

Intramuscular Injection of Hypertonic Saline: *In vitro* and *in vivo* Muscle Tissue Toxicity and Spinal Neurone c-fos Expression

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(Received July 5, 2004; Accepted January 25, 2005)

Abstract: Intramuscular injection of hypertonic saline (4–6% NaCl) is widely used to induce muscle pain in volunteers. The quality of the pain is comparable to clinical muscle pain with localised and referred pain. The objective was to evaluate the muscle toxicity of hypertonic saline by characterisation of 1) cytotoxicity *in vitro*, 2) local muscle toxicity in rabbits and 3) number of spinal dorsal horn neurones expressing c-fos after intramuscular injection in pigs as an indicator of nociception. Rat myocyte cultures and erythrocyte suspensions were treated with hypertonic NaCl solutions. The creatine kinase activity remaining in the myocytes and haemolysis were measured. Groups of six rabbits were given an intramuscular injection of 0.5 ml of 0.9, 3 or 6% NaCl. Three days later, creatine kinase activity was determined in injection site muscle tissue and normal contralateral muscle. The amount of injection site muscle tissue totally depleted of creatine kinase was calculated. Groups of two pigs were given an intramuscular injection of 3.0 ml of 6% NaCl. The spinal cord was sampled 1, 2 or 3 hr later and processed for stereological quantification of the number of dorsal horn neurones expressing c-fos. Saline was not toxic *in vitro* at 0.9–6%, but toxic to erythrocytes at 7% or higher and rat myocytes at 15% or higher. No muscle toxicity was seen in rabbits. The number of dorsal horn neurones expressing c-fos was not above basal level. In conclusion, 6% saline caused no *in vitro* or *in vivo* toxicity in sensitive models. Consequently, the pain caused by intramuscular injection of hypertonic saline is most likely not related to tissue damage. Consistently, intramuscular injection of 6% NaCl did not activate dorsal horn neurones in pigs to express c-fos beyond basal level.

Hypotonic or hypertonic sodium chloride or potassium chloride causes pain when injected into human muscles or other deep tissues (Stohler *et al.* 1992; Graven-Nielsen *et al.* 1997a & b; Leffler *et al.* 2000; Svensson *et al.* 2000). Hypertonic saline has by far been the most used chemical substance to induce muscle pain and intramuscular injection of 4–6% sodium chloride in volunteers is a widely used method to induce human muscle pain (Graven-Nielsen *et al.* 1997a & b). The quality of the pain is comparable to clinical muscle pain with localised and referred pain (Graven-Nielsen *et al.* 1997a & b). The dominant sensation to hypertonic saline injected intramuscularly is deep diffuse pain after some latency corresponding with a major activation of C-fibres. Intramuscular injection of hypertonic saline is claimed to be a safe technique causing no adverse effects (Stohler & Lund 1994; Graven-Nielsen *et al.* 1997a & b). However, since the injection is rather painful, the injected hypertonic saline may have caused damage to the local tissue at the injection site.

Intramuscular injection has been used as a route for administration of drugs to humans for about one hundred and fifty years. In contrast to the early days, intramuscular injection is considered today to be a simple and safe routine pro-

cedure which seldom or never causes complications or side-effects. However, a survey of the literature indicates that complications are not as infrequent as generally believed (Svendsen 1988). Several clinical and in particular clinical chemical observations indicate that a range of different and normally used injection formulations cause local damage to the muscle tissue at the injection site (Svendsen 1988).

Determination of the amount of local muscle tissue totally depleted of creatine kinase activity in rabbits is a useful model for muscle tissue toxicity (Svendsen *et al.* 1979). *In vitro* methods with determination of cytotoxic potency of drugs in cell cultures (fibroblasts) or haemolytic potency in erythrocyte suspensions are useful as screening models for prediction of local muscle damaging potential (Svendsen *et al.* 1985). Another *in vitro* model using cultured rat myocytes has shown promising correlation with the *in vivo* toxicity in rabbit muscle (Kato *et al.* 1992; Anonymous 1994; Edwards & Svendsen 2000). Until recently no easy method was available for identification of nociception related to intramuscular injections. However, c-fos expression in spinal cord dorsal horn neurones seems to be a useful indicator of spinal nociception and intensity of the painful stimulus (Hunt *et al.* 1987). Since then, many studies have confirming this result (Harris 1998).

