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Altered Mechanosensitive Properties of Vagal Afferent Fibers Innervating the Stomach Following Gastric Surgery in Rats

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Abstract

Background and Aims—Several types of gastric surgeries have been associated with early satiety, dyspepsia and food intolerances. We aimed to examine alterations in gastric vagal afferents following gastric surgery-fundus ligation.

Methods—Six week old, male Sprague-Dawley rats underwent chronic ligation (CL) of the gastric fundus. Sham rats underwent surgery, but without ligation. Another group of rats underwent acute ligation (AL) immediately prior to experiments. Animals were allowed to grow to age 3–4 months. Food intake and weights were recorded post-operatively. Gastric compliance and gastric wall thickness was measured at baseline and during gastric distension (GD). Extracellular recordings were made to examine response characteristics of vagal afferent fibers to GD and to map the stomach receptive field (RF). The morphological structures of afferent terminals in the stomach were examined with retrograde tracings from the nodose ganglion.

Results—The CL group consumed significantly less food and weighed less than control. The mean compliance of CL group was significantly less than control, but higher than the AL. The spontaneous firing and responses to GD of afferent fibers from the CL rats were significantly higher than AL rats. There was a marked expansion of the gastric RF in the CL rats with significant reorganization and regeneration of intramuscular array (IMA) terminals. There was no difference in total wall or muscle thickness among the groups.

Conclusion—CL results in aberrant remodeling of IMAs with expansion of the gastric RF and alters the mechanotransduction properties of vagal afferent fibers. These changes could contribute to altered sensitivity following gastric surgery.

Keywords

Vagal Afferents; Stomach; Satiety; Fundus ligation; Gastric surgery

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Introduction

Sensorimotor dysfunction is a common clinical problem following surgery of the upper gastrointestinal (GI) tract. Surgical procedures that involve manipulation of gastric tissue such as cutting, suturing or banding can result in significant morbidity. For example, mobilization and wrapping of the gastric fundus in adults and children has been associated with significant post-surgical hypersensitivity including early satiety, post-prandial fullness or nausea (Remes-Troche et al. 2007; Mousa et al. 2006; Kornmo and Ruud 2008). Similarly, food intolerance following gastric banding is a leading cause of band removal and has no clear etiology (Dargent 2008). While it is generally speculated that these abnormalities are a result of abnormal gastric accommodation or gastric emptying, very little is known about sensory neuroplasticity that may occur following gastric surgery.

Vagal afferent fibers innervating the stomach wall have been characterized using both electrophysiological recordings and morphologically using anterograde dye tracing methods. Vagal afferents project from the stomach muscle wall, myenteric plexus and mucosal villi to central terminals in the nucleus of the solitary tract (NTS) and from there to higher brain centers involved in processing non-painful, physiological stimuli leading to behavioral responses and sensations such as satiety, nausea, mood and cognition (Berthoud and Neuhuber 2000). In addition, approximately 5% of projections terminate in the cervical spinal cord where they are believed to be involved in referred pain (Chandler et al. 1996). While it is generally held that spinal visceral afferents are primarily involved in conveying painful stimuli, there is growing evidence that gastric vagal afferents may contribute to hypersensitivity related functional disorders. For example, gastric ulceration in rats results in sensitization of mechanosensitive vagal afferents and hypersensitivity to gastric distension (Kang et al. 2004; Ozaki et al. 2002).

Afferent nerve endings that account for the receptive fields are distributed throughout the stomach. These receptive fields are activated by normal physiological events either through chemical stimulation (chemosensitive) or stretch (mechanosensitive). The distribution and properties of various types of receptive fields have been previously characterized in the rat. Within the stomach wall there are at least two types of morphologically distinct classes of vagal afferents that respond to stretch or mechanical distension. These afferents predominate either within the myenteric plexus or circular and longitudinal smooth muscle layers of the stomach and are known as intraganglionic laminar endings (IGLEs) or intramuscular arrays IMAs, respectively (Phillips and Powley 2000). These afferents exhibit widespread regeneration when partially axotomized proximal to the stomach (Phillips et al. 2003). More importantly, a recent study demonstrated reorganization and regeneration of these vagal afferents proximal to either a small incision in the ventral wall of the stomach or to suture material (Phillips and Powley 2005). These studies suggest important morphological alterations that occur following surgical manipulation; however, how this translates to functional changes has not been characterized.

