus block in rats of different age, corresponding to human neonate, childhood, adolescence and adulthood. We then examined pro-inflammatory cytokines and activated caspase 3 to determine the neurotoxicity effects. We found that high dose of dexmedetomidine significantly reduced IL-6 and TNF-α levels in all aged rat brachial plexus compared to saline treatment, and these levels are similar to that of control brachial plexus at postnatal day 14, 18 and adulthood. Caspase 3 level is not significantly different between dexmedetomidine and control group, except that it is higher in dexmedetomidine treated group at postnatal day 5. We found that this neurotoxicity effect of dexmedetomidine is only present at a high dose. Dexmedetomidine shows minimal neurotoxicity in neonate rats during brachial plexus block when moderate doses are administered. This observation warrants more detailed clinical studies to determine the safety of using dexmedetomidine for brachial plexus block in infant or early childhood patients.

1. Introduction

Dexmedetomidine is an α2-receptor agonist with both sedative and analgesic effects. When applied systemically by intravenous injection, dexmedetomidine showed a dose-dependent sedative effect as well as decrease of blood pressure in healthy human subjects (Kauppila et al., 1991). It has been used as a sedative agent in postoperative patients requiring intensive care (Venn et al., 1999). In addition to systemic application, dexmedetomidine is more frequently used as a local anesthetic adjuvant in combination with other anesthetic agents, leading to increased duration of block as well as reduced amount of total anesthetics required (Brummett et al., 2008; Brummett et al., 2011; Fritsch et al., 2014; Knight et al., 2015).

Local anesthesia is a widely used technique to reduce perioperative pain and surgical stress post-surgery (Kessler et al., 2015). One concern with local anesthesia is that it may cause toxicity. When applied to *in vitro* neuronal cultures, lidocaine, bupivacaine, mepivacaine and ropivacaine all caused growth cone collapse and neurite degeneration (Radwan et al., 2002). In a neuroblastoma cell line, it was found that all local anesthetics induced neurotoxicity in a dose dependent manner and they caused neuronal death even when clinically used concentrations were applied (Werdehausen et al., 2009). Since local anesthetics exert their analgesic effects by blocking voltage-gated sodium channels, toxicity is also frequently observed in the central nervous system and the cardiovascular system (Graf, 2001; Ruetsch et al., 2001). Application of dexmedetomidine to these local anethesthetics may overcome toxicity issues.

When added as a peripheral nerve blockage adjuvant, dexmedetomidine does not cause detectible neurotoxicity (Knight et al., 2015). On the contrary, dexmedetomidine has been shown to exert a protective effect on multiple organs with the presence of α2-receptor in these organs (Weerink et al., 2017). Dexmedetomidine has been shown to reduce bupivacaine and levobupivacaine induced convulsion in rats (Tanaka et al., 2005). Neuroprotective effects have also been observed in various animal models and *in vitro* studies, including brain injury, hyperoxia-induced toxicity in neonate rats as well as attenuation of isoflurane-induced neurocognitive impairments (Sanders et al., 2009; Schoeler et al., 2012; Sifringer et al., 2015).

Brachial plexus block provides effective anesthesia during upper limb surgery. It has been shown that dexmedetomidine prolongs axillary brachial plexus block when administered with ropivacaine (Zhang et al., 2014). However, it is not clear whether dexmedetomidine can be safely applied to achieve brachial plexus block in younger patients. In the current study, we explored the neuroprotective and neurotoxic

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effects of dexmedetomidine in neonate, juvenile, adolescent as well as adult rats. Our finding that dexmedetomidine induces minimal toxicity in neonate rats warrants further research on whether dexmedetomidine is a safe local anesthetic agent for peripheral nerve blockage in infants and early childhood patients.

2. Materials and methods

2.1. Rats and experimental grouping

All animal experiments were conducted in accordance with guidelines set by USA National Institutes of Health and were approved by Quanzhou First Hospital's Institutional Animal Care and Use Committee. Sprague Dawley rats of indicated ages were used in the study. Rats were purchased from SLAC Company (Shanghai, China) and housed in an environmentally controlled animal facility with free access to water and food. To determine the age dependent neuroinflammatory effects of dexmedetomidine, 15 rats at each indicated age (postnatal day 5, postnatal day 14, postnatal day 28, and adulthood) were used in the study and divided into three groups with 5 rats per group: saline, 0.5% ropivacaine +60 μg/kg dexmedetomidine, and 60 μg/kg dexmedetomidine. To determine the dose dependent neurotoxic effects of dexmedetomidine, 24 rats at postnatal day 5 were randomly divided into three groups (with 8 rats per group): 20 μg/kg dexmedetomidine, 40 μg/kg dexmedetomidine, and 60 μg/kg dexmedetomidine. Of these rats, 5 per group were used to evaluate caspase-3 levels and 3 per group were used for hematoxylin and eosin (H&E) staining.

2.2. Brachial plexus block

Brachial plexus block was performed according to a previously described procedure (He et al., 2015). Briefly, rats were anesthetized with 1% isoflurane. A 1 cm infraclavicular incision was cut followed by a blunt dissection of the muscle to reveal the subclavian artery. Brachial plexus was exposed after dissection near the artery. Left brachial plexus blockage was performed by injecting saline, dexmedetomidine alone, or a combination of dexmedetomidine and ropivacaine into the perineural space below the fascia covering the nerve. Right brachial plexus was not injected and used as a control. After the procedure, the surgical wound was sutured and the animal was sent to recovery.