The aim of the present study was to evaluate the safety of intramuscular injection of hypertonic saline by characterisation of 1) the cytotoxicity produced by hypertonic saline solutions in *in vitro* systems with cultured rat myocytes and

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erythrocyte suspensions, 2) the muscle toxicity caused by intramuscular injection of hypertonic saline in rabbits and 3) the number of spinal dorsal horn neurones expressing *c-fos* after intramuscular injection of hypertonic saline in pigs as a measure of spinal nociception.

Materials and Methods

Rat myocyte test. One day old Wistar rats of the stock Br/Han: WIST@Mol(GALAS) were obtained from M&B A/S, Denmark. The rats were anaesthetised using an intraperitoneal injection of a lethal dose of pentobarbital. When breathing had stopped, all the muscle tissue was collected from the leg between the thigh and ankle joints. The muscle tissue was incubated in a dissociation medium (0.25% trypsin and 0.05% deoxyribonuclease I, both Sigma-Aldrich Denmark A/S, Denmark) at 37° with agitation. After 10 min., the medium was replaced with fresh dissociation medium and incubated for a further 30 min. Then the cells were suspended in culture medium (Dulbecco's MEM/Ham F12 supplemented with 10% foetal bovine serum and 50 µg/ml gentamycin, all Invitrogen A/S, Denmark) by vigorous pipetting.

The cell suspension was filtered through sterile cloth to remove large cell clumps, centrifuged at 500 ×g for 5 min. at room temperature and the cell pellet was re-suspended in culture medium. After a second centrifugation, the cell pellet was re-suspended in culture medium. The cells were incubated at 37° in a humidified atmosphere of 5% carbon dioxide in air in plastic Petri dishes for a few minutes to allow the fibroblasts to attach. The non-adherent cells were harvested and diluted to 2 × 10⁵ viable cells/ml. Aliquots of the cell suspension were cultured in multi-well, collagen-coated culture plates (conditions as above) for 8 days. The culture medium was replaced with selection medium (as culture medium, but with only 5% foetal bovine serum) after three days of the incubation period.

The cell cultures were treated by replacing the selection medium with aqueous solutions of NaCl (>99.5%, GR for analysis; Merck KGaA, Germany) at concentrations in the range 0.9 to 15%. At least four cultures were treated at each test point. Untreated control cultures and positive control cultures, treated with acetic acid (glacial acetic acid, Merck KGaA, Germany, diluted to 0.5% v/v with 0.9% NaCl), were also prepared.

The cultures were incubated (conditions as above) for a treatment period of 60 min. Then the cells were rinsed with phosphate buffered saline solution (PBS, Merck KGaA, Germany) to remove any creatine kinase that had been released and lysed for 15 minutes with lysis buffer (0.4% Triton X-100, Sigma-Aldrich Denmark A/S, Denmark, in PBS). The creatine kinase activity of the liquid in each well was then measured using a commercial assay kit with a Cobas Mira S Instrument (both Roche A/S, Denmark).

Haemolysis test. Solutions of NaCl were prepared at concentrations in the range 0.9 to 15% w/v. Fresh, heparinised rabbit blood was added (20 µl blood/ml) and the mixtures were incubated at 37° for 60 min. Four preparations were analysed for each concentration.

After centrifugation at 500 ×g for 5 min. at room temperature to sediment intact cells, the absorbance of the supernatant liquid was measured at 545 nm using a Multiskan Ascent instrument (LabSystems Oy, Finland). The amount of haemolysis was expressed as a percentage of the complete haemolysis produced by incubating blood in distilled water (20 µl blood/ml).