Similar to gastric banding in humans, our rat model of fundus ligation is likely to affect satiety, alter gastric accommodation and compliance and allow us to investigate changes in intrinsic vagal innervation. We hypothesized that gastric injury through fundus ligation results in neuroplastic changes in vagal afferents terminals and subsequent alterations in the mechanosensory properties of these afferent fibers. Therefore our aim was to investigate changes that result from fundus ligation including: 1) satiety 2) gastric compliance 3) reorganization and regeneration of vagal afferent terminals in the stomach wall 4) gastric receptive fields and 5) functional alteration of vagal afferent fibers innervating the stomach.

Methods

Animals

This study was carried out in male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA). Rats were kept in controlled conditions with a 12 hour light/dark schedule and had access to both food and water ad libitum. Twenty-four hours before surgery the animals were placed in a wire bottom cage and access to food, but not water, was denied in order to empty the stomach. The Institutional Animal Care and Use Committee at Medical College of Wisconsin approved all experimental protocols.

Gastric Ligation

All chronic ligation surgeries were performed in 6-week-old rats under deep anesthesia using xylazine (10mg/kg, i.p.) and ketamine (100mg/kg, i.p.). Antibiotic (Enrofloxacin 2.5mg/kg, sc) was given preoperatively to prevent infection. For chronic fundus ligation (CL), a small (1–2cm) incision was made along the midline of the stomach in order to expose the stomach. The transitional region between the fundus and glandular portion of the stomach was tightly ligated using 2.0 silk thread (fig 1). Sham animals had the same abdominal incision but without fundus ligation. All rats received carprofen (50mg/ml) post-operatively (0.1ml, sc) daily for 4 days. Following surgery rats were housed separately and denied food for 12 hours. They were then housed in pairs 5–7 days after surgery. In a group of sham animals, acute fundus ligation (AL) was also performed 30 minutes prior to compliance and electrophysiological recording in order to compare changes to CL animals.

Weight and Food Measurements

To determine the effects of fundus ligation on satiety and feeding pattern, food intake was recorded weekly for 8 weeks during a 1 hour and 3 hour period starting one week after surgery. All measurements were done in a metabolic cage the morning following overnight fast in both sham and CL rats. The weights of both sham and CL rats were recorded preoperatively and postoperatively every week at the same time (8–10am) for 13 weeks.

Gastric Compliance

At age 3–4 months old, rats (sham or CL) were anesthetized with pentobarbital sodium (Ovation Pharmaceuticals, Inc. Brown Deer, IL; 45–50 mg/kg i.p.) and maintained under anesthesia via intravenous infusion of pentobarbital (5–10mg/kg/hour). The right femoral vein was cannulated in order to infuse anesthetic. The left carotid artery was cannulated and attached to a pressure transducer for blood pressure measurements throughout the experiment. The trachea was intubated but the rats were not mechanically ventilated during these experiments. A transverse incision was made in the abdomen and the proximal duodenum was cannulated through a small (1–2mm) incision slightly below the pylorus with the catheter tip in the stomach. The catheter was connected to a pressure transducer in order to monitor intragastric pressure (IGP) on-line via CED1401 data acquisition system. A 6 French 2.0mm foley catheter was inserted through the mouth into the esophagus and the balloon was distended slightly above the gastroesophageal junction. Baseline IGP measurements were taken and a pressure-volume curve was constructed using increasing volumes of saline. Following baseline measurements, 2.5ml were infused every 3 minutes allowing for gastric accommodation. IGP was recorded following 2.5, 5.0, 7.5, 10 and 12.5ml. Two trials were run in each animal and the stomach was given 30 min to rest before each distension sequence. Similar experiments were carried out in sham animals following acute ligation of the fundus (AL). A maximum of 10 ml of intragastric saline was given in the AL group because diminished compliance that resulted in gastric perforation of two animals tested with a volume of 12.5. A pressure-volume relationship was obtained with increasing gastric volume. Gastric compliance was defined as the ratio

between the pressure change and the change in infused volume and calculated using the following equation:

$$\text{Gastric Compliance} = 1 / [(P_1 - P_0) / (V_1 - V_0)]$$

Vagal Afferent Recordings

Rats (3–4 months old) were initially anesthetized with pentobarbital sodium (Ovation Pharmaceuticals, Inc. Brown Deer, IL; 45–50 mg/kg i.p.) and maintained under anesthesia via intravenous infusion of pentobarbital (5–10mg/kg/hour). The right femoral vein was cannulated to infuse anesthetic and other fluids. The left carotid artery was cannulated and connected to a pressure transducer to monitor arterial blood pressure. The trachea was intubated for mechanical ventilation. Rats were paralyzed by injecting galamine (1mg/kg, i.v.). Supplemental doses of anesthetic were given through out the experiment to maintain paralysis.

