Dexmedetomidine provides cortical neuroprotection: impact on anaesthetic-induced neuroapoptosis in the rat developing brain

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Background: Recent evidence has demonstrated the anti-apoptotic of dexmedetomidine in different brain injury models. Herein, we investigated whether dexmedetomidine could directly protect against cortical injury in vitro and in vivo.

Methods: Apoptosis was induced by staurosporine or wortmannin treatment in cortical neuronal cultures in vitro or by 6 h of isoflurane (0.75%) administration to post-natal day 7 rat pups in vivo. Dexmedetomidine was then applied in escalating doses to assess the neuroprotective potential of this agent. Cell survival was quantified using an MTT assay in vitro and in vivo apoptosis was assessed using cleaved caspase-3 immunohistochemistry. Cortical Western blots were conducted for the cellular survival proteins Bcl-2 and phosphorylated extracellular signal-regulated protein kinase (pERK)1 and 2.

Results: In vitro dexmedetomidine dose-dependently prevented both staurosporine- and wortmannin-induced injury in cortical neuronal cultures, indicating that dexmedetomidine can prevent apoptosis when applied directly. In vivo isoflurane induced cortical neuroapoptosis compared with air (327 ± 80 vs. 34 ± 9 caspase-3-positive neurons; P < 0.05). Dexmedetomidine inhibited isoflurane-induced caspase-3 expression (P < 0.05), although the protection achieved did not completely attenuate the isoflurane injury (P < 0.05 vs. air). Isoflurane treatment decreased Bcl-2 and pERK protein expression relative to air, an effect reversed by dexmedetomidine treatment.

Conclusions: Dexmedetomidine prevents cortical apoptosis in vitro and in vivo. However, using higher doses of dexmedetomidine does not further increase protection against isoflurane injury in the cortex than previously observed.
**In vitro experiments**

Neuronal cells derived from cerebral neocortices were harvested from 16-day-old embryonic mice by a caesarean section for pregnant BALB/c mice as described above. The cells were plated at a density of $1.2 \times 10^5$ cells/cm$^2$ on 24-multiwell plates pre-coated with poly-l-lysine (Cater, Cambridge, MA), and the cultures were maintained at $37^\circ$C in a humified 5% CO$_2$ and air environment and fed with neurobasal media supplemented with B27 (× 1) and glutamine (25 μM). The cultures were maintained at $37^\circ$C in a humified 95% air–5% CO$_2$ atmosphere. On day 5 after neuronal plating, 100 μl/10 ml cytosine arabinoside (10 μM, Sigma, Poole, UK) was added to the cell cultures to halt non-neuronal cell division. Neuronal cell cultures were ready to use on day 7. Once prepared, the cells were treated with the protein kinase inhibitors staurosporine (200 nM) or wortmannin (3 μM) to induce apoptosis over 24 h$^{9-11}$ and simultaneously exposed to various doses of dexmedetomidine (0.1–100 μM). Cell survival was assessed by a tetrazoium salt (MTT) assay using colorimetry to provide quantitative data. Further, the other cohort cell cultures treated with dexmedetomidine (1 μM) alone or in combination with staurosporine (200 nM) were labelled with Hoechst 33258 (10 μg/ml in PBS; Sigma, Poole, UK) and propidium iodide (10 μg/ml in PBS; Sigma, Poole, UK) staining to visually identify dying cells under a fluorescence microscope.

**In vivo experiments**

Seven-day-old Sprague–Dawley rat pups were exposed to 6 h of isoflurane 0.75% in oxygen (25%) or air in a temperature-controlled chamber ($n = 6$ per group). Three doses of saline or dexmedetomidine (25, 50 or 75 μg/kg) were administered by an intraperitoneal (i.p.) injection over the 6-hour exposure (at 0, 2 and 4 h) as per our previous protocol.

The animals were sacrificed (with sodium pentobarbital 100 mg/kg by an i.p. injection) at the end of gas exposure and perfused transcardially with heparinized saline followed by paraformaldehyde 4% in 0.1 M buffer. After removal of the brain and storage overnight at 4 °C in paraformaldehyde, it was transferred to sucrose solution (30%) with phosphate buffer and sodium azide 1% and maintained at 4 °C until the brains were sectioned and stained immunohistochemically for caspase-3.

