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ORIGINAL PAPER

# Correlation of Enteric NADPH-d Positive Cell Counts with the Duration of Incubation Period in NADPH-d Histochemistry

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**Abstract** Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) staining can be used in the enteric nervous system to determine nitrergic neuronal counts, critical in motility disorders such as intestinal neuronal dysplasia and hypoganglionosis. The reported incubation periods of specimens with NADPH-d staining solution has varied from 2 to 24 h. The aim of this study is to investigate the impact of the incubation period on the overall NADPH-d positive cell counts in porcine rectal submucosal plexus. The submucosal plexus of rectal specimens from 12-week-old pigs ($n$=5) were studied. Conventional frozen sections were used to identify nitrergic neurons while whole-mount preparations were used to quantify the effect of prolonged duration of incubation on positively identified ganglion cells with NADPH-d histochemistry. The same submucosal ganglia on the conventional sections, and a minimum of 12 ganglia per whole-mount preparation specimen were photographed sequentially at 2, 6, and 24 h and used to count the number of nitrergic cells per ganglion. The same staining solution was used throughout the experiment. Results were analysed using a one-way ANOVA test. Prolonged incubation with the staining solution revealed new NADPH-d positive cells in the ganglia on the conventional sections. The total number of neurons counted in the 12 adjacent ganglia in the whole-mount specimens was 180±55, the mean neuronal cell per ganglion was 15±8 after 2 h of incubation. This increased to 357±17, and to 29±12 after 6 h ($p$<0.05). A further increase was observed of 515±19 and 43±17 after 24 h ($p$<0.05). When the photomicrographs were retrospectively analysed, not even the outline of the neuronal cells that stained with prolonged incubation was evident at the earlier time points. NADPH-d positive cell counts increase in proportion to the duration of incubation in NADPH-d histochemistry. Comparative studies attempting to quantify nitrergic cell counts in dysmotility disorders must take into account the variability in NADPH-d positive cell count associated with prolonged incubation in NADPH-d histochemistry.

**Keywords** Positive cell counts · Incubation time · NADPH-d histochemistry

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## Introduction

The intrinsic innervation of the human gut can be divided into three major components with respect to cholinergic and catecholaminergic phenotypes: cholinergic, catecholaminergic (or adrenergic), and noncholinergic, nonadrenergic (NANC). Bult et al. have previously provided evidence that nitric oxide (NO) is released on stimulation of NANC nerves [1]. Since then, substantial evidence has emerged indicating that NO acts as a NANC neurotransmitter in the gut and mediates relaxation of the smooth muscle of the gastrointestinal tract [2–4].

Deficiency of nitrergic innervation has been shown in different tissues from patients with Hirschsprung's disease [5], infantile hypertrophic pyloric stenosis [6], and internal anal sphincter achalasia [7] suggesting that a lack of NO

release may be involved in the pathophysiology of these disorders.

NO is synthesised by the neuronal nitric oxide synthase (NOS) in nitrergic neurons. Calcium and nicotinamide adenine dinucleotide phosphate (NADPH) are required for this synthesis. There are two techniques available to identify and quantify nitrergic neurons: NADPH-d histochemistry and NOS immunohistochemistry. Dawson et al. has previously reported that NOS staining is identical to NADPH-d staining [8].

Nitrergic neuronal counts, quantified by NADPH-d histochemistry, have been used as a means of defining motility disorders such as intestinal neuronal dysplasia and hypoganglionosis [9, 10]. Several reports in recent years have attempted to quantify nitrergic neurons within the ENS using the NADPH-d histochemical technique [11, 12]. However, the duration of staining has varied markedly between these studies.

The first empirical study was undertaken by Scherer-Singer to determine the optimum conditions for the NADPH-d histochemical procedure in rat brain. The dye nitro blue tetrazolium was tested at different concentrations, the effects of pH on the staining was examined. Regarding the duration of the incubation it was only postulated: "Intense NADPH-diaphorase positive neurons were readily detected after 15–30 min incubation at 37°C" [13].