Rabbit muscle test. The study was performed in 18 specified pathogen-free female albino rabbits of the stock New Zealand White from Charles River, Germany. On allocation to the study, the animals weighed approximately 2.5 kg. The animals were randomly divided into three groups with six animals in each group. The animals were allowed a five day acclimatisation period before the commencement of treatment. The animals were housed in an animal room provided

with filtered air with a temperature of 21 ± 3° and relative humidity of 55% ± 15%. The room was illuminated to give a cycle of 12 hours light and 12 hr darkness. The animals were kept in PPO/HIPS (Nor-yl®) rabbit cages and had *ad libitum* access to drinking water and a pelleted complete diet "Altromin 2123" from Chr. Petersen A/S, Denmark.

The fur on the back of the animals was clipped before the start of treatment. The animals were then anaesthetised by an intramuscular injection of Hypnorm®, Janssen, Belgium, 0.5 ml/animal (fentanyl citrate, 0.315 mg/ml and fluanisone, 10 mg/ml), and the back of the animals were disinfected with 70% ethanol. Group 1 was treated with physiological saline (0.9% sodium chloride solution, Fresenius Kabi Norge A/S, Norway), group 2 with a 3% sodium chloride solution and group 3 was treated with a 6% sodium chloride solution. The dose formulations (0.5 ml/animal) were injected as centrally as possible in the right dorsal longissimus muscle. The needle was introduced at the intersection of the transverse plane through the lumbar region halfway between the last rib and the iliac crest and a sagittal plane in the middle of the muscle. This corresponded to about 4 cm ahead of the crest of the ilium and 2 cm laterally from the spinal process of the lumbar vertebrae. The needle was inserted about half-way (1.5 cm) into the vertical plane of the muscle. The thickness of the muscle at the injection site was evaluated by palpation immediately before the injection.

Three days after the injection, the animals were anaesthetised (50 mg/kg pentobarbital (mebumalum NFN) intravenously) and killed by exsanguination. The skin of the back was removed, and the fascia covering the muscle was cut along its insertion to the spinal processes and the crest of the iliac bone and then moved laterally. The muscle was cut transversely in front of the crest of the ilium and at a level in line with the curvature of the ribs. This piece of the muscle was removed after a vertical cut along the spinal processes and a horizontal cut along the transverse processes. Before serial sectioning of the removed muscle, a cross section was made in the middle of the muscle where the injection site was planned. The individual serial sections were cut thinly so that macroscopic changes could be identified. The area where macroscopic changes occurred was isolated by careful dissection from the surrounding normal muscle tissue.

The muscle tissue sample with macroscopic changes and a normal muscle tissue sample taken from the contralateral side were weighed and frozen until analysis for creatine kinase activity. The samples were homogenised (Ultra-Turrax homogeniser, 4 × 15 sec. in an ice bath) in a 0.1 M imidazole acetate buffer adjusted to pH 6.7 by addition of acetic acid. The homogenate was centrifuged (4°) for 20 min. at 1100 ×g and 2 ml of the supernatant was centrifuged (4°) for 5 min. at 8000 ×g. The second supernatant was diluted with the buffer and the creatine kinase activity in the diluted sample was determined with reagents from Roche using a Cobas Mira S. The amount of muscle tissue at the injection site depleted of creatine kinase was calculated as follows:

$$\frac{\text{Control tissue creatine kinase activity} - \text{injection site creatine kinase activity}}{\text{Control tissue creatine kinase activity}} \times \text{g changed muscle at injection site}$$

