Nociception after intraperitoneal injection of a sodium pentobarbitone formulation with and without lidocaine in rats quantified by expression of neuronal c-fos in the spinal cord – a preliminary study

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Summary

After a search on Medline, it appears that intraperitoneal injection of sodium pentobarbitone is often used for anaesthesia and euthanasia of rodents. In the present pilot study in rats, spinal nociception after intraperitoneal injection of sodium pentobarbitone, with and without lidocaine, was examined by estimation of the number of c-fos-expressing neurones in the spinal dorsal horn. One group of rats received an intraperitoneal injection of 0.4 mL/kg sodium pentobarbitone (100 mg/mL; n = 4). Another group of rats received a similar intraperitoneal injection of sodium pentobarbitone formulated with lidocaine 10 mg/mL (n = 4); a control group received a similar intraperitoneal injection of 0.9% saline (n = 4). After 3 h, the animals were re-anaesthetized and perfused with 4% formaldehyde, and the spinal cord was collected and processed by immunohistochemistry for stereological quantification of the number of neurones with c-fos-like immunoreactivity (FLI).

Intraperitoneal injection of the sodium pentobarbitone formulation caused a significantly increased number of neurones with FLI in the spinal cord (3930 ± 247; mean ± SEM; P < 0.001) compared with the saline control group (765 ± 131). The lidocaine added to the sodium pentobarbitone formulation significantly reduced the number to 2716 ± 393 (P < 0.05). In conclusion, intraperitoneal injection of sodium pentobarbitone caused a significant increase in nociception which was lowered by adding lidocaine to the formulation, although it was still significantly higher than the control level. Further studies are needed with the aim of optimizing the lidocaine concentration and also to examine the effect of the combination of lidocaine with a long-acting local anaesthetic agent, e.g. bupivacaine.

Keywords  Nociception; intraperitoneal injection; sodium pentobarbitone; lidocaine; rats

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this route of injection, there are issues of absorption, misplacement and irritation to be taken into consideration. All these factors add to the variation in pharmacokinetic and pharmacodynamic endpoints to be measured. Visceral absorption can vary considerably depending on where the injection is placed in the abdominal cavity (Claassen 1994). In case of visceral absorption, the drug will subsequently enter the portal vein and may be subject to first-pass metabolism in the liver. Likewise, the extent of visceral absorption varies in different parts of the abdominal cavity and at the diaphragm level, additional lymphogenic absorption may take place (Tsilibary & Wissig 1987, Abu-Hijleh et al. 1995).

Drug formulations of e.g. barbiturates may cause irritation to the parietal and visceral peritoneum and the underlying tissues after intraperitoneal injection. In addition, intraperitoneal injections of different irritating formulations have been used as a tool to induce peritoneal sclerosis in mice and rats (Gotloib et al. 2005). This irritation may affect the absorption rate and, in addition, cause peritoneal and visceral pain. Intraperitoneal injection of isotonic saline has been shown to be potentially stressful to the animals as indicated by expression of c-fos in discrete areas of the brain of mice (Ryabinin et al. 1999) or by elevation of nociceptive thresholds after intraperitoneal injection of hypertonic saline (Wright & Lincoln 1985).

After a search on Medline, it appears that intraperitoneal injection of sodium pentobarbitone is often used for anaesthesia and euthanasia of rodents. Ambrose et al. (2000) have shown that addition of lidocaine to a solution of sodium pentobarbitone used for intraperitoneal euthanasia reduced distress and writhing behaviour in rats.

Based on the findings of Ambrose et al. (2000), this study was undertaken to quantify the nociceptive spinal input by estimation of the number of spinal nociceptive neurones expressing c-fos-like immunoreactivity (FLI), in rats after intraperitoneal injection of sodium pentobarbitone in order to quantify the nociceptive response. In addition, the number of FLI spinal neurones was estimated after intraperitoneal injection of the same concentration of sodium pentobarbitone to which 1% lidocaine had been added to see if lidocaine was able to reduce the nociceptive response. If so, the addition of lidocaine would improve animal welfare and thus potentially be a substantial procedure refinement.

Materials and methods

Animals

Twelve male Wistar Hanover GALAS rats (obtained from HanTac:WH, Taconic M&B A/S, Ry, Denmark), weighing 350–400 g, were housed under controlled temperature (21 ± 1°C) with a fixed light/dark cycle (12 h:12 h, light on at 08:00 h) in cages of three rats. Commercial feed (Altromin 1324; Brogaarden, Gentofte, Denmark) was available ad libitum until 18 h before the intraperitoneal injection procedure, and tap water was available ad libitum until the experiment was commenced. The rats were fasted for 18 h to ease correct intraperitoneal injection.