Following a ventral midline incision in the neck, the sternocleidomastoid, sternohyoid, and omohyoid muscles were removed and the right vagus nerve was exposed. The skin was reflected laterally and tied to the stereotaxic frame to make a pool for warm mineral oil (37° C). Following removal of the carotid sheath, the nerve was dissected and decentralized close to its entry to the nodose ganglia. The perineural sheath was removed in the pool of warm mineral oil over a micro-base plate and the nerve was split from the bundle to obtain a single-unit. Electrical activity of the single unit was recorded as previously described (Sengupta et al. 2004).

In all animals, the abdomen was opened and the proximal duodenum was cannulated through a small (1–2mm) incision slightly below the pylorus with one catheter tip in the stomach and the other connected to a distension control device. IGP was monitored via a pressure transducer. A 6 French 2.0mm foley catheter was inserted through the mouth into the esophagus and the balloon was distended slightly above the gastroesophageal junction in order to maintain a closed system during gastric distensions. Fundus ligation was performed acutely, 30 minutes prior to electrophysiological experiments in a separate group of sham animals (AL). The fibers innervating the stomach were identified with a test distension of 30 mmHg. Fibers that responded to distension were further tested with a stimulus response function (SRF) to graded intensities of gastric distension of 10, 20, 30 and 40 mmHg. Each distension was given for 30 seconds with a three-minute interval between distensions. After completion of the SRF, the abdomen was re-opened and the stomach receptive field (RF) was found using a blunt probe. Following the recordings, animals were euthanized with 0.2ml/kg of Beuthanasia-D (390 mg pentobarbital+50 mg phenytoin sodium+2% benzyl alcohol USA) intravenously.

Gastric Afferent Morphology

Groups of sham and CL rats (n=3 in each group) received bilaterally nodose ganglion injections of 5% dextran-tetramethylrhodamine-biotin, 10,000 MW (Invitrogen) to obtain complete Golgi-like fills of individual vagal sensory terminals. Fourteen days post-injection, rats were perfused with 4% paraformaldehyde and whole mounts consisting of the smooth muscle layer of the ventral and dorsal stomach walls were prepared. Terminals were visualized using Vectastain Elite ABC kits (Vector Laboratories) followed by DAB as the chromagen.

Gastric Ultrasound

High frequency intraluminal ultrasonography was used to measure gastric wall thickness at baseline and during distension at 2.5, 5, 7.5, 10 and 12.5 ml of saline in sham, AL and CL rats (n=5 in each group). The probe was sited in the greater curvature of the stomach. Images were acquired using a 1.2 mm diameter 40 MHz intra-coronary artery ultrasound probe (Atlantis SR

Pro, Boston Scientific, Natick MA) and recorded on SVHS tape. A 40mHz transducer was used to generate adequate resolution of the stomach wall to measure wall and muscle thickness. This resulted in a limited imaging field. Resolution was poor at lower frequencies. Care was taken to image all animals at a similar location in the stomach fundus. Images were then digitized and the thickness was measured using graphing software SigmaScan Pro. The inner mucosal surface, inner muscular layer, outer muscular layer, and outer serosal surface were identified. Total wall thickness was calculated by measuring the distance from the inner mucosal surface to the outer serosal surface using a line that bisected the center of the stomach lumen. The muscular thickness was determined by measuring the distance from the inner muscle layer to the outer muscle layer.

Statistics

The cumulative food intake data at each time point from surgery and weekly weights were analyzed by two-way repeated measures ANOVA or Student's *t*-test. Compliance data were analyzed by 1-way analysis of variance followed by the Tukey test. The resting activity of vagal fibers was counted for 30 seconds prior to gastric distension and the response was determined as the increase in discharge during distension above the resting activity in impulses per second. Results were analyzed using one-way ANOVA for repeated measures or Student's *t*-test. For gastric wall and muscle thickness, the average of 10–12 measurements was used to determine the thickness (total wall and muscle layer) at each distension level). The data was distributed normally; therefore a two-way ANOVA for multiple measures was used to compare groups. All data are reported as mean standard error of the mean. A *p*-value of less than 0.05 was considered significant.