**Immunohistochemistry**

The brain was sliced at 30 μm intervals beginning at −3.6 mm from the bregma; the sections were then transferred to a six-well plate containing PBS. Sections were dried at 37 °C for 24 h and then immunostained while adherent to the slides, before pre-incubation with hydrogen peroxidase 0.3% in methanol for 30 min and then rinsed in PBS. The sections were then incubated overnight at 4 °C with rabbit anti-cleaved caspase-3 (1:2500; New England Biolab, Hitchin, UK) and then washed three times in PBS with Triton 3% at room temperature. Biotinylated secondary antibodies (1:200; Sigma, St. Louis, MO) and the avidin–biotin–peroxidase complex (Vector Laboratories, Orton Southgate, Peterborough, UK) were applied. The sections were again washed in PBS before incubating with 0.02% 3,3′-diaminobenzidine (DAB) with nickel ammonium sulphate in 0.003% hydrogen peroxide (DAB kit, Vector Laboratories). The sections were dehydrated through a gradient of ethanol solutions (70–100%) and then mounted (floating section) and covered with a cover slip.

**Immunoblotting**

Three experimental groups were included in the Western blotting experiments: (1) air plus i.p. saline, (2) isoflurane (0.75% in oxygen) plus i.p. saline and (3) isoflurane (0.75% in oxygen) plus i.p. dexmedetomidine (25 μg/kg). After experimental exposure, Sprague–Dawley rat pups ($n = 3$ per group) were sacrificed, their brains were immediately removed and the cortex was separated, harvested and frozen at −80 °C. The samples were then homogenized (Polytron homogenizer by Kinematica, Bethlehem, PA) in ice-cooled lysis buffer (20 mm Tris-HCl, 150 mm NaCl, 1 mm Na$_2$DTA, 1 mm EGTA, 1% Triton, 2.5 mm sodium pyrophosphate, 1 mm β-glycerophosphate, 1 mm Na$_3$VO$_4$, 2 mm dl-dithiothreitol, 1 mm phenylmethanesulphonyl and 1 μg/ml leupeptin; pH 7.5) and centrifuged at 3000 g for 10 min at 4 °C to remove cellular debris. The supernatant was further centrifuged twice, initially at 12,000 g for 15 min at 4 °C and a second time at 20,000 g for 45 min at 4 °C. The supernatant, stored at −80 °C, and subsequently used to blot Bcl-2 and phosphorylated extracellular signal-regulated protein kinase (pERK)1 and 2 (Cell Signalling). The protein concentration of lysates was determined using the Bradford protein assay (Bio-Rad, Herts, UK). Protein extracts (10 μg per sample) were denaturated in NuPAGE LDS.
Sample buffer (Invitrogen, Paisley, UK) at 70 °C for 10 min and were then loaded on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Buckinghamshire, UK) and incubated with a blocking solution composed of 5% fat dry milk in Tween-containing Tris-buffered saline (pH 8.0, 10 mm Tris, 150 mm NaCl, 0.1% Tween). The blocked membrane was incubated overnight at 4 °C with the following antibodies: Bcl-2, pERK1 and 2. After washing the membrane for 20 min with four changes of Tween-containing Tris-buffered saline, it was incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody directed at the primary antibody. The bands were then visualized with enhanced chemiluminescence (New England Biolab) and exposed onto a Hyperfilm ECL film (Amersham Biosciences).

**Statistics**

Both *in vitro* and *in vivo* results are presented as mean ± SD. The number of caspase-3-positive neurons in the cortex in each brain slice (four brain slices/brain) was counted per animal by an observer blinded to the experimental protocol. Statistical analysis was performed by analysis of variance, followed by *post hoc* Newman–Keuls testing using the Instat (London, UK) program. A *P* < 0.05 was set to indicate statistical significance.