Spinal *c-fos* expression in pigs. Six pigs weighing from 18 to 20 kg received a single injection of 3.0 ml of sterile hypertonic saline (6%) in the left lumbar muscle (*m. longissimus dorsi*) midway between the caudal point of the costal arch and the iliac crest. The optimal injection depth aiming at the centre of the muscle was initially determined to be approximately 1.75 cm by ultrasound scanning. Prior to perfusion the pigs were sedated with azaperone (Stresnil, 6 mg/kg, intramuscularly) and anaesthetised with pentobarbital (10–15 mg/kg, intravenously), while heparin (500 i.e./kg, Leo Pharma, Denmark) was injected intravenously immediately before initiation of the perfusion. One, two or three hours after the injection the pigs were perfused by pumping 3 l of 4% formaldehyde buffer (pH 7.0, Lillys solution, Bie & Berntsen, Denmark) through a carotid artery, while the blood was led out from the ipsilateral jugular vein. Fol-

lowing perfusion the three most caudal thoracic and the lumbar segments of the spinal cord were removed, and then fixated in 4% formaldehyde for 24 hr at 4°. Thereafter, the spinal cord was cryoprotected for 24 hr in 10% sucrose (4°) and for 72 hr (4°) in 30% sucrose and frozen at -80°.

Subsequently, the spinal cord was cut into equidistant blocks with a length of 5 mm. The beginning of the first block was chosen at random within 0–5 mm cranially to the most caudal thoracic nerve root. In this way, between 15 and 17 blocks were obtained per animal. A cryostat section (100 µm) was obtained from the cranial end of each block. In this way, 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).

Each section was immunohistochemically stained for Fos protein by the avidin-biotin-peroxidase (ABC) method of Hsu *et al.* (1981) modified for pigs, as previously described (Lykkegaard *et al.* 2005). The sections were incubated for 10 min. in a blocking solution of hydrogen peroxide in potassium phosphate buffered saline (KPBS, pH 7.4) and incubated for 24 hr (20°) with polyclonal rabbit-anti-human-Fos antibody (Oncogene Research Products, Cambridge, MA, USA) diluted 1:8000 in KPBS containing 1% bovine serum albumin 0.3% Triton X-100 and 10% goat serum (AB-buffer). Subsequently, the sections were washed in KPBS-buffer containing 0.25% bovine serum albumin and 1% Triton X-100 (washing-buffer) for 24 hr, and incubated for 60 min. in biotinylated goat-anti-rabbit-IgG (DAKO A/S, Denmark) diluted 1:600 in AB-buffer. Following washing in washing buffer, KPBS and again washing buffer, the sections were incubated for 60 min. in avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) diluted 1:400 in washing buffer. Subsequently, the sections were washed once in washing buffer and twice in KPBS before they were developed for four minutes in VIP chromogen (SK 4600, Vector Laboratories, Burlingame, CA, USA), 3 drops per 5 ml of KPBS. Finally, the sections were washed in distilled water to stop the staining process and mounted on gelatine-subbed slides and air dried over night at 4°. The sections were then dehydrated in graded alcohol (70%, 96% and 99%), cleared in xylene and coverslipped with Eukitt (O. Kindler GmbH 6 CO, Freiburg, Germany).

The number of Fos-positive neurones in the spinal dorsal horn was estimated using a light microscope. The spinal dorsal horn was defined as the grey matter dorsally to a vertical line extending from the central canal to the lateral border of the grey matter, including Rexed laminae I through VI. The Fos-positive neurones were counted by optical disector counting rules (Gundersen *et al.* 1988; West & Gundersen 1990). Thus, a neurone was counted when the nucleus came into focus, appearing dark purple with a well-defined border, within the counting frame when zooming down through the section. The counting procedure was blinded, meaning that the investigator was unaware of the origin of the sections during the procedure.

