Neurotoxicity of bupivacaine and liposome bupivacaine after sciatic nerve block in healthy and streptozotocin-induced diabetic mice

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Abstract

Background

Diabetes mellitus and the associated neuropathic complications have become a steadily increasing global health burden. Diabetic patients are estimated to require surgery at least twice as often as nondiabetic patients. Neuropathy may change the way nerves respond to nerve blocks. There is currently no consensus on whether regional anaesthesia techniques should be adopted in these patients. Long-acting local anaesthetics (e.g. bupivacaine HCl) or sustained-release formulations of bupivacaine (e.g. liposomal bupivacaine) could prove neurotoxic in the presence of diabetic neuropathy. The aim of the study was to assess neurotoxicity of liposome bupivacaine in streptozotocin (STZ)-induced diabetic mice after sciatic nerve block using a reduction in fibre density and decreased myelination assessed by G-ratio as an indicator of local anaesthetic neurotoxicity.

Results

Prior to performing sciatic nerve block, higher levels of fasting glucose were recorded in diabetic mice compared to nondiabetic mice (P < 0.001). Likewise, significant differences were noted in the tail flick and plantar test thermal latencies between the groups (P < 0.001) which confirmed the presence of peripheral sensory neuropathy in diabetic mice. In both, diabetic and nondiabetic mice, sciatic nerve block with 0.25% bupivacaine HCl resulted in a significantly greater G-ratio (axon diameter/large fibre diameter) and an axon diameter compared to nerves treated with 1.3% liposomal bupivacaine or saline (0.9% sodium chloride) (P < 0.01). Moreover, sciatic nerve block with 0.25% bupivacaine HCl resulted in higher fibre density and large fibre and axon diameters compared to the control (untreated) sciatic nerves in both STZ-induced diabetic (P < 0.05) and nondiabetic mice (P < 0.01). No evidence of acute or chronic inflammation was observed in any of the treatment groups.

Conclusions

Under the conditions of this study, sciatic nerve block with bupivacaine HCl, but not liposome bupivacaine or saline, resulted in histomorphometric indices of neurotoxicity. The presence of diabetes did not appear to affect the severity of the histologic findings.
The incidence of diabetes has been steadily increasing, and in the next two decades, it is estimated that over 640 million people worldwide will be affected [1]. Although only 10% of patients with diabetes report symptomatic peripheral neuropathy, as many as 50% may already have subclinical neuropathy [2]. Patients with diabetes require surgical procedures more frequently than healthy patients, and due to their comorbidities, peripheral nerve blocks are often recommended as an alternative to general anaesthesia, particularly for lower extremity surgery [3]. In diabetic rats, a prolonged application of high doses of local anaesthetics perineurally has been associated with neurotoxicity [4–6]. In humans, it is not well established if the nerve blocks can exacerbate a pre-existing diabetic neuropathy [7].

In the last decade, extended release local anaesthetic formulations have been developed to increase the duration of peripheral nerve blocks and to reduce the risk of systemic or local tissue toxicity [8]. Bupivacaine liposome injectable suspension (DepoFoam bupivacaine, EXPAREL®, Pacira Pharmaceuticals, Inc., San Diego, CA, USA) is an extended-release formulation of bupivacaine encapsulated in multivesicular liposomes that has been approved by the U.S. Food and Drug Administration for wound infiltration and interscalene brachial plexus block [9, 10]. Studies to date have found no evidence of neurotoxicity of liposomal bupivacaine used for peripheral nerve blocks or epidural applications in animals and humans [11–17]. However, long-acting local anaesthetics (e.g. bupivacaine HCl) or sustained-release formulations of bupivacaine (e.g. liposomal bupivacaine) could prove neurotoxic in the presence of a pre-existing neuropathy [5]. The aim of this study was to assess the neurotoxic effects of liposomal bupivacaine and bupivacaine hydrochloride (HCl) following perineural injection for sciatic nerve block in streptozotocin (STZ)-induced diabetic mice. We hypothesized that perineural injections of bupivacaine HCl and liposomal bupivacaine would result in a reduction of fibre density and decreased myelination in diabetic nerves assessed by G-ratio.

