Metoclopramide does not increase gastric muscle contractility in newborn rats

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Running Title: Metoclopramide and Newborn Gastric Muscle Contraction
Abstract

Feeding intolerance resulting from delayed gastric emptying is common in premature neonates. Metoclopramide (MCP), the most frequently used prokinetic drug in neonates, enhances gastric muscle contractility through inhibition of dopamine receptors. Although its therapeutic benefit is established in adults, limited data are available to support its clinical use in infants. Hypothesizing that developmentally-dependent differences are present, we comparatively evaluated the effect of MCP on fundus muscle contractility in newborn, juvenile and adult rats. The muscle strips were either contracted with electrical field stimulation (EFS) to induce cholinergic nerve-mediated acetylcholine release or carbachol, a cholinergic agonist acting directly on the muscarinic receptor. Although in adult rats MCP increased EFS induced contraction by 294±122% of control (P<0.01), no significant effect was observed in newborn fundic muscle. MCP had no effect on the magnitude of the carbachol-induced and/or bethanechol-induced gastric muscle contraction at any age. In response to dopamine, a 80.7±5.3% relaxation of adult fundic muscle was observed, as compared with only a 8.4 ± 8.7%, response in newborn tissue (P<0.01). Dopamine D₂ receptor expression was scant in neonates and significantly increased in adult gastric tissue (P<0.01). In conclusion the lack of MCP effect on the newborn fundic muscle contraction potential relates to developmental differences in dopamine D₂ receptor expression. To the extent that this novel data can be extrapolated to neonates, the therapeutic value of MCP as a prokinetic agent early in life requires further evaluation.

Key Words: Prokinetic, Gastric emptying, Dopamine
Introduction

Feeding intolerance and impaired gastric emptying are often observed early in life, especially in neonates. Prokinetic pharmacological agents are used in premature infants to promote gastric emptying and thus enhance feeding tolerance, yet currently only limited and conflicting data are available on their clinical benefit early in life (19).

Amongst the prokinetic agents, metoclopramide is one of the most commonly used drugs to facilitate enteral nutrition tolerance in neonates (20). Metoclopramide enhances proximal gastrointestinal motility primarily via its inhibition of dopamine D₂ receptors, as well as serotonin 5-HT₄ receptor agonist and 5-HT₃ receptor antagonist properties (28). Endogenously generated dopamine promotes gastrointestinal muscle relaxation (16), thus pre- and postsynaptic dopamine D₂ receptors inhibition by metoclopramide enhance gastric emptying. Although widely used as a prokinetic agent in adults, concerns about the drug-induced side effects, such as tardive dyskinesia, have resulted in avoidance of its prolonged clinical use (16).

The dopamine receptor distribution is tissue specific and developmentally regulated. When compared with adult values, dopamine receptors expression is reduced in the newborn renal and brain tissues (7, 33). Reduced gastric tissue receptor expression early in life, as compared with adulthood, would render the dopamine relaxant effect on the gastrointestinal muscle contractility of limited physiological significance. In addition, given its functional dependence on dopamine receptors, maturity related differences in D₂ expression would impact on the metoclopramide effect as a prokinetic agent early in life. To the best of our
knowledge, developmental dopamine D2 receptor expression comparative data for the gastrointestinal tract is not available.

Hypothesizing that a lower expression of D2 receptor in the newborn, as compared with adult gastric tissue, results in developmental-dependent differences in its prokinetic activity, we comparatively evaluated the metoclopramide effect across ages in rats. We demonstrated that this prokinetic agent does not potentiate muscle contraction in the newborn due to a deficiency in the developmentally regulated dopamine D2 receptor expression in gastric tissue.

Methodology

Chemicals and reagents

All chemicals and reagents were obtained from Sigma Aldrich (Oakville, ON, Canada), unless otherwise indicated.

Animals

All procedures were conducted in agreement with the Canadian Council on Animal Care regulations and the study protocol was approved by the Hospital for Sick Children’s Animal Care Committee.

Sprague Dawley rats (Charles River, ON, Canada) bred in house were utilized. All animals were fed regular rodent pellets and housed under standard lighting and temperature conditions. Newborn (3-7 days of age), juvenile (13-21 days) and adult (> 30 days) of both sexes
were studied. The animals were sacrificed by pentobarbital sodium i.p. injection (60 mg/kg) and the gastric tissues quickly excised after death. Gastric tissue samples obtained for Western blots were snap-frozen in liquid nitrogen and stored for later processing.