The total number of Fos-positive neurones within the spinal dorsal horn was estimated by the optical fractionator method (Gundersen 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: $N = N' \times f$ (Gundersen *et al.* 1988). In actual practise this was achieved by counting Fos-positive neurones in a known fraction of the thickness (H/h) of the individual sections, under a known fraction of the sectional area (a(step)/a(frame)), on a known fraction of the sections (T/t) from the spinal cord. The total number of Fos neurones N(Fos) was then calculated as the product of the number of Fos-positive neurones counted with the optical disector N'(Fos), and the inverse sampled fractions, by the equation:

$$N(\text{fos}) = N'(\text{Fos}) \times \frac{a(\text{step})}{a(\text{frame})} \times \frac{H}{h} \times \frac{T}{t}$$

The fraction of the sectional area and the fraction of the thickness were selected by the use of a computer assisted stereological toolbox

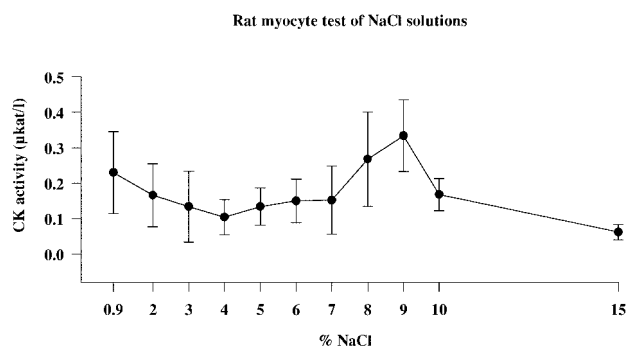


Fig. 1. Creatin kinase (CK) activity remaining in cultured rat muscle cells exposed to solutions with increasing NaCl concentration (mean $\pm$ S.D.). Each mean value represents the mean of five determinations, $n = 5$. There was no statistically significant difference in CK activity data between 0.9–10% concentrations. The results obtained after the concentration 15% were statistically significant lower than all other concentrations ($P < 0.05$, ANOVA).

(C.A.S.T.-Grid, Olympus, Denmark), as described by West *et al.* (1996).

Ethics. The two types of animal experiments were carried out under the authority of licenses from the Experimental Animal Inspectorate, Ministry of Justice, Denmark. The regulations in Denmark conform to the EU directive 86/609.

Statistics. Data was processed to give group mean values and standard deviations where appropriate.

Thereafter each continuous variable was tested for homogeneity of variance with Bartlett's test. If the variance was homogeneous, analysis of variance was carried out for the variable. If any significant differences were detected, possible intergroup differences were assessed with Dunnett's test. If the variance was heterogeneous, each variable was tested for normality by the Shapiro-Wilk method. In case of normal distribution, possible intergroup differences were identified with Student's *t*-test. Otherwise the possible intergroup differences were assessed by Kruskal-Wallis's test. If any significant intergroup differences were detected, the subsequent identification of the groups was carried out with Wilcoxon Rank-Sum test. For all tests, the level of significance was defined as $P < 0.05$ or $P < 0.01$.

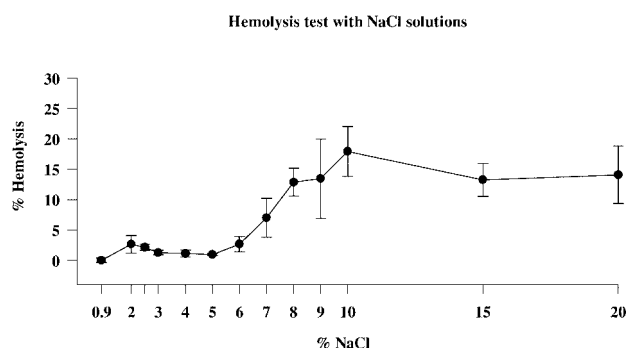


Fig. 2. Haemolysis of erythrocytes exposed to increasing concentrations of saline. Each mean figure represents two testing episodes each with three tests of the same concentration; $n = 6$. The haemolysis after concentrations 2–6% were all higher than the haemolysis after the 0.9% concentration ($P < 0.05$). Haemolysis after concentrations 7–20% were all higher than the haemolysis after the 6% concentration (6 versus 7%, $P < 0.05$; 6 versus 8–20%, $P < 0.01$, Wilcoxon Rank Sum test).