Using the NADPH-d histocehmistry and nNOS immunohistochemistry double labelling technique, Timmerman validated the Scherer-Singler method in the enteric nervous system and found complete overlap of the two techniques in the small bowel of the pig. The duration of the incubation with NADPH-d staining was 10–15 min [14].

Kuo et al. studied the relation of duration of the incubation and optical density and number of positively stained cells within the rat CNS and found that increasing the length of incubation only increased the optical density of NADPH-d staining, while the number of NADPH-d positive cells counted was relatively consistent across incubation times [15]. The effect of the duration of the incubation in NADPH-d solution on the number of positively stained cells in the ENS has not been studied.

In this study we have investigated the impact of the incubation period with NADPH-d solution on the number of NADPH-d positive cells in the submucous plexus counted over a period of 24 h.

## Materials and Methods

Pig tissue was provided by the Institute of Experimental Clinical Research, Skejby Sygehus, University of Aarhus Denmark. The study was approved by the Danish authorities of animal protection, permission number 200601-068. The entire gastrointestinal tract was removed and subsequently fixed using perfusion fixation with 4% paraformaldehyde (PFA) and stored in PBS at 4°C until needed.

The outer rectal submucous plexus was studied in specimens from 12-week-old pigs ($n$=5). Conventional 8 μm thick transverse frozen sections were used to identify nitrergic neurons while whole-mount preparations were used to quantify the effect of prolonged incubation duration on the number of positively identified cells. In whole-mount preparations, the mucosal layer was removed, the submucosa was peeled off and the specimens were subjected to NADPH-d histochemistry.

For NADPH-d histochemistry, the sections and whole-mount preparations were incubated in 1 mg/ml β-NADPH (Sigma), 0.25 mg/ml nitro blue tetrazolium, and 0.3% Triton-X in 0.05 mol/l Tris–HCL buffer (pH 7.6) at 37°C. Incubation of the whole-mounts was performed using a free-floating technique in a 12-well cell culture dish.

The submucous ganglia seen in conventional sections were photographed after 2 and 24 h using a Leica DLMB light microscope with an attached digital camera. The whole-mount specimens were mounted sequentially at 2, 6, and 24 h on polysine slides (BDH) using the original staining solution as mounting medium, and covered with cover glass slips. The same 12 ganglia were sequentially photographed in each specimen, and the number of NADPH-d positive cells per ganglion was counted on the micrographs, regardless of the staining intensity. The same staining solution was used throughout the experiment. Results were analysed using a one-way ANOVA test.

## Results

After 2 h of incubation, clearly labeled NADPH-d ganglion cells appeared in the conventional sections. All the positive cells stained with a similar staining intensity. After 24 h, the neurons that had stained after 2 h became more intensely stained and new weakly stained cells appeared in some ganglia, which were not seen on the previous micrographs (Fig. 1).

After 2 h of incubation with the NADPH-d solution, nitrergic cells were clearly identified within all ganglia in the whole-mount specimens (Fig. 2). The primary nerve bundles were also seen. The total number of positively identified cells was 180±55 in the 12 adjacent ganglia examined. The mean neuronal cell count per ganglion was 15±8. The total number of positively stained cells counted after 2 h staining nearly doubled after 6 h of incubation (357±17). The mean neuronal cell count also increased significantly to 29±12 ($p$<0.05). The NADPH-d positive cells that were seen following the 2-h incubation period