The experiment was approved by the Danish Animal Experiment Inspectorate.

Groups

One group of rats (n = 4) received an intraperitoneal injection of sodium pentobarbitone (100 mg/mL), and another group of rats (n = 4) received an intraperitoneal injection of the same formulation of sodium pentobarbitone (100 mg/mL) to which lidocaine hydrochloride had been added at a concentration of 10 mg/mL. A third group of rats (n = 4) received an intraperitoneal injection of physiological saline (0.9% sodium chloride). All formulations were given intraperitoneally at a dose volume of 0.4 mL/kg in the lower quadrant on the right side of the rat’s abdomen. The concentrations used of sodium pentobarbitone and lidocaine hydrochloride were chosen because the very same concentrations had been used in the previously reported study by Ambrose et al. (2000). The rats recovered from the anaesthesia before
being re-anaesthetized for perfusion fixation. One animal from the sodium pentobarbitone + lidocaine group was excluded due to technical problems.

Formulations

The pentobarbitone formulation contained sodium pentobarbitone (final concentration 100 mg/mL), propylene glycol (400 mg/mL) and 96% ethanol (150 mg/mL). The pH in the solution was around 10. The sodium pentobarbitone formulation with lidocaine was composed identically (100 mg/mL), but furthermore contained 10 mg/mL lidocaine hydrochloride. Sodium pentobarbitone and lidocaine hydrochloride were of Ph Eur grade. The formulations used were prepared by the pharmacy of the university. A commercially available physiological saline solution (0.9%) for injection was used in the control group.

Perfusion fixation/euthanasia

Two hours and 45 min after the intraperitoneal injection of the test formulations (i.e. the rats had recovered), the animals were re-anaesthetized by another intraperitoneal injection of a similar formulation of sodium pentobarbitone in a dose volume of 0.6 mL/kg. After 15 min, the animals were killed through perfusion with 4% formaldehyde buffer [pH 7.0; Bie & Berntsen, Rødovre, Denmark] via the aorta and an incised right atrium of the heart. The flow of formaldehyde buffer was 29 mL/min for 20 min.

Tissue preparation – dehydration and cryopreservation

Following perfusion fixation, the spinal cord was removed from the last cervical vertebra to the first lumbar vertebra and fixed for 24 h in a 4% formaldehyde buffer. The tissue was then dehydrated in 10% sucrose in potassium phosphate-buffered saline (KPBS) for 24 h, followed by 72 h in 30% sucrose in KPBS, all at 4°C. Finally, the right side of the spinal cord was marked by making a groove of a few millimetres, and the tissues were stored at −80°C.

Tissue preparation – spinal cord sections

The specimen of the spinal cord was cut into segments of 5 mm length. Following fixation in Tissue Tec™ (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), 100 μm thick slices were made from the cranial end of each segment using a cryostat (Microm HM 500 OM; Microm Laborgeräte GmbH, Walldorf, Germany). These slices were selected for stereological quantification of the number of neurones with FLI. Thus, all parts of the selected spinal cord had an equal opportunity of being sampled and a systematic uniform random sampling (SURS) scheme was achieved [Gundersen et al. 1988].

