Normal glucose enhances neuronal regeneration after lidocaine-induced injury

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Background. Local anaesthetics exhibit direct neurotoxic effects on neurones. Numerous studies have investigated the factors that may reverse this neuropathology, but the effects of glucose conditions on neuronal regeneration after lidocaine-induced injury have not been examined by observing living neurones. The present study investigated the effects of different glucose conditions on neurite length, growth cone regeneration, and cell death in dorsal root ganglia (DRG) neurones after lidocaine-induced injury in vitro.

Methods. DRG explants were isolated from chick embryos at embryonic day 8 and cultured in media containing low, normal, or high glucose concentrations (10, 25, or 40 mM) for 24 h. Tissues were exposed to lidocaine 8 mM for 1 h, then rinsed and incubated for a further 24 h. Neurite length and growth cone collapse assays were performed to assess neuronal growth and regeneration. Lactate dehydrogenase (LDH) and caspase assays were also performed to detect neuronal cell death.

Results. Addition of lidocaine for 1 h resulted in >97% growth cone collapse and neurite destruction under all three glucose conditions. Two hours after rinsing out the lidocaine, significant reversal of growth cone collapse and neurite elongation was observed under all glucose conditions. Growth cone collapse was higher under low-glucose condition (P<0.05). High glucose negatively affected neurite length more than growth cone collapse. At 24 h, LDH release with both low- and high-glucose conditions was higher than with normal glucose (P<0.05). Low- and high-glucose conditions increased caspase 3/7 activation.

Conclusions. Normal glucose is optimal for neuronal recovery after lidocaine-induced injury in vitro.

Br J Anaesth 2010; 104: 482–6

Keywords: anaesthetics local; recovery, neurological; toxicity

Accepted for publication: January 20, 2010

Although local anaesthetics are widely used clinically, numerous studies have revealed neurotoxic effects when these agents are applied to peripheral neurones in high concentrations or for long durations.1 2 Previous studies from our laboratory have demonstrated local anaesthetic toxicity in growing and regenerating neuronal tissues.3 4 This is of potential clinical importance, as local anaesthetics are often applied repeatedly or continuously at sites where peripheral nerves may still be growing or regenerating, as in paediatric or trauma patients. Neurotoxicity caused by local anaesthetic administration is not a novel concept. Transient neurological symptoms (TNS) were reported after continuous spinal anaesthesia with 5% lidocaine or 1% tetracaine5 or bolus injection of 5% lidocaine into the lumbar subarachnoid space.6 7 Although TNS might represent the lower end of the toxicity spectrum,8 the results of numerous clinical studies suggest that lidocaine is associated with a higher incidence of TNS than bupivacaine.9 In addition, this neurotoxicity has been proven to be dose-dependent in both in vitro and in vivo studies.10 11 Persistent neurological deficits were observed with bolus injection of high concentrations of lidocaine and tetracaine.
into the subarachnoid space of the spinal cord in rabbits, but not with clinically used concentrations.\textsuperscript{12}

Glucose concentration is one of the factors known to affect neuronal growth and regeneration. The aim of the current study was to examine the effects of glucose concentration on neuronal growth and regeneration after lidocaine toxicity. The dorsal root ganglion (DRG) and associated cell bodies are well known to be particularly vulnerable to the metabolic effects of high-glucose concentrations, as the DRG is positioned outside the spinal cord and thus is not protected by the blood–nerve barrier.\textsuperscript{13} Sensory neurones located in the DRG of streptozotocin (STZ)-induced diabetic rats undergo apoptotic cell death \textit{in vivo}.\textsuperscript{14} When DRG neurones were treated with high-glucose concentrations \textit{in vitro}, significant apoptosis and impaired neurite outgrowth were observed.\textsuperscript{14} In addition, Oztürk and colleagues\textsuperscript{16} described higher numbers of dead and apoptotic DRG neurones with increased glucose concentrations. Several recent studies have reported glycaemic control and proper local tissue care as the main lines of treatment for diabetic neuropathy.\textsuperscript{17, 18} However, the effects of glucose concentration on neuronal regeneration after lidocaine-induced injury have not been clarified in observations of living neurones. The current study therefore investigated the effects of different glucose conditions on neurite length, growth cone regeneration, and cell death in DRG neurones after injury induced by lidocaine \textit{in vitro}.