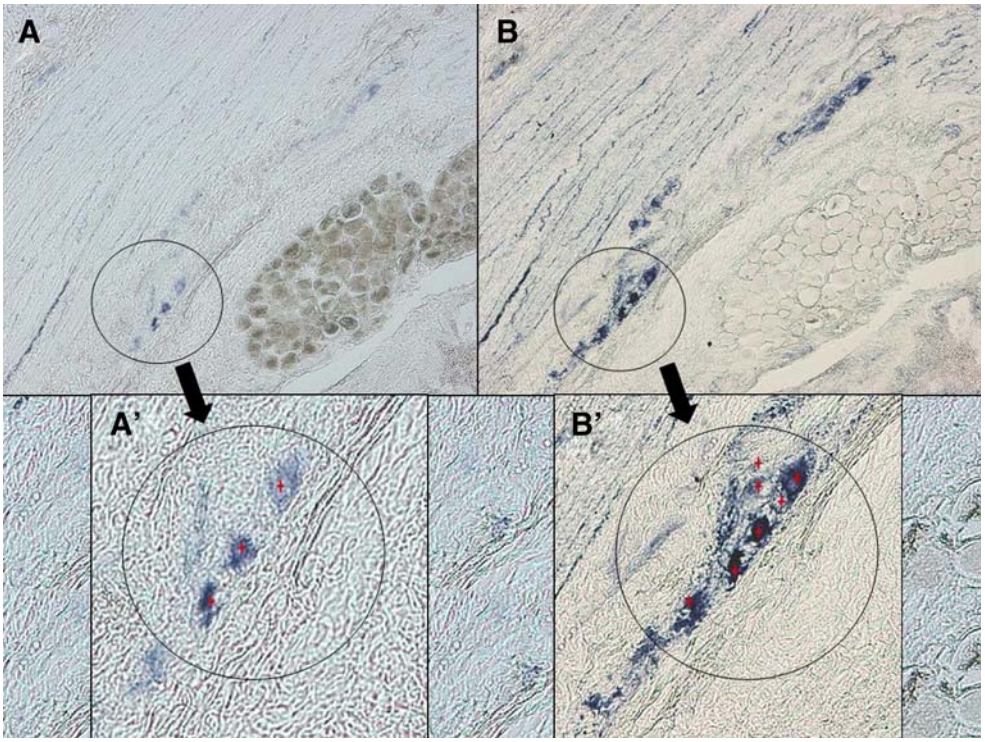



**Fig. 1** Conventional frozen section of the rectum of 12-weeks-old pig: **a**–**a′**: the same submucosal ganglion after 2 h incubation with NADPH-d staining solution. **b**–**b′**: the same submucosal ganglion after 24 h incubation with NADPH-d staining solution. Note the increased number of cells marked on **b′** picture

were notably more intensely stained after 6 h of incubation, while those cells that only appeared after the 6 h period were relatively pale in appearance. The visible density of the primary nerve bundles increased and secondary bundles became evident (Fig. 2).

The total number of positively identified cells further increased by 44% up to 515±19 after 24 h staining and the mean neuronal cell count also increased significantly ($p<0.05$) to 43±17 (Table 1). The optical density of the neurons and nerve bundle increased as well.