Gastric fundus and antrum smooth muscle mechanical properties

Gastric longitudinally oriented fundus and circular antrum muscle strips (average 2mm wide and 5mm long) were obtained as described by others (4, 8, 27) and maintained in ice-cold oxygenated Krebs-Henseleit solution (NaCl, 115 mM; NaHCO$_3$, 25 mM; NaHPO$_4$, 1.38 mM; KCl, 2.51 mM; MgSO$_4$ · 7 H$_2$O, 2.46 mM; CaCl$_2$, 1.91 mM; and dextrose, 5.56 mM). The mucosa and sub-mucosa were removed from all muscle strips by sharp dissection.

The muscle strips were secured at either end with 6x10mm flat surface tissue clips, suspended between two flat lead electrodes, and submerged into a 25 mL tissue bath (Radnoti LLC, Monrovia, California) filled with Krebs-Henseleit solution at 37°C, pH 7.4 and bubbled with 95% O$_2$/5% CO$_2$. One end of the muscle strip was fixed to the bottom of the tissue bath and the top clamp tied to the magnesium arm of an electromagnetic muscle lever system using 7-0 braided silk. Changes in force were recorded digitally for further measurement (LabChart 7, ADinstruments, Colorado Spring, CO, USA).

The muscle strip was equilibrated in the bath for the first 45 minutes, replacing the Krebs solution every 15 minutes and stretched to its optimal length by incrementally increasing the force to a pre-determined resting tension of either 6 mN (adult), 4 mN (juvenile) or 3 mN (newborn). All force measurements were obtained at the optimal resting tension. Some of the
muscle strips were stimulated with KCl (128 mM) at the beginning of the experiment and the subsequent agonist-induced force normalized to the KCl response. The contraction potentials of the muscle strips were evaluated through carbachol and bethanechol dose-response curves and electrical field stimulation (EFS). EFS was employed as previously reported (34), using a commercially available stimulator (Cibertec, Madrid, Spain) as follows: 80 V [voltage] stimulation, 0.5 msec pulses with 20-second trains of at a frequency of 5 Hz. Three stimulations obtained 10 min apart were employed and averaged to determine the EFS-induced force increase. The onset of fundic and antral muscle contraction is evident immediately following the initiation of EFS stimulation, a pattern similar to what has been reported by others (5, 13, 22). In order to evaluate the metoclopramide effect on the gastric fundic muscle contraction potential, the strips were evaluated in the absence and presence of the drug at a bath concentration of 1 mcg/ml.

**Western Blot Analysis**

Gastric tissue frozen samples were lysed in 10 mM Tris-HCl pH 7.4 lysis buffer containing 1% Triton X-100 and protease/phosphatase inhibitor cocktails (Thermo Fisher Scientific Inc., Rockford, IL), and centrifuged at 14,000 g for 30 min. Protein concentrations were determined via the Bradford assay (6). Equivalent amounts of lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to polyvinylidene di-fluoride membranes and blotted. Membranes were treated with 5% skim milk and exposed at 4°C overnight for the anti-D2 Dopamine receptor (Alpha Diagnostic, 1:1000; catalog # D2R11-A, San Antonio, TX) and α-tubulin (1:10,000; catalog #ab7291-100, Cambridge, MA) antibodies. Appropriate IgGs
conjugated with HRP were used as secondary antibodies. The enhanced chemiluminescence (ECL, Perkin Elmer, Shelton, Connecticut, USA) reagent was used for detection and the band intensities were quantified by ImageJ software (NIH).

**Measurement of stomach weight and small bowel transit time**

Overnight fasted rats of each aged were weighed prior to the experiment and randomly assigned to receive either vehicle (0.9% NaCl; Control) or metoclopramide (1 mL/kg, ip). After 75 minutes, 10% powdered charcoal suspended in 5% gum arabic (1 ml volume) was administered via gavage into the stomach under isoflurane anesthesia. The animals were killed with a barbiturate overdose precisely 45 minutes after the charcoal meal delivery and the stomach as well as the small bowel was removed. The distance traveled by the charcoal meal through the intestine was measured and expressed as a percentage of the total small intestinal length. This parameter was used as a measurement of gastrointestinal transit time as reported by others (23, 26). In addition, we elected to measure the charcoal meal stomach content to ascertain the gastric emptying time since we determined in a pilot study that overnight fasted rats (12 hours) had an empty stomach.

