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A ROLE FOR NITRIC OXIDE IN THE PATHOGENESIS OF NECROTIZING ENTEROCOLITIS:

Discovery, evaluation and design of a novel prophylactic therapy

by

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A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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January 2000

ABSTRACT

Necrotizing enterocolitis (NEC) is an acute, self-limiting, inflammatory bowel disease of unclear pathogenesis affecting premature infants. It is the most common acquired surgical emergency in the neonatal period. NEC is characterized by necrosis of the bowel wall and may lead to peritonitis, stricture formation with bowel obstruction and lower gastrointestinal hemorrhage. Approximately 10% of premature babies develop NEC with up to 94% of cases seen in infants born at less than 36 weeks gestation. As more and more premature infants survive the neonatal period, it is expected that the incidence of this disease will increase.

There is no *specific* treatment for NEC, and as such, therapy has not changed over the past two decades. The initial management of early NEC is medical and essentially supportive: bowel rest, naso-gastric suction, intravenous hydration and nutrition, and antibiotics, with serial abdominal examinations and plain abdominal films to monitor outcome. Non-response to medical treatment or bowel perforation, as demonstrated by the presence of free air on plain abdominal radiographs, warrants surgical intervention in 23-70% of patients.

The two primary risk factors consistently identified for the development of NEC are prematurity and enteral feeding. Intestinal dysmotility associated with prematurity and mucosal damage by intraluminal substrate must thus contribute to the pathogenesis of NEC.

Nitric oxide (NO), a short-lived labile gas produced in most biological systems through the conversion of L-arginine to L-citrulline by a specific family of synthases (NO synthase), is a key factor in gastrointestinal physiology and gut responses to injury. Of particular interest to us is evidence that NO mediates i) inhibitory nerve-related relaxation of intestinal smooth muscle and may have a role in ii) regulation of gut mucosal blood flow, and iii) mucosal protection. The constitutive isoform of this synthase (cNOS) generates small amounts of NO in physiological signalling processes.

The inducible isoform (iNOS) generates large amounts of NO and is associated with infection and inflammation. NO is also synthesized in non-vascular sites including nerve cells of the central nervous system and the gastrointestinal tract.

Animal studies suggest that NO plays an important role in protecting the gut from damage. Smooth muscular sphincteric function as well as coordinated peristalsis is also mediated by NO release, which is a neurotransmitter of the enteric neurons distributed within the myenteric and submucosal nerve networks throughout all regions of the gut wall.

Strategies to prevent NEC will target the premature infant and will probably focus on varying aspects of gastrointestinal maturation, enteral and parenteral nutrition, mucosal defense and immunity, inflammatory mediators and infectious agents. Thus, given this background, an experimental model of NEC which incorporates both the intraluminal and the dysmotility components leading to gut injury was modified and adapted to the premature piglet. Briefly, the protocol involves laparotomy of anesthetized animals, creation of closed *loops* from terminal ileum to proximal colon which *are injected with acidified casein test solution separated by saline injected control loops.* After 3 hours, both macro- and microscopic examination of test loops reveals lesions similar to human NEC.

Because NO is involved in neural control of intestinal motility and mucosal protection, we sought to evaluate the effects of manipulation of the nitrergic system on experimentally induced NEC in this model. We hypothesized that inadequate production of NO in premature and neonatal infants is a major pathogenetic factor for NEC. We demonstrated that stimulation of NO production in our acute model of intestinal inflammation by either intravenous administration of the NO synthase substrate, L-arginine or the NO donor, sodium nitroprusside significantly attenuates NEC-induced intestinal damage in both term and pre-term neonatal Yorkshire piglets. This was acheived without adverse systemic effects. Administration of the NO synthase inhibitor

L-NAME (i.v.) caused hemorrhagic congestion of the gut wall. Treatment with D-arginine or D-NAME had no effect on the extent of NEC induced lesions.

Developmental changes in piglet intestinal motility were then documented *in vivo*, as well as those modifications induced by systemic stimulation or inhibition of NO availability. Our results have shown the presence of an ontogenically determined pattern of fasting intestinal motor activity, which is more susceptible to nitrergic manipulation in earlier developmental stages as documented through motility responses of the terminal gut.

Futher documentation on the ontogeny of nitrergic innervation of the piglet gut was obtained by histochemical staining of NO neurons in fresh laminar preparations of the intestine using NADPH dependant diaphorase histochemistry. The frequency of NO neurons was determined at 3 different stages of development (premature, term neonatal and 2 week old piglets) in the myenteric nerve plexus and in the Henle's plexus of the submucosa. Premature animals showed the highest frequency of NO neurons in all regions. However, these neurons increase in the cranio-caudal direction in all age groups, with the distal gut (cacum and colon) showing the highest frequency. In addition, the number of NO neurons shows a developmental decrease in the myenteric plexus accompanied by a concomitant increase in their numbers in the submucosa. The combined motility and histochemistry results confirm the presence of an ontogenically determined pattern of nitrergic enteric network maturation which may render the distal gut of more immature animals more susceptible to variations in NO production.

NO synthase activity was then measured in different regions of the developing piglet gut as well as in various layers of the piglet gut wall and compared to the activity found in NEC-induced gut specimens (test loops) at different stages of development and in different gut regions. Total NOS, iNOS and cNOS activity was assayed using the conversion of ^{14}C L-arginine to ^{14}C citrulline in full thickness gut, and

in muscular and mucosal layers separated under the dissecting microscope. As with NO neuron frequency, total NOS activity was highest in the muscularis of prematures and increased, with development, in the mucosa of term 1 week old piglets. Whereas cNOS was highest in the muscularis of prematures, iNOS predominated in 1 week old mucosa. High mucosal iNOS with age, may be the result of induction by intraluminal substrate (1 week old animals were suckling). Although mucosal cNOS lacked regional variability within each group, premature mucosal cNOS was significantly higher in the proximal small bowel compared to 1week olds. This relatively lower physiologic level of cNOS in the distal gut of premature animals may explain the anatomical predilection of NEC.

In premature animals, iNOS in test loops showed a significant increase in activity 3 hours after NEC induction compared to similarly treated 3 day old piglets. Test loop iNOS levels continued to rise significantly 6 hours after NEC induction in prematures. However, premature animals delivered by maternal prostaglandin injection to induce labour failed to demonstrate such a rise in iNOS levels. In 3 day old animals, iNOS levels also rose significantly 16 hours after NEC induction compared to the 3 hour group. Thus in conclusion, iNOS production is increased in premature compared to term animals, and continues to rise in the presence of ongoing intestinal damage regardless of developmental status. In addition, maternal administration of prostaglandins seems to attenuate this NEC-induced rise in iNOS activity.

Having determined the attenuation of NEC-induced gut damage in both term and premature piglets by the systemic administration of the NO synthase substrate, L-arginine or the NO donor sodium nitroprusside (SNP) in our model, we subsequently sought to determine the optimal timing of treatment and the minimal dose required for efficacy in premature piglets. These were important considerations in the possible development of a *prophylactic* therapeutic regimen for NEC, since not every premature, enterally fed infant develops NEC.

Prophylactic intravenous administration of L-arginine (20-600 mg/kg/hr) or SNP (titrated at 3-10 μg/kg/min to maintain blood pressure) 3 hours prior to NEC induction was more effective than administration at the time of, or 3 hours after NEC induction in premature piglets.

Further studies were then undertaken in order to identify the possible toxicity, if any, associated with chronic prophylactic intravenous administration of a clinical dose of L-arginine in the premature piglet by monitoring systemic and metabolic side effects through biochemical analysis of liver, kidney, and pancreatic function and venous blood gases. No adverse effects were documented.

The potential therapeutic applications of these findings in preventing or attenuating NEC-induced intestinal damage in premature infants led to the development of a, now ongoing, clinical trial evaluating the effects of intravenous L-arginine administration in premature infants. It is expected that, based on preliminary data, a multi-centre clinical trial may lead to the prophylactic use of L-arginine as a treatment modality for NEC.

ACKNOWLEDGEMENTS

It would have been impossible for me to have even fathomed the completion of this work had it not been for the ongoing support, advice, encouragement and, at times, blind faith of Dr. Anthony Krantis. I could not have been more fortunate in finding such a mentor. Thank you.

I would like to thank my friend and colleague, Dr. Juan Bass for planting the seed of interest in research and the Bass family for their care and nurturing during its growth.

In addition, I am grateful for the emotional support and encouragement given me by my colleague and chief in Montreal, Dr. Salam Yazbeck and his counterpart in Ottawa, Dr. Pierre Soucy. There were times, gentlemen, when I wasn't quite sure whether I was going to make it !

I would like to thank Dr. Doris Yuen, a fellow clinician, for her ongoing support and collaboration with the clinical trial and for her friendship.

My thanks go to Kim Nichols who initially took me under her wing and "taught me the ropes" in the lab, to Ian Glasgow who introduced me to the "necessary evil" of the computer and essentially bailed me out when I had computer trouble, and to Alexander Aurora for his help in refining the NOS assay.

I must extend my thanks to Anu Pandeya and Kim Wong for their excellent laboratory technical support, and to Jirina Sistek, Pierre Bradley and the Animal Care technicians for their excellent work in, and dedication to, a labour-intensive project. It was well worth it after all!

To Miza Bosc-Davie for her enthusiasm, rigor and technical prowess, all of which facilitated the peri-operative aspects of dealing with immature fragile animals, and to Drs. Julie Lebeau and Marilyn Keany for their collaboration and veterinary expertise, I extend my gratitude.

The completion of this manuscript was facilitated through the gracious technical computer support of Dr. Marisa Tucci and the secretarial assistance of Jocelyne Vachon and Anna DeNardis.

Last, but not least, this research would not have been possible without the collaboration of Agriculture Canada, Ottawa in the insemination of sows and the procurement of accurately timed premature piglets.

We are grateful to the following institutions for their financial support of this research: Children's Hospital of Eastern Ontario Research Institute

Fonds de la Recherche en Santé du Québec

Kiwanis Medical Foundation

Power Corporation of Canada.

DEDICATION

To Carolyn, David, Nancy, and Juan.

The surgical investigator must be a bridge-tender, channeling knowledge from basic science to the patient's bedside and back again. He is thus a bastard, and is called this by everybody. Those at one end of the bridge say that he is not a very good scientist, and those at the other end say that he does not perform enough operations. It is much harder to stay in the middle of the bridge than it is to retreat to one end or the other. But all of the fundamental advances in surgery from Vesalius to Halsted to Cushing have been made by those willing to maintain this uncomfortable posture - the bridge builder.

Francis D. Moore, 1958

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LIST OF ABBREVIATIONS

Ach	acetylcholine
AchE	acetylcholinesterase
ALT	alanine-amino-transferase
ANOVA	analysis of variance
AST	aspartate-amino-transferase
BUN	blood urea nitrogen
CHEO	Children's Hospital of Eastern Ontario
cm	centimetre
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
C-prem	cesarian-derived premature piglet
dl	decilitre
EDRF	endothelium
ENS	enteric nervous system
ET-1	endothelin-1
g	gram
Hg	mercury
HP	Henle's plexus
IFNγ	interferon-gamma
IJP	inhibitory junction potential
iNOS	inducible nitric oxide synthase
i.v.	intra venous
kg	kilogram
LBW	low birth weight
LPS	lipopolysaccharides
mg	milligram
ml	millilitre
μl	microlitre
mm	millimetre
mM	millimole
MMC	migrating myo-electric complex
MP	myenteric plexus
NADHd	nicotinamide adenine dinucleotide diaphorase
NADPHd	nicotinamide adenine dinucleotide phosphate diaphorase
NANC	non-adrenergic, non-cholinergic
NBT	nitro blue tetrazolium
NEC	necrotizing enterocolitis
ηg	nanogram
ηm	nanometre
ηmol	nanomole
NO	nitric oxide
OGH	Ottawa General Hospital
PAF	platelet activating factor
PMN	polymorphonuclear leukocyte
SCFA	short-chain fatty acids
SNP	sodium nitroprusside
TNFα	tumor necrosis factor-alpha
TPN	total parenteral nutrition
VIP	vaso-active intestinal polypeptide

CHAPTER 1

GENERAL OVERVIEW

1.1. NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis (NEC) is an acute, self-limiting, inflammatory bowel disease of premature infants of unclear pathogenesis. It is the most common acquired surgical emergency in the neonatal period (Kleinhaus et al. 1992). NEC is characterized by necrosis of the bowel wall and may lead to peritonitis, stricture formation with bowel obstruction and lower gastrointestinal hemorrhage. Approximately 10% of premature babies develop NEC with up to 94% of cases seen in infants born at less than 36 weeks gestation (Stoll 1994). The incidence varies with gestational age and birthweight, with the highest incidence in infants weighing less than 1000 g (42.1/1000). Estimates of NEC disease burden in the United States vary between 1,200 to 9,600 cases per year with a case fatality rate of 108 to 2,688 per year (Stoll 1994). The age of onset is inversely related to birthweight and gestational age. Since technology allows more and more immature neonates to survive, these infants are at the greatest risk for developing NEC. Due to its high morbidity and mortality (up to 20%) (Stoll 1994), NEC is responsible for a large part of neonatal care costs. Because NEC usually develops during the first 2 weeks after birth, it is precisely those infants recovering from the systemic effects of prematurity who are most at risk.

The gastrointestinal manifestations of NEC include abdominal distension and tenderness, emesis and hemorrhage, progressing to peritonitis and intestinal perforation. Systemically, signs and symptoms are those of neonatal sepsis and shock (metabolic acidosis, hypovolemia, thrombocytopenia and leukopenia). The diagnosis is confirmed radiologically by the presence of pneumatosis intestinalis (intramural bowel air) or portal venous air or both.

There is no *specific* treatment for NEC, and as such, therapy has not changed over the past two decades. The initial management of early NEC is medical and essentially supportive: bowel rest, naso-gastric suction, intravenous hydration and nutrition and antibiotics, with serial abdominal examinations and plain abdominal films to monitor outcome. Non-response to medical treatment or bowel perforation, as demonstrated by the presence of free air on plain abdominal radiograph, warrants surgical intervention in 23-70% of patients (Stoll 1994). Ricketts and Jerles (Ricketts and Jerles 1990) reported that 93% of infants weighing less than 1000 g had surgery after 10 days of age compared to only 26% of infants weighing more than 1500 g. Complications of surgical treatment further add to the morbidity and mortality of NEC.

Macroscopically, the intestine affected by NEC is hemorrhagic and necrotic with peritonitis and pseudomembranous exudate. The terminal ileum and proximal colon are the sites of prediliction for NEC. Surgical treatment usually involves resection of variable segments of small intestine and colon (sometimes resulting in short bowel syndrome) and construction of enterostomies. The bowel ends are eventually reanastomosed (Kleinhaus, Weinberg et al. 1992).