Immunohistochemical staining

All sections were immunohistochemically stained for FLI as free-floating sections by the avidin–biotin–peroxidase (ABC) method [Hsu et al. 1981]. The sections were placed in wells (one section per well) in a multiwell PVC tray, which was submerged into the different solutions, that penetrated to the well through 1 mm holes (diameter) in the bottom of the tray. At first, the tissue was incubated for 10 min in a blocking solution of 1% hydrogen peroxide in KPBS before being incubated for 24 h in primary antibody diluted 1:8000 in an AB-buffer (KPBS; 1% bovine serum albumin [Sigma-Aldrich, St Louis, MO, USA]; 0.3% Triton X-100 [AppliChem, Darmstadt, Germany] and 10% goat serum [Sigma-Aldrich, St Louis, MO, USA]). The primary antibody was a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 4–17 of human c-fos [PC38, Oncogene®, San Diego, CA, USA]. The sections were then washed in washing buffer (KPBS containing 0.25% bovine serum albumin and 1% Triton X-100) for 24 h, whereafter the sections were incubated for 60 min in biotinylated goat-anti-rabbit immunoglobulin G [IgG] [DAKO A/S, Glostrup, Denmark] diluted 1:600 in AB-buffer. Following washing in washing buffer, in KPBS and again in washing buffer, the sections were incubated in ABC complex (Vectastain Elite ABC Kit, PK-6100 standard,
Vector\textsuperscript{\textregistered} Laboratories, Burlingame, CA, USA) diluted 1:400 in washing buffer for 60 min. Thereafter, the sections were washed in washing buffer and KPBS before they were developed in VIP chromogen \textit{(Peroxidase Substrate Kit VIP SK-4600, Vector\textsuperscript{\textregistered} Laboratories, Burlingame, CA, USA)}, 3 drops/5 mL of KPBS for 4 min. The staining process was stopped by washing in distilled water and afterwards the sections were mounted on gelatine-subbed slides to be air dried overnight at 4°C. Finally, sections were dehydrated in graded alcohol (70%, 96% and 99%) followed by clearing of the sections in xylene to be coverslipped with Eukitt (O Kindler GmbH & Co, Freiburg, Germany). All incubations and procedures were carried out at room temperature except where otherwise stated.

\textbf{Quantification of FLI neurones}
The number of FLI neurones in the selected areas of the stained tissue sections was counted using a light microscope. The spinal dorsal horn was defined as the grey matter dorsal to a vertical line extending from the central canal to the lateral border of the grey matter. Thus, Rexed laminae I through approximately VI was included (Bonica 1990).

A nucleus was counted when it appeared dark purple with a well-defined border. In lighter labelled cells, the unlabelled nucleolus could be identified. The counting procedure was blinded, meaning that the investigator was unaware of the origin of the sections during the counting procedure. The FLI neurones were counted by optical dissector counting rules (Gundersen \textit{et al.} 1988, West & Gundersen 1990). Thus, a neuron was counted when the nucleus came into focus within the counting frame when zooming down through the section.

The total number of neurones with FLI in both spinal dorsal horns was estimated by the optical fractionator method \textit{(Gundersen et al. 1986)}. This method gives an unbiased estimate of the total number of cells \(N\) derived from the number of cells in the sampled fraction \(N'\) and the inverse sampling fraction \(f\) as in the formula:

\[
N = N' \times f \quad \text{(Gundersen \textit{et al.} 1988)}.
\]

Practically, this was achieved by counting Fos-LI neurones in a known fraction of the thickness \(H/h\) of the individual sections, under a known fraction of the sectional area \(a(\text{step})/a(\text{frame})\), on a known fraction of the sections \(T/t\) from the spinal cord or paraventricular nucleus [PVN]. The total number of FLI neurones \(N_{\text{FLI}}\) was then calculated as the product of the number of FLI neurones counted with the optical dissector \(N'_{\text{FLI}}\), and the inverse sampled fractions, by the equation

\[
N(\text{FLI}) = N'_{\text{FLI}} \times \frac{a(\text{step})}{a(\text{frame})} \times \frac{H}{h} \times \frac{T}{t}.
\]

The fraction of the sectional area and the fraction of the thickness were selected by the use of a computer-assisted stereological toolbox \textit{(CAST-Grid, Olympus, Denmark)}, as described by West \textit{et al.} (1996).

\textbf{Data analysis}
Differences between groups were evaluated by using one-way breakdown analysis of variance \textit{(ANOVA)}. \textit{Post hoc} tests were done with the least significant difference \textit{(LSD)} test. \(P\) values less than 0.05 were considered statistically significant. Data are presented as mean \(\pm\) SEM.

\textbf{Results}
Fos-positive neurones were found in laminae I, II, V and X of the spinal dorsal horn. The largest number of neurones with FLI was found in the right dorsal horn reflecting that the injection was applied on the right side of the abdomen. The FLI neurones were found in the entire length of the spinal cord examined.

The number of FLI neurones was significantly elevated \(P<0.001\) in the two groups given intraperitoneal injection of the sodium pentobarbitone formulations compared with the group receiving physiological saline \textit{(Figure 1)}. The number of FLI neurones in the group receiving sodium pentobarbitone with lidocaine was significantly lower \(P<0.05\) compared with
the group receiving sodium pentobarbitone alone. Numerically, the number of FLI neurones in the sodium pentobarbitone/lidocaine group was about two-thirds that of the sodium pentobarbitone alone group.

Discussion

Although the data obtained from this pilot study are limited, we find that they are valid for the following discussion.