The charcoal meal method could not be utilized in newborn pups due to the fact that their stomach is filled with breast milk to capacity. In a pilot experiment we observed that any attempt to gavage the charcoal meal into the newborn pups led to esophageal reflux and lung aspiration. Instead, we utilized a previously described method (18), where the pups are separated from their mother at a precise time and their stomach content weighed 2 hours later. Normal saline vehicle and metoclopramide (1 mL/kg, ip) were administered to the
newborn animals immediately after maternal separation and they were kept in a 37°C environment until killed with barbiturate overdose. The stomach content was normalized to the pup body weight.

**Data Analysis**

Data were evaluated by one or two-way analysis of variance (ANOVA) for repeated measures with multiple comparisons obtained by the Tukey-Kramer test, or unpaired Student’s t-test, when appropriate. Statistical significance was determined at P<0.05. All statistical analyses were performed with the Number Cruncher Statistical System software (NCSS, Kaysville, Utah, USA). Data are presented as means±SEM.

**Results**

**Metoclopramide does not potentiate the EFS-induced force in newborn fundic muscle**

Metoclopramide significantly increased (P<0.01) the EFS-induced fundic muscle contraction in juvenile and adult rats. In contrast, following EFS stimulation the drug had no effect on the newborn gastric muscle force generation expressed as active tension (peak – resting tension)(Figure 1, Panel A), or relative to KCl-induced response (Figure 1, Panel B).

In order to determine whether the metoclopramide-dependent potentiation of the muscle contraction relates to its effect on the nerve terminals, or the smooth muscle cell, we further evaluated its effect on the muscarinic agonists. As shown in Figure 2, metoclopramide had no effect on the bethanechol-induced (Panels A-C) muscle contraction of all ages indicating
that it acts directly on the nerve terminals. Similar lack of metoclopramide effect on force generation was obtained following carbachol stimulation (data not shown).

Lastly, we further confirmed that the age differences in the metoclopramide effect on gastric muscle were not unique to the fundus. EFS-stimulated newborn antrum strips force generation was not affected, whereas juvenile antrum samples exhibited potentiation of the response in the presence of metoclopramide (Figure 3, Panel A). As observed for the fundi, the newborn and juvenile antrum muscle bethanechol-induced force dose-response was not altered following pre-incubation with metoclopramide (Figure 3, Panels B-C).

*Maturational dependent dopamine receptor expression differences account for the absent metoclopramide effect in newborn fundic muscle*

Given that the metoclopramide prokinetic effect appears to be modulated via inhibition of dopamine receptors, we comparatively evaluated the dopamine effect on newborn, juvenile and adult pre-contracted (carbachol $E_{75}$) fundic muscle. As shown in Figure 4 (Panel A), the dopamine ($10^{-4}$ M) fundic muscle relaxant effect was absent in the newborn and maximal in adult tissue. In response to bethanechol pre-contraction a significant ($P<0.01$) age-dependent difference in relaxation was observed when newborn and adult fundic muscle strips were exposed to incremental dopamine concentrations (Figure 4, Panel B).

We hypothesized that the absent dopamine-induced relaxant effect and failure of metoclopramide to enhance fundic muscle contraction early in life was related to limited expression of dopamine receptors in the neuromuscular plate. As such, we proceeded to comparatively evaluate fundic tissue for dopamine receptor expression. We choose to solely
evaluate the dopamine D₂ receptor expression, since this isotype was reported by others to be involved in the mechanism of metoclopramide action (17). D₂ receptor expression was 3-fold greater in adult gastric tissue, when compared with newborn samples (Figure 5).

**Metoclopramide does not enhance newborn gastric emptying**

In order to confirm the in vitro maturational-dependent differences in the metoclopramide effect on gastric muscle contraction, we proceeded to test its effect in vivo. Whereas metoclopramide significantly enhanced (P<0.01) gastric emptying in juvenile and adult rats, it showed no effect in the newborn animals (Figure 6, Panel A).