Microscopic findings typically include intestinal mucosal edema, hemorrhage, inflammation, coagulation/ischemic necrosis and bacterial overgrowth. Evidence supporting an acute inflammatory reponse lies in the the presence of neutrophils in surgically obtained peritoneal fluid of NEC infants even though inflammatory neurtrophilic infiltration of the bowel is uncommon (Ballance et al. 1990).

Death from NEC results from refractory shock, disseminated intravascular coagulation, multiple organ faiure, intestinal perforation with sepsis, extensive bowel necrosis, and complications resulting from short bowel syndrome after surgery. Birth weight and gestational age are extremely imporant determinants of mortality risk from NEC. Case-fatality rates for infants weghing less than 1500 g range from 10% to 44%. Those for infants weighing more than 2500 g range from 0% to 20% (Stoll 1994). Wiswell et al (Wiswell et al. 1988) reported that 4.7% of full-term infants died from NEC compared to 11.9% of preterm infants.

Thus, based on epidemiological evidence, we can conclude that *prematurity is the primary risk factor for NEC.* The role of immaturity of the gut with regards to mucosal permeability, intestinal blood flow, enzyme function and motility has been investigated (for reviews see Stoll et al 1994). Perinatal risk factors, many of which produce varying degrees of mesenteric hypoxia and ischemia, are also implicated in the pathogenesis of NEC but as coincidental events. For instance, "ischemic" NEC lesions are different in anatomical location and appearance from "classical" NEC lesions. The former results in generalized necrosis and "gun shot"-like perforations of the gut without anatomical prediliction. The latter results in necrosis localized to the ileo-caecal area (Ballance, Dahms et al. 1990). In addition, the occasional epidemic occurence of NEC

seems to point to an infectious etiology, although this has of yet to be confirmed. Finally, 95% of affected neonates have been fed cow's milk-based formula. Available data point to the ingestion of bovine casein as an important etiologic factor and to the relative protection afforded by maternal breast milk.

In conclusion, it is likely that strategies to prevent NEC will target the premature infant and will probably focus on varying aspects of gastrointestinal maturation, enteral and parenteral nutrition, mucosal defense and immunity, inflammatory mediators and infectious agents.

1.2. INTESTINAL MOTILITY AND ONTOGENY

Intestinal motility is organized such that digestion is optimized and that net propulsion occurs aborally. The intestine possesses both intrinsic and extrinsic innervation. The primary control of motility rests with the intrinsic innervation composed of neurons of the myenteric plexus, located between the circular and longitudinal smooth muscle layers, and the submucous plexus, located between the circular muscle and mucosal layers. (Furness and Costa 1980; Weisbrodt 1981). These two nerve layers are interconnected and functionally integrated. Extrinsic innervation to the bowel is from the vagus nerve and from the celiac and superior mesenteric ganglia and serves to modulate gut functions. Inputs from the central, sympathetic and enteric nervous systems inhibit motor activity. Parasympathetic nerve inputs are excitatory. In addition, various gastrointestinal peptides modulate motility.

In 1969, Szurszewski (Szurszewski 1969) described in the small intestine of fasted dogs an electric complex of intense spiking activity migrating down the small

bowel. Upon reaching the ileum, another complex developed in the duodenum. Feeding interrupted this cycle with irregular spiking activity. This phenomenon is known today as the interdigestive migrating myoelectric complex (MMC) (Code and Marlett 1975). The MMC is thought to have a housekeeping role, cleansing the small intestine of residual food and preventing bacterial overgrowth in the fasted state. Code and others (Carlson et al. 1972; Marik and Code 1975) divided the complex into 4 phases: phase one is characterized by the relative absence of action potentials and has been proposed to be the result of quiescent enteric cholinergic neurons in the absence of local reflex activity of the empty bowel lumen (Weisbrodt 1981)); phase two shows presistent irregular action potential activity due to the irregular contractions of circular smooth muscle and an increased proportion of slow waves; phase three shows a sudden onset and end to the activity front and large action potentials are present on every slow wave of that period resulting in regular spiking activity and regular contractions of the circular muscle layer; during phase four, action potentials rapidly decline in incidence and intensity and disappear completely. This cycle repeats itself every 90 to 100 minutes, however it is abolished by feeding (Weisbrodt 1981).

In summary, small intestinal motor activity is thus divided into 2 phases: the above mentioned cyclical fasting pattern which is disrupted after a meal and replaced by a second type of continuous activity which promotes mixing and segmentation of the luminal contents. This *postprandial* pattern normally lasts 3-4 hours in the adult human, at which time the fasting interdigestive pattern resumes. Approximately every 90 minutes an organized band of phasic contractions passes from the stomach, along the

small intestine to the ileum (phase 3). These contractions are preceded by a period of irregular contractile activity (phase 2) and followed by a period of quiescence (phase1).

Successful post-natal adaptation in the very premature infant is frequently precluded by the immaturity of gastrointestinal function (Bisset et al. 1989). By 28 weeks gestation, absorptive (McNeish et al. 1979), and digestive (Grand et al. 1976) functions are moderately developed. Adequate motor function lags many weeks behind (Dunn 1963), and thus limits tolerance to enteral feeding. Intraluminal manometric studies of fasting motor patterns in preterm infants show a gestationally dependent process of maturation (Bisset et al. 1988). Between 28 and 32 weeks post conceptional age, intestinal motor activity is characterized by low amplitude random disorganized contractions. Clustered phasic activity, 50% of which are propagated aborally, occur between 28 and 35 weeks, and are characterized by short (4 min) bursts of organized contractions. Prolonged phasic activity with longer periods (12 min) of propagated motor activity (90% aborally) appear at 34-36 weeks, and MMC activity occurring every 25 min appears between 37 and 42 weeks (Bisset, Watt et al. 1988). In preterm infants, motor quiescence duration increases and clustered contractions are sustained for longer periods decreasing their overall duration and leading to the appearance of migratory activity with increasing age (Berseth 1989; Berseth 1992). This increase in fasting intestinal motor activity with age is likely related to the development of the enteric nerve networks.

Postprandial intestinal motor activity, on the other hand , has been found to be mainly influenced by the volume of feeds and the duration of exposure to enteral feeds (Bisset, Watt et al. 1989). Consequently it is proposed that early introduction of enteral nutrition to the premature infant may enhance the motor response of the small

intestine to feeds (Bisset, Watt et al. 1989). Berseth and colleagues (Berseth 1992) studied fasting motor activity manometrically via measurement of intraluminal pressure changes in the upper gut of premature infants born at 28 to32 weeks' gestation. Motor activity was compared at 1, 2, and 4 weeks after birth. Enteral feeding was found to hasten the maturation of fasting motor activity patterns. Unfed infants were found to have significantly less MMC's than fed ones of similar age and gestation.

Similar alterations in intestinal motor activity patterns have been observed in asphyxiated term infants who show feeding intolerance (Berseth and McCoy 1992). With resolution of these abnormal motor activities, feeding tolerance begins. Because levels of plasma gastrin and gastric inhibitory peptide do not differ in feeding-tolerant and intolerant infants, hormonal regulation of intestinal motor activity may not be a key factor in the development of feeding intolerance. In addition, contraction amplitude (force of muscular contraction) does not differ in these infants, indicating that a lack of intrinsic muscle function may not be contributory. Thus, the inference is that immaurity of neural regulation of intestinal motility is implicated in oral feeding intolerance. Infants become feeding-tolerant over a 2 to 4-week period, indicating that this immaturity is transient (Berseth and Nordyke 1992).

Morriss et al. (1990) report that preterm infants who later developed NEC, have an earlier increase in duodenal contraction rate than did healthy infants of the same gestational age. There was no marked increase in peak intraluminal duodenal pressures in the NEC infants. Thus it is unlikely that intestinal hypertonicity may be a factor in the pathogenesis of intestinal ischemia and possibly NEC. One may argue, on the other

hand, that the maturation of motility in the duodenum does not necessarily reflect events in the distal small intestine and colon where NEC usually occurs.

Because increasing gastric residues (feeding intolerance) and abdominal girth (paralytic ileus) are early signs of NEC, abnormal intestinal motor activity is most probably involved in this pathological entity. Whether motor abnormalities are pathogenetic for this disease or are a result of intestinal inflammation and sepsis has not been demonstrated experimentally to date. However, having taken into consideration the fact that prematurity and enteral feeding are the two primary risk factors for developing NEC, enteric nervous system (ENS) immaturity causing impaired clearance of intestinal contents and facilitating bacterial overgrowth could initiate the cascade of events leading to NEC.

1.3. ENTERAL FEEDING AND INTESTINAL CONTENTS

Not every premature, enterally fed infant develops NEC. Therefore, how can the relationship between prematurity and oral feeding and the development of NEC be elucidated?

In general, there are 2 types of presentations of NEC. The "early" type occurs in critically ill unfed premature babies usually within the first 7 days of life. These infants have multiple system involvement. Because their intestinal tract is only beginning to be colonized, bacteria are not likely to be involved primarily. Hypoxia leading to ischemic intestinal injury seems the most likely cause resulting in a more generalized pattern of necrosis (Kliegman et al. 1994). "Late" or classic NEC is usually seen in healthier, growing, fed premature infants. By this time, intestinal bacterial

colonization has occurred. The lesions are predominant in the terminal ileum and proximal colon (Lawrence et al. 1982). It is hypothesised that undigested components of feedings act as substrate for bacteria thereby causing bowel injury. This is more compatible with the anatomic prediliction of "late" NEC.

Clark et al (1985) have analyzed intestinal contents from the site of perforation at surgery or autopsy from fed and unfed NEC patients. Unfed infants had no carbohydrate, few bacteria, no dietary protein and intraluminal pH ranging from 5.5-7.5. Fed infants on the other hand, had carbohydrate, various bacteria , pH between 3.5 and 5.0 and protein including dietary casein, the main protein found in cow's milk-based formula, in excess of 5 g/dl.

Evidence for bacterial and carbohydrate involvement in classic NEC includes the following: hydrogen is a component of the gas found in intestinal lymphatics causing pneumatosis intestinalis (Kliegman, Walker et al. 1994). This results from intestinal fermentation of carbohydrate by enteric bacteria. Jackson et al (1991) have demonstrated bacterial translocation to mesenteric lymph nodes in neonatal rabbits whose intestine was inoculated with E. coli. Interestingly, unfed infants who have a slower rate of bacterial colonization of their bowel and who subsequently develop NEC, rarely have pneumatosis intestinalis.

Organic acids produced from bacterial fermentation of carbohydrate include lactic, acetic, propionic, succinic, butyric and formic acids (Murray 1990). These organic acids may be cleared by 3 potential mechanisms. First, some are absorbed and metabolized or excreted in the urine (D- lactate). Second, intestinal motility allows a portion of these organic acids to be excreted in the stool. Finally, excess acids can be

buffered by mucosal surface proteins and by other undigested dietary carbohydrates. Once organic acids overwhelm these clearing mechanisms, intestinal pH decreases (Kien 1990).

Bovine milk proteins present in infant formula have different buffering capacities. Casein, the most common, buffers in the range of pH 5.1-5.6. Whey proteins buffer between pH 4.0-4.4 (Miller et al. 1990). Therefore between pH 4.5-5.1 dietary proteins have little buffering capacity. Furthermore, Sibbons et al (1992) report that high molecular weight milk fats and some proteins stagnant in distended intestinal lymphatics provide excellent substrate for gas (hydrogen) production by proliferating facultative anaerobic bacteria. This gas is radiologically and histopathologically observed as pneumatosis intestinalis. The fat-protein substrate is also a good medium for lipid peroxidation and for oxygen free radical generation, which may be involved in the pathogenesis of NEC (Gollin and Marks 1993).

In order to test the possible intraluminal pathogenesis of NEC, Clark and Miller (Clark, Thompson et al. 1985; Clark et al. 1988) developed a rabbit model where, under general anesthesia, intestinal loops were flushed with saline and injected with various solutions. No lesions were observed with carbohydrate alone at different doses, protein alone, infant formula, saline solution and pH as low as 3, carbohydrate and pH $<$ 5 or hydrolyzed protein at pH $<$ 5. Using protein, especially casein combined with organic acid of pH $<$ 5, there was a progression of intestinal damage documented biochemically and histologically, starting with fluid secretion and leading to villus necrosis, bowel wall edema, focal hemorrhage and transmural necrosis causing systemic effects and death.

Thus, it is clear from the preceding, that in order to understand the multifactorial pathogenesis of neonatal NEC, the complex interaction between intestinal contents and clearing mechanisms in the immature human gut must be analyzed.

1.3.1. Bacterial Flora

Healthy babies acquire a complex *enteric bacterial flora* within days after birth. Sick infants, requiring intensive care acquire these organisms slowly, especially if parenterally fed (Bennet et al. 1986). During the first weeks of oral feeding, when NEC most often occurs, only one or a few species of bacteria are present in stool. They may exist in greater numbers than occur in healthy babies and constitute a spontaneous form of bacterial overgrowth in the distal bowel. With time, the pattern of bacterial colonization becomes more normal, although administration of broad spectrum antibiotics can reverse or interrupt the process.

In 1982, investigators (Lawrence, Bates et al. 1982) recognized that bacterial overgrowth by cytotoxin-producing colonies could lead to NEC. However, certain organisms can be present without causing harm (Scheifele et al. 1987; Scheifele 1990). In comparing the effects of ischemia, bacteria and substrate on the pathogenesis of intestinal necrosis in germ-free rats, Musemeche (1986) concluded that the presence of bacteria was the most crucial factor in the development of bowel necrosis in their model.

The relevance to the development of NEC of bacterial fermentation of carbohydrate in the premature colon has been examined. Between one half and two thirds of ingested carbohydrate is malabsorbed in the neonate and is fermented by colonic bacteria (MacLean and Fink 1980). Lactose is the sole carbohydrate in human milk,

cow's milk and most commonly used infant formulas. Dietary lactose is hydrolyzed by intestinal brush-border lactase. Fetal lactase activity develops late in gestation. Despite this, premature infants thrive on formulas containing lactose as the sole source of carbohydrate (MacLean and Fink 1980). Lactose and other carbohydrates not fully hydrolyzed in the small intestine reach the colon where bacteria ferment them to short chain fatty acids (SCFA), lactate, other acids and various gases including carbon dioxide, methane, and hydrogen. Most short chain fatty acids and some gases are absorbed in the colon. Much of dietary ATP is salvaged via the process of bacterial fermentation. Thus, colonic bacteria in premature infants serve a beneficial role (Kien et al. 1989). If, however, intestinal motility is impaired, bacterial overgrowth may cause increased fermentation and decreased intraluminal pH in the distal ileum and colon with subsequent mucosal injury (Pomare et al. 1985).

Disruption of the oxidation of SCFA's (Roediger and Nance 1986) or bacterial depletion and loss of fermentation end products (Soergel et al. 1989) predisposes to inflammation and diarrhea. These findings support the hypothesis that NEC involves the generation of inflammation by the local disruption of intestinal metabolism. In the premature neonate, the normal sequence of bacterial colonization and the subsequent production of normal bacterial products is modified, the consequences of which may certainly predispose to NEC.

