The role of nitric oxide synthase in infantile hypertrophic pyloric stenosis

Irnak M. Barbosa, Saulo M.R. Ferrante, Carlos A. Mandarim-de-Lacerda

Abstract

Objective: to experimentally reproduce, in rats, the findings corresponding to the histopathology of infantile hypertrophic pyloric stenosis (IHPS), using nitric oxide synthase (NOS) inhibitor (L-NAME).

Methods: L-NAME was administered to pregnant rats (L-NAME group), from the 14th gestational day on in order to reproduce the model of NOS inhibition in the production of IHPS. This group was then compared to control animals. After birth, all the animals in the L-NAME group were maintained under NOS inhibition until the 42nd day of life, when they were sacrificed. The control animals, which did not receive any kind of drug, were also sacrificed on the 42nd day of life. The animals and their internal organs were analyzed and weighed. The pyloric region was technically prepared and observed through light microscopy.

Results: the L-NAME group presented lower body and intestinal weight and higher gastric weight than the control group. Light microscopy revealed hypertrophy of the circular smooth muscle layer of the pyloric muscle in L-NAME animals.

Conclusions: this work reproduced an experimental model of an IHPS study, confirming the effect of NOS blockade on the pyloric musculature.

Introduction

Infantile hypertrophic pyloric stenosis (IHPS) is a common disorder with an incidence of 0.3% in newborn infants. It occurs mainly from the third to the sixth week of life, and is characterized by hypertrophic circular pyloric muscle and by developing with pyloric obstruction and persistent vomiting.

Until the present moment, the etiology of IHPS is not fully understood. It is believed, however, that it involves an association of hereditary and environmental factors. Many hypotheses have been presented involving pyloric muscle innervation, hormone control, extracellular matrix proteins, peptidergic and nitrenergic innervation, and, more recently, smooth muscle growth factor alterations.1,2 Currently, it is understood that primary abnormalities of innervation of enteric nerves, associated with absence of nitric oxide synthase (NOS) and interstitial cells of Cajal,3 are more promising theories for explaining the pathophysiology of IHPS.
Recent studies have shown that nitric oxide (NO) is an unstable substance, synthesized from L-arginine by NO synthase (NOS), and that it is one of the main relaxant transmitters of gastrointestinal smooth muscle. The neuronal nitric oxide synthase is found in both the central nervous system and in the peripheral nervous system. The activity of NOS in the gastrointestinal tract has been detected by immunohistochemistry in the myenteric plexus and in muscle planes (circular and cross-sectional). Huang et al. were able to generate mutant mice by homologous recombination with alteration of the locus of the NOS gene. The mice presented lack of NOS activity and grossly enlarged stomachs with hypertrophy of the circular muscle layer.

The objective of our study is to experimentally reproduce the histopathological findings corresponding to IHPS in cases of inhibition of NOS.

Materials and methods

We used Wistar rats from the Fiocruz central animal facilities, state of Rio de Janeiro, Brazil. Three pregnant females, one with 14 days of gestation and two with 16 days of gestation, were named D14 and D16 (NOS inhibitor group) and D16c (control). Until the end of gestation, the rats remained at the animal facilities of the Morphometry Laboratory of the Universidade Estadual do Rio de Janeiro (UERJ). The facilities were controlled for temperature, lighting, and humidity. Rats were fed with appropriate feed (Novilab TM) and given potable water ad libitum.

From the beginning of the study until the end of gestation, rats D14 and D16 received nitric oxide synthase inhibitor L-NAME (N(G)-nitro-L-arginine methyl ester hydrochloride; Sigma Chemical Co.; St. Louis; 98H1427) at 50 mg/kg/day. The inhibitor was diluted in drinking water administered at sufficient minimal daily amounts. There was no significant difference on the volume intake of animals. Rat D16c was not administered L-Name (control).

D14, D16, and D16c litter sizes were of, respectively, seven, six, and five pups. On the 21st day of life, pups were removed from their mothers, weighed, followed-up for another 21 days, and then sacrificed. After weaning, all pups were fed with the same diet fed to their respective mothers until weaning. L-NAME was administered to D14 and D16 pups, but not D16c, with drinking water for another 21 days and at 50 mg/kg/day.

