Abstract

**Background**: Previous studies suggest that dexmedetomidine has a protective effect against local anaesthetic-induced nerve injury in regional nerve blocks. Whether this potentially protective effect exists in the context of diabetes mellitus is unknown.

**Methods**: A diabetic state was established in adult male Sprague–Dawley rats with intraperitoneal injection of streptozotocin. Injections of ropivacaine 0.5%, dexmedetomidine 20 μg kg⁻¹ (alone and in combination), or normal saline (all in 0.2 ml) were made around the sciatic nerve in control and diabetic rats (n=8 per group). The duration of sensory and motor nerve block and the motor nerve conduction velocity (MNCV) were determined. Sciatic nerves were harvested at post-injection day 7 and assessed with light and electron microscopy or used for pro-inflammatory cytokine measurements.

**Results**: Ropivacaine and dexmedetomidine alone or in combination did not produce nerve fibre damage in control non-diabetic rats. In diabetic rats, ropivacaine induced significant nerve fibre damage, which was enhanced by dexmedetomidine. This manifested with slowed MNCV, decreased axon density, and decreased ratio of inner to outer diameter of the myelin sheath (G ratio). Demyelination, axon disappearance, and empty vacuoles were also found using electron microscopy. An associated increase in nerve interleukin-1β and tumour necrosis factor-α was also seen.

**Conclusions**: Ropivacaine 0.5% causes significant sciatic nerve injury in diabetic rats that is greatly potentiated by high-dose dexmedetomidine. Although the dose of dexmedetomidine used in this study is considerably higher than that used in clinical practice, our data suggest that further studies to assess ropivacaine (alone and in combination with dexmedetomidine) use for peripheral nerve blockade in diabetic patients are warranted.

**Keywords**: diabetes mellitus; local anaesthetics; peripheral nerve injuries; neurotoxicity
Ultrasound-guided regional anaesthesia techniques facilitate nerve blocks have gained popularity. The addition of adjuvants such as dexmedetomidine to local anaesthetics (LA) improves the quality and prolongs the duration of the block. The potential neurological toxicity of LA–adjuvant combinations should be ruled out before adopting them into clinical practice.

Most animal studies have not observed long-term neurotoxicity with common peripheral nerve block adjuvants. Dexmedetomidine, an α2 adrenoceptor agonist, decreases perineural inflammation and attenuates bupivacaine-induced nerve injury in rat sciatic nerve block models. However, these studies were carried out in non-diabetic rats. Diabetic individuals undergoing peripheral nerve block might be more sensitive to LA neurotoxicity. One animal study showed that lidocaine 4% induced significantly more oedema in diabetic nerves than in controls. Another study showed that diabetic rats receiving lidocaine 1% plus clonidine 7.5 μg ml⁻¹ or ropivacaine 0.5% alone had more abnormal myelinated axon profiles than non-diabetic rats. Clinical studies have also shown that diabetic neuropathic nerves exhibit complex functional changes and seem to be more sensitive to LA. The neuroprotective effects of dexmedetomidine against LA-induced nerve injury could translate into significant clinical benefit. The aim of the current study was to investigate potential neurotoxicity and underlying mechanisms of perineural ropivacaine and dexmedetomidine alone or in combination in normal or diabetic rats.

**Methods**

**Animals and diabetes mellitus model**

Experimental procedures and protocols used in this investigation were approved by the Institutional Animal Care and Use Committee of Peking University Third Hospital, Beijing, China. Male Sprague–Dawley rats (Vital River Laboratories, Peking, China) weighing 192–252 g were housed in an animal care facility thermostatically maintained at 20°C with a 12-h light–dark cycle. To induce a diabetic state, a single 70 mg kg⁻¹ dose of streptozotocin i.p. (Sigma Co., St. Louis, MO, USA) (freshly dissolved in 0.05 M, pH 4.0, citrate buffer) was given. Diabetic rats were confirmed by blood glucose concentration measurements in samples obtained from the tail vein 3 weeks after injection. Rats whose blood glucose concentration was >350 mg dl⁻¹ were considered to be diabetic. Rats in the control group received vehicle citrate buffer injection.

**Verification of diabetic neuropathy**

Tactile allodynia in response to electronic von Frey stimulation of hind paw plantar surface was used as the criterion for diabetic neuropathy. The pressure was gradually increased and maintained for 1–2 s until a brisk lifting of the hind paw was considered as a positive response. Each hind paw was assigned six stimulation sites for 30 s intervals. The average of the six stimulation sites was calculated as the withdrawal threshold.