Results

Animal characterization

Prior to induction of diabetes, no significant difference in mean body mass was observed between the diabetic [24.6 (1.5) g] and nondiabetic groups [24.7 (2.0) g]. Four weeks after STZ treatment, a lower
mean body mass [21.3 (1.9) g] was recorded in diabetic compared to nondiabetic group [27.7 (2.1) g], \(P < 0.001\). At the same time, fasting glucose levels were higher in diabetic [32.4 (2.0) mmol l\(^{-1}\)] compared to nondiabetic mice [6.8 (0.9) mmol l\(^{-1}\)] \(P < 0.001\).

Before application of STZ, no differences were noted in paw withdrawal test thermal latencies between the groups. By contrast, following the STZ treatment and prior to sciatic nerve block, significant differences were observed in tail flick and plantar test thermal latencies between the groups (Fig. 1). The success of sciatic nerve block was confirmed in all animals using a paw withdrawal test.

Histopathological evaluations

For each animal, the treated and untreated sciatic nerve tissue specimens were analysed. Data are presented in Table 1. After bupivacaine HCl treatment, the sciatic nerves of diabetic and nondiabetic mice showed a significantly lower fibre density and a myelin width, and higher axon and large fibre diameters compared to untreated control nerves. After liposomal bupivacaine and saline treatments, by contrast, no differences were observed in morphometric parameters compared to untreated control nerves in both diabetic and nondiabetic mice. Thus, the presence of diabetes did not affect the severity of morphometric changes among the groups (Fig. 2). There was also no evidence of inflammation observed in any specimen; inflammatory cells were scarce, occurring only as discrete leucocytes in a few specimens (Fig. 3).

Discussion

The results of this study suggest that bupivacaine HCl but not liposomal bupivacaine used for sciatic nerve block had a neurotoxic potential as evident from the lower nerve fibre density and the higher G-ratio as indicator of nerve demyelination. However, the presence of STZ-induced diabetes did not appear to affect the severity of pathohistological changes of nerves.

In contrast to our results, no changes indicative of neurotoxicity after application of bupivacaine HCl and liposome bupivacaine were reported in other studies on healthy rats [11, 13, 16]. Moreover, both local anaesthetic formulations were reported to be safe for use in brachial plexus nerve block in rabbits and dogs [12], and liposome bupivacaine was reported to be safe for use in sciatic nerve block.
in pigs [14]. Similarly, a summary analysis of clinical trials of off-label liposome bupivacaine use for peripheral nerve block in 335 patients without neuropathy found that liposome bupivacaine had a similar safety and side effect profile to bupivacaine HCl and saline [15].

Peak local anaesthetic concentration may play a role in nerve injury in subjects with diabetic neuropathy [12]. In STZ-induced diabetic rats, nerve oedema, degeneration and demyelination of myelinated nerve fibres were observed after application of 2% and 4% but not 1% lidocaine [18]. These data led to the widely accepted belief that the risk for local anaesthetic-induced nerve injury was higher in diabetic animals [4]. Similarly, myelin sheet thinning was found after 0.5% ropivacaine, 1% lidocaine with clonidine, and 1% lidocaine with epinephrine in STZ-induced diabetic rats [5]. Furthermore, the duration of peripheral nerve block could also contribute to neurotoxicity [5]. A small increase in local nerve toxicity was also observed in obese diabetic rats with subclinical diabetic neuropathy after 2% lidocaine injections [19].

Diabetic neuropathic nerves exhibit complex functional changes [7]. In our study, diabetic mice showed early functional sensory impairment without morphological correlates, consistent with findings in STZ-induced diabetic rats [5, 20]. In contrast, in 6 week-old male STZ-induced diabetic mice of the same strain used in another study, thin, disorganized and demyelinated sciatic nerve fibres were observed [21]. Given that axon and myelin sheet growth are not yet completed in the 6 week old mice [22], the difference in animal age at the time of STZ application (6 weeks in Pan et al. [21] and 8 weeks in our study), may be responsible for the differential effects of STZ and hyperglycaemia on the sciatic nerves.