At 42 days of life, animals were sacrificed by inhalation of sulfuric ether and intracardial injection of approximately 0.5 ml of KCl. We removed the stomach, the pylorus, and the small intestines, which were all measured and weighed. The intestines (from the duodenum to the ileocecal valve) were removed, rid of intestinal material (cleaned), and measured. Next, fragments from the whole extension of the wall of the pyloric region were fixed in buffered formalin at 10% at room temperature for 48 hours. Subsequently, fragments were embedded in paraffin wax and divided into sections of 3 micrometers with 10 micrometers of thickness. Sections were prepared and stained by hematoxylin-eosin and trichromic (Masson).

We calculated descriptive statistics. Differences between groups were tested using the nonparametric Mann-Whitney test. Significance was considered for P = 0.05.

Results

Groups were assessed according to weight of pups. Comparison of weight on the 21st day of life of L-NAME groups (D14 and D16) and controls (D16c) was not significant (Figure 1). On the 42nd day, however, this comparison indicated a significant difference on weight gain between the experimental (L-NAME) and control groups (Figure 2). We observed values for weight and dimension of bodily structures removed from the 18 pups. Average ± standard deviation of length of small intestines and of weight of the stomach were, respectively 90.72 ± 5.89 cm and 1.50 ± 0.27 g. In the L-NAME groups (D14, D16), the average stomach weight was higher than in the control group.

![Graph showing body weight on the 21st day after birth](image)

The difference in the comparison of stomach weight to body weight of the L-NAME and control groups was statistically significant (Figure 3). The average intestine weight was 7.20 ± 0.87 g. The difference in the relation weight-to-small intestine length of the experimental and control groups was also statistically significant (Figure 4).
Histological examination of circular pyloric muscle of the 13 pups that received L-NAME indicated a statistically significant difference in relation to thickness of the pylorum. Cross-sections indicated that hypertrophy of the circular muscle layer of the pylorum presented the largest diameter, which was twofold the thickness of pylorum in controls (Figure 5).

Discussion

The etiology of IHPS is still not fully understood despite the advancements in molecular biology and genetic engineering. More recent studies have indicated abnormalities of the enteric nervous system (ENS) in patients with IHPS both quantitatively and qualitatively. This discussion was initiated by Belding and Kernohan, who posited that patients with IHPS presented a decrease in the number of ganglial cells and neural fibers as a result of a degenerative process. These findings were later confirmed by Sptiz and Kaufmann.

Friesen and Boley, following a comparative analysis of neural cells of IHPS children and normal fetuses, suggested that IHPS patients present immaturity of ganglial cells. Later on, others observed abnormalities of nerve terminations of the pylorum and of nerve supporting cells, in addition to ultrastructure abnormalities of the myenteric plexus and pyloric muscle.

Table 1 - Average weight of pups and of their stomach and intestine

<table>
<thead>
<tr>
<th>Variable</th>
<th>D14 (n=7)</th>
<th>D16 (n=6)</th>
<th>D16c (n=5)</th>
<th>P ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (±SD, g) on the 21st day</td>
<td>29±2.62</td>
<td>53.6±4.26</td>
<td>59.3±5.35</td>
<td>0.06</td>
</tr>
<tr>
<td>Weight (±SD, g) on the 42nd day</td>
<td>77.4±9.83</td>
<td>144.6±23.07</td>
<td>168.8±27.52</td>
<td>0.04</td>
</tr>
<tr>
<td>Weight of small intestine (±SD, g)</td>
<td>6.50±0.84</td>
<td>6.035±0.28</td>
<td>8.160±0.24</td>
<td>0.006</td>
</tr>
<tr>
<td>Weight of stomach (±SD, g)</td>
<td>1.504±0.27</td>
<td>1.691±0.20</td>
<td>1.250±0.08</td>
<td>0.006</td>
</tr>
</tbody>
</table>

* standard deviation; †: grams; ‡: significance level (P< 0.05), nonparametric Mann-Whitney test
New techniques have allowed for identification of gastrointestinal peptides and description in better detail of peptidergic innervation. Malmfors and Sandler\(^1\) described abnormalities of peptidergic innervation by showing reduction in nerve fibers specific for the vasoactive intestinal peptide (VIP), substance P, and enkephalin in IHPS patients. VIP is found in high concentrations in the pylorus and is related to relaxation of gastrointestinal tract smooth musculature. It is also probably one of the elements responsible for control of the pyloric sphincter.\(^{13}\) Grider\(^{14}\) showed that there is a synergic activity between VIP and NO with in vitro analyses of smooth muscles fibers of the pylorus. The author concluded that VIP stimulates production of NO. In this sense, VIP maintains and amplifies relaxation of smooth muscle cells and is released presynaptically by NO stimulation. NO also acts on the initial stage of relaxation.