**Measurement of the motor nerve conduction velocity**

After induction anaesthesia with 3 vol% isoflurane and then maintenance with 1–1.2% isoflurane in oxygen via mask, the left and right hind limbs were shaved. Electrical stimuli were produced using a Cadwell Cascade Elite electrophysiological monitor (Cadwell Co., Kennewick, WA, USA) driven by a 50 μs, 20 mA monophasic stimulus. The active stimulation electrode was inserted first at the ankle and then at the sciatic notch; the stimulation reference electrode was inserted 5 mm proximal to the stimulation electrodes. The record electrode was inserted at the muscle between the first and second plantar pad of the hind limb; the record reference electrode was inserted 5 mm proximal to the record electrodes. To calculate the motor nerve conduction velocity (MNCV), the distance between stimulation sites was divided by the difference in latency to the evoked motor response. Repeated baseline measurements were made at 1 min intervals for 5 min to ensure that the response amplitudes were within ±10% of the average and that the response was not decaying over time.

**Sciatic nerve block**

Sciatic nerve blockade was performed at the 5th week after induction of diabetes mellitus as described. Animals were briefly anaesthetised with 1.2–1.5 vol% isoflurane in oxygen by mask and placed in the lateral position. The thigh was laterally incised and muscle fascia separated by blunt dissection to expose the sciatic nerve. The experimental drug (0.2 ml) was injected into the perineural space below the clear fascia covering the nerve using a tuberculin syringe with a 30 G needle. Ropivacaine 1% (Naropin; Astra Zeneca PLA, Wuxi, China) and dexmedetomidine 100 μg ml⁻¹ (Aibeining; Jiangsu Hengrui Medicine Co., Ltd, Lianyungang, China) were used to prepare a final concentration of ropivacaine 0.5% and a dose of dexmedetomidine 20 μg kg⁻¹ (alone or in combination). Drugs were diluted with normal saline to 0.2 ml. Except for the naïve controls treated with saline (n=4), rats treated with ropivacaine and dexmedetomidine alone or in combination were randomly assigned. For each set of experiments, eight control rats and eight diabetic rats received blocks on both sides. S, R, D, RD represented control rats receiving saline, ropivacaine, dexmedetomidine, or ropivacaine with dexmedetomidine, whereas S-DM, R-DM, D-DM, RD-DM represented diabetic rats receiving saline, ropivacaine, dexmedetomidine, or ropivacaine with dexmedetomidine alone or in combination, respectively.

After recovery from general anaesthesia, a sensory response was evaluated by observing foot withdrawal when a pin was applied to the plantar midline surface of each hind paw (tibial nerve distribution). Motor response was evaluated by observing the toe spreading reflex (peroneal nerve and tibial nerve).
motor fibres), which is a vestibular reflex induced by lifting the rat and producing a response with the toes extended and spread.\(^9,\)\(^13,\)\(^18\) For both tests, responses were rated as present or absent. Sensory and motor blockade were tested every 30 min until full recovery.

**Histomorphometry and determination of pro-inflammatory cytokine concentration**

Seven days after sciatic nerve block, rats were anaesthetised with 1.2 vol% isoflurane in oxygen by mask, and MNCV was measured as described above. The sciatic nerve was gently exposed over a length of 20 mm from the sciatic notch to the mid-thigh,\(^9,\)\(^13\) and the nerve was harvested after euthanasia with an overdose of pentobarbital (120 mg kg\(^{-1}\), i.p.). Left limb nerve samples were homogenised in protease inhibitor cocktail (Plygen) and centrifuged at 1,000 g of 3000 rpm for the machine used. The supernatant was used to measure interleukin (IL)-1 and tumour necrosis factor (TNF)-\(\alpha\) using custom enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK). Right limb nerve samples were stained with toluidine blue to determine the number of differently sized axons\(^19\) and the ratio of the inner to outer diameter of the myelin sheath, and was used to assess axonal myelination. Ten field micrographs of one section of each rat were obtained under 100\(^\times\) magnification. Electron microscopy was performed to assess axonal degeneration and demyelination with image analysis performed by a histologist blinded to the experimental groups.

**Statistical analysis**

Normal distribution of data was tested with the Shapiro–Wilk test. Normally distributed data were expressed as mean (standard deviation, SD), whereas non-parametric data were expressed as median (range). Normally distributed data were analysed with analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. Non-parametric data were analysed using ANOVA followed by a Kruskal–Wallis test. A \(P\) value <0.05 was considered statistically significant. Describe power analysis here [part of Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines].