**Results**

Dexmedetomidine increased the survival of cortical neurons *in vitro* in a dose-responsive manner against apoptosis induced by either wortmannin or staurosporine. Specifically, dexmedetomidine 0.1–100 μM protected against wortmannin-induced apoptosis (*P* < 0.01, Fig. 1), increasing cellular survival 71–141% over the control. Dexmedetomidine 1–100 μM protected against staurosporine-induced apoptosis (*P* < 0.05), increasing cellular survival by 16–32% over the control (Fig. 1). The results for staurosporine were confirmed visually by both Hoechst and propidium iodide staining. While all cell nuclei were labelled with Hoechst, nuclei with features suggestive of neuronal death (pyknosis, i.e., condensation of chromatin) were stained brightly, which was confirmed with the specific cell death staining marker of propidium iodide (Fig. 2).

In *vivo*, all neonatal rats survived the experiments. Isoflurane induced neuroapoptosis throughout the cortex compared with air (327 ± 80 vs. 34 ± 9; *P* < 0.05) (Fig. 3). Dexmedetomidine (25 μg/kg) did not induce apoptosis (51 ± 19; *P* > 0.05 vs. air) but reduced isoflurane-induced caspase-3 expression in the cortex. Dexmedetomidine 25 μg/kg reduced the number of caspase-3-positive neurons to 188 ± 29 (*P* < 0.05). However, escalation of doses to 50 μg/kg (189 ± 59) and 75 μg/kg (166 ± 23) did not further improve this protection (*P* > 0.05 relative to dexmedetomidine 25 μg/kg).

Western blots showed that exposure to isoflurane (0.75% in oxygen) reduced the cellular survival proteins pERK1 and 2 and Bcl-2 relative to air.
This effect was reversed in the presence of 25 μg/kg dexmedetomidine, indicating that dexmedetomidine maintained trophic cellular signalling through pERK1/2 and Bcl-2 in the presence of isoflurane (Fig. 4).

**Discussion**

Our *in vitro* data importantly demonstrate that dexmedetomidine exerts direct neuroprotective effects to prevent cultured cortical neuronal apoptosis when temperature, oxygen and glucose supply are closely controlled. *In vivo*, we have confirmed that dexmedetomidine at 25 μg/kg inhibits isoflurane-induced cortical injury as reported previously; however, in this study, we have been unable to demonstrate that higher doses provide superior protection against isoflurane-induced apoptosis. We have also shown that isoflurane-induced apoptosis involves a reduction in the anti-apoptotic signalling pathways mediated by pERK1 and 2 and Bcl-2 similar to injury of the neonatal brain.

Fig. 2. Cortical neuronal co-cultured cells were labelled with Hoechst and propidium iodide staining to visually identify dying cells following staurosporine (200 nM) treatment. Dexmedetomidine (1 μM) qualitatively reduced the observed fluorescence from the dying cells. Scale bar = 100 μm. Arrow, died cell; arrow head, normal cell.
with propofol and ketamine\textsuperscript{13} and that dexmedetomidine can reverse this effect, likely through demonstrated effects on pERK signalling.\textsuperscript{14}

**Models employed**

The \textit{in vitro} model of cortical apoptosis used is a validated model of neuronal apoptotic injury,\textsuperscript{9–11} and it is incorporated to show further proof that dexmedetomidine can inhibit cortical apoptosis via a direct effect. However, we merely used this model to validate whether dexmedetomidine could act directly on cortical neurons to inhibit apoptosis. We chose the tetrazoium salt assay (MTT) as our outcome measure as we wished to show that cellular survival was increased in these validated models of apoptosis\textsuperscript{9–11}; the injury was further confirmed visually (Fig. 2). Our \textit{in vivo} model is also a validated model of neuronal apoptotic cell death, with caspase-3 able to identify apoptotic neurons in this context.\textsuperscript{1–5} Because of the size of subject, we were unable to measure systemic physiological parameters such as blood glucose, gases and pressure during our \textit{in vivo} work; however, in our \textit{in vitro} models (these included herein and isoflurane injury of organotypic hippocampal slices), oxygen, glucose and temperature are controlled. Therefore, this suggests that dexmedetomidine does not alter systemic physiology to prevent apoptosis, rather dexmedetomidine acts directly to institute neuroprotection.\textsuperscript{5,8} Unfortunately, due to technical problems, quantitative data for the Western blots are not available; however, dexmedetomidine has previously been shown to upregulate both pERK\textsuperscript{14} and Bcl-2\textsuperscript{15} and therefore we have included the data for completeness.