2.3. Real time – PCR (RT-PCR)

Rats were sacrificed 4 h after surgery with excess pentobarbital sodium (100 mg/kg). After 1 cm infraclavicular incision, the brachial plexus was exposed. Then, blunt dissection of the muscle was conducted to reveal the subclavian artery. Following the dissection, the brachial plexus was clearly identified near the artery by an operating microscope. Using the injection site as the center, brachial plexus was quickly harvested on ice. Half of the sample was placed into a 1.5 ml tube with RNAlater (Cat. No. 76104, Qiagen, USA), and stored at −80 °C. Total RNA was isolated using the RNeasy Mini Kit (Cat. No. 74104, Qiagen, USA). RNA concentration was determined through NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). An equivalent amount of RNA from each sample was used as template for reverse transcription. Quantitative real-time RT-PCR was performed according to standard protocol using the QuantiTect SYBR Green RT-PCR Kit (Cat. No. 204245, Qiagen, USA) to measure the mRNA levels of *TNF*-α and *IL-6*. The quantity of target mRNA was normalized to a house-keeping gene, *GAPDH*, which was served as an internal control. Primers for rat *TNF*-α, *IL-6* and *GAPDH* were used in this study and the sequences of these primers were as following. Primers for *TNF*-α are 5′-TTC TGT CTA CTG AAC TTC GGG GTG ATG GGT CC-3′ and 5′-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3′; primers for *IL-6* are 5′-GAC TGA TGT TGT TGA CAG CCA CTG C-3′ and 5′-TAG CCA CTC CTT CTG TGA CTC TAA CT-3′; primers for *GAPDH* are 5′-CCT TCA TTG ACC TCA ACT AC-3′ and 5′-GGA AGG CCA TGC CAG TGA GC-3′.

2.4. Western blot

As described above, brachial plexus was extracted and the other half sample was homogenized in lysis buffer containing Protease Inhibitor Mixture (*Ca*. No. 04693159001, Roche, Germany) and phosphatase inhibitors. Total protein concentrations were measured using the bicinchoninic acid (BCA) method. Equal amount of proteins were resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were subsequently transferred to a nitrocellulose membrane. Immunoblots were blocked with 10% skim milk in Tris-buffered saline (TBS), incubated with primary antibodies diluted in 1% skim milk in TBS at 4 °C overnight, rinsed three times with TBS + Tween 20 (TBST) and then incubated with secondary antibodies. Proteins were detected using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate assay kit (Cat. No. N5514-25TAB, Sigma, St. Louis, MO) and quantified using Adobe Photoshop software (Adobe Systems Inc.). The following antibodies were used in our study: cleaved caspase-3 (Asp175) antibody (1: 1000, Cat. No. 9661, cell signaling technology, USA), β-actin (1: 1000, Cat. No. 3700, Cell Signaling Technology, USA), p-p38 MAPK (1: 500, Cat. No. 4511, cell signaling technology, USA) and total p38 MAPK (1: 500, Cat. No. 8690, Cell Signaling Technology, USA) P-p38 MAPK bands were normalized relative to total p38 MAPK, and caspase-3 bands relative to β-actin.

2.5. Histopathological assessment

Four hours after treatment, 3 rats per group at postnatal day 5 were euthanized with excess pentobarbital sodium (100 mg/kg). Brachial plexus was isolated and fixed overnight in 4% paraformaldehyde at 4 °C, embedded in paraffin blocks and sectioned into 4 μm thick longitudinal sections. Brachial plexus slices were stained with hematoxylin and eosin (H&E) using standard procedures as described previously (Fischer et al., 2008).

2.6. Statistical analysis

One-way ANOVAs were used to determine the difference among each treatment group at the same age followed by *Bonferroni post hoc* tests. Two-way ANOVAs were used to determine the age × treatment interaction, followed by *Bonferroni post hoc* tests. Paired *t*-tests were used for left and right brachial plexus comparison. All analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and data were presented as mean ± standard error of mean (SEM). The difference was regarded statistically significant when p value < 0.05.

3. Results

3.1. Dexmedetomidine reduced inflammation in brachial plexus block

To determine the neuroprotective effects of dexmedetomidine for brachial plexus block in young rats, we examined the levels of pro-inflammatory cytokines IL-6 and TNF-α. We found that saline injection significantly increased IL-6 and TNF-α levels at all age groups (Fig. 1A–H, Fig. S1A–D). Injection of both dexmedetomidine and ropivacaine significantly reduced IL-6 and TNF-α levels compared to saline alone. Importantly, dexmedetomidine alone at 60 μg/kg further reduced IL-6 and TNF-α levels, which were not significantly different from that of control brachial plexus at postnatal day 14, 28 and adulthood, with the only exception of postnatal day 5. We found that dexmedetomidine alone at 60 μg/kg induced IL-6 and TNF-α production significantly higher than that of control brachial plexus at postnatal day 5. Additionally, we examined the effects of dexmedetomidine at lower doses, including 20 μg/kg and 40 μg/kg, and found that dexmedetomidine at these lower doses reduced IL-6 and TNF-α production at