**Results**

**Diabetes mellitus model**

Five weeks after streptozotocin injection, body weights of diabetic rats were significantly reduced to 246 (49) g compared with controls [346 (23) g; \(P<0.05\)]. Diabetic rats also had polyuria. Before streptozotocin injection, rats in the diabetic group had a mean force withdrawal threshold of 40.0 (1.6) g, which decreased to 32.9 (5.3) g \(P<0.05\) 35 days after streptozotocin injection, indicating tactile allodynia.

**Duration of sciatic nerve block**

Animals had sensory and motor nerve block lasting for \(>100\) min after sciatic nerve block with ropivacaine 0.5% (dexmedetomidine alone and saline did not produce nerve block). Ropivacaine, with or without dexmedetomidine, produced longer mean duration of sensory nerve block in diabetic rats than controls. Sensory and motor block with ropivacaine 0.5% lasted 2.0 (1.8–2.1) h in controls, but this was extended by 2 h in diabetic animals \(P<0.05\). The addition of dexmedetomidine to ropivacaine produced a small but significant increase in sensory and motor block in controls, but extended sensory and motor block in diabetic rats by more than 60 h (Fig. 1; median and range of these data are shown in Supplementary Table S1).

**Motor nerve conduction velocity**

Before sciatic nerve block, rats in the control group had a mean MNCV of 41.1 (5.5) m s\(^{-1}\), whereas those in the diabetic group had a mean MNCV of 33.3 (4.5) m s\(^{-1}\) \(P<0.05\). With the exception of the RD-DM group, the MNCV tested at 1 week after the procedure returned to baseline (Fig. 2).

![Fig 1](image-url). Duration of sensory and motor block produced by ropivacaine alone or ropivacaine in combination with dexmedetomidine in diabetic and control rats. After receiving sciatic nerve block with ropivacaine 0.5% or ropivacaine 0.5% with dexmedetomidine 20 \(\mu\)g ml\(^{-1}\), sensory block duration was evaluated. (a) Evaluation using foot withdrawal response and motor block duration. (b) Evaluation using the toe-spreading reflex. All responses were rated as present or absent. Data shown are median (inter-quartile range, IQR) \((n=4\) in the naive control and \(n=8\) in other groups). \(*P<0.05\), \(**P<0.01\).
Pro-inflammatory cytokines

One week after sciatic nerve block, nerve tissue in all control rats showed no significant difference in the amounts of IL-1β and TNF-α (Fig. 3a and d). There was also no significant difference in cytokine levels in the nerves from rats treated with saline, dexmedetomidine, or ropivacaine in diabetic rats (P>0.05). Nerves in the ropivacaine plus dexmedetomidine group had higher levels of these two pro-inflammatory cytokines compared with saline, dexmedetomidine, and ropivacaine group in diabetic rats (P<0.05; Fig. 3b and e). Moreover, nerves in the ropivacaine plus dexmedetomidine group had higher levels of these two pro-inflammatory cytokines compared with the ropivacaine group of diabetic rats (P<0.05; Fig. 3c and f).

Histology

In the control groups, there was no significant difference in the number of axons per field 1 week after sham nerve block (axon density; Fig. 4a and b). Axon density was significantly decreased in diabetic rats blocked with ropivacaine plus dexmedetomidine, but there was no significant difference in the other three groups (Fig. 4c). Compared with control groups, axon density was significantly decreased in diabetic rats with ropivacaine injection (Fig. 4d). The G ratio of axons was lower in diabetic rats compared with controls for each diameter, and nerve block with ropivacaine plus dexmedetomidine yielded a lower G ratio for diameters of 2–3, 3–4, and 4–5 μm than ropivacaine alone in the diabetic rats (Fig. 5).

Electron microscopy

Segmental demyelination and degeneration of axonal fibres was found in all treatment groups. In all diabetic rats, there were larger areas of demyelination and degeneration of axons. These changes were more pronounced in the ropivacaine groups (R-DM and RD-DM); the most severe damage was seen in the RD-DM group with full-thickness demyelination and vacuole formation (Fig. 6). The severe nerve damage induced by ropivacaine and dexmedetomidine alone or in combination shown was confirmed by our colleagues (Y.B. Sun, D.X. Wang, and S.L. Wang, Peking University) as an independent group. Using the identical experimental protocol, similar results were found (Supplementary Tables S2 and S3, and Figs S1 and S2).