Endogenous intestinal defense mechanisms, akin to the proline-arginine-rich antibacterial peptide isolated from pig intestine (Agerberth et al. 1991; Shi et al. 1996) may protect against both Gram-negative and Gram-positive bacteria, and may be diminished in the premature infant. In addition, sepsis has been shown to impair gut

amino acid absorption and to adversely affect barrier and metabolic functions of the gut , thus contributing to associated morbidity and mortality (Sodeyama et al. 1993).

Much evidence exists regarding the importance of preserving intestinal flora and its beneficial effects on the gut. Antibiotic administration, and intestinal starvation, despite parenteral nutition, in premature infants leads to mucosal atrophy, promoting translocation of potentially pathogenic microorganisms (Bengmark and Jeppsson 1995) .

1.3.2. Human milk

The hypothetical protection afforded by human milk against NEC continues to be investigated. Clinical studies of the effect of human milk feedings on NEC are difficult to perform, due to the unpredictable and sporadic occurrence of this disease. Two retrospective studies comparing formula-fed to human milk-fed infants who developed NEC were contradictory (Kliegman et al. 1979; Moriartey et al. 1979). In a large prospective, controlled, randomized trial of preterm infants weighing less than 1850 g, human milk-fed infants had a decreased occurrence of NEC compared to formula-fed infants who were more than 30 weeks gestation at birth (Lucas and Cole 1990). Exclusively formula-fed babies develop NEC 6-10 times more often than those fed breast milk alone, and 3 times more often than those fed formula and breast milk (Barlow et al. 1974).

Because there is no single model of NEC, laboratory sudies of the effects of human milk on NEC are inconclusive. In the rat, milk leukocytes attenuated disease in a neonatal model of hypoxia-induced enterocolitis (Pitt et al. 1977). In humans, feeding

breast milk with preserved milk leukocytes had no effect on NEC (Kliegman, Pittard et al. 1979).

It is well established that human milk protects the neonate against infectious illness caused by specific agents. In developing countries, protection against diarrhea caused by a variety of agents is clinically documented (Victora et al. 1987; Velasquez et al. 1992). The purported anti-infectious properties of human milk are based on the presence of increased secretory IgA in colostrum and milk, the presence of macrophages, polymorphonuclear leukocytes and lymphocytes which can participate in host defense mechanisms, the presence of bifidus factor, a microbial growth factor which might favour the selective growth of nonpathogenic bacteria and the presence of lactoperoxidase, lactoferrin, and lysozyme which have antibacterial activities in vitro (Hanson and Winberg 1972; Goldman and Smith 1973).

Studies indicate that human milk is also anti-inflammatory. TNF , one of the numerous cytokines involved in the initiation and propagation of acute inflammation is present with variable bioactivity in human milk (Rudloff et al. 1992). Several factors in human colostrum potently inhibit another cytokine, interleukin-2, (T-cell growth factor) (Hooton et al. 1991). Colostrum also contains functionally active antioxidants, two of which are uric acid and an ascorbate-like component (Buescher and McIlheran 1988; Buescher et al. 1989; Buescher and McIlheran 1992). Thus, reactive oxygen species such as superoxide and hydrogen peroxide produced by polymorphonuclear leukocytes (PMNs) are consumed. Colostrum interferes with cellular functions in acute inflammation such as PMN adhesion, orientation, deformability and enzymatic activities (Thorpe et al. 1986; Buescher 1991). In a rat model of acute inflammation induced by

the injection of carrageenan in subcutaneous air pouch, the anti-inflammatory activity of human colostrum has been demonstrated using PMN accumulation. The decrease in the inflammatory response was about 50%, comparable to the administration of known anti-inflammatory agents indomethacin or dexamethasone (Murphey and Buescher 1993). Lysozyme, also present in human milk, has been shown to function as a negative feedback sysytem for inflammation (Gordon et al. 1979).

It is presumed that breast milk provides IgA in the gut lumen which has been shown to decrease enteric infection and to be protective for NEC (Tacket et al. 1988; Ruiz-Palacios et al. 1990; Wolf and Eibl 1991) Human milk also contains growth factors which enhance neonatal intestinal development and maturation (Morriss 1986).

In conclusion, laboratory and clinical evidence would indicate that human milk feeding affords protection against the development of NEC.

1.3.3. Bovine casein

Bovine casein is implicated in a number of undesireable effects on the premature intestine. Casien precipitates from raw cow's milk at pH 4.6 at 20^{o} C . Casein is absorbed on the neutrophil cell surface and ingested. In combination with fat, it inhibits both phagocytosis and intracellular killing of Staphylococcus aureus by neutrophils (Paape and Guidry 1977). In the presence of glycine, casein derivatives enhance beta-lactamase production by Enterobacter cloacae (Gatus et al. 1986). Casein digests and hydrolysates induce increased carbohydrate fermentation by enteric gram-negative rods by inducing the lactose operon and by increasing beta-galactosidase activity (Carbonaro et al. 1988). This results in a more rapid fermentation of

carbohydrate which lowers intraluminal pH. Thus, SCFA absorption by colonocytes is altered. This may be a possible etiological factor in NEC (Clark, Thompson et al. 1985). Because alpha and beta-caseins are chemotactic for leukocytes, casein injection into the peritoneal cavity is routinely used to harvest peripheral neutrophils of experimental animals (Balcom et al. 1985). Casein also induces the activity and release of phospholipase A_2 which promotes arachidonic acid metabolism by the cyclo-oxygenase and lipo-oxygenase pathways in neutrophils and other cells (Chang et al. 1986; Chang et al. 1987). Lipo-oxygenase products, leukotrienes, may exacerbate the inflammatory response through cellular activation and recruitment and by modulating local vascular tone and permeability (Samuelsson et al. 1987; Pace-Asciak and Asotra 1989). Tryptic digests of bovine casein potentiate the proinflammatory responses to bradykinin. Since bradykinin is a potent proinflammatory agent involved in inflammatory bowel disease (Leduc and Zipser 1989), bovine casein or its tryptic hydrolysate can exacerbate intestinal inflammation (Miller, Witherly et al. 1990).

In the model which reproduces the intraluminal biochemistry of infants with NEC, the greatest damage induced by the combination of protein with organic acids was with that using casein (Clark, Thompson et al. 1985). The low intraluminal pH resulting from carbohydrate fermentation by enteric bacteria can increase epithelial permeability (Powell 1981). The presence of casein initiates a local inflammatory response and cellular necrosis. Casein alone or in combination with bacterial toxins is probably the cause of NEC-associated neutropenia. Here, activated neutrophils adhere to the vascular wall and/or migrate into tissues, thereby decreasing circulating numbers (Balcom, Clark et al. 1985).

Of the biologically active fragments of casein, beta-casomorphins (BCM) are the best characterized. They are derived from beta-casein; the bovine casein derived beta- casomorphins being more potent than those from human milk. BCM's stimulate electrolyte absorption and inhibit intestinal motility (De Ponti et al. 1989). The effect on intestinal motility is due to a reduction in periodicity rather than the amplitude of peristaltic waves (Kromer et al. 1980). In newborns, the gastrointestinal tract is more permeable (Berezin et al. 1989) and may allow for increased uptake of BCM's.

1.4. INTESTINAL ISCHEMIA

The histology of NEC clearly indicates that ischemia is a feature of this disease. Whether circulatory phenomena are primary or secondary to the pathogenesis of NEC is still being debated. No discussion of NEC would be complete without a description of the most widely accepted theory on the pathogenesis of NEC, the diving reflex theory proposed by (Lloyd 1969). This theory proposes that neurogenic redistribution of cardiac output away from splanchnic organs leads to bowel injury in newborn infants. This theory resulted from clinical reports of perinatal asphyxia in most infants with bowel perforation (Stevenson et al. 1969; Hopkins et al. 1970), and from laboratory evidence of selective reduction of blood flow to the terminal ileum and colon of newborn swine during sustained asphyxia with histologic evidence of bowel necrosis (Touloukian et al. 1972; Alward et al. 1978). The parallel was drawn to the physiology of diving mammals and thus became known as the diving relfex theory. Over the past three decades, accumulated data on intestinal hemodynamics refute the diving reflex theory on the pathogenesis of NEC.

That an extrinsic vascular effector system (adrenergic neurons) compromises intestinal oxygenation leading to necrosis was the basis of the theory. Thus intestinal oxygenation must depend on blood flow, and sustained adrenergic stimulation causes intestinal ischemia and hypoxia. The preceding were disproved.

Intestinal blood flow reduction leads to active and passive microvascular changes to preserve tissue oxygenation. Firstly, the surface area available for O_2 diffusion is increased by the opening of pre-capillary sphincters and secondly, the capillary to cell pO_2 is maintained by a concomitant passive decrease in cell pO_2 which at rest is nearly one order of magnitude above the level required for cellular oxidative metabolism (Granger and Nyhof 1982). Thus, intestinal oxygen uptake is independent of flow unless severe ischemia occurs (Kvietys and Granger 1981). The rapid increase in intestinal vascular resistance on stimulation of postganglionic, periarterial mesenteric nerves, or noradrenaline infusion decreases blood flow and tissue oxygenation (Shepherd et al. 1973). This effect is not sustained even with continuous stimulation or infusion. Resistance and blood flow are restored to baseline within minutes in a process called autoregulatory escape mediated by a vasodilator mechanism intrinsic to intestinal parenchyma (Shepherd and Granger 1973). Its existence has been confirmed in the newborn gut (Nowicki et al. 1991).

Clinical epidemiologic investigations using case-control studies did not identify asphyxia, hypotension or hypoxia as key risk factors in the pathogenesis of NEC (Stoll et al. 1980; Kliegman and Fanaroff 1984). Therefore, intestinal perfusion is only transiently compromised and of insufficient magnitude to induce gut injury without coexisting factors.

Historically, there are two other common circulatory events that have been linked to NEC. The first is umbilical vessel catheterisation. Exchange transfusions through an umbilical vein catheter causes an acute elevation of intestinal venous pressure and a reflex vasoconstriction in intestinal resistance vessels (Burrows and Johnson 1983). This is mediated via the myogenic response which is the inherent capacity of vascular smooth muscle to contract in response to a stretch stimulus (Folkow 1964). This response reduces flow to the gut thus reducing portal venous pressure and has been demonstrated in newborn swine (Crissinger et al. 1988; Nowicki et al. 1991). However, myogenic vasoconstriction does not occur in very young infants and has been shown to have no effect on gut oxygenation. The potential adverse effects of umbilical artery catheterisation on intestinal blood flow have also been investigated. Administration of pharmacologic agents for treatment of sick preterm infants might cause vasospasm in the gut (Book and Herbst 1980). In addition, catheters positioned in the thoracic aorta can be thrombogenic with embolization within the watersheds of the mesenteric and renal arteries. Emboli have not been demonstrated on pathological investigation of the intestinal circulations of NEC infants (Tyson et al. 1976).

The second is the reported increased incidence of NEC in polycythemic infants (Leake et al. 1975). The associated hyperviscosity impairs erythrocyte mobility within capillary networks decreasing cardiac output and regional perfusion resulting in intestinal necrosis. Experimental evidence in support of this was provided in puppy and newborn swine models of polycythemia induced by exchange transfusions (LeBlanc et al. 1984; Nowicki et al. 1984). Nevertheless, the role of hyperviscosity in NEC is questionable, since most NEC babies are not polycythemic.

Although past circulatory theories lack substantiation through clinical and laboratory investigation, it is a well known observation that NEC is most common in the terminal ileum and proximal colon, regions which lie in the watershed between the superior and inferior mesenteric arteries (Stevenson et al. 1971). Observations of circulatory physiology have revealed age-dependent differences which may be relevant to NEC. Investigators have shown a change in the rate of intestinal oxygen consumption in the neonatal period. Intestinal oxygen uptake nearly doubles in newborn lambs (Edelstone and Holzman 1981), whereas oxygen use in newborn swine is high and decreases by the end of the first postnatal month (Crissinger, Kvietys et al. 1988). Due to the marked increase in intestinal work of motility, absorption, secretion and in growth, oxidative demands peak in the early newborn period. Indeed, vascular resistance in newborn swine intestine is low, and blood flow is high, in order to insure a high oxygen diffusion gradient from capillary to cell. However, newborn swine do not demonstrate the decrease in gut vascular resistance in response to reduction in arterial pressures which exists in weaned piglets (Nowicki et al. 1988; Nowicki and Miller 1990). Nevertheless, newborn intestine does show a vasodilatory response to sustained nerve stimulation: the escape process referred to earlier.

The relevance of these observations to the pathogenesis of NEC may be explained as follows. The high rate of oxygen consumption in newborn intestine might increase its susceptibility to hypoxia, which is accentuated by the relative absence of a vascular regulatory response to arterial hypotension and hypoxia (Nowicki and Miller 1988; Nowicki, Miller et al. 1991; Nowicki and Miller 1992).

Vascular tone is also regulated by endothelial production of vasoactive agents affecting adjacent vascular smooth muscle (Furchgott 1983; Ignarro 1989). Nitric oxide (NO; see below) is one such agent and may have an age-dependent role in postnatal vascular tone regulation (Rees et al. 1989; Nowicki and Edwards 1992). Inhibition of NO formation increases vascular resistance to a greater degree in 3-day-old denervated intestinal segments than in 35-day-old swine. Basal production of NO may be greater in younger subjects, and if so, may explain why resting vascular resistance is low in newborn piglet gut. Ischemia or hypoxia has been shown to cause endothelial cell damage (Ku 1982). This damage decreases NO production and enhances neutrophil adhesion, in turn leading to increased vascular resisitance and decreased blood flow (Ambrosio et al. 1989; Tsao and Lefer 1990). Thus, events leading to intestinal injury may cause local endothelial damage, impaired NO production, microvascular dysfunction, and intestinal ischemia and necrosis: this is referred to as NEC.

Flow reduction damages the intestine, but restoration of flow greatly accentuates tissue injury. Generation of oxygen free radicals upon reintroduction of oxygen has been found to be the cause of subsequent tissue damage. These radicals are derived from two sources. One results from the enzymatic breakdown of hypoxanthine, which accumulates during ischemia, by tissue xanthine oxidase (Granger et al. 1981; Parks et al. 1982). The other from activated neutrophils which adhere to the microvasculature during ischemia/reperfusion (Grisham et al. 1986; Granger et al. 1989). Post-natal piglet intestine was originally shown to be relatively deficient in xanthine oxidase activity and resident granulocyte population (Crissinger et al. 1989). However, ischemia/reperfusion intestinal damage was later demonstrated by these same

investigators in postnatal swine intestine. More importantly, intraluminal exposure to cow's milk-based formula greatly exacerbated mucosal injury, and this, only in very young animals (Crissinger and Granger 1989). These findings point to the link between feeding and ischemia in immature gut as possible prerequisites for the development of NEC.