**Methods**

DRG explants were isolated from lumbar paravertebral sites of chick embryos at embryonic day (E)8, plated on laminin-coated cover slips, and cultured in F-12 medium (GIBCO BRL, Life Technologies, Grand Island, NY, USA) supplemented as described by Bottenstien and colleagues\textsuperscript{19} with 100 \textmu g ml\textsuperscript{-1} bovine pituitary extract (Kojin Bio Co., Chiba, Japan), 2 mM glutamine (MP Biomedicals, Germany), 100 units ml\textsuperscript{-1} penicillin, 100 \textmu g ml\textsuperscript{-1} streptomycin (GIBCO BRL Life Technologies), and 20 ng ml\textsuperscript{-1} mouse 7S nerve growth factor (Invitrogen, Tokyo, Japan). D-Glucose was added to the culture media to produce final concentrations of 10, 25, or 40 mM. Cultures were then incubated at 37°C in 5% CO\textsubscript{2}. Next, tissues were exposed to lidocaine 8 mM added to the culture medium for 1 h. The medium was then replaced with lidocaine-free media containing the same glucose concentration. Lactate dehydrogenase (LDH) assays were undertaken to detect cell death before the addition of lidocaine, and 8 and 24 h after washout of the lidocaine, using the LDH-cytotoxicity test (Wako Chemicals, Osaka, Japan). For each experiment, a negative control in which DRG explants were not exposed to lidocaine, using the growth cone collapse assay as long as they were not in contact with or in close proximity to neighbouring growth cones or neurites. Fifty growth cones were randomly chosen and viewed for scoring.

Scoring criteria for growth cone collapse assay were based on the method reported by Raper and Kapfhammer.\textsuperscript{20} Briefly, growth cones without filopodia or lamellipodia were considered to be collapsed and were given a value of 1. Growth cones with intact filopodia and lamellipodia were considered not collapsed and given a value of 0. Growth cones without filopodia and with shrunken lamellipodia were considered partially collapsed and given a value of 0.5. The total percentage of growth cone collapse was calculated as \text{(number of collapsed growth cones/total of 100 growth cones)}\times 100.

**Cytotoxicity assay**

DRG explants were cultured in media containing the three different glucose concentrations used in the morphological assessment and were incubated for 24 h at 37°C in 5% CO\textsubscript{2}. Next, tissues were exposed to lidocaine 8 mM added to the culture medium for 1 h. The medium was then replaced with lidocaine-free media containing the same glucose concentration. Lactate dehydrogenase (LDH) assays were undertaken to detect cell death before the addition of lidocaine, and 8 and 24 h after washout of the lidocaine, using the LDH-cytotoxicity test (Wako Chemicals, Osaka, Japan). For each experiment, a negative control in which DRG explants were not exposed to lidocaine (the medium was replaced with lidocaine-free medium) and a positive control in which DRG explants were continuously exposed to lidocaine for 24 h were included.

**Caspase 3/7 activation**

For evaluating apoptosis in DRG neurones in the different glucose conditions we used, DRG explants cultured in the
different glucose conditions for 24 h. The activity of caspase 3/7 was determined using the caspase-Glo 3/7 assay (Promega, Madison, WI, USA). Enzyme activity was normalized to the amount of protein in the sample. The protein concentration was determined by the Bradford–Lowry method using the reagents and protocol supplied by Bio-Rad Laboratories (CA, USA). Samples were read in a 96-well plate in an absorbance plate reader (Bio-Rad Laboratories).