Results

Weight and Food Intake

Food consumption was measured during a one-hour interval on a weekly basis following surgery. The CL group consumed significantly less food during both the one and three hour period measured over the first five weeks. After five weeks, there was no significant difference and thus measurements were not continued beyond 8 weeks (fig 2A and 2B). Body weights of both sham and CL animals were recorded over a 13 week period starting with pre-operative weights. Although weights of the CL increased at a similar rate to sham animals, their average weight was lower throughout the testing period (fig 3, *p*<0.05).

Compliance

Pressure-volume relationships were recorded during progressive gastric distensions with increasing volumes of saline (fig. 4). Gastric compliance in the CL group was significantly reduced compared to naïve animal with a significant increase in IGP starting at 10ml (fig. 4). However, compliance was significantly lower in the AL group compared to the CL group and sham. IGP was significantly greater in the AL group starting at 5ml saline and the stomach could accommodate a maximum of 10ml.

Electrophysiology

A total of 33 mechanosensitive vagal fibers from 27 rats were identified that responded to gastric distension. In all groups, vagal fibers exhibited spontaneous firing without distension. All fibers exhibited an intensity dependent increase in response to graded gastric distension (fig. 5). The spontaneous firing of vagal afferent fibers from the CL rats (3.75 ± 0.95 impulses/s, *n*=16) was significantly higher than AL rats (1.3 ± 0.31 impulses/s, *n*=13). Similarly, responses of vagal afferent fibers from CL rats to gastric distension were higher at distending pressures 10 mmHg compared to AL rats (*p*<0.05). The spontaneous firing (0.99 ± 0.2

impulses/s, $n=6$) and response of vagal afferent fibers to gastric distension from the sham rats was no different from the AL group ($p>0.05$) (fig. 6). The receptive fields (RF) of 22 fibers were mapped by probing the serosal surface of the stomach with a fine-tip glass probe after completion of the SRF. There was a marked expansion of the RF in the CL rats compared to AL. In some CL rats, the RF expanded to the ventral aspect of the stomach, an area usually not innervated by the right vagus (table 1).

Gastric Afferent Morphology

The morphology of vagal sensory terminals near the ligation site was investigated both in CL ($n=3$) and sham ($n=3$) rats. Plasticity of vagal sensory afferents was evident 6–8 weeks following fundus ligation in the proximal corpus near the fundus in the dorsal wall of the stomach. Disorganized, hyperplastic IMAs were seen near the site of the fundus ligation approximately 4–5mm from the scar region. Compared to control, telodendria lacked the uniform/parallel characteristics of IMAs and in some animals, dystrophic afferent neurites terminating in a putative growth cone profile were seen. Additionally, IMAs in circular smooth muscle were particularly elongated compared to sham animals (fig 7A–C).

Ultrasound Measurements of Gastric Wall Thickness

Ultrasonography was employed to study the changes in the rat gastric wall thickness proximal to the ligated site (fig 8). There was no difference in total wall thickness between sham ($n=4$), AL ($n=3$), and CL rats ($n=4$) at baseline or at any distending volume. Table 2 shows the mean total wall thickness for all three groups at each distension volume. There was also no difference in the mean muscle thickness of the sham, AL and CL animals at baseline or during distensions ($p>0.05$, data not shown).

Discussion

In the current study, ligation of the gastric fundus was used as a model of gastric surgery to investigate long-term alteration in vagal afferents that may ultimately be involved in hypersensitivity and clinical symptoms following gastric injury. Our study showed that chronic fundus ligation results in: 1) decreased food consumption 2) structural changes of vagal afferent terminals in the stomach wall with regeneration and reorganization of IMAs, 3) expansion of the stomach RF and 4) hyperexcitability of vagal afferents innervating the stomach. To our knowledge, this is the first report of chronic, functional alterations in gastric vagal afferents that results from remodeling of sensory neurons following surgery.