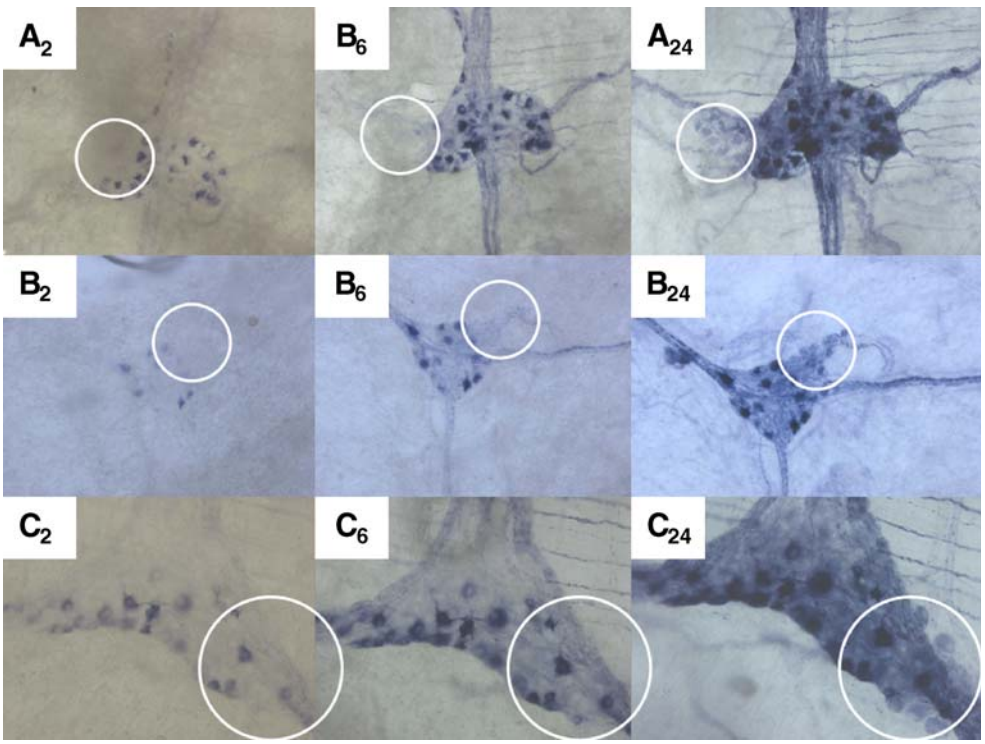
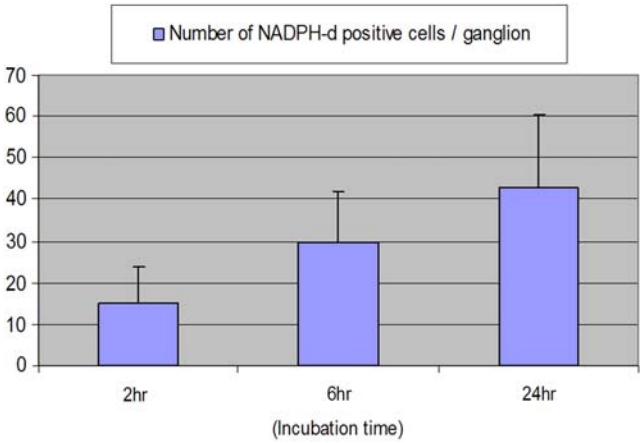



**Fig. 2** Whole-mounts specimens of the rectum of 12-weeks-old pigs: photographs of three different ganglia (**a**, **b**, **c**) after 2 h (*left column*), 6 h (*middle column*) and 24 h (*right column*) incubation. The *circles* indicate the area where the new cells appeared during the prolonged incubation

**Table 1** Number of NADPH-d positive cells per ganglia counted in whole-mounts specimens after 2, 6, 24-h duration of incubations. The cells were counted in 12 adjacent ganglia




## Discussion

NADPH-d histochemistry with various incubation duration is used to quantify nitrergic neurons of the ENS, both in the clinical practice and research situations. It is believed that the number of NADPH-d positive neurons is independent of the optical density of the staining, based on observations in the CNS [15]. Using conventional sections to identify NADPH-d positive cells and whole-mount preparations to quantify them, we have discovered in this study that NADPH-d positive cell counts in the ENS increase in proportion to the duration of incubation of NADPH-d histochemistry.

We believe that the duration of the incubation in NADPH-d histochemistry may influence the histological diagnosis in situations where NADPH-d positive cell counts are used as diagnostic criteria for motility disorders. For example, a longer incubation period of NADPH-d staining of a normal specimen may result in a false positive result, whereas a short duration of incubation of a specimen with IND may result in a false negative diagnosis of giant ganglia.

In published studies involving counting of myenteric ganglia on NADPH-d stained specimens, groups of NADPH-d positive neurons were considered ganglia when there were more than three positive cells in one group and the distance between the cells exceeded the two cell lengths [11]. Weakly stained NADPH-d positive cells that only appear after a long duration of incubation may connect two or more previously separately counted groups of NADPH-d positive neurons (ganglia) and therefore altering the final count of the ganglia per area in the specimen. Thus, the effect of the duration of incubation on NADPH-d staining should be considered in studies quantifying positively stained neurons.