An additional observation points to the implication of circulatory mechanisms in the pathogenesis of NEC. Antenatal cocaine exposure has been linked to severe NEC in both preterm and full-term infants (Czyrko et al. 1991). Cocaine inhibits presynaptic reuptake of noradrenaline and dopamine. An excess of these agents at postsynaptic receptors might accentuate vasoconstriction leading to ischemia. Intravenous infusion of cocaine in newborn (Hebra et al. 1993) and adult animals (Nalbandian et al. 1985) causes intestinal vasoconstriction which is different from that resulting from adrenergic nerve stimulation, in that escape does not take place.

The circulatory evidence to date is inconclusive as to whether ischemia is a primary or secondry factor in NEC pathogenesis. Primary injury to the intestinal vasculature may lead to ischemia and gut damage or mucosal damage may initiate a cascade of reactions through neutrophil aggregation and emigration within the bowel (infants with increasing severity of NEC become neutropenic), oxygen free radical production leading to endothelial cell damage, and modification of basal endothelial cell NO production leading to localized ischemia.

1.5. INFLAMMATION AND GUT INJURY

Regardless of the multifactorial etiology of NEC, it is reasonable to assume that gut injury results from a "final common pathway" which most likely involves the production of inflammatory mediators. Many such mediators have been studied using animal models of intestinal damage. Some substances were found to attenuate intestinal injury such as prostaglandin E_1 (PGE_1) and NO (Miller et al. 1988) Others, such as complement, endothelin-1 (ET-1) (Miura et al. 1991), leukotrieneC4 (LTC_4) (McIntyre et al. 1986), O_2 radicals (Miller, McNeill et al. 1988)), platelet activating factor (PAF), and tumor necrosis factor (TNFα) (Sun and Hsueh 1988) potentiated such injury.

PAF is the most extensively studied of such mediators with regards to NEC. It is a phospholipid mediator with many biological effects (Hanahan 1986; Whatley et al. 1987) whose synthesis is regulated by the enzyme phospholipase A_2, associated with arachidonic acid production and degraded by acetlyhydrolase (Blank et al. 1981; Farr et al. 1983). PAF has a very short *in vivo* half-life and is synthesized by many cell types including endothelial cels, eosinophils, hepatocytes, keratinocytes, macrophages, and neutrophils and is found in virtually all biological fluids and tissues. The biological effects of intravenously administered PAF in animal models include: systemic hypotension, bronchconstriction, pulmonary artery hypertension, neutrophil and platelet aggregation and degranulation, capillary leak, and ischemic bowel necrosis (Handley et al. 1986).

Factors relevant to NEC pathogenesis such as lipopolysaccharide (LPS)(Chang, Kudo et al. 1987) and hypoxia (Prevost et al. 1984; Caplan et al. 1992) increase PAF production which in turn stimulates other mediators such as TNFα (Valone

and Ruis 1992), complement (Sun and Hsueh 1991), oxygen radicals (Kubes et al. 1991), catecholamines (Hsueh et al. 1988), prostaglandins (Zimmerman et al. 1985), thromboxane (Tanizawa and Tai 1989) and leukotrienes (Hsueh et al. 1986). Ischemic bowel necrosis similar to human NEC has been shown following intra-aortic PAF in a rat model (Hsueh et al. 1986). Endotoxin-induced (LPS) intestinal injury is associated with increased PAF in gut homogenates and can be prevented by PAF receptor antagonists (Whittle et al. 1987). Models of ischemia/reperfusion of the mesenteric circulation have also shown an increase in PAF plasma levels and bowel necrosis which is prevented by PAF antagonists (Kubes et al. 1990). More interestingly, hypoxia and low dose LPS causing hypotension, acidosis, hemoconcentration and intestinal injury in the rat, have all been ameliorated by PAF antagonists (Caplan et al. 1992). Thus, evidence to date support PAF's role as a major mediator of intestinal damage, regardless of etiology.

Studies in human neonates with NEC have shown significantly higher plasma PAF levels and lower plasma acetylhydrolase activity compared to age-matched controls (Caplan et al. 1990). Ontogenically, PAF acetylhydrolase has been shown to be suppressed in premature infants, and that adult levels are attained at about 6 weeks of life, possibly explaining the susceptibility to NEC of these premature infants. In addition, feeding alone was shown to increase circulating levels of PAF (MacKendrick et al. 1993). Of interest is the fact that human milk contains PAF-acetylhydrolase which is lacking in cow's milk preparations.

Secondary mediators involved in PAF-induced gut injury include leukotrienes, prostaglandins, noradrenaline (Hsueh, Gonzalez-Crussi et al. 1986), and complement activation (Llausas-Magana et al. 1988). Protective roles in these models

have been demonstrated by oxygen radical scavengers (Cueva and Hsueh 1988), neutrophil depletion (Musemeche et al. 1991; Sun and Hsueh 1991) and, more recently, NO (MacKendrick et al. 1993).

TNFα, a protein originally described as a factor causing tumor necrosis in mice, is secreted from macrophages and other cells after endotoxin stimulation (Tracey et al. 1986; Beutler and Cerami 1987). Its administration to animals causes shock and tissue injury. Necrosis, though, is not gut-specific. PAF increases TNFα synthesis and TNFα stimulates PAF production, thereby potentiating their effects. TNFα levels in infants with NEC are higher than controls (Caplan et al. 1990). But, because histological examination shows injury to be limited to the gut in NEC, the multi-systemic necrosis induced by TNFα makes this mediator less likely to be NEC-specific.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide secreted by endothelial cells (Yanagisawa et al. 1988; Rubanyi and Botelho 1991) which participates in blood pressure regulation, persisitent pumonary hypertension of the newborn, congestive heart failure and renal failure. It also stimulates the inflammatory cascade (Takayasu et al. 1989). Hypoxia increases ET-1 levels in vivo and in vitro (Isozaki-Fukuda et al. 1991; Elton et al. 1992). Exogenous ET-1 causes intestinal injury in rats which can be prevented by PAF antagonists (Miura, Kurose et al. 1991). The possible role of this peptide in NEC has yet to be elucidated. More recently, in a study of human NEC, the only cytokine found to be consistently elevated in premature infants with NEC was interferon gamma (IFNγ(Ford et al. 1997)

In conclusion, factors implicated in NEC pathogenesis promote the generation of PAF and other inflammatory mediators which may be the final common

pathway to intestinal damage. Experimental evidence seems to point to ontogenically determined regulatory mechanisms which affect these mediators, and may thus explain in part the premature infant's predisposition to NEC.

1.6. NITRIC OXIDE (NO)

In 1980, it was demonstrated that a substance that relaxed vascular smooth muscle was released by the endothelial lining of blood vessels (Furchgott and Zawadzki 1980). This substance was referred to as endothelium-derived relaxing factor (EDRF). Its identity remained unknown until 1987 when it was demonstrated by 2 independent groups that NO was released from vascular endothelial cells and that the effects of NO and EDRF were indistinguishable (Ignarro et al. 1987; Palmer et al. 1987). Today, EDRF is thought to be endothelium-derived NO.

NO is a short-lived labile gas produced in most biological systems by converting L-arginine to L-citrulline by a specific family of synthases (NO synthase). The constitutive isoform of this synthase (cNOS) generates small amounts of NO in physiological signalling processes. The inducible isoform (iNOS) generates large amounts of NO and is associated with infection/inflammation. NO is also synthesized in non-vascular sites including nerve cells of the CNS and the gut (for reviews see Ignarro 1990; Moncada and Higgs 1991; Snyder and Bredt 1992; Rodeberg et al. 1995).

NO exists as a free radical in the form of a highly diffusible gas. In contrast to other signaling molecules in the mammalian system, NO has a unique chemical nature since it can diffuse rapidly through both lipid (membrane) and aqueous environments. This property of NO allows for its three-dimensional spread, the extent of

which is limited only by its half-life (ibid) which, in vivo, may be as short as a few seconds or longer if it is transported by a carrier molecule (i.e. cysteine or glutathione). Interestingly, based on the half-life of NO and its ability for diffusion, it has been estimated that NO may spread across as many as 2 million synapses in the CNS (Bruhwyler et al. 1993).

NO is a key factor in gastrointestinal physiology and gut responses to injury. Of particular interest to us is evidence that NO mediates i) inhibitory nerve-related relaxation of intestinal smooth muscle and may have a role in ii) regulation of gut mucosal blood flow, and iii) mucosal protection.

Exogenously applied NO mimics the responses to non-adrenergic, non-cholinergic (NANC) inhibitory nerve stimulation, which include hyperpolarization of the membrane potential, and produces inhibitory junction potentials (IJPs) similar in amplitude to NANC induced IJPs. These responses, which are evoked following applied NO and NANC inhibitory nerve stimulation, are reduced by oxyhemoglobin which binds and inactivates NO. NANC nerve stimulation results in the release of NO (Boeckxstaens et al. 1991).

There is, at present, extensive literature describing the disposition of NO synthesizing elements and related substances as well as enteric innervations targeting gut NO neurons and mucosal cells in many species including the human, rodent and piglet (Nichols et al. 1992; Sanders and Ward 1992; Nichols et al. 1993a; Nichols et al. 1993b; Nichols et al. 1994a; Nichols et al. 1994b). Krantis and coworkers found extensive NO innervation of all layers of the gut wall and this led directly to the discovery of NO elements in the submucosal nerve networks and provided the first histoanatomical

confirmation of NO synthase activity in enteric blood vessels (Bredt et al. 1990; Nichols et al. 1992; Nichols et al. 1993a; Nichols et al. 1994b).

Furthermore, NO may protect gut mucosa by maintaining mucosal perfusion, inhibiting neutrophil adhesion to mesenteric endothelium, blocking platelet adhesion and preventing mast cell activation (Gryglewski et al. 1988; Moncada et al. 1988; MacNaughton et al. 1989; Bath et al. 1991; Whittle et al. 1992). Excessive NO in the more advanced stages of inflammation is toxic and causes intestinal barrier dysfunction (Boughton-Smith et al. 1990; Deitch 1990; Hutcheson et al. 1990; Kubes 1992). High concentrations of NO disrupt the actin cytoskeleton, inhibits ATP formation and dilates cellular tight junctions, leading to intestinal hyperpermeability (Salzman 1995).

At rest, mucosal perfusion is regulated by mesenteric vascular endothelial NO. Excessive NO induced by inflammatory states causes mucosal hyperemia. Investigation into the role of NO in inflammatory conditions involving tissue damage and increased vascular permeability due to vasoactive mediators, toxic oxygen radicals or neutrophils provides important clues to the pathogenesis of inflammatory bowel disease (IBD), NEC (neonatal IBD), infectious colitis and ischemic gastrointestinal injury.

1.6.1 Mucosal protection

Nitric oxide plays an important role in protecting the intestine from blood-borne toxins and tissue destructive mediators (Hutcheson, Whittle et al. 1990; Whittle et al. 1990; Kubes and Granger 1992). Inhibitors of NO synthesis decrease blood flow and enhance endotoxin-induced damage and plasma leakage in the small intestine (Kubes

1993; Miller et al. 1993). Generators of NO attenuate endotoxin-induced intestinal damage (Boughton-Smith et al. 1990; Hutcheson et al. 1990; Kubes, Arfors et al. 1991). Exogenous NO-donating compounds can modulate electrolyte transport in guinea pig intestine in vitro (MacNaughton 1993). Inhibition of NO production has been shown to increase epithelial permeability which is both circulating leukocyte dependent and independent (Kubes 1992; Kubes and Granger 1992; Miller et al. 1993). Endogenous NO protects against platelet-activating factor-induced bowel injury in the rat (MacKendrick, Caplan et al. 1993) and reduces reperfusion-induced mucosal barrier dysfunction independent of intestinal blood flow alterations (Payne and Kubes 1993).

The mechanism by which endogenous NO protects intestinal mucosa and microvasculature is unclear. Endotoxin can stimulate the formation of oxygen radicals, such as superoxide anion which may mediate intestinal injury. Clark et al. (1988) demonstrated the contribution of locally generated superoxide anion in their model of NEC, and the protective effects of superoxide dismutase. It is hypothesized that endogenous NO may act as an antioxidant and can interact with superoxide anion to produce a less toxic species reducing microvascular damage produced by endotoxin (Miller et al. 1993; Payne and Kubes 1993). In addition, by inhibiting neutrophil adhesion to vascular endothelium and hence emigration from blood vessels, NO may protect the intestine from endotoxin-induced damage by attenuating neutrophil-induced injury (Madden et al. 1988). Thus endogenous formation of NO may maintain the integrity of intestinal mucosal microvasculature following acute endotoxin challenge. The highest levels of constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) activity have been shown in intestinal villus cells. Whereas constitutively formed

NO is physiological, LPS-induced NO causes a decrease in gut epithelial cell viability in the rat (Tepperman et al. 1993).

Myers et al. (1992) have shown that low concentrations of endotoxin decrease both vasodilator bioactivity due to NO and endothelial production of NO. Since endotoxin directly inhibits NO and EDRF release, endotoxemia-induced systemic hypotension may not be due to acute release of an endothelium-derived vasodilator. Alternate sources of endotoxin-mediated nitro-vasodilators such as macrophages, monocytes or vascular smooth muscle may cause this systemic hypotension. Moncada's group demonstrated that endotoxin stimulated inducible NO synthase (iNOS) in rats (Hutcheson et al. 1990; Moncada and Higgs 1991). Dexamethasone, an inhibitor of the iNOS, was found to decrease NO synthesis induced by bacterial lipopolysaccharide. This suggests that iNOS activity contributes to the hypotension and tissue damage of endotoxic shock.

Using a trinitrobenzenesulphonic acid model of chronic IBD in guinea pigs, Miller et al. (1993) showed that enhanced NO synthesis promoted mucosal injury, whilst under physiological conditions NO is anti-inflammatory. It thus appears that NO is both beneficial and detrimental to gut mucosal integrity. By therapeutically inhibiting iNOS without affecting cNOS, Miller et al (1993) propose that IBD may possibly be better controlled. Interestingly, in a separate study of inflammation in their rabbit model of NEC, Miller et al (1993) also observed an increased production of NO. However, in a feline model of PAF-induced inflammation, Kubes and co-workers (1995) showed that high concentrations of NO did not affect microvascular or mucosal integrity.

Endothelial NO can attenuate sympathetic neurogenic vasoconstriction in rat intestine microvasculature (Nase and Boegehold 1996; Nase and Boegehold 1997). Ontogenically, NO has been recently shown to be a major vasodilator in the fetal lamb gut at mid-gestation, with decreased effects later in development (Fan et al. 1998). In swine, NO has a more important role in the regulation of intestinal vascular resistance in the early postnatal period (Nankervis and Nowicki 1995). In addition, postnatal gut circulation shows progressive vasoconstriction at mechanically reduced flow rates which is age specific in swine, and occurs in 3- but not 35-day old piglets. These changes in vascular resistance may be due to lower gut NO production in the younger animals (Nowicki 1998).