Barajas-Lopez\(^{15}\) and Serio\(^{16}\) showed that interstitial cells of Cajal (ICC) operate as the pacemaker cells regulating electrical waves in intestinal smooth musculature. Langer\(^3\) observed a decrease in ICC count in IHPS patients. This finding was confirmed by immunohistochemistry using a specific antiserum raised against c-kit, a tyrosine kinase receptor expressed by interstitial cells.\(^{17}\)

NO, initially identified as an endothelium derived relaxing factor (EDRF),\(^{18,19}\) is known as a powerful noncolinergic, nonadrenergic neurotransmitter that is involved in inhibitory innervation of gastrointestinal tract smooth musculature.\(^5,20\) IHPS patients have been reported with lack of NADPH-diaphorase (enzyme considered identical to NOS in the central and peripheral nervous systems). Biopsy from pyloric muscle sections from IHPS children presented decreased NOS in neural fibers of the circular muscle layer of the pylorus. These biopsies also indicated that NOS activity was preserved in the myenteric plexus.\(^{21,22}\) Mutagenic rats (knockout) with lack of NOS are fertile and present no abnormalities of the CNS. However, they do present grossly enlarged stomachs with hypertrophy of the circular muscle layer of the pylorus.\(^6\) Kusafuka and Puri\(^{23}\) showed lower levels of messenger RNA in neuronal nitric oxide synthase gene in IHPS patients.

![Figure 4 - Bar graph (mean±standard deviation) showing the weight/small intestine length ratio for sacrificed rats in D16 (L-NAME) and D16c (control) groups](image)

![Figure 5 - Photomicrographs of the pyloric region (trichromic (Masson) staining, bar: 0.2mm). Transition between pylorus and duodenum in control animals (A) and in L-NAME animals (B). The arrows indicate the initial portion of the duodenum (Brünner’s glands)](image)
The formation of NO increases during gestation, which explains maternal vasodilatation during this period. The use of NOS inhibitors at the end of the gestation in rats did not affect the health of the mother; however, it did affect the litter with intrauterine growth retardation and ischemic alterations with hemorrhagic disruptions of hind limbs.\textsuperscript{25,26} In addition, studies have also observed growth retardation during the neonatal period in rats administered L-NAME, which was associated with increase in stomach size, decrease in weight of intestines, and hypertrophy of the muscular layer of the pylorus. The comparison of brain weight in relation to the vitals indicated malnutrition in rats receiving L-NAME due to pyloric obstruction.\textsuperscript{26}

In our study, we observed that the animals gained weight from weaning (21st day) to sacrifice (42nd day), which can be explained by the delayed effect of NOS inhibition and by the transition from fluid diet (mother’s milk) to solid diet (feed). The increase in stomach weight and decrease in small intestine weight, associated with hypertrophy of circular muscle layer of the pylorus confirmed the findings of Voelker et al.\textsuperscript{26}

The results of our study corroborate the theory that tries to explain pathophysiology of IHPS as a consequence of lack of the nonadrenergic and noncholinergic neurotransmitter in the pylorus, which Furchgott\textsuperscript{18} and Ignarro\textsuperscript{19} described as being NO. Vanderwinden\textsuperscript{21} was the first to associate lack of NOS with IHPS, which was later confirmed by other authors.\textsuperscript{6,22,23} The normalization of pyloric innervation and NOS activity in patients who were submitted to surgical treatment suggests that lack of NOS in IHPS patients is transient.

Though further and more specific studies are needed for assessing the importance of NO in the etiopathogeny of IHPS, our study suggests that inhibition of NOS, which leads to loss of relaxation of smooth musculature of the intestines, is a central element in this process.

References


Correspondence:
Dr. Irnak Marcelo Barbosa
Rua General Osório, 109/302
CEP 28625-630 - Nova Friburgo, RJ, Brazil