In our study, the number of small sciatic nerve fibres was lower following bupivacaine HCl compared to saline application in nondiabetic mice. However, this effect was not replicated in other morphometric studies after perineural bupivacaine HCl application in rat, rabbit or dog model [11–13]. Given that the small nerve fibres are usually first to be affected by diabetes [23], bupivacaine HCl may further promote small nerve fibre degeneration after nerve blockade in subjects with diabetic neuropathy.

We did not observe any signs of inflammation in diabetic and nondiabetic nerves, which is in line with
McAlvin et al. [13]. In contrast, using an open approach for sciatic nerve block in nondiabetic rats, infiltrations with macrophages, lymphocytes and fibroblasts were observed after both liposomal bupivacaine and bupivacaine HCl injections [11]. These differences could be attributed to differences in animal model and nerve block technique. We used a percutaneous rather than an open technique to minimize the nerve injury due to procedure itself [5]. Furthermore, it may not be suitable to study the inflammatory changes of the peripheral nerves in a STZ-induced diabetic model, since STZ causes depletion of immune cells in the peripheral nervous system up to 3 weeks after treatment [24]. A longer-term longitudinal study in a high-fat-diet-induced diabetes type 2 model could be more informative in assessing possible inflammatory effects.

Any extrapolation of our results to clinical practice of perineural application of bupivacaine and liposomal bupivacaine must be considered within the context of a number of limitations. The unifascicular sciatic nerve in mice may be more sensitive to neurotoxic effects compared to multifascicular nerves with abundant connective tissue within epineurium in humans. Although large animal models may better resemble the multifascicular sciatic nerve seen in humans, there are difficulties in establishing diabetes and diabetic neuropathy in larger animals [25]. Further, the STZ-induced diabetic mouse model does not correlate well with all aspects of type 1 or type 2 diabetes in humans [24]. High-fat-diet-induced diabetes is thought to better represent the more prevalent type 2 diabetes in humans, however there is no diabetic mouse model that intimately mirrors the human pathophysiology of diabetes [26]. And finally, given that only females were used in our study, another study in males is warranted as sex differences in STZ sensitivity have been noted in rodent models [27].

Conclusions
Under the conditions of our study, application of bupivacaine HCl, but not liposome bupivacaine, resulted in histological evidence of neurotoxicity in both STZ-induced diabetic and nondiabetic mice. The presence of diabetic neuropathy did not appear to increase the severity of the neurotoxic changes.

Methods
The study was carried out in accordance with the recommendations of the Guide for the Care and the Use of Laboratory Animals of the National Institutes of Health (National Research Council (U.S.) [28], the Committee for the Update of the Guide for the Care and Use of Laboratory Animals, and the Institute for Laboratory Animal Research (U.S.), 2011). The study was approved by the Ethical Committee for laboratory animals of the Republic of Slovenia (Permit Number: U34401-21/2013/6) following European directives on the use of laboratory animals in research and the ARRIVE guidelines.

**Animal housing and induction/confirmation of diabetes**

Six weeks old C57BL/6j-OlaHsd female mice ($n = 36$, weight 25-30 g) were obtained from Harlan Laboratories – Envigo (Italy) and reared at the Centre for Laboratory Animals of the Biotechnical Faculty of the University of Ljubljana. All mice were housed individually in ventilated cages (IVC system) with temperature maintained at $23 \pm 1^\circ$ C, humidity maintained at 40-60%, and a 12-hour light/12-hour dark cycle.

At the age of 8 weeks, after 2 weeks of quarantine and acclimatization period with free access to clean water and standardized diet (Mucedola, Milan, Italy), diabetes type 1 was induced in mice by intraperitoneal injection of 200 mg kg$^{-1}$ STZ in accordance with the protocols for achieving STZ-induced diabetes in mice [21, 29]. STZ is an alkylating agent that induces degeneration in pancreatic $\beta$ islets [18, 30]. Diabetes was confirmed by measuring a fasting glucose level in blood samples collected from the tail vein using Bayer Contour glucose meter (Ascensia Diabetes Care Holdings AG, Switzerland) three weeks after STZ injection. Animals with a fasting glucose level of more than 25 mmol l$^{-1}$ were considered diabetic, while those with less than 8 mmol l$^{-1}$ were considered nondiabetic [21].