While it has been suggested that vagal nerve injury may contribute to postoperative hypersensitivity following gastric surgery, a decrease in gastric compliance has been speculated to play a major role (Mousa H et al. 2006). Interestingly, it has been reported that compliance of the stomach following fundoplication is no different from that in healthy volunteers (Wijnhoven et al. 1998). This observation suggests a change in the visco-elastic properties of the stomach wall over time and clearly shows the ability of the stomach to adapt to its modified anatomy. It is likely that feeding gradually increases the size of the stomach, leading to improved accommodation and compliance over time. In our model of fundus ligation we found that food consumption in the CL rats was lower than the sham group only during the first 5 weeks postoperatively. Food consumption was recorded for one and three hours following overnight fast and total daily food consumption was not measured. It is possible that the CL animals could have adapted and changed to a continuous “grazing” feeding behavior throughout a 24-hour period that is different from the sham animals. Thus, recording intake for 24 hours could potentially show no difference in overall food consumption between the groups. This would also explain how the weight loss was seen only in the first week following surgery. Nevertheless, the fact that CL animals ate less during the 1 and 3 hour periods tested suggests

a possible change in gastric accommodation and/or hypersensitivity. Gastric compliance in the CL group did not normalize compared to sham animals over time and this is what somewhat expected. Ligation results in functional removal of the fundus, limiting the stomach's ability to accommodate. More importantly however, the compliance in the CL group was significantly greater than the AL animals. This is an important finding, particularly as it pertains to both the higher spontaneous and distension-induced activity of the vagal afferents in the CL group compared to the AL or Sham. In other words, the altered mechanosensitivity of CL rats is a result of vagal hyperexcitability and not a decrease in compliance. An interesting observation was that neither the gastric wall nor smooth muscle appeared to have undergone significant hypertrophy following surgery which also suggests no alteration in wall tension during distension.

Injury to tissue can trigger the production of multiple inflammatory mediators such as cytokines and growth factors that lead to altered gene transcription and ultimately changes in neuronal properties (Bielefeldt et al. 2003). In the current study, the observed neuroplasticity in the gastric wall is likely to explain the functional changes in the vagus nerve and may translate to hypersensitivity and early satiety. The hypersensitivity to gastric distension may also be a pathophysiological mechanism in dyspepsia and help explain food intolerances that develop years after gastric banding (Piessevaux et al. 2001; Tack et al. 2004; Dargent 2008). It can be speculated that increased resting activity of vagal afferent fibers alone could contribute to altered sensation from the proximal gastrointestinal tract.

The morphological characteristics of primary afferents have been recently described on the basis of tracing experiments from the nodose ganglia (Berthoud and Powley 1992; Neuhuber 1987; Phillips et al. 1997). At least two types of mechanosensitive vagal afferent endings have been described in the stomach, (IGLEs and IMAs). The IMAs, found in the longitudinal and circular muscle layers have been suggested to be in-series tension receptors that respond to stretch of smooth muscle while the IGLEs, found in the myenteric plexus, may represent additional mechanoreceptors that detect shearing forces between the muscle layers (Blackshaw et al. 1987; Phillips and Powley 2000). Tracing of individual afferent fibers has also demonstrated striking differences in the distribution of the two within the stomach wall. IMAs are rarely found in the antrum, moderately dense in the corpus, but abundant in the forestomach. Conversely, IGLEs are abundant in the antrum and corpus, but less numerous in the forestomach (Phillips and Powley 2000). Based on this distribution, it is likely that the majority of neurons recorded in the present study were from IMAs, since no neurons were recorded from the antrum and the majority was recorded from the forestomach, an area innervated by only 13% IGLEs. While previous studies have suggested that IMAs may not function as tension receptors and that their involvement in mechanosensitivity is strictly as high threshold units, more studies are needed to clarify their precise characteristics (Zagorodnyuk, 2001). A striking observation from the current study is that IMAs developed hyperplastic dystrophic terminals near the ligation site, features of regenerating and proliferating terminal branches. These branches appeared to have undergone reorganization forming irregular and disorganized terminals with growth cone-like extensions. Previous studies have also documented similar plasticity and morphological changes that occur following gastric injury such as cutting or suturing the gastric wall (Phillips and Powley 2005). Abnormal regenerative nerve sprouting has also been shown following nerve transection in animal models of neuropathic pain along with increased excitability and discharge of C-fibers (Jänig 1988; Han et al. 2000). Further, the expanded receptive fields in the stomach of CL animals may very well correlate with the elongated IMAs noted in the circular smooth muscle.