We further ascertained whether metoclopramide has any effect on intestinal transit time, since others evaluated the effectiveness of this drug in adult rats by feeding a charcoal meal and assessing the distance that it traveled through the small bowel (26). We reasoned that such measurement does not allow proper differentiation between the effects of metoclopramide on gastric emptying, when compared with small bowel transit time. In fact, metoclopramide does not alter the juvenile and adult rat intestinal transit time (Figure 6, Panel B). Such measurements could not be obtained in newborn rats since their stomach remains full after 3-4 hours of fasting not allowing for the administration of the charcoal meal.

**Discussion**

Metoclopramide is commonly used for the treatment of gastrointestinal dysmotility in adults given its effect of smooth muscle contraction that results in reduce transit time (16). Feeding intolerance and gastrointestinal functional dysmotility are commonly observed in
premature neonates (20). Yet, the therapeutic benefit of metoclopramide in neonates with feeding intolerance has been poorly evaluated (19). In this study, we observed that the metoclopramide effect is absent in the newborn, as opposed to adult rat gastric fundus muscle contraction. This metoclopramide gastric muscle potentiation effect is only observed following electrical field stimulation and not direct muscarinic receptor agonist activation with carbachol or bethanechol. We further confirmed that the age-difference in metoclopramide effect on fundic muscle contraction relates to the lower dopamine D₂ receptor expression in the newborn, when compared with adult tissue.

Endogenous dopamine production has been demonstrated in the gastrointestinal tract of the rat (32) and reduces gastric muscle contraction by diminishing the nerve terminal acetylcholine release (29, 30). To date several dopamine receptor subtypes are known to exist in the adult rat gastrointestinal tract (D₁(A/B), D₂, D₃ and D₄ and D₅) (31).

Metoclopramide potentiates gastrointestinal muscle contraction though the inhibition of dopamine D₂ receptors (3, 21, 35). In spite of publications dating back 30 years showing enhanced gastric emptying in response to pharmacological doses (11, 12), the therapeutic benefit of metoclopramide towards feeding intolerance in neonates is controversial (10, 19). Yet, a recent study in the United States reported that close to 50% of the premature infants discharged home at a post-conceptional age of >42 weeks were prescribed anti-reflux and prokinetic medications including metoclopramide (20).

In adult rats, metoclopramide enhances gastrointestinal transit (9) and gastric fundus contraction (1). Developmentally, the newborn rat gastrointestinal system is comparable to human preterm infants (25). Immaturity of dopamine receptors account for the lesser
In the present study we have clearly shown that the dopamine regulation of gastric motility is not present in the newborn rat. As shown in Figure 7, metoclopramide enhances gastrointestinal motility by offsetting the dopamine-induced relaxant effect on gastric smooth muscle nerve terminals. The present study clearly indicates that its therapeutic benefit is dependent on the presence and activity of gastric tissue dopamine receptors.

In rodents the dopamine D2 receptor subtype is the primary regulator of gastrointestinal tract motility (17). Dopamine, via its D2 receptor, blocks the nerve terminal acetylcholine generating effect on the smooth muscle muscarinic receptors that modulate the neuro-regulated muscle contraction (14, 15, 24). In contrast, metoclopramide does not affect the stimulation response induced by muscarinic receptor agonists such as carbachol and bethanechol that directly contract the smooth muscle (Figure 7).

To the extent that these animal data can be extrapolated to the clinical setting, the use of metoclopramide as a prokinetic agent in neonates, especially when premature, is ill justified. Unfortunately, there are no effective and safe drugs presently capable of accelerating gastric emptying and enhancing intestinal peristaltic activity in neonates.

In summary, we documented in the present study a lack of effect of metoclopramide on newborn rat gastric muscle contraction. Maturational-related changes in dopamine D2 receptors expression in the stomach tissue account for the age-difference in the muscle contraction potentiation effect of metoclopramide. These data raise concerns regarding the clinical use of metoclopramide as a prokinetic in neonates.
Grants

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Figures

Fig. 1. A: Fundic muscle contraction in the absence and presence of metoclopramide (MCP) following electrical field stimulation (EFS) of newborn (N=26 and 18), juvenile (N=18 and 9) and adult (N=12 and 11) animals. B: EFS-induced force normalized to KCl-induced contraction (Newborn N=4; Juvenile N=8; Adult N=3). Figure insert shows representative myograph tracings with the arrow-heads indicating initiation of the EFS contraction. ** P<0.01 when compared with same age control samples by two-way ANOVA with Tukey-Kramer multiple comparison testing.