**Verification of diabetic neuropathy**

To confirm the presence of peripheral sensory neuropathy, tail flick and paw withdrawal tests were performed using Combination Plantar/Tail Flick Analgesia Meter (IITC Life Science, California, USA) with infrared intensity set at 40% and 50%, and cut-off times of 4.00 and 15.00 seconds, respectively [31]. The paw withdrawal test was performed two days before the STZ application and two days prior
to the sciatic nerve block, whereas the tail flick test was performed two days prior to the sciatic nerve block. Heat stimulation was repeated 3 times at 5 minute-interval; the mean value of the two measurements was used as the baseline [32]. The plantar method is based on Hargreaves method of quantifying the heat thresholds in the hind paws of rodents upon application of radiant or infrared heat stimulus [33]. The tail flick test involved the application of a heat stimulus to the tail after which the time for the tail to “flick” or twitch was recorded. We used a tail temperature option with an automatic temperature trigger at the start of the tests. Once the pre-set temperature was reached, the timer was automatically triggered and stopped after the tail flicks and the light had stopped. The automatic readouts of the start and end temperatures, and the test time improved a repeatability of the measurements. This option has solved the problem associated with “tail temperature prior to and at the end of testing” [34]. While the recent reviews discussed advantages as well as disadvantages of both tests, the two methods are still considered as relevant stimulus-evoked nociception tests [35].

**Study groups**

Eighteen STZ-induced diabetic and eighteen nondiabetic mice were randomized into the three treatment groups. According to the group assignment, both diabetic and nondiabetic groups included 6 mice treated with 35 mg kg\(^{-1}\) 1.3% liposomal bupivacaine (EXPAREL), 6 mice treated with 7 mg kg\(^{-1}\) 0.25% bupivacaine HCl (AstraZeneca UK Ltd, UK), and 6 mice treated with saline (NaCl Braun, 9 mg ml\(^{-1}\) injection solution, B Braun Melsungen AG, Germany).

**Sciatic nerve block**

The mice were anaesthetized with isoflurane up to 4% in a nitrous oxide/oxygen mixture (N2O/O2) via a face-mask. Sciatic nerve blocks were performed by injecting local anaesthetics or saline perineurally using a 29-gauge needle (Omnican\(^{®}\)A, B. Braun Melsungen AG, Germany) while held in a lateral recumbent position with paws in a right angle with the trunk. The needle was introduced posteromedially towards the greater trochanter in an anteromedial direction. After encountering the ischial tuberosity, 85 µl of testing solution was injected by a single research staff member, blinded to the study group assignment [13, 36, 37]. The success of the sciatic nerve block was evaluated 20
min after using the paw withdrawal test.

**Histopathological evaluation of the sciatic nerve**

The animals were sacrificed by cervical dislocation one week after the nerve block in order to allow enough time for nerve pathohistological changes to manifest [38]. At the site of local anaesthetic injection and contralaterally, five mm-long sections of the sciatic nerve were harvested and processed for Epon-embedding for histomorphometric evaluations. After initial fixation in Karnovsky’s KII Solution (2.5% glutaraldehyde, 4.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4), the nerve sections were post-fixed in an 1:1 solution of 2% aqueous osmium tetroxide and 3% potassium ferrocyanide. Dehydration was accomplished with graded ethanol solutions and propylene oxide following Epon embedding. A high-resolution light microscope (Eclipse E800; Nikon, Tokyo, Japan) was used to study the prepared 0.5 µm toluidine blue stained cross-sections with images captured by a digital camera (DXM1200F™, Nikon, Tokyo, Japan) connected to the microscope. Images were analysed by a single operator blinded to group assignment.

Morphometric analysis was performed using the Ellipse program (ViDiTo, version 2.0.7.1, 2004, Košice, Slovakia) [14]. Randomly selected areas of the nerve were analysed. The outer border of the nerve fibres and the inner border of the myelin sheaths were assessed at high magnification followed by measurement of the nerve fibre density, proportion of large fibres (percent of fibres where the myelin sheet is visible and can be circumscribed), large fibre diameter, axon diameter and myelin width. Furthermore, G-ratio defined as the ratio of the inner to the outer diameter of the myelin sheath was also calculated [39]. The images were analysed by a trained evaluator blinded to group assignment.