The statistical analyses were made with SAS® procedures (version 8.2) described in “SAS/STAT® User's Guide”, SAS OnlineDoc®, 1999, SAS Institute Inc., Cary, North Carolina 27513, USA.

Results

Hypertonic saline did not reduce the creatine kinase-activity by concentrations up to 10% (fig. 1). A concentration of 15% caused release of creatine kinase from the myocytes to a higher extent than 0.9% NaCl. The mean value of the negative control was 0.52 ± 0.19 ukat/l, whereas the positive control caused 100% release. There was no statistically significant difference in creatine kinase activity at saline concentrations in the range 0.9–10%. The results obtained after treatment with 15% saline were statistically significantly lower than all other concentrations ($P < 0.05$).

Hypertonic saline caused statistically significantly increased haemolysis at concentrations of 7% and above compared to the concentration 6% (fig. 2). The negative control, 0.9% NaCl caused no haemolysis whereas the low concentrations from 2% to 6% caused low grade haemolysis with no difference between the concentrations applied. However, the results obtained with the concentrations 2–6% were statistically significantly higher than the results obtained with 0.9%. The positive control, distilled water, caused 100% haemolysis.

In the rabbits 0.5 ml of hypertonic saline at concentrations of 3 or 6% did not cause depletion of creatine kinase activity different from that caused by isotonic saline (fig. 3). There was no group-related statistically significant difference between the values obtained.

Although the number of observations at each time point was very low, the number of c-fos expressing dorsal horn neurones was not above basal level at any time point after intramuscular injection of 3.0 ml saline at the concentration of 6% (table 1).

Discussion

Based on a muscle pain model in humans with local microdialysis determinations of sodium and potassium concentrations and visual analogue score it was concluded that the pain activation and cessation were related to increased intramuscular sodium and potassium, respectively (Graven-Nielsen *et al.* 1997a & b). The present studies show that hypertonic saline at concentrations up to 6% caused no evidence of *in vitro* toxicity in rat myocytes or red blood cells and no evidence of *in vivo* muscle toxicity in rabbits. Consequently, it is likely that the pain caused in humans by intramuscular injection of hypertonic saline is not caused by tissue damage, which supports the conclusion of Graven-Nielsen *et al.* (1997a & b).

It has been shown previously that the *in vitro* rat myocyte assay is sensitive to the toxic effects of injectable formulations of different drugs and preservatives (Edwards & Svendsen 2000). The assay correctly identified that chlorpromazine, benzyl alcohol and m-Cresol are potent muscle



Fig. 3. Gram muscle tissue depleted of CK activity after intramuscular injection of 0.5 ml of 0.9, 3 or 6% saline in rabbits (n = 6). There is no group-related statistically significant difference between the values obtained (Kruskal-Wallis test).

toxins. In this assay, hypertonic saline with concentrations up to 10%, there was no evidence of damage to the myocytes. However, as can be seen from the standard deviation values, the robustness of the assay could be better. To compensate, the assay was repeated five times with each concentration.

The susceptibility of erythrocytes to toxic components of intramuscular drug formulations has previously been shown by Svendsen *et al.* (1985). The studies illustrated a good relationship between haemolysis and *in vivo* muscle toxicity in different animal models. In this assay, hypertonic saline with concentrations lower than 7%, there was no evidence of damage to the erythrocytes. It is evident from the standard deviation values that this assay is much more robust than the rat myocyte assay. In spite of this the assay was conducted at two different occasions with three repetitions at each occasion. This was considered necessary because the results obtained with concentrations from 2–6% saline were all statistically significantly higher than the result obtained with 0.9% saline but they were not different from each other.

The validity of the rabbit muscle test with determination of release of creatine kinase activity from local muscle tissue has been illustrated by Svendsen *et al.* (1979). Hypertonic saline in concentrations up to 6% did not give evidence of muscle damage in this *in vivo* model. In this animal model there can be a relatively high variation in the lower range areas as in the present study. With formulations that cause damage to the local muscle tissue the relative variation is considerably lower (Svendsen *et al.* 1979 & 1985). Histological examination was not included because it is documented

Table 1.