**Statistical analysis**

Data are presented as mean [standard deviation (SD)] of six independent measurements for neurite length, LDH leakage, growth cone collapse, and four independent measurements for caspase 3/7 activation. Chronological changes in neurite length, LDH leakage, growth cone collapse, and washout effects were analysed using one-way analysis of variance (ANOVA) for repeated measurements. Mean values were compared using two-way ANOVA. Post hoc analysis was performed using the Scheffe test and values of $P<0.05$ were considered significant.

**Results**

Exposure of DRG explants to lidocaine 8 mM for 1 h resulted in >97% growth cone collapse under each of the three glucose conditions (Figs 1 and 2). A significant reversal in growth cone collapse was observed 2 h after washing the lidocaine out, with the percentage of growth cone collapse decreasing from >97% to 25.6 (7.3)%, 20.6 (5.7)%, and 13.6 (11.2)% at low, normal, and high glucose concentrations, respectively. This reversal was continued at 8 and 24 h after wash-out of lidocaine (Figs 1 and 2). The percentage of growth cone collapse was higher under low-glucose conditions than under normal and high-glucose conditions in both control (see Supplementary material online, Appendix 1) and 1 h exposure groups (Fig. 2).

In addition, we found that 1 h exposure to lidocaine also reduced the rate of neurite elongation for 8 h after washing lidocaine out, particularly with normal and low-glucose concentrations. Elongation rate then recovered to control levels. Neurite length at high glucose concentration was lower than at normal and low glucose concentrations across the entire experiment. Lidocaine exposure did not significantly worsen this (see Supplementary material online, Appendix 2).

Although the percentage of growth cone collapse was largely unaffected by the high-glucose condition (Fig. 2), the deleterious effects on neurite length were significant (see Supplementary material online, Appendix 2). After 24 h of continuous exposure to 8 mM lidocaine, total growth cone collapse and complete destruction of neurites were observed.

LDH release increased in a time-dependent manner regardless of the presence or absence of lidocaine. At 24 h after removing lidocaine from cultures, LDH was higher under both low- and high-glucose conditions than under the normal glucose condition, either with or without 1 h lidocaine exposure (Fig. 3) (Supplementary material online, Appendix 1). When DRG explants were continuously exposed to lidocaine for 24 h, LDH leakage reached a significant level after 8 h exposure under low-glucose conditions ($P=0.0175$), whereas significant LDH leakage was only seen at 24 h under normal and high-glucose conditions (see Supplementary material online, Appendix 3).

Caspase 3/7 activation as a marker of apoptosis increased in both low- ($P=0.041$) and high-glucose conditions ($P=0.006$) compared with normal glucose.

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**Fig 1** DRG cultured for 20 h, immediately before exposure to lidocaine (A–C), collapsed growth cones and shrinkage of neurites after exposure to lidocaine for 1 h in the three glucose conditions (D–F). Recovery of the collapsed cones 2 h after washing lidocaine out (G–I). Growth cones 24 h after wash (J–L). Neurite length 24 h after wash (M–O).

**Fig 2** Growth cone collapse after 1 h exposure to lidocaine. *Significant difference compared with values in normal glucose ($P<0.05$).
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Fig 3 LDH leakage after 1 h exposure to lidocaine at different glucose concentrations. *Significant difference compared with the values in normal glucose (P<0.05). +Significant difference compared with pre-exposure value (P<0.05).

Discussion

A vast number of clinical studies have reported strict glycaemic control and adequate tissue care as the most successful lines of treatment for diabetic neuropathy. Neuronal growth cones play an important role in path finding and in the establishment of cytoarchitecture, and might be the most sensitive structure to toxicity in growing or regenerating neourone, due to their unique biochemical characteristics. For this reason, growth cone collapse is considered a morphological parameter for studying the effects of drugs or factors on neuronal growth. The current study used lidocaine toxicity as a model of neuronal injury. Exposure of DRGs to lidocaine 8 mM for 1 h resulted in >97% growth cone collapse and neurite destruction under all three glucose concentrations investigated. Previous experiments from our laboratory have used a lower concentration of lidocaine 4 mM, and found 87% growth cone collapse. This lidocaine-induced neurotoxicity is attributable to direct neurological damage, and increases in intracellular calcium to toxic levels. Total growth cone collapse, complete destruction of neurites, and significant LDH leakage were observed after 24 h of continuous exposure to lidocaine 8 mM. This suggests that exposure to lidocaine for long periods or at a high concentration causes membrane disruption as one aspect of direct toxicity.