Histopathological evaluation was also employed to assess inflammatory cell infiltration in the histological specimens. Frozen samples of the sciatic nerve were sliced into 10 µm transverse sections processed for immunohistochemistry for leucocyte receptor-type tyrosine-protein phosphatase C (CD45) labelling with anti-CD45 antibody (MCA1388, Bio-Rad Laboratories Inc., San Francisco, CA, USA) and revealed by a secondary antibody P0260 (Dako, Glostrup, Denmark). Positive and negative tissue controls were included with each batch of slides as a check on correct tissue
preparation and staining techniques. Sections of mouse thymus served as positive control for the presence of leukocytes. For negative controls, the sections in which the primary antibody was replaced with phosphate-buffered saline were used. The images were analysed by a trained evaluator blinded to group assignment.

**Statistical analysis**

The Shapiro-Wilk test was used to evaluate the groups for normality. If normality and equal variance assumptions were met, differences in histomorphometric parameters among treatment groups were tested by two-way analysis of variance (ANOVA) followed by Bonferoni *post-hoc* tests that corrected the *p*-values for the subgroup analyses. The dependent *t*-test for paired samples was used to test differences in histomorphometric parameters between treated and untreated sciatic nerves in the same animal. One-way ANOVA, followed by Tukey post-hoc tests was used for paw withdrawal test. Independent *t*-test was used to compare tail flick test results, body mass and fasting glucose. Statistical analysis was performed with the IBM SPSS Statistics for Windows, version 25 (IBM Corp, NY, USA). Differences were deemed statistically significant at *P* < 0.05. Data are presented as means (standard deviation).

The sample size calculation was based on the primary research hypothesis that the STZ-induced diabetic and nondiabetic nerves would differ in their fibre density as an indicator of local anaesthetic neurotoxicity [40]. Using the difference in mean fibre density (14,000 fibres per mm$^2$), pooled standard deviation (1600 fibres per mm$^2$), Type I alpha (0.01), and a desired power (0.90), the sample size was estimated at 6 animals in each treatment group for this two-sided test of a completely crossed 2x3 ANOVA (diabetic/nondiabetic by liposomal bupivacaine/bupivacaine HCl/saline).

**Abbreviations**

ANOVA – analysis of variance

BHCL – bupivacaine hydrochloride

HCl - hydrochloride

IVC – individually ventilated cages

LB – liposome bupivacaine
STZ - streptozotocin

Declarations

**Ethics approval and consent to participate**

The study was carried out in accordance with the recommendations of the Guide for the Care and the Use of Laboratory Animals of the National Institutes of Health (National Research Council (U.S.) [28], the Committee for the Update of the Guide for the Care and Use of Laboratory Animals, and the Institute for Laboratory Animal Research (U.S.), 2011). The study was approved by the Ethical Committee for laboratory animals of the Republic of Slovenia (Permit Number: U34401-21/2013/6) following European directives on the use of laboratory animals in research and ARRIVE guidelines.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

Dr. Admir Hadzic has received research funding and honoraria as a consultant to PACIRA Pharmaceuticals. The other authors declare no competing interests.

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**Authors’ contributions**

LM, SH, EC designed the study, conducted the study, collected data, wrote the manuscript, read and approved the final version of the manuscript; VM, TSP collected data, wrote the manuscript, read and approved the final version of the manuscript; NU analysed data, wrote the manuscript, read and approved the final version of the manuscript.
approved the final version of the manuscript; AH, MK designed the study, wrote the manuscript, read and approved the final version of the manuscript.