Of further note, rat intestinal lymphatic vessel endothelium has been shown to release endothelial-dependent vasodilators which dilate nearby arterioles and cause lymphatic vessels to constrict (Bohlen and Lash 1992). This may be an additional mode of mucosal protection by which NO would attenuate bacterial translocation into gut lymphatics.

1.6.2. Motility

Nerve-mediated relaxation and associated membrane hyperpolarizations (inhibitory junctional potentials, IJP's) occur in the intestinal muscle of humans and of a number of mammals. These relaxations are primarily related to the release of a non-adrenergic, non-cholinergic (NANC) neurotransmitter. However, the nature of this neuroeffector system is unclear. Although there is evidence for ATP and the peptide VIP as putative inhibitory transmitters released by the NANC's, NO is thought to mediate

NANC neural inhibition of gastrointestinal smooth muscle. (Christinck et al. 1991; Berdeaux 1993).

Smooth muscular sphincteric function as well as coordinated peristalsis is also mediated by NO release, which is the principal neurotransmitter of the enteric nervous system (Bult et al. 1990; Burleigh 1992; Sanders and Ward 1992). By using L-arginine analogues, NO has been shown to be involved in mediating relaxation in the lower esophageal sphincter (Tottrup et al. 1991), stomach (Desai et al. 1991; Ozaki et al. 1992), small intestine (Gustafsson et al. 1990; Toda et al. 1990), sphincter of Oddi (Kaufman et al. 1993), ileocaecal junction and colon (Thornbury et al. 1991), and internal anal sphincter (Gillespie et al. 1989; Rattan and Chakder 1992) in many species and in the human colon and jejunum (Burleigh 1992).

Neuronal NO synthesis occurs whenever the local calcium concentration exceeds approximately 500nM (Schmidt et al. 1992). NO is proposed to diffuse from these sites when synthesized to act on its target, soluble guanylyl cyclase. It is still not entirely certain whether NO is released mainly from somata (thereby controlled by release of intracellular calcium stores), from axon terminals or varicosities (thus controlled via voltage-gated calcium channels) or from dendrites (thereby controlled by transmitter-gated channels). Electron microscopic reports on enteric neurons (Llewellyn-Smith et al. 1992) suggest that NO synthase may be concentrated immediately beneath the plasma membrane (associated with subcellular organelles including mitochondria, endoplasmic reticulum and Golgi apparatus), where it would be directly exposed to calcium entering through transmitter-gated channels. Findings indicate that the classical concept of storage and quantal release of NO as a transmitter may not be applicable to

NO releasing neurons. Unlike the classical transmitters like acetylcholine (ACh) and putative peptide transmitters like vasoactive intestinal polypeptide (VIP), NO is not pre-stored and released by directed vesicular exocytosis but is produced on demand (Sanders and Ward 1992).

1.7. HYPOTHESIS

NO has thus a dual role in the neural control of intestinal smooth muscle relaxation and in gastrointestinal mucosal protection. Because the two primary risk factors for the development of NEC are prematurity, related to intestinal dysmotility, and enteral feeding, resulting in mucosal damage, we chose to investigate the nitrergic system and its possible implication in the pathogenesis of NEC.

The nature of NO production in NEC, where the associated inflammation is established to be "acute", has not been determined. Deficient constitutive NO production may lead to acute intestinal inflammation due to gut dysmotility and ineffective mucosal protection of premature infants. Chronicity of gut inflammation results from over-stimulation of iNOS production. Our hypothesis is that the susceptibility of premature infants to NEC lies in inadequate NO availability which is constitutively derived.

1.8. AIMS

The aims of this body of work are to:

i. Develop an intraluminal model for NEC adapted to the premature and term neonatal piglet, which most closely approximates the human condition. The

development of such a model of NEC which is amenable to *in vivo* pharmacological manipulations is essential. We will adapt an intraluminal model of NEC to the immature piglet. We aim to show this model to be a reproducible one, similar both macroscopically and microscopically to human NEC. In addition, the piglet's gastrointestinal system physiologically and histologically resembles the human's.

ii. Elucidate the role of the nitrergic system in the pathophysiology of NEC by investigation of the effects of systemic pharmacological manipulation of NO availability on disease outcome. In so doing, we aim to affect outcome in NEC by modifying the current treatment of this disease based on observed pathogenetic mechanisms. This will involve the systemic administration of pharmacological agents which affect NO availability either by increasing or decreasing its production by specific isoforms of NO synthase, or by donating NO directly Systemic administration of these agents will be carried out at various times prior to or after NEC induction and for a variable duration in the anesthetized piglet at different stages of post-conceptional and post natal development. The effects of such manipulations will be studied macroscopically (primary tool used to determine the viability of intestinal segments at the time of surgery for resection in NEC) and histologically, in order to quantify their effects on NEC-induced intestinal damage and to objectively compare outcomes.

iii. Determine the ontogeny of the enteric nervous system (ENS), particularly as it relates to intestinal motility, NO positive neurons and the development of intestinal muscular and mucosal NO synthase enzyme activities. This will be achieved through an *in vivo* investigation of intestinal motility carried out in the anesthetized piglet at different stages of development. Histological evaluation of the morphology of the

developing piglet enteric nervous system as it relates specifically to the nitrergic system will also be undertaken. This will include histochemical staining of NO positive neurons in the various layers of the gut wall and will be used to study the effects of developmental age on their numbers and disposition. In addition, biochemical analysis of NO synthase enzyme activities in the intact and NEC-injured gut wall will provide added insights into the functional aspects of these neurons, and their variability, if any, with ontogeny.

iv. If a beneficial effect on NEC-induced intestinal damage is observed with any of the pharmacological manipulations, we aim to document the specific toxic effects of chronic intravenous administration, if any, of such an agent in the premature piglet. That study will be undertaken with the specific aim of evaluating the potential use of this therapy in infants susceptible to NEC.

CHAPTER 2

ANIMAL MODELS OF NECROTIZING ENTEROCOLITIS

2.1 BACKGROUND

Many experimental models have been developed using different animal species of varying ages and different methods to induce gut injury. These models are based on features that reflect the diverse etiologies of NEC based on clinical, epidemiological, pathological, and experimental evidence. Most models have used variations of mesenteric vascular occlusion or hypothermia and hypoxia to induce lesions characteristic of NEC. More recently, investigators have incorporated combinations of prematurity, enteral feeding, infection, stress, hypoxia, inflammation and mucosal insult resulting in intestinal damage similar to human NEC. Such models are of particular relevance given the accumulated data on NEC.

Hypoxia in neonatal rats which are later enterally fed has been shown to cause intestinal disease similar to NEC (Barlow, Santulli et al. 1974; Israel et al. 1991). Hypothermia, intravenous infusion of proinflammatory agents such as PAF, TNF (Tracey, Beutler et al. 1986; Kliegman and Walsh 1987; Sun and Hsueh 1988) and bacterial lipopolysaccharide (LPS) (Mathan et al. 1988) have all been shown to cause bowel ischemia and necrosis similar to NEC. Dose-related intestinal injury has been shown with the injection of PAF in the splanchnic circulation (Caplan and Hsueh 1990). Intravenous administration of LPS or nitric oxide synthase inhibitor prior to PAF

administration, decreased the dose of PAF required to induce damage and resulted in hypotension, leukopenia, and hemoconcentration (MacKendrick, Caplan et al. 1993). When enterocolitis was caused by PAF alone, antioxidants which attenuate reperfusion injury, such as superoxide dismutase, catalase, and allopurinol, decreased the extent of gut damage, thus suggesting a role for reactive oxygen metabolites in the induction of NEC lesions.

Using the hypoxic neonatal rat model, protection had been observed with the feeding of maternal rat milk, lactobacilli or oral immunoglobulins (Barlow, Santulli et al. 1974). This protection was subsequently shown to be dependent on the presence of intact milk leukocytes in the feedings (Pitt, Barlow et al. 1977). Using a similar model, antepartum glucocorticoid-enhanced intestinal maturation in rat pups by the treatment of rat dams with glucocorticoids during gestation attenuated hypoxia-induced NEC-like disease in these pups (Israel, Schiffrin et al. 1991).

The sporadic epidemic occurrence of NEC seemed to point to an infectious etiology. Researchers examined the gut of neonatal animals challenged with micro-organisms isolated from NEC patients and found that in germ-free neonatal rats, strains of C. difficile, C. perfringens, Pseudomonas aeruginosa, or Staphylococcus aureus caused bowel injury (Lawrence, Bates et al. 1982; Scheifele 1990). However, these models do not approximate the majority of NEC cases in that induced lesions are not specifically localized to the ileo-caecal area. Furthermore, not all preterm infants who sustain ischemic insults develop NEC. Only approximately 10% of cases of neonatal NEC have an ischemic or infectious etiology (Kliegman and Fanaroff 1984).

Generally, very little attention has been paid to the predilection of NEC for the ileo-caecal region of the premature human intestinal tract or to the acute, self-limiting nature of this disease. Most animal models of NEC involve some form of ischemic insult resulting in more generalized intestinal necrosis (Kliegman and Fanaroff 1984; Krasna et al. 1986; Sibbons et al. 1992) differing from the classical anatomical predilection of NEC in the terminal ileum and colon. This anatomical predisposition can be tentatively explained by considering the aforementioned risk factors for NEC in the following manner: the immature human gastrointestinal tract has been shown to be dysmotile, with a gestationally timed pattern of development of intestinal motor activity resulting in a normal cycling pattern of fasting motor activity by approximately 37 weeks gestation (Bisset, Watt et al. 1988; Berseth 1994). Thus, the resulting lack of fully matured clearance mechanisms in the premature gastrointestinal tract may lead to bacterial overgrowth in the terminal ileum and colon. In addition, full lactase activity is only partially developed in the premature infant (MacLean and Fink 1980; Kien, Heitlinger et al. 1989). Infant formula, containing lactose as the sole cabohydrate source, overwhelms this partially developed lactase activity, allowing enteric bacteria to ferment excess carbohydrate to short chain fatty acids, thus decreasing intraluminal pH in the terminal ileum and colon predisposing to mucosal injury. Impaired clearance of intraluminal contents exacerbates this effect.

Well-known models of chronic intestinal inflammation utilizing intraluminal trinitrobenzenesulphonic acid (Miller, Sadowska-Krowicka et al. 1993) or more recently, the sulphydryl blocker, iodoacetamide, (Rachmilewitz et al. 1997) approximate more closely Crohn's disease. Histological findings in these modes are

characterized by multifocal mucosal erosions, ulcerations with granulomas and giant Langhans cells characteristic of a chronic ongoing inflammatory process. These findings are very dissimilar to NEC histology as prevously described in Chapter 1.

The anatomic location of classic NEC seems to be somewhat reproducible using intravenous TNFα administration. In this model (Tracey, Beutler et al. 1986; Sun and Hsueh 1988), the caecum displayed more severe injury than did other areas of bowel. In investigating the acute nature of NEC in the LPS and PAF model, animals pretreated with vinblastine which causes neutropenia, and thus a decrease in acute inflammatory cells (PMNs), failed to develop intestinal lesions (Musemeche, Caplan et al. 1991).

The most attractive models of NEC involve the intraluminal induction of intestinal injury using neonatal animals. Such models are based on the two key risk factors for NEC: prematurity and enteral feeding. Only one such model had been described prior to developing ours (detailed description to follow). Based on the analysis of intestinal contents of NEC infants at surgery or autopsy, (Clark, Thompson et al. 1985; Miller, McNeill et al. 1988; Clark and Miller 1990) developed a rabbit model, whereby the intraluminal injection of a solution consisiting of protein and organic acids at an acidic pH induced gut lesions which were similar to human NEC (see Chapter 1). Use of the antioxidant superoxide dismutase, but not allopurinol, attenuated intestinal injury (Clark, Thompson et al. 1985).

Prior to the publication of our results, there had been no other reported models of NEC which addressed the intraluminal pathogenesis of NEC and its relation to the ontogenic development of the gastrointestinal tract in the immature piglet. Subsequently, Adzick and co-workers (Graf et al. 1997) confirmed its reproducibility.

However, to date, the developmental aspect of this model, as it pertains to the pathogenesis of NEC, has yet to be addressed by other investigators.

2.2 CHOICE OF ANIMAL SPECIES

The porcine intestine, as previously stated, resembles the human's both physiologically and histologically. Despite this, the anatomical disposition of the porcine digestive tract is somewhat different from that found in the human. The length of small bowel is greater in the pig, and the ileo-caecal valve is situated in the left iliac fossa, as opposed to the human condition where this joining of the small and large bowel is found in the right iliac fossa. Finally, in lieu of an appendix in the right lower quadrant of the abdomen, as in the human, the pig possesses a very large caecum in the left lower quadrant and its colon is spiralled in a corkscrew fashion in the left hemi-abdominal cavity rather than framing the small bowel, extending from the right, at the ileo-caecal valve, to the left before emptying into the rectum, as in the human.

Despite these minor anatomical differences, the microscopic classification of the various types of neurons by Stach (1989), based on their role in the enteric nervous system, uses porcine histology and closely resembles neuronal types found in human gut. Furthermore, the layers of the gut wall in this species are histologically similar to the human and easily separated from one another under the dissecting microscope, thus permitting their independent histochemical and biochemical analysis. In addition, porcine gut is easily amenable to histochemical staining (Stach 1989), providing clear images which are easily quantifiable at microscopy.

2.3 MATURITY OF ANIMALS

In developing the NEC model, we sought to reproduce the human condition as accurately as possible while at the same time allowing for the utilization of piglets of sufficient maturity to survive the experimental protocol. In the initial testing of our model, weanling piglets aged 3 to 4 weeks were used. As anesthetic experience was gained in the oro-tracheal intubation of these animals, and the efficacy and reproducibility of this model was documented, we undertook experiments using more immature animals. Newborn animals, born at term and only a few hours old were able to withstand the induction of NEC under general anesthesia for 3 to 6 hours inclusively and were susequently recovered and observed for a total of 16 hours after NEC induction. The only clinical side effects were mild nausea and 1 to 3 episodes of bilious vomiting, expected findings since the experimental protocol requires the construction of bowel loops, effectively creating a distal bowel obstruction (see model below).

The adaptation of this model to the premature piglet required some trial and error regarding the timing of prematurity. Consultation with veterinarians at Animal Care, University of Ottawa and Agriculture Canada helped in establishing the proper post-conceptional age for our test piglets. This age was calculated by extrapolation from human gestation of 40 weeks. Based on the assumption that the experimental procedures could be safely tolerated by a 36 week old post-conceptional age infant (10% earlier than full term), the initial timing of delivery for the piglets was calculated to be about 10-12 days prior to term, the gestational period in pigs being approximately 115 days. Disease-free, Yorkshire sows were specifically inseminated at Agriculture Canada, Ottawa for this study, and the duration of gestation was calculated after ultrasonographic

confirmation of pregnancy. Premature piglets were harvested by hysterectomy of the preganant sow. No medications were used in this procedure, the sow being sacrificed by bullet head wound, since the potential interference of placental transfer of drugs to the unborn piglets was undesirable. The newborn premature piglets were transported to our Animal Care facility by heated truck, in crates especially prepared for them with blankets and hot water bottles. Because premature piglets, as premature infants, are susceptible to hypothermia which leads to shock and metabolic acidosis these precautions were also maintained in the operating room. In addition, heating lamps were used, and rectal temperature was continuously monitored during all procedures.