Number of c-fos expressing neurones after intramuscular injection of 3 ml 6% saline in pigs.

Hours after injection	Number of pigs	Number of c-fos expressing neurones (range)
1	2	630–1,167
2	2	1,111–2,270
3	2	0–276
0.9% saline control ^a	4	2,400 ± 800

^a Data (mean ± S.D.) from Lykkegaard *et al.* (2005).

by the literature cited that there are only limited histological changes, when the creatine kinase depletion from the local muscle tissue is very low, which was the case in the present study. For this reason it was not considered ethically acceptable to include further animals for the purpose of taking muscle tissue samples for histology.

Several of the mean results show relatively large standard deviations. The number of animals used and the size of the standard deviation should always be balanced, but the important principle is that the minimum numbers of animals necessary to answer the question investigated should be used. More animals could have been used, but that would probably not have altered the conclusion.

The choice of animal species was related to the test method used. For each individual test, the species included in the present study was the same species as that used for the development of the original test. The test method was followed exactly as described in the literature instead of including new species. The species included in each test is considered the most susceptible animal species. For the c-fos evaluation the pig was included because this group has accumulated a considerable spinal c-fos database in the pig.

As mentioned in the introduction, there are strong evidence supporting a link between nociception and c-fos expression. This makes the identification of neurones with Fos protein a tool for cellular mapping of activity in neurones following noxious stimuli and the quantification of these neurones can give information regarding the magnitude of nociceptive activity. When using the c-fos technique, some important considerations should be taken into account. First of all, the nociceptive stimulus must have a certain strength and duration for c-fos expression (Bullitt *et al.* 1992; Abbadie *et al.* 1994). Secondly, the number of c-fos positive neurones at the level of the spinal cord is not necessarily equivalent to the level of pain perceived at the level of the cortex. Intramuscular injection of up to 6% saline in six pigs seemed not to elevate the number of spinal dorsal horn secondary neurones expressing c-fos. This group has been working with stereological counting of c-fos expressing neurones in lumbar spinal cord of pigs for a number of years and from one study to another found very consistent basic results (Lykkegaard *et al.* 2005). For this reason it was considered justified to refer to data from previous studies rather than including further animals in the present study. Upon a nociceptive input c-fos expression is at its maximum about 2 hr after the input and subsequently declines with a half-life of about 2 hr (Hunt *et al.* 1987). In order to cover a broader time-span pigs were killed 1, 2 or 3 hr after injection of hypertonic saline. Since at any of these time points the number of c-fos expressing neurones was within the control level, it can be argued that it has been documented in six pigs that intramuscular injection of 6% saline did not elevate the number of spinal dorsal horn secondary neurones expressing c-fos.

It is well documented that intramuscular injection of hypertonic saline causes pain in humans (Graven-Nielsen *et al.* 1997a & b; Leffler *et al.* 2000). However, the nociceptive

transmission expected to be caused by intramuscular injection of hypertonic saline in pigs seemed not to activate nociceptive dorsal horn neurones to a degree causing c-fos expression. Consequently, it is suggested that the pain caused in humans by intramuscular injection of hypertonic saline, being diffuse and with some latency, may be mediated by saline-induced c-fibre action potentials rather than saline-induced tissue injury and that this stimulation is too small or too short to activate c-fos expression in dorsal horn nociceptive neurones. In this study there was only little indication whether the injections in the rabbits or pigs were painful. However, it is assumed that the injections were painful because there seems to be no anatomical or physiological differences indicating species differences. In addition, the rabbits were anaesthetised while receiving the injection and pigs have difficulties expressing the sensation of pain.

Acknowledgements

Signe Klasttrup, M.Sc., Scantox A/S, Denmark, is thanked for her contributions to the statistics used in the present study.

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