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Tables

Table 1 Histomorphometric parameters of the sciatic nerve after treatment with bupivacaine hydrochloride (BHCl), liposome bupivacaine (LB) and saline in STZ-induced diabetic mice and nondiabetic control mice.
|                         | Treated nerves | Control nerves |
|-------------------------|----------------|----------------|
|                         | NaCl           | Saline         | LB             | Saline         | BHCl           |
| Fibre density (mm⁻²)    |                |                |                |                |                |
| Diabetic                | 23724 (6888)   | 22862 (4349)†  | 30259 (8511)   | 26232 (4743)   | 31717 (8258)   |
| Nondiabetic             | 22492 (4881)   | 21582 (6144)†† | 21983 (3408)   | 25969 (2950)   | 29522 (3558)   |
| Large fibre area per total area |                |                |                |                |                |
| Diabetic                | 65.20 (2.54)   | 62.63 (7.00)   | 64.03 (2.50)   | 67.93 (1.39)   | 64.58 (3.00)   |
| Nondiabetic             | 66.91 (3.61)   | 63.95 (6.46)   | 65.71 (2.53)   | 65.87 (2.39)   | 65.19 (2.86)   |
| Large fibre diameter (µm) |                |                |                |                |                |
| Diabetic                | 5.58 (0.65)    | 5.58 (0.54)†   | 4.97 (0.72)    | 5.37 (0.48)    | 4.81 (0.68)    |
| Nondiabetic             | 5.82 (0.69)    | 5.8 (0.75)††   | 5.68 (0.42)    | 5.27 (0.38)    | 4.94 (0.21)    |
| Axon diameter (µm)      |                |                |                |                |                |
| Diabetic                | 3.13 (0.41)    | 3.53 (0.31)# †† | 2.79 (0.68)    | 2.97 (0.55)    | 2.85 (0.31)    |
| Nondiabetic             | 3.26 (0.49)    | 3.55 (0.50)††  | 3.25 (0.17)    | 2.98 (0.09)    | 2.78 (0.16)    |
| Myelin width (µm)       |                |                |                |                |                |
| Diabetic                | 1.23 (0.18)    | 1.01 (0.17)*    | 1.09 (0.06)    | 1.14 (0.12)    | 1.06 (0.19)    |
| Nondiabetic             | 1.28 (0.19)    | 1.12 (0.29)*    | 1.21 (0.22)    | 1.15 (0.20)    | 1.08 (0.07)    |
| G-ratio                 |                |                |                |                |                |
| Diabetic                | 0.56 (0.03)    | 0.63 (0.04)**## | 0.56 (0.06)    | 0.55 (0.06)    | 0.60 (0.09)    |
| Nondiabetic             | 0.56 (0.04)    | 0.62 (0.06)**## | 0.58 (0.05)    | 0.57 (0.04)    | 0.56 (0.02)    |

Values are means (SD), n = 6 for each study group. From two-way ANOVA: *P < 0.05, **P < 0.01 for BHCl- versus saline-treated nerves, and #P < 0.05, ##P < 0.01 for BHCl- versus LB-treated nerves. From dependent t test: †P < 0.05, †† P < 0.01 between treated and control nerves.

Figures
Figure 1

Paw withdrawal test before streptozotocin (STZ) treatment and two days prior to sciatic nerve block and tail flick test two days prior to sciatic nerve block in STZ-induced diabetic (●) (n=18) and nondiabetic (■) (n=18) mice. *P<0.0001 vs nondiabetic mice prior to sciatic block and diabetic and nondiabetic mice prior to STZ treatment (one-way ANOVA);

#P<0.001 vs nondiabetic mice (independent t-test)
Figure 2

Cross section of the right sciatic nerve seven days after administration of saline, 0.25% bupivacaine hydrochloride (BHCl) and 1.3% liposome bupivacaine (LB) at the sciatic nerve in streptozotocin (STZ)-induced diabetic (a, e, i) and nondiabetic mice (c, g, k). Untreated nerve from the left leg served as controls (b, f, j, d, h, l). Staining with toluidine blue. Bar – 50 µm
Figure 3

Cross-section of the right sciatic nerve seven days after administration of saline, 0.25% bupivacaine hydrochloride (BHCl) and 1.3% liposome bupivacaine (LB) at the sciatic nerve in STZ-induced diabetic (a, c, e) and nondiabetic mice (b, d, f) demonstrating rare leucocyte (arrows) infiltration. Immunoreactivity for CD45 is presented. Bar – 50 μm

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
ARRIVE Guidelines Checklist Markova.pdf