Despite our best efforts, we found this post-conceptional age to be too premature for Yorkshire piglets. These animals were weak and dyspneic, and were hence used for harvesting of gut for our biochemical and histochemical developmental studies. More mature animals only 5-7 days premature were vigorous and easily able to withstand our experimental protocol.

2.4 MATERIALS AND METHODS

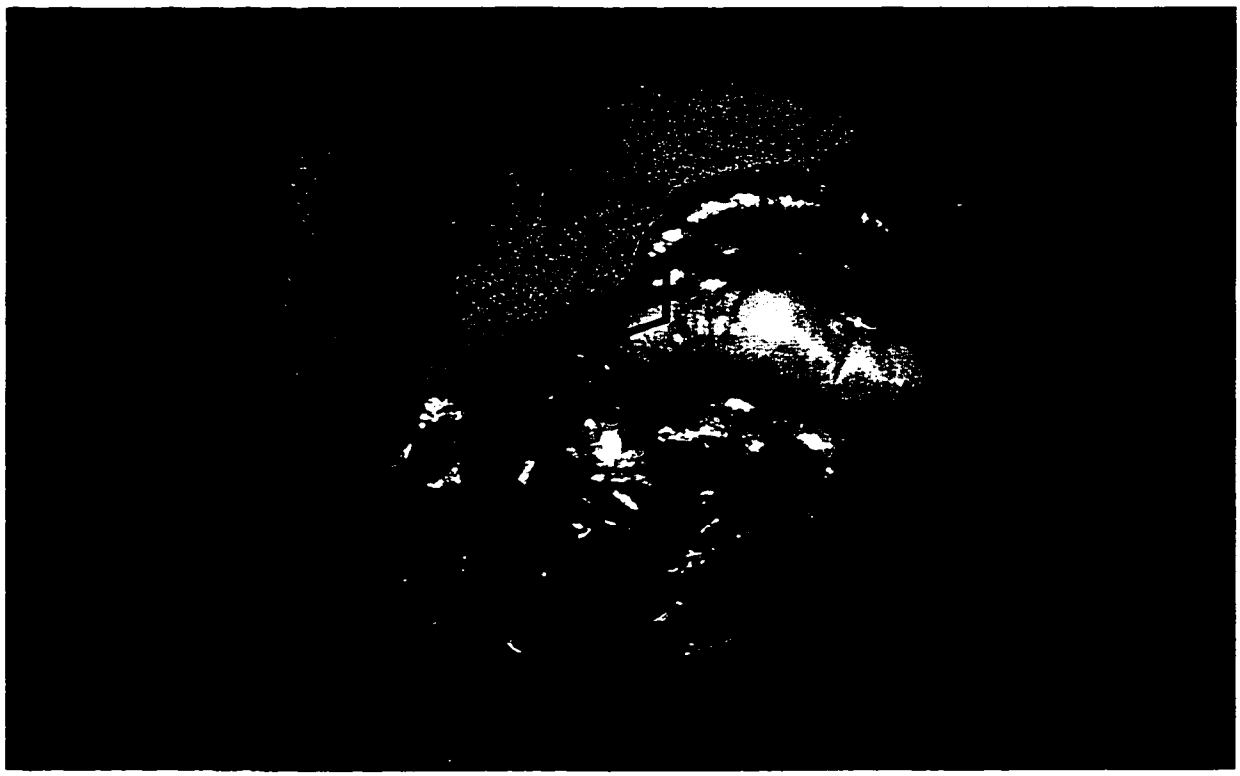
NEC Model: A modification of the model described in the rabbit by Clark et al (1985) was adapted to the pig. The experimental protocol was approved by the Animal Care Committee of the University of Ottawa, Canada. Newborn, term, Yorkshire suckling piglets (n=8, supplied by the Center for Food and Animal Research, Agriculture Canada, Ottawa) were divided into 2 age groups: Group 1= <3 days old; Group 2= 2 weeks old (see Chapter 3 for *premature* piglet results). Animals were anesthetized using midazolam, 0.1mg/kg i.m. and a halothane (1-2.5%) and oxygen

(97.5-99%) mixture via endotracheal tube. A midline laparotomy was performed. Without causing ischemia, a ligature was placed at the proximal ileum, 40 cm from the ileo-caecal valve and flushed distally by transmural injection of warm sterile 0.9% saline using a 26-gauge needle. Six intestinal loops were created (4 ileal, 1 caecal, 1 colonic) by placing ligatures at consecutive 10-cm. intervals. Loops were injected transmurally with sterile 0.9% saline (control), or test iso-osmolar solution consisting of bovine casein 10 mg/ml and calcium gluconate 50 mg/ml acidified to pH 4.0 with propionic acid using a 24-gauge needle. During injections, care was taken to avoid over-distention of bowel loops, eliminating a possible ischemic component to the induced damage. The abdominal cavity was then closed. During the experiment, all animals received 5 ml/kg/hour of 0.9% saline i.v. or subcutaneously. Body temperature was maintained at 37° C using a heated matress and warming lamp and verified via retal thermometer. After 3 hours, the intestinal segments were inspected macroscopically for lesions and harvested. Animals were then euthanized with 3-5 ml of intra-cardiac sodium pentobarbital (65 mg/ml).

All tissues were prepared for microscopic analysis by clearing the lumen of material with gentle perfusion of 0.9% saline solution followed by immersion in 10% formalin. Transverse frozen sections of treated and control intestine were then cut, and stained with hematoxylin and eosin following standard procedures.

Intestinal segments harvested from the <3 day old (Group 1, n = 24 loops) and the 2 week old (Group 2, n =24 loops) piglets were sectioned and evaluated microscopically. The degree of necrosis was scored using a scale of 0 - 4 after the method of Clark (Clark et al. 1988) as follows: 0= villi completely intact; 1= villus tip necrosis with preservation of villus crypts; 2= necrosis of villus tips and crypts with loss

Fig 2.1: Photograph of the macroscopic appearance of piglet gut 3 hours after NEC induction. Arrows show acidified casein-induced damage in the terminal ileum and colon



of mucosal and submucosal architecture; 3= necrosis extending to the muscularis; 4= transmural necrosis. The presence of hemorrhage, inflammatory cell infiltrate and lymphatic distention was noted.

Statistical analysis: The mean and standard error of the mean was calculated for each gut loop after grading for damage. Statistical analysis was undertaken using the Instat statistical computer program. Paired, one-tailed t-test was used to compare control or test loops between the two age groups. One way analysis of variance (ANOVA) and Bonferroni post test were used to compare differences among the control or test loops within the same group. P value of <0.05 was considered significant.

Chemicals: Bovine casein, (SIGMA, St.Louis, MO); calcium gluconate, propionic acid, (BDH Inc., Canada); midazolam, (Hoffman-LaRoche Ltd. Canada); halothane, (Ayerst Laboratories, Canada); sodium pentobarbital, (MTC Pharmaceuticals, Canada).

2.5 RESULTS

All piglets remained stable for the duration of the experiments based on maintenance of vital signs within normal limits. Upon macroscopic inspection, necrotic damage was consistently induced in all loops of intestine injected with the acidified casein test solution (Fig.2.1). Damage was more apparent in the younger age group. More particularly, the test loops remained distended after the 3 hour time period. This finding is indicative of mucosal damage with resultant loss of absorptive capacity. The control loops were collapsed. Upon inspection of the luminal aspect of the bowel wall, observed lesions were patchy with areas of confluence characterized by mucosal

Fig.2.2: Micrographs of hematoxylin and eosin stained sections of piglet intestine.

A : saline control loop of terminal ileum taken from a 2 week old piglet.

B : acidified casein injected loop of terminal ileum from 2 week old piglet.

C : saline control loop of terminal ileum taken from 18 hour old piglet.

D : acidified casein injected loop of terminal ileum from 18 hour old piglet.

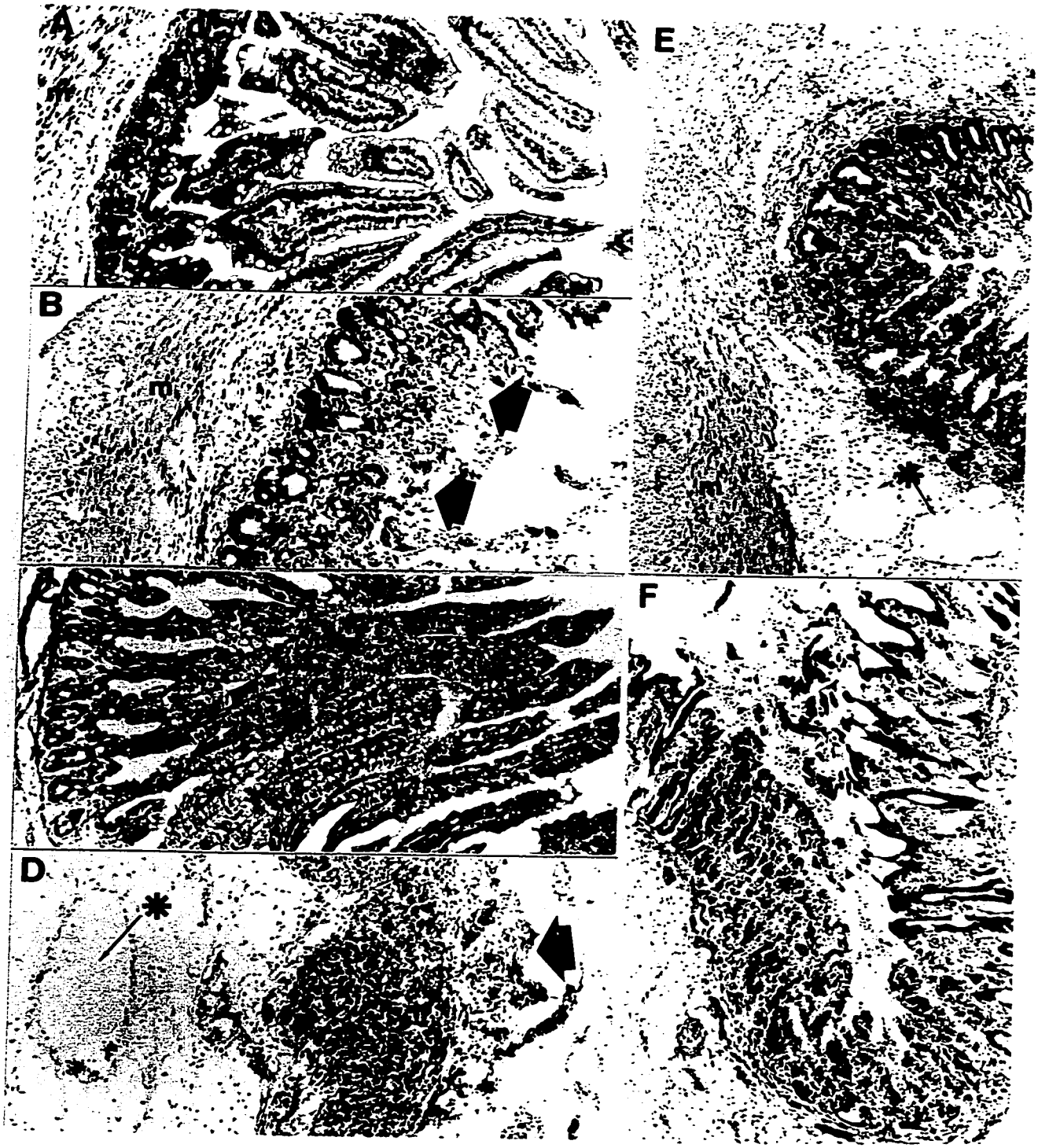
E: acidified casein injected loop of colon from 2 week old piglet.

F: acidified casein injected loop of colon from 18 hour old piglet.

Arrowheads indicate mucosal damage in **B** and damage extending through the submucosa in **D**.

* denotes lymphatic distention; **m** denotes muscularis in **A**

Magnification: x125


A
B
m
D
E
m
F

hyperhemia or frank necrosis extending below the mucosa and in some cases through the muscle layers.

Microscopic evaluation of these segments revealed areas of necrosis, submucosal edema, inflammatory cell infiltrate and lymphatic distention (Fig.2.2: B,D,E,F). In the <3 day olds, intestinal damage extended through the muscle layers (Fig.2.2: D&F) with an average grade of 3.25 ± 0.13. In the 2 week olds, damage included the mucosa with loss of submucosal architecture, generally sparing the muscle layers (Fig.2.2: B&E) with an average grade of damage 2.43 ± 0.14.

Comparison of the extent of injury in the NEC-induced loops between the two age groups revealed a significant ($p<0.05$) difference (Fig.2.3). Anatomically matched NEC loops showed significant ($p<0.05$) damage in the <3 day old group in the terminal ileum, 10 cm from the ileo-caecal valve and in the colon (Fig.2.4). This difference in damage (although not significant: $p= 0.057$) was also evident in the more proximal ileum, 30 cm from the ileo-caecal valve. There was no significant regional difference among the NEC damaged loops of ileum and colon within the same age group. Similarly, no significant difference was evident among saline control loops within and between groups.