Discussion

Previous studies have shown that addition of dexmedetomidine to perineural LA alleviates oxidative stress and reduces peripheral nerve injury in non-diabetic models.7–9 Our aim was to verify that dexmedetomidine attenuates ropivacaine-induced neurotoxicity in a streptozotocin model of diabetes mellitus. However, although dexmedetomidine itself did not cause sciatic nerve injury in diabetic rats, it drastically enhanced the nerve injury induced by ropivacaine with more significant histopathological changes and greater pro-inflammatory cytokine levels.

In line with previous studies,9,13,18 our data show that ropivacaine 0.5% alone injected into the sciatic notch produces longer sensory and motor blockade in diabetic rats compared with non-diabetic rats. We also showed that dexmedetomidine added to ropivacaine significantly enhanced sensory and motor blockade of the sciatic nerve and prolonged the duration of block in all rats; these changes were significantly more pronounced in diabetic rats. Dexmedetomidine alone did not produce nerve blockade, as reported previously.5,16,22 The median and range of duration of both sensory and motor block in the R, RD, and R-DM groups in our current study were of a similar order of magnitude to those reported in studies where equivalent doses of ropivacaine and dexmedetomidine were used. Any variability is likely to reflect differences in methodology in assessing block duration between studies. A summary of relevant previous studies is shown in Supplementary Table S4 for comparison. Pre-existing neuropathy (such as in diabetes mellitus) as a possible risk factor for LA-induced nerve injury has been described.8,9,13,23–25 However, this is the first study to describe the profound enhancement of block duration and significant neurotoxicity-enhancing effects of dexmedetomidine when added to perineural ropivacaine in a diabetes model.

The mechanisms of action underlying these observations have not yet been identified. The large increase in the duration of nerve block in diabetic rats is likely caused by metabolic factors.9,12 Streptozotocin-induced diabetic rats have altered neural protein kinase C activity26 and altered potassium and sodium conductance leading to decreased hyperpolarization-activated cation current (Ih) in large sensory axons27 that may contribute.

It has been shown that dexmedetomidine enhances LA action via peripheral α2A adrenoceptor agonism,28 and increases the duration of analgesia by inhibition of Ih by a mechanism independent of the α2A receptor.16 This double inhibition of Ih might explain the large increase in block duration (by >60 h) in the RD-DM group.5,6,25 A previous study reported a correlation between block duration and nerve fibre damage in diabetic rats13 although a causative link has not been established, and our findings support that observation.

Ropivacaine plus dexmedetomidine produced severe demyelination and degeneration of axons in diabetic rats. We
speculate that the pre-existing neuropathy induced by diabetes, with complex functional changes such as altered ionic currents and aberrant calcium homeostasis explains this. Inflammation also clearly plays a role as evidenced by the increase in pro-inflammatory cytokines in the RD-DM group. Another possibility is that the higher dose of dexmedetomidine used in our study causes receptor-mediated vasoconstriction with reduced perineural blood flow that potentiates...
ropivacaine-induced neurotoxicity. α2 receptor-independent upregulation of norepinephrine release by high-dose dexmedetomidine may also contribute.31

We observed neither an increase in perineural inflammation with LA treatment in non-diabetic rats nor a significant reduction in perineural inflammation with dexmedetomidine addition in non-diabetic rats, as has been reported.4,5 Previous studies also found that perineural administration of dexmedetomidine 20 μg kg⁻¹ reduced sciatic nerve levels of IL-6 and TNF-α at both the mRNA and protein levels.6 The different time points at which the tissue samples were harvested are likely to explain this (7 days after injection in our study vs between 24 h and 14 days in previous work; see Supplementary Table S4). Brummett and colleagues4 showed that these anti-inflammatory effects of dexmedetomidine were only evident at 24 h and that there was no significant difference in inflammation between groups at 14 days; this fits with our results and suggests an acute anti-inflammatory effect only in non-diabetic rats. Furthermore, when dexmedetomidine was administered systemically it increased pain threshold, suppressed microglia activation, and decreased pro-inflammatory cytokines in the spinal cord in streptozotocin-induced diabetic rats.32 This finding of a protective rather detrimental effect with systemic dexmedetomidine is consistent with protective effects afforded by intravenous infusion of dexmedetomidine.33