We selected 25 mM of glucose to represent normal plasma glucose as previously defined. On the basis of this definition, we selected 40 mM to represent high glucose and 10 mM to represent low glucose. Hyperosmolality caused by increasing glucose concentration up to 50 mM has no significant effect on neuronal growth. In our media, osmolarity was measured with a vapour pressure osmometer (Wescor Inc., USA) and found to be within the acceptable range for neuronal growth (298, 310, and 316 mOsm for low, normal, and high glucose media, respectively).

The percentage of growth cone collapse was higher at all time end-points under low-glucose conditions compared with normal and high-glucose conditions. This effect of low glucose could be due to ATP depletion and an increase in caspase-3 cleavage. Although high-glucose conditions in the current study did not lead to significant growth cone collapse, total neurite length was significantly impaired. This discrepancy suggests that the high-glucose condition might affect growth cone function more than morphology. Our findings are consistent with those of Russell and colleagues who reported that maximum neuronal growth of cultured DRG explants from E15 rats occurred at a glucose concentration of 30 mM, and that growth was significantly reduced in medium containing either 20 or 40 mM glucose.

DRG sensory neurones are the primary site of diabetic neuronal complications, as these cells are particularly vulnerable to oxidative stress caused by hyperglycaemia. We found that low- and high-glucose conditions increased caspase 3/7 activation significantly in DRG neurones. The effects of high-glucose concentration on DRG neurones depend on the duration of exposure since an increase in the apoptosis of DRG neurones of STZ-diabetic rats was observed after 1, 3, and 12 months of diabetes. In addition to time dependence, these deleterious effects also depend on glucose concentration, as neuronal apoptotic nuclei increased in a concentration-dependent manner when DRG neurones were cultured in media containing glucose concentrations >30 mM.

The present study identified that both low- and high-glucose conditions significantly increased LDH leakage 48 h after culture, but not before that. After exposure to lidocaine for 1 h, LDH levels in low- and high-glucose media were notably higher than that in the normal glucose medium at 24 h after washing. In addition, when DRGs were continuously exposed to lidocaine for 24 h, the increase in LDH release reached a significant level by 8 h under low-glucose conditions, but by 24 h under normal and high-glucose conditions. DRG explants cultured under low-glucose conditions seem more vulnerable to the toxic effects of lidocaine than DRG explants cultured under normal or high-glucose conditions.

In summary, we found that the highest percentage of growth cone collapse and the highest level of LDH leakage were observed under low-glucose conditions, whereas the shortest neurite length was identified under high glucose. Caspase 3/7 activation has been confirmed where the shortest neurite length was identified under high glucose. Caspase 3/7 activation has been confirmed in both high- and low-glucose conditions. We therefore conclude that normal glucose is the most appropriate for neuronal growth and recovery after lidocaine-induced toxicity. Moreover, when DRG explants were exposed to lidocaine for only 1 h, reversal of toxicity was satisfactory. Although in vitro laboratory data cannot be directly applied to clinical settings, our findings suggest that tightly regulated glycaemic control could facilitate optimal recovery after local anaesthetic-induced neuronal injury.

Supplementary material

Appendices 1, 2, and 3 are available as Supplementary material at British Journal of Anaesthesia online.
Funding
Supported, in part, by a Grant-in-Aid (Scientific Research Grant #20390411-90 to S. Saito) from the Ministry of Education, Science and Culture of Japan.

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