2.6 DISCUSSION

The piglet NEC model used in this study involves the intraluminal injection of a solution similar to the intestinal contents of NEC infants, and therefore more closely resembles the human condition. It is postulated that the organic acid (propionic acid) used in the test solution contributes to mucosal damage and facilitates

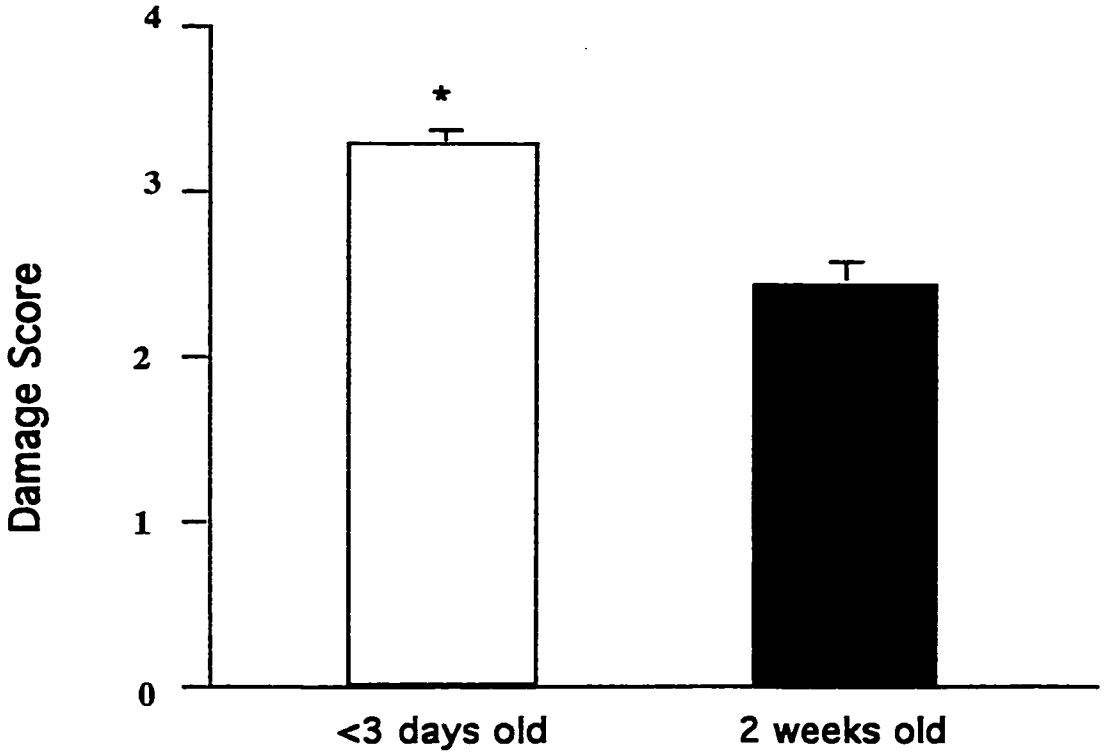


Fig.2.3: Histogram of the intensity of acidified casein-induced damage assessed in H&E stained sections of intestinal loops from < 3 day old (n=12) versus 2 week old piglets (n=12). 0 = villi completely intact; 1 = villus tip necrosis with preservation of villus crypts; 2 = necrosis of villus tips and crypts with loss of mucosal and submucosal architecture; 3 = necrosis extending to the muscularis; 4 = transmural necrosis

* denotes a statistically significant ($p<0.05$) increase in damage between the groups

transepithelial migration of casein, the main protein in cow's milk-based infant formula, causing recruitment of lymphocytes and release of inflammatory mediators (Van Epps et al. 1990). In addition, casein has been shown to increase carbohydrate fermentation by enteric gram-negative bacteria to short-chain-fatty acids further lowering intraluminal pH (Carbonaro, Clark et al. 1988). Indeed, our results show the histological presence of pneumatosis intestinalis, the radiological hallmark for NEC, which is the result of hydrogen gas in lymphatics produced by bacterial fermentation of carbohydrate. This finding has not been demonstrated in another previously reported study using piglets, where NEC was induced using luminal perfusion of a variety of full-strength infant formulas together with ischemia to produce necrosis (Crissinger et al. 1994). The intraluminal infusate used in the present study is based on the intestinal contents of NEC infants (Clark et al 1985) and is certainly more physiological. In addition, in their study, Crissinger et al (1994) did not recover the piglets from surgery. The piglet model used in our study can be applied to conscious animals, since we have recovered neonatal piglets for up to 16 hours. The use of closed intestinal loops in our piglet NEC model incorporates dysmotility, an important feature of the immature human gastrointestinal tract. Evidently, the resulting impaired clearance of luminal contents favours bacterial proliferation and translocation through organic acid damaged mucosa. Furthermore, one of the biologically active fragments of bovine casein, β-casomorphin, has been shown to inhibit intestinal motility (De Ponti, Marcoli et al. 1989; Miller, Witherly et al. 1990).

It is well-known that NEC lesions show anatomic predilection for the terminal ileum and proximal colon. Within our piglet model, we were able to choose this

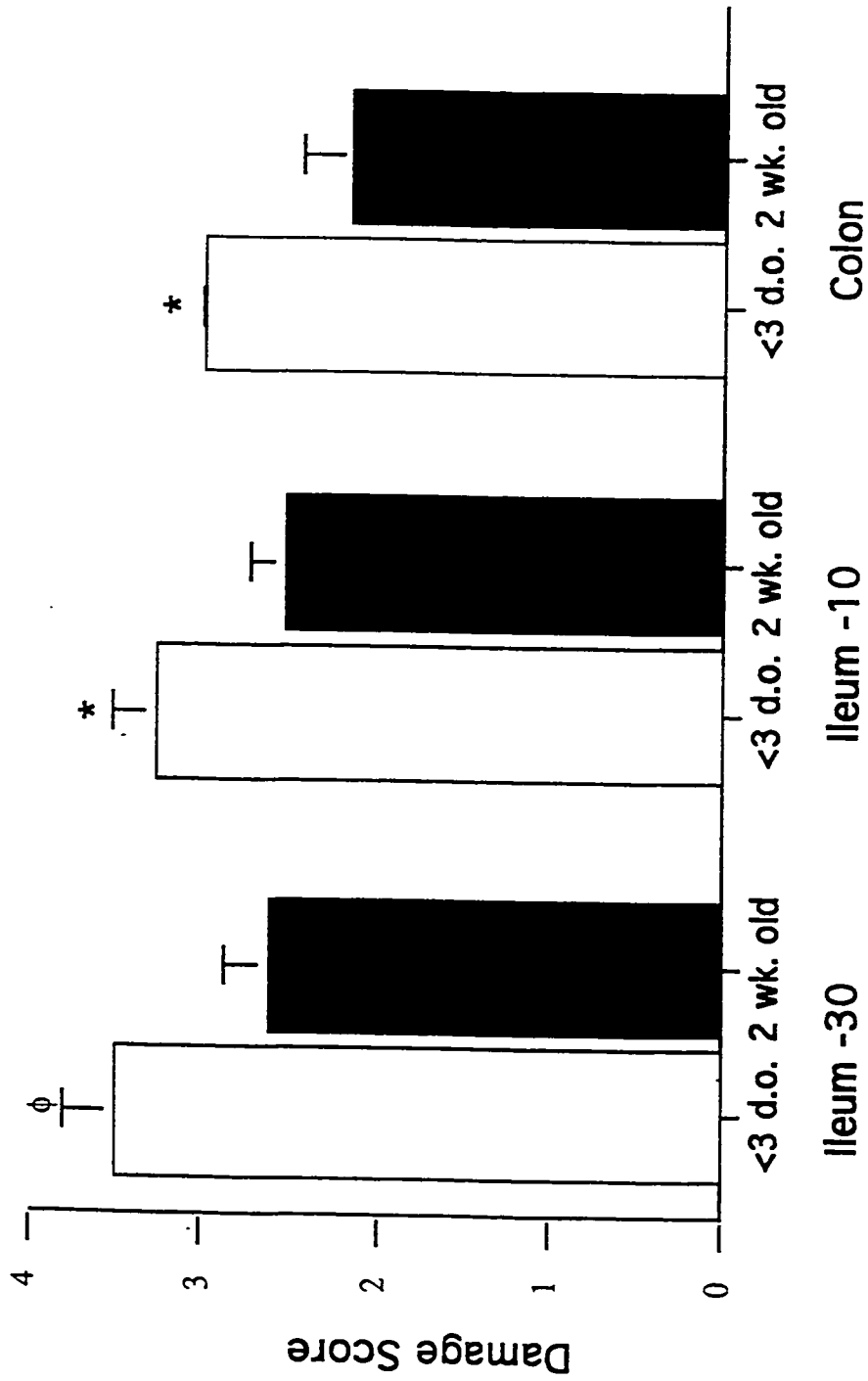
Fig.2.4: Histogram of the depth of acidified casein-induced damage in H&E stained sections of anatomically matched intestinal loops in < 3day old animals versus 2 week olds.

Ileum-30 = ileum 30 cm from the ileo-caecal valve

Ileum-10 = ileum 10 cm from the ileo-caecal valve.

* denotes a statistically significant ($p<0.05$) increase in depth of damage in the younger animals

ϕ although not significant, the p value obtained =0.057


Damage Score
0
1
2
3
4
ϕ
*
*
<3 d.o. 2 wk. old
<3 d.o. 2 wk. old
<3 d.o. 2 wk. old
<3 d.o. 2 wk. old
Ileum -30
Ileum -10
Colon

anatomic area and to subsequently demonstrate this increased susceptibility to damage in the younger animals.

In another study of NEC (Gollin and Marks 1993) using a mature rat model, mucosal injury was mild and limited to villus blunting 3 hours after acidified casein injection. These findings are in contrast to those reported by Clark et al (1985) in weanling rabbits infused with acidified casein solution and to our findings in neonatal piglets (described herein), where more necrosis was evident 3 hours after a single injection of identical test solution. This discrepancy in the extent of injury may be species related and, more particularly, developmentally related, as demonstrated by the results of this study. Findings in our laboratory applying this model to premature piglets further validate this notion (see Chapter 3). In using this intraluminal model to compare the outcome between two age groups of neonatal piglets, we have been able to reproduce in a laboratory setting one consistent epidemiological finding: that the incidence of NEC is highest in the least mature infants (Kliegman and Fanaroff 1984).

In summary, our in vivo model of NEC in the developing neonatal piglet incorporates two of the most common epidemiological risk factors for NEC: immaturity, with its resulting intestinal dysmotility, by the use of intestinal closed loops, and enteral feeding, via the use of the intra-luminal injection of a solution mimicking the intestinal contents of NEC infants. Because of its simplicity, reproducibility, consistency and applicability to conscious neonatal animals, and histological similarity to the human disease, we believe this model of NEC to be a valuable tool enabling in vivo pharmacological manipulation in the study of this disease. As such the use of this model will facilitate the study of a multitude of possible etiological mechanisms which in turn

may lead to the potential development of therapeutic modalities for this common disease of uncertain pathogenesis.

CHAPTER 3

NECROTIZING ENTEROCOLITIS AND THE NITRERGIC SYSTEM

Having mastered the the technical aspects of the aforementioned experimental model of NEC, we proceded with the actual investigation of the effects of manipulation of the nitrergic system on intestinal lesions resulting from intraluminal injection of the acidified casein test solution.

Initial experiments were performed on term neonatal suckling Yorkshire piglets up to 2 weeks of age. Subsequent studies were undertaken on premature piglets born approximately 5 days before term and delivered by cesarian section. The extent of prematurity required to withstand the experimental protocol had been determined by trial and error as previously discussed.

Given the conflicting reports in the literature regarding the role of NO in inflammation, it was quite impossible to predict whether increasing or decreasing NO availability would attenuate NEC-induced intestinal lesions. However, the acute nature of this inflammation led us to hypothesize that increasing NO availability would have a beneficial effect on gut lesions. The following is original data first published in 1995 (appended). Of note is the fact that this was the first time since this disease entity had been described that a potentially specific treatment for NEC had been alluded to. These findings were subsequently substantiated in a rabbit model using the NO donor,

nitroglycerine (Graf et al. 1997). Nevertheless, there has not been any published documentation using premature animals to date, and as such our findings are unique.

3.1 THE USE OF L-ARGININE IN THE TREATMENT OF EXPERIMENTAL NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis (NEC) is the most common acquired surgical emergency in the neonatal period (Cohen et al. 1991). In North America, approximately 10% of premature babies develop NEC with 90% of cases seen in those born at less than 36 weeks gestation (Kliegman 1990). The disease usually develops during the first 2 weeks after birth. Gastrointestinal manifestations include abdominal distension and tenderness, emesis and hemorrhage, progressing to peritonitis and intestinal perforation with the terminal ileum and proximal colon being the sites of predilection.

Prematurity is the primary risk factor for NEC. It is well-known that premature infants characteristically display intestinal dysmotility which assumes a more normal pattern with the passage of time (Bisset et al. 1988). Successful post natal adaptation in the very premature infant is frequently precluded by the immaturity of gastrointestinal function. By 28 weeks gestation, absorptive (McNeish et al. 1979), and digestive (Grand et al. 1976) functions are moderately developed. Adequate motor function lags many weeks behind, and may thus limit tolerance to enteral feeding. Studies of fasting motor patterns in preterm infants show a gestationally dependent process of maturation with full activity appearing only between 37 and 42 weeks (Bisset et al. 1988). This change in fasting intestinal motor activity with age is likely related to the development of the enteric nerve networks.

The second most important risk factor for the development of NEC is enteral feeding. Ninety-five percent of affected neonates have been fed cow's milk-based formula (Goldman 1980), and available data points to the ingestion of bovine casein as a potential etiologic factor (Clark et al. 1985; Miller et al. 1990). Using a rabbit model of NEC, Clark et al. (1985) found that intestinal loops injected with protein, especially casein combined with organic acid of pH < 5, resulted in a progression of intestinal damage, starting with fluid secretion and leading to villus necrosis, bowel wall edema, focal hemorrhage and transmural necrosis causing systemic effects and death. Thus, it is clear from the preceding, that in order to understand the pathogenesis of neonatal NEC, the complex interaction between intestinal contents and clearing mechanisms in the immature human gut must be analyzed.

L-arginine supplemented diets have recently been shown to improve survival in gut-derived sepsis and peritonitis. This beneficial effect of L-arginine seems to be mediated by improvement of bactericidal capacity via the NO synthase catalyzed arginine-nitric oxide (NO) pathway (Gianotti et al. 1993). Secondly, even though the capacity of newborn piglet enterocytes for net L-arginine production has been demonstrated (Blachier et al. 1993), repeated stress has been shown to significantly decrease plasma arginine levels in young rats (Milakofsky et al. 1993). This may be of particular pertinence to the premature neonate.

There are two forms of NO synthase, a calcium dependent constitutive synthase found in neurons and vascular endothelial cells, and an inducible form found in macrophages (Berdeaux 1993). Calcium dependent NO synthase activity has been localized in arterioles of the guinea-pig, rat and human submucosa, and in neurons and

fibres throughout the wall of the stomach and intestine (Nichols et al. 1992; Cuffari et al. 1993; Nichols et al 1993b; Nichols et al. 1994a; Nichols et al. 1994b). Of particular interest to us is evidence that NO is a putative transmitter of enteric non-adrenergic, non-cholinergic (NANC) inhibitory motor nerves mediating reflex peristalsis (Boeckxstaens, Pelckmans et al. 1991; Grider and Jin 1993) and has a role in regulation of gastrointestinal mucosal blood flow, and mucosal protection (Boughton-Smith et al. 1990; Hutcheson et al. 1990; Whittle et al. 1990; Kubes 1992; Kubes and Granger 1992; Tepperman et al. 1993). Because of the proposed dual role of NO with regards to intestinal motility and mucosal protection, and because two of the primary risk factors for NEC are immaturity, as it relates to intestinal dysmotility, and intraluminal substrate which predisposes to mucosal damage, we chose to look at the effect of the NO synthase substrate, L-arginine on the development of NEC in neonatal piglets, using a modification of the Clark et al (1985) intraluminal NEC model. The porcine gastrointestinal system is histologically and physiologically very similar to the human system. The advantage of this acute model is that it mimics most closely the cases of NEC associated with feeding. In addition, the anatomical location can be controlled, as opposed to other models of mesenteric ischemia where vascular compromise leads to more diffuse gut necrosis.