The presence of the FLI neurones in laminae I, II, V and X of the spinal dorsal horn is in agreement with the fact that these laminae mainly receive input from visceral nociceptive fibres [Menétrey et al. 1989, Hammond et al. 1992, Clement et al. 2000]. The FLI neurones were found in the entire length of the spinal cord examined which is in accordance with findings described in the above-referred papers and in accordance with a global intraperitoneal distribution of the injected fluid.

The dose volume of 0.4 mL/kg was chosen as the smallest dose to obtain short-lasting anaesthesia, and, in fact, the anaesthesia lasted for 30–45 min in the present study. Menétrey et al. (1989), Hammond et al. (1992) and Clement et al. (2000) applied in their studies a dose volume of 0.5 mL/rat. In the behavioural study of Ambrose et al. (2000), a dose volume of 1.5 mL/kg was applied in order to obtain a lethal effect.

The number of FLI neurones was lower in the group receiving the sodium pentobarbitone/lidocaine formulation. These results are in agreement with Ambrose et al. (2000) who observed reduced distress and writhing after administration of sodium pentobarbitone with lidocaine compared with sodium pentobarbitone alone. In that study, the concentrations of sodium pentobarbitone and lidocaine were similar to the present study but given in a higher dose volume. Thus, the positive effect of the addition of lidocaine has been shown at a neuronal and behavioural level. In a study with intraperitoneal injection of a formulation of acetic acid in rats, Hammond et al. (1992) showed that spinal c-fos expression and pain behaviour were reduced in animals pretreated with morphine as an analgesic agent. The pain behaviour was significantly reduced when the number of c-fos-expressing neurones was reduced to 50%, and the pain behaviour was almost eliminated when c-fos expression was further reduced to 35%. It seems possible that acetic acid induces somewhat more intensive nociception than sodium pentobarbitone since intraperitoneal injection of acetic acid is routinely used as a standard algesic test where the animals also show writhing behaviour.

It takes up about 2 h after an acute noxious stimulus for the maximal level of FLI neurones to be reached [Hunt et al. 1987, Morgan & Curran 1991]. The rats in the present study were killed 3 h after the injection. This extra hour was chosen in order to include potential peritoneal inflammatory nociceptive input to the spinal cord. It can only be speculated whether this was obtained in particular because the half-life of Fos is about 2 h, meaning that peritoneal...
inflammatory c-fos expression may have compensated the disappearance of immediately induced c-fos expression. The fact that c-fos expression is at its maximum 2 h after a nociceptive input justifies the use of a second intraperitoneal injection of sodium pentobarbitone to obtain anaesthesia while the animals were perfused. Thus, the potential nociceptive input on c-fos expression of the second injection was negligible.

The nucleus tractus solitarius is another target of visceral sensory nociceptive input via the vagus nerve. In this nucleus, Menetrey and Basbaum (1987) showed increased c-fos expression after intraperitoneal injection of acetic acid. Since this nucleus was not included in the present study, we may have underestimated the total nociceptive input generated by intraperitoneal injection of the sodium pentobarbitone solutions.

In addition, misplacement of the injection, e.g. into the intestines, liver, spleen, seminal vesicle or other abdominal organs, is another factor of concern when giving intraperitoneal injections. It has been reported that misplacement in mice or rats varies from 10% to 24% (Lewis et al. 1966, Steward et al. 1968, Miner et al. 1969, Arioli & Rossi 1970, Schneider & Sheider 1970, Walvoort 1991, Claassen 1994). However, with a two-person procedure, one holding the animal and the other injecting the animal, the incidence of error was substantially reduced in one study (Arioli & Rossi 1970), whereas optimization of the procedure in other studies did not reduce the incidence of errors (Miner et al. 1969, Walvoort 1991).

According to textbooks in pharmacology, the onset of the local anaesthetic effect of lidocaine is very fast. This was the reason for adding lidocaine to the sodium pentobarbitone formulation. However, the duration of effect of lidocaine is, on the other hand, short lasting (about 30-60 min) compared with bupivacaine (3-4 h), which has a slower speed of onset. For this reason, it seems logical to combine lidocaine and bupivacaine in cases where sodium pentobarbitone is used in animals that are to recover from anaesthesia.

It is concluded that addition of lidocaine to formulations of sodium pentobarbitone may improve animal welfare by reducing the nociceptive input from sodium pentobarbitone. However, further studies are needed to optimize the lidocaine concentration and a long-acting local anaesthetic agent like bupivacaine.

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