**Cortical injury**

As cortical injury appears to be particularly important in studies evaluating anaesthetic-induced apoptosis in primate brains,\textsuperscript{7} we wished to explore dexmedetomidine’s effects further in this brain region. In our previous study, we found that dexmedetomidine attenuated the isoflurane injury completely (i.e. reduced the level of apoptosis to non-injured levels) in the thalamus and hippocampus but not in the cortex.\textsuperscript{5} However, our present data do not suggest that increasing the dose of dexmedetomidine to \(25\)\textsuperscript{m}g/kg confers further protection against isoflurane injury of the neonatal rat cortex. Thus, maximal cortical neuroprotection against isoflurane-induced apoptosis was not achieved with dexmedetomidine therapy; combination therapies may be needed to reduce this form of isoflurane injury. Our \textit{in vitro} model data suggest that dexmedetomidine can act locally to prevent cellular apoptosis, supporting our recent demonstration that dexmedetomidine can inhibit isoflurane injury in organotypic hippocampal cultures \textit{in vitro}. We cannot extrapolate from our experiments
herein that dexmedetomidine can act directly within the central nervous systems to inhibit cortical neuron apoptosis induced by isoflurane (i.e. by direct cellular actions). However, we have provided further evidence that dexmedetomidine can prevent apoptotic cell death against different modalities of injury [now including protein kinase inhibitors as well as isoflurane (in hippocampal cultures)] and excitotoxic injury in vitro. Furthermore, as disruption of protein kinase signalling appears to be intrinsic to staurosporine-, wortmannin- and isoflurane-induced apoptosis, there may be some overlap in their mechanisms of injury, although this is speculative at this stage and will require further evaluation. Herein, we demonstrate that similar to the intravenous anaesthetics ketamine and propofol, isoflurane apoptosis is also associated with downregulation of pERK and Bcl-2 signalling. We have also shown that similar to other neuroprotective agents in this context, such as lithium, dexmedetomidine neuroprotection is associated with an upregulation of these critical effectors (Fig. 4), consistent with dexmedetomidine’s ability to regulate the pERK neuroprotective signalling cascade.

\(\alpha_2\)-adrenoceptor agonists, such as dexmedetomidine, are used clinically for analgesia, anxiolysis, sedation and sympatholysis, as well as anaesthetic-sparing and haemodynamic-stabilizing effects. Although the present study demonstrated that dexmedetomidine exerts anti-apoptotic effects in vitro and in vivo against cortical neuron apoptosis, the potential use of dexmedetomidine as a neuroprotectant especially in paediatric anaesthesia requires further study. However, while dexmedetomidine inhibited apoptosis it was unable to completely attenuate the cortical injury induced by isoflurane; in the future, studies should assess a multi-modal strategy to reduce cortical brain injury induced by anaesthetics. However, before any translation is made to patients, both confirmation of a clinical injury and primate safety and efficacy studies are required. Nonetheless, herein, we reconfirm our recent finding that dexmedetomidine can reduce isoflurane-induced neuroapoptosis in the developing rat cortex and exerts anti-apoptotic effects in vitro. As dexmedetomidine is the first agent to show neurocognitive protection against isoflurane-induced neuroapoptosis, future studies should compare efficacy against dexmedetomidine for this injury. If a clinical injury from anaesthetics is proven in children, we hope that dexmedetomidine may be part of a neuroprotective regimen to enhance the safety profile of paediatric anaesthesia in the future.

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**References**


12. Jawad N, Rizvi M, Gu J, Adeyi O, Tao G, Maze M, Ma D. Neuroprotection (and lack of neuroprotection) afforded by...


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