The class of NADPH-d positive neurons has been subdivided based on the different staining intensity in the CNS and ENS as well. Three intensities of reactivity of NADPH-d histochemistry (strong, weak or negative) were detected in the neurons of nodose ganglia of rat, dog and guinea pig [16]. Strong, lightly and very lightly stained NADPH-d positive cells were identified with different shapes in the monkey amygdaloid complex [17]. In the submucosa of the porcine rectum, only about one third of neurons were stained strongly, with the remaining neurons moderately or weakly stained [12].

The NADPH-d positive cells could also be classified on the basis of their function. The most well known functional group of nitrergic neurons is the group of inhibitory motor neurons that mediates relaxation of the smooth muscle of the gastrointestinal tract. Apart from this, some elements of the sensory innervation of the circular musculature may derive from nitrergic neurons. The rest of the nitrergic neurons may serve as inhibitory interneurons and control other (probably excitatory motor) neurons in the myenteric plexus [18]. In the rat cerebellum, double immunofluorescence labelling using anti-glial fibrillary acidic protein (GFAP) and anti neuronal NOS antibodies have revealed that a minor population of glial cells presents a strong neuronal NOS immunostaining [19]. However, to date, there have been no reports of myenteric glial cells in the ENS containing NOS.

Cells of the CNS and ENS from various functional groups contain NOS, and therefore presumably NADPH-d at differing levels. Increased NADPH-d staining activity has been found to be associated with increased NOS immunoreactivity in pigs with *Schistosoma japonicum* infection [20]. Thus, the intensity of NADPH-d staining may represent the activity or content of NOS within the cell. It has to be considered that NADPH is a general mitochondrial enzyme, which is naturally present, usually at lower level, in all eukaryotic cells.

According to these observations, NADPH-d staining activity may support functional separation of nitregic NADPH-d positive cells. We hypothesize that neurons that contain NADPH-d to a lesser extent or with weaker activity may reduce NBT slowly and therefore become visible only after longer incubation times. In this study, the cells that appeared after 2 h of incubation containing the higher amount of NO/NADPH-d most likely are inhibitory motorneurons. The cells that appeared after 6 and 24 h of incubation may be interneurons, sensory neurons or glial cells. Exact origin of the weakly stained cells needs to be further classified by double labeling techniques.

In our study, the longest incubation time extended that used by Kou et al. (24 vs. 8.5 h). In contrast to this study, we have already observed a significant increase in the number of NADPH-d positive cells after 6 h. Thus, the

difference between the two studies doesn't originate solely from the longer incubation period. Kou et al. does not specify the staining intensity observed (weakly or strongly stained neurons) within any one specimen at a given time. There is probably a homogenous group of NADPH-d positive neurons in the rat striatum, which might explain the different results of the two studies.

Whole-mount preparations, providing three-dimensional views of the ganglia, are more accurate for cell counting compared to conventional sections. A similar technique, exposing all three dimensions of the ganglia, is not available in the CNS as yet. Kou et al. [15] were therefore only able to use conventional sections in their study. Although we were also able to identify new weakly stained cells appearing in some ganglia in the conventional sections after only 24 h, inaccuracy due to differing thickness of cut cells in conventional sections for cell counting may be another reason for any discrepancy between the two studies. NADPH-d positive cell counts in the ENS increase proportionally with the duration of incubation of NADPH-d histochemistry. Comparative studies attempting to quantify NADPH-d positive cell counts in dysmotility disorders must take into account the variability in NADPH-d positive cell count associated with prolonged incubation in NADPH-d histochemistry.

NADPH-d staining with short incubation period (30 min) is suitable only for qualitative demonstration of the of the ganglion cells within the enteric nervous system. However quantitative analysis of ganglion cells based on the NADPH-d staining is more reliable with extended (24-h) incubation duration.