The dose of dexmedetomidine used in our study is much higher than that being used clinically. Because of interspecies variability and fundamental differences between human and animal models of disease, drug doses that need to be used in animal models are often much higher than those used clinically (often up to 10-fold higher). The literature on translating doses from animal models to human trials has repeatedly shown that it is far more complex than scaling dosage based on body mass.34,35 Therefore, it is invalid to assume that a 20 μg kg⁻¹ dose of dexmedetomidine in a rodent is equivalent to a 20 μg kg⁻¹ dose in a human. Our choice of dose was determined by those used in a number of studies conducted previously to allow for comparison with the established literature (Supplementary Table S4). Moreover, the same dose used in normal controls did not cause any damage, indicating its inherent neurotoxic effect in the diabetic condition. An open wound model was used in the present study to avoid unintentional intra-nerve injection, but the potential impact of local tissue damage on inflammatory state in those animals cannot be ruled out. However, cytokines were not significantly increased in sham surgery controls, suggesting again that the disease conditions rather than other factors resulted in the toxic effects of the treatments. The streptozotocin-induced diabetes model is not an ideal model to mimic the human neuropathy of type 2 diabetes mellitus, so one should consider this in interpreting our

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**Fig 4.** Axon density of all groups at 1 week after injection. (a) Toluidine blue staining of rat sciatic nerves at 1 week after injection. (b) Axon density (number of axons per field) in normal rats. (c) Axon density in diabetic rats. (d) Axon density in rats with ropivacaine with or without dexmedetomidine injection. Data shown are mean (standard deviation, SD) (n=4 in the naive control and n=8 in other groups); *P<0.05, **P<0.01.
Lastly, whether $\alpha_2$ adrenoceptors are involved in dexmedetomidine-induced neurotoxicity is not unknown; if nerve fibre damage already exists in the diabetic condition, introducing another chemical of $\alpha_2$ adrenoceptor antagonist may cause further nerve damage rather than reduce the toxicity.

In summary, high-dose perineural dexmedetomidine enhanced LA-induced nerve injury in a rat diabetes model. Because of these unexpected findings, further study is necessary to investigate the effects of clinically relevant doses of dexmedetomidine in a model of type 2 diabetes mellitus and its mechanisms. It is premature to extrapolate the present results to clinical recommendations about the risks and benefits of this adjuvant to LA when performing regional anaesthesia in patients with pre-existing neuropathy. Establishing a dose–response relationship for these observations and further work looking at the underlying mechanisms will help clarify these important questions.

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**Fig 5.** G ratio in different sizes of axons. (a) G ratio for axon diameters of 1–2 $\mu$m. (b) G ratio for axon diameters of 2–3 $\mu$m. (c) G ratio for axon diameters of 3–4 $\mu$m. (d) G ratio for axon diameters of 4–5 $\mu$m. (e) G ratio for axon diameters of 5–6 $\mu$m. Data are derived from 10 field micrographs of one section in each rat obtained under 100× magnification. The mean G ratio of those 10 fields was calculated for further analysis and expressed as mean (standard deviation, SD) (n=4 in the naive control and n=8 in other groups); *$P<0.05$. D-DM, dexmedetomidine in diabetic rats; R-DM, ropivacaine in diabetic rats; RD-DM, ropivacaine plus dexmedetomidine in diabetic rats; S-DM, saline in diabetic rats.
Authors’ contributions

Performed experiments: Z.Y.Y., J.G., Y.B.S., S.L.W.
Data analysis: Z.Y.Y., J.G., Y.B.S., S.L.W., M.L., D.M.
Design of the study: M.L., D.M.
Interpretation of data: M.L., D.M.
Project supervision: M.L., D.M.
Writing and revising paper: All authors.

Declaration of interest

D.M. is a board member of British Journal of Anaesthesia. All other authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2018.08.022.

References


Fig 6. Electron microscopy of rat sciatic nerves at 1 week after injection. Tissue from all rats (n=4 in naïve control treated with saline and n=8 in all other groups) was used for electron microscopic analysis; one representative micrograph from each group is presented. Segmental demyelination (*) can be seen in all groups. The S-DM and D-DM groups displayed larger areas of demyelination and degeneration of axons (#). These changes were more obvious in the R-DM group. In the RD-DM group there was full-thickness demyelination and vacuole formation (△), which indicated more severe nerve injury. D-DM, dexmedetomidine in in diabetic rats; R-DM, ropivacaine in diabetic rats; RD-DM, ropivacaine plus dexmedetomidine in diabetic rats; S-DM, saline in diabetic rats.

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