Chronic ligation of the gastric fundus results in plasticity of vagal afferent terminal in the stomach wall and significantly alters the response properties of gastric mechanoreceptors. Based on these findings, we can speculate that functional changes in gastric vagal afferent

fibers may play an important role in the development of postoperative dyspepsia and hypersensitivity. Because of the widespread practice of gastric surgeries such as gastrostomy tube placement, fundoplication and most recently, bariatric surgeries, more studies are needed to explore post-operative changes that may occur in humans.

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Figure 1.
Schematic diagram of fundus ligation

Figure 2.

Food consumption measured weekly for 1 hour (**A**) and during 3 hours (**B**) following overnight fast. Consumptions was greater in the sham animals compared to CL throughout the first 5 weeks measured ($p < 0.05$)

Figure 3. Mean total body weight of sham rats and chronic fundus ligated rats. CL animals had lower weights compare to naïve at all weeks measured following surgery ($p<0.05$).

Figure 4.

Pressure-volume curves obtained after instillation of increasing volumes of saline in AL, CL and sham rats. Values are expressed as mean and SEM. * $p < 0.05$ vs. chronic ligated, # $p < 0.05$ vs sham.

Figure 5.

Example of typical response of mechanosensitive afferents near the corpus and response to GD (10–40mmHg). In each panel, the top tracing represents the frequency histogram (1s binwidth), the middle tracing is the nerve action potential and the lower tracing is the distension pressure. The spontaneous firing and response to GD was significantly higher in CL animals compared to AL.

Figure 6.

Summary data of stimulus response function of vagal afferents to GD in CL AL and sham (non-ligated) rats. The spontaneous firing of neurons in the CL animals was significantly higher than AL. Also, the response to mechanical distension was significantly higher in CL animals compared to AL at pressures ≥ 20 mmHg. There was no difference in the spontaneous firing of neurons or response to distension between AL and sham group. Values are expressed as mean and SEM. * $p < 0.05$.

Figure 7.

Vagal sensory terminal remodeling associated with fundic ligation. Afferent terminals, labeled with Dextran-Biotin, illustrating the patterns observed in the proximal corpus near the fundus in the dorsal wall of the stomach of sham (**A**) and fundic ligated rats (**B, C**). **A**: An intramuscular array (IMA). The IMA terminal runs diagonally from the upper right to the lower left of the panel. This example is a characteristic IMA, in that it consists of multiple telodendria running parallel to each other (and smooth muscle fibers) in a single muscle layer. **B**: A disorganized, apparently hyperplastic IMA near the site of the fundic ligation surgery. Originating from a single axon, the terminal is morphologically similar to an IMA, yet the telodendria lack the uniform/parallel characteristics of an IMA. **C**: Another type of dystrophic afferent neurite terminating in a putative growth cone profile near the site of the fundic ligation. Scale bars = 50 μ m.

Figure 8.

Ultrasound images of the gastric wall in CL rats. **A.** The gastric image is from the corpus at a time when there was no volume distension and demonstrates the measure of wall thickness.

B. Total wall thickness was calculated by measuring the distance from the inner mucosal surface to the outer serosal surface using a line that bisected the center of the stomach lumen.

Table 1

Proportion of vagal units with evidence for expanded receptive fields in the rat stomach of AL and CL fundus.

Receptive Field	Acute Ligation	Chronic Ligation
> 1 cm	0/10	10/12
< 1 cm	10/10	2/12
Receptive Field on Ventral Side	0/10	4/12

Table 2
Average gastric wall thickness (mm) during volume distension of the stomach in acutely ligated (AL), naïve, and chronically ligated (CL) animals.

	2.5 ml	5.0 ml	7.5 ml	10.0 ml	12.5 ml
AL	1.4 ± 0.12	1.2 ± 0.06	1.0 ± 0.12	0.86 ± 0.08	0.83 ± 0.06
Naïve	1.9 ± 0.18	1.8 ± 0.20	1.6 ± 0.20	1.36 ± 0.14	1.16 ± 0.14
CL	1.9 ± 0.22	1.8 ± 0.55	1.5 ± 0.45	1.2 ± 0.40	1.15 ± 0.35