## References


1. Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345:346–347
2. Boeckxstaens GE, Pelckmans PA, Bult H, De Man JG, Herman AG, Van Maercke YM (1990) Non-adrenergic non-cholinergic relaxation mediated by nitric oxide in the canine ileocolonic junction. Eur J Pharmacol 190:239–246
3. Stark ME, Bauer AJ, Sarr MG, Szurszewski JH (1993) Nitric oxide mediates inhibitory nerve input in human and canine jejunum. Gastroenterology 104:398–409
4. Burleigh DE (1992) Ng-nitro-L-arginine reduces nonadrenergic, noncholinergic relaxations of human gut. Gastroenterology 102:679–683
5. Hanani M, Louzon V, Udassin R, Freund HR, Karmeli F, Rachmilewitz D (1995) Nitric oxide-containing nerves in bowel segments of patients with Hirschsprung's disease. EJ Pediatr Surg 30:818–822
6. Vanderwinden JM, Mailleux P, Schiffmann SN, Vanderhaeghen JJ, De Laet MH (1992) Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. N Engl J Med 327:511–515
7. Hirakawa H, Kobayashi H, O'Briain DS, Puri P (1995) Absence of NADPH-diaphorase activity in internal anal sphincter (IAS) achalasia. J Pediatr Gastroenterol Nutr 20:54–58
8. Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci U S A 88:7797–7801
9. Puri P (2000) Newer neuronal markers. In: Holschneider AM, Puri P (eds) Hirschsprung disease and allied disorders. Harwood, Amsterdam, p 275
10. Holschneider AM, Puri P (2000) Intestinal neuronal dysplasia in markers. In: Holschneider AM, Puri P (eds) Hirschsprung disease and allied disorders. Harwood, Amsterdam, p 150
11. Wester T, O'Briain DS, Puri P (1999) Notable postnatal alterations in the myenteric plexus of normal human bowel. Gut 44:666–674
12. Montedonico S, Sri Paran T, Pirker M, Rolle U, Puri P (2006) Developmental changes in submucosal nitrergic neurons in the porcine distal colon. J Pediatr Surg 41:1029–1035
13. Scherer-Singler U, Vincent SR, Kimura H, McGeer EG (1983) Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J Neurosci Methods 9:229–234
14. Timmermans JP, Barbiers M, Scheuermann DW, Stach W, Adriaensen D, Mayer B, De Groodt-Lasseel MH (1994) Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. Ann Anat 176:515–525
15. Kuo H, Grant S, Muth N, Hengemihle J, Ingram DK (1994) The correlation between neuron counts and optical density of NADPH-diaphorase histochemistry in the rat striatum: a quantitative study. Brain Res 660:57–65
16. Koike S, Hisa Y, Uno T, Murakami Y, Tamada Y, Ibata Y (1998) Nitric oxide synthase and NADPH-diaphorase in neurons of the rat, dog and guinea pig nodose ganglia. Acta Otolaryngol Suppl 539:110–112
17. Pitkanen A, Amaral DG (1991) Distribution of reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) cells and fibers in the monkey amygdaloid complex. J Comp Neurol 313:326–348
18. Wilhelm M, Batori Z, Pasztor I, Gabriel R (1998) NADPH-diaphorase positive myenteric neurons in the ileum of guinea-pig, rat, rabbit and cat: a comparative study. Eur J Morphol 36:143–152
19. Arbones ML, Ribera J, Agullo L, Baltrons MA, Casanovas A, Riveros-Moreno V, Garcia A (1996) Characteristics of nitric oxide synthase type I of rat cerebellar astrocytes. Glia 18:224–232
20. Balemba OB, Mortensen K, Semuguruka WD, Hay-Schmidt A, Johansen MV, Dantzer V (2002) Neuronal nitric oxide synthase activity is increased during granulomatous inflammation in the colon and caecum of pigs infected with *Schistosoma japonicum*. Auton Neurosci 99:1–12