Fig. 2. Newborn, juvenile and adult fundic muscle bethanechol-induced (A-C; N=4, 4 and 3 respectively) dose-response in the absence and presence of metoclopramide (MCP).

Fig. 3. Newborn and juvenile antral muscle contraction in the absence and presence of metoclopramide (MCP) following electrical field stimulation (EFS) (A; N=7 and 3 respectively) and bethanechol dose response (B-C; N=3 for each age). ** P<0.01 when compared with same age control samples by two-way ANOVA with Tukey-Kramer multiple comparison testing.

Fig. 4. A: Newborn (N=7), juvenile (N=3) and adult (N=4) carbachol pre-contracted fundic muscle relaxation response to a single dose dopamine molar concentration (10^{-4} M). B: newborn (N=4) and adult (N=4) fundic muscle dose-dependent relaxation response to dopamine. ** P<0.01 when compared with newborn tissue by one-way (A) or two-way (B) ANOVA with Tukey-Kramer multiple comparison testing. Figure insert shows representative myograph tracings with the arrow-heads indicating exposure to dopamine.
Fig. 5. Newborn, juvenile and adult (N=3 per age) gastric tissue dopamine D<sub>2</sub> receptor expression normalized to α-tubulin. Figure insert illustrates the Western blot. ** P<0.01 and *P<0.05 versus adult by one-way ANOVA with Tukey-Kramer multiple comparison testing.

Fig. 6. A: Stomach content/body weight ratio in the absence (saline vehicle), or presence of metoclopramide (MCP) in newborn (N=5), juvenile (N=6) and adult (N=4) animals. B: Distance travelled by charcoal meal expressed as a percentage of total small bowel length in the absence (Vehicle), or presence of metoclopramide (MCP) in juvenile (N=6) and adult (N=9) animals. **P<0.01 as compared with age-matched control by two-way ANOVA with Tukey-Kramer multiple comparison testing.

Fig 7: Mechanism depicting the effect of metoclopramide on the dopamine D<sub>2</sub> receptor and electrical field stimulation (EFS), as well as vagal efferent-induced acetylcholine (ACh) release. Carbachol acts directly on the smooth muscle muscarinic receptor to promote a dopamine D<sub>2</sub> receptor independent contraction.


Figure 1

A

EFS-Induced Force (mN)

- Newborn
- Juvenile
- Adult

Control
MCP

B

Force (EFS/KCl)

- Newborn
- Juvenile
- Adult

Control
MCP
Figure 2

A  Newborn

B  Juvenile

C  Adult
Figure 3

A

![Bar graph showing Force (EF/KCl) for newborn and juvenile groups.]

- **Newborn**
- **Juvenile**

B

![Line graph showing Force (Bethanecol/KCl) for newborn group.]

- **Control**
- **MCP**

C

![Line graph showing Force (Bethanecol/KCl) for juvenile group.]

- **Control**
- **MCP**
Figure 4

A

Relaxation (% of Max Contraction)

Newborn  Juvenile  Adult

B

Relaxation (% of Max Contraction)

log [Dopamine] (M)

Adult  Newborn
Figure 5

The bar graph shows the expression levels of D$_2$ receptor normalized to Tubulin in different developmental stages: Newborn, Juvenile, and Adult. The expression levels are indicated on the y-axis, and the stages are shown on the x-axis.

- **: Statistically significant difference
- *: Statistically significant difference

The Western blot image at the top of the figure confirms the expression patterns of D$_2$ Receptor and Tubulin across the developmental stages.
Figure 6

A

![Bar graph showing Stomach Content/Body Weight for Newborn, Juvenile, and Adult stages with Vehicle and MCP treatments.](image)

B

![Bar graph showing Bowel Transit Time (% of Total Length) for Juvenile and Adult stages with Vehicle and MCP treatments.](image)