3.1.1 Methods

NEC induction : A modification of the model of necrotizing enterocolitis (NEC) previously described in the rabbit by Clark et al (1985) was adapted to the pig. See Chapter 2, section 2.4 for details. Newborn Yorkshire piglets (n=32, supplied by the

Center for Food and Animal Research, Agriculture Canada, Ottawa) of low and normal birth weight, from 0 to 4 weeks of age (Group 1), were used. Three or 16 hours later the bowel segments were inspected macroscopically for lesions and harvested. The 16 hour group was allowed to recover. Buprenorphine 4-8 μg / kg i.m. was administered every 8-12 hours for analgesia. The animals were euthanized with 3-5 ml intra-cardiac sodium pentobarbital (65mg/ml) after harvesting.

All tissues were prepared for microscopic analysis by clearing the lumen of material with gentle perfusion of 10% formalin and then immersing the tissue in 10% formalin. Transverse frozen sections of treated and control intestine were then cut, mounted onto subbed slides and counterstained with H&E following standard procedures.

These sections were evaluated microscopically and the degree of necrosis was scored using a scale of 0-4 after the method of Clark et al (1988) as per Chapter 2, section 2.4.

NEC treatment: For the treatment groups, piglets of less than 72 hours of age were used. The left internal carotid artery was cannulated for continuous blood pressure monitoring. Upon closure of the abdomen, a continuous peripheral i.v. infusion of L- arginine (n=6), an NO synthase substrate (Group 2), or D-arginine (Group 3), its inactive dextrorotatory isomer, (n=4) at a rate of 600 mg/kg/hr or L-NAME (n=6), an NO synthase inhibitor (Group 4), or D-NAME (Group 5), its inactive dextrorotatory isomer, (n=4) at 20 mg/kg/hr was started. Gut segments were harvested and processed for evaluation 3 hours later.

Statistical analysis: The mean and standard error of the mean was calculated for each gut loop in all groups after grading for damage. Statistical analysis

was undertaken using the Instat statistical computer program. One way analysis of variance (ANOVA) and corrected p value was used to analyse the difference among treatment groups. Repeated measures ANOVA was used to compare casein loops within the same treatment group. Paired, one-tailed t test was used to compare control and casein loops within the same treatment group. A one sample, two-tailed t test was used to compare saline control loops to normal gut. P value <0.05 was considered significant.

Chemicals: Bovine casein, L-arginine, D-arginine, L-NAME, D-NAME (SIGMA, St.Louis, MO); calcium gluconate, propionic acid, (BDH Inc., Canada); midazolam, (Hoffman-LaRoche Ltd. Canada); halothane, (Ayerst Laboratories, Canada); buprenorphine, (Reckitt & Colman Pharmaceutical Division, England); sodium pentobarbital, (MTC Pharmaceuticals, Canada).

3.1.2 Results

NEC induction: In the 0 to 4 week old piglets (Group 1), acidified casein injected intestinal loops displayed macroscopic damage indistinguishable from neonatal NEC. Intestinal injury ranged from mucosal sloughing to full thickness necrosis and perforation, with more extensive damage occurring at 16 hours and in the younger animals. In the 0 to 3 day olds of Group 1, at 3 hours after NEC induction, histological grading of H&E stained gut sections from acidified casein injected loops revealed damage that extended to the muscularis. The average grade was 3.25 ± 0.13 and was characterized by patches of necrosis, submucosal edema and inflammatory cell infiltrate (Fig.3.2 b).

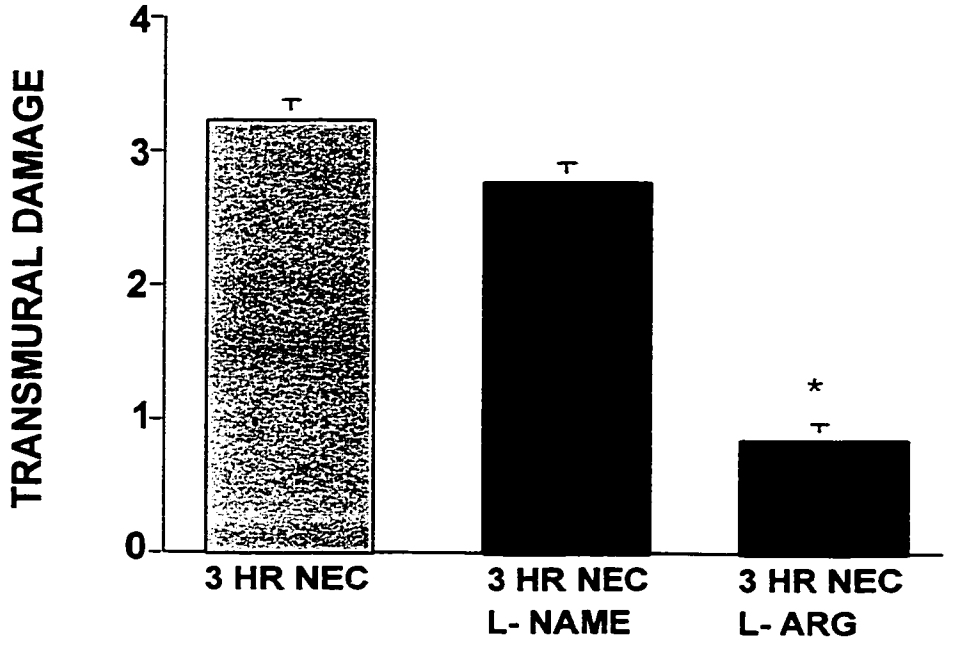


Fig. 3.1 Histograms of transmural damage scored in piglets with NEC. Treatment groups (n=4) are shown in the abscissa. Damage was graded from 0-4 in H&E stained frozen sections, with 0 representing completely intact villi; 1, representing villus tip necrosis with preservation of villus crypts; 2, representing necrosis of villus tips and crypts with loss of mucosal and submucosal architecture; 3, representing necrosis extending to the muscularis; 4, representing transmural necrosis. L-NAME treated animals displayed similar levels of damage to the NEC group. L-arginine treatment resulted in significant (* $p<0.0001$) attenuation of lesions with reduction of damage by 74% as compared to the untreated NECgroup.

Figure 3.2 Micrographs of H&E stained frozen sections taken from the distal ileum of piglets (<72 hours of age).

a: saline control

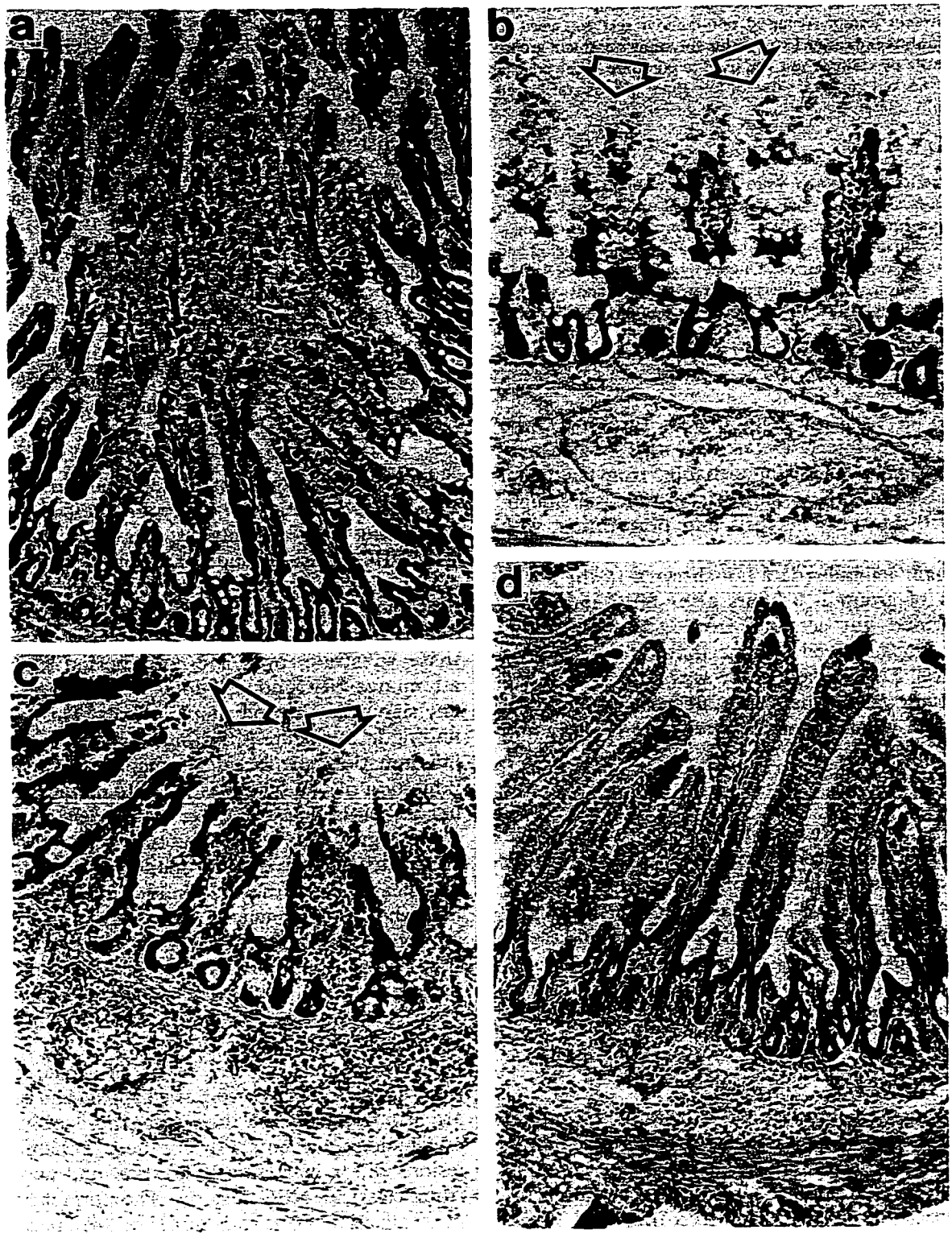
b: acidified casein-induced NEC

c: acidified casein-induced NEC in animals treated with i.v. L-NAME (20 mg/kg/hr for 3 hours)

d: acidified casein-induced NEC in animals treated with i.v. L-arginine (600 mg/kg/hr for 3 hours).

In micrograph **b & c** mucosal damage (indicated by open arrows) is extensive and extends to the villus crypts. In animals treated with L-arginine **(d)** damage is attenuated, limited to the villus tips.

Magnification: x125


a
b
c
d

L- NAME treatment: Continuous IV infusion of L-NAME caused a mild sustained increase (approximately 5 -10 mm Hg) in systolic and diastolic blood pressure. After 3 hours, the L-NAME treated, 0 to 3 day old piglets (Group 4) showed macroscopic hemorrhage in the acidified casein injected loops. Intestinal damage (2.78 ± 0.15) was similar to the untreated NEC group ($p>0.05$), but was characterized by marked hemorrhagic congestion (Fig.3.2 c).

L- Arginine treatment: Continuous IV infusion of L- arginine (Group 2) caused a sustained mild decrease (approximately 5 mm Hg) in systolic and diastolic blood pressure. All L-arginine treated animals demonstrated an absence of macroscopic lesions in the NEC-induced intestinal loops. Histologically, damage was greatly attenuated (by 74% compared to the untreated NEC Group 1 of the same age; $p<0.0001$) with an average grade of 0.86 ± 0.12. Necrosis was limited primarily to the villus tips. Inflammatory cell infiltrate and mild sub-mucosal edema were still present (Fig.3.2 d). These results are summarized in Fig.3.1.

Saline control segments: In all groups, the saline control intestinal loops showed an absence of macroscopic damage, with mean histological grades of necrosis of 0.17±0.08 for the NEC group, 0.11±0.10 for the L-NAME treated group and 0.06±0.06 for the L-arginine treated group ($p>0.05$ for all comparisons). None of the saline control loops differed significantly ($p>0.05$) from corresponding normal gut (Fig.3.2 a).

D-NAME and D-Arginine treatment: In these animals (Groups 3&5) the NEC-induced intestinal loops showed macroscopic tissue damage which was similar to the untreated NEC group (see Appendix VII). The mean histological grades of damage were 2.90±0.17 and 3.00±0.05 respectively.

3.1.3 Discussion

Our results demonstrate that in neonatal Yorkshire piglets, the intraluminal injection of acidified casein, by mimicking intraluminal substrate found in infants with NEC, causes tissue damage which is macroscopically and histologically similar to "classical" neonatal NEC. More importantly, we have shown that continuous intravenous infusion of L- arginine, an NO synthase substrate, over a three hour period significantly attenuates NEC-induced intestinal damage, without any significant hemodynamic effects. This particular finding in our acute intraluminal model of intestinal inflammation corroborates prior reports where inhibition of NO synthesis in acute models of intestinal ischemia is associated with increased gut injury (Payne and Kubes 1993). On the other hand, studies with chronic animal models using long term chemical administration to induce intestinal damage, have demonstrated the beneficial effects of the NO synthase inhibitor L-NAME on intestinal cytoprotection (Miller et al. 1993; Miller et al. 1993). This may be due to the protective effects of basal constitutive NO production in the acute setting, and the deleterious effects of induction of NO synthesis in the chronic setting (Miller, Chotinaruemol et al. 1993).

Perinatal risk factors, many of which produce varying degrees of mesenteric hypoxia and ischemia, are also implicated in the pathogenesis of NEC but as coincidental events. For instance, "ischemic" NEC lesions differ in anatomical location and appearance from "classical" NEC lesions. The former results in generalized necrosis and "gun shot "- like perforations of the gut limited to the given territory of mesenteric vascularization. The latter results in necrosis commonly localized to the terminal ileum and colon (Rowe 1986). In this study, precautions were taken during the surgical

procedure to eliminate the possibility of vascular compromise contributing to gut wall damage. Firstly, gut loops were tied off without injury to mesenteric vessels, and secondly, the quantity of intraluminal fluid injected was insufficient to distend the bowel segment to the point of ischemia.

Inflammation is necessary for the repair of injured tissue and for the control of infection. Leukocyte adhesion is key to initiating this process. Uncontrolled leukocyte infiltration is associated with many inflammatory disorders and may contribute to gut damage associated with NEC, also known as acute neonatal inflammatory bowel disease. Recent evidence indicates that NO prevents such leukocyte adherence (Gaboury et al. 1993). This may be one mechanism whereby NO production attenuates mucosal damage in our model.

It has been proposed that basal levels of constitutive NO production act to protect intestinal mucosa (Whittle et al. 1990; Kubes 1992), while induction of NO synthesis by bacterial endotoxin or cytokine(s) promotes intestinal damage (Tepperman, Brown et al. 1993). We hypothesize, therefore, that in premature infants, levels of constitutive NO have not reached "term" levels thus rendering their gut susceptible to damage. In support of this hypothesis is the finding that NO synthase activity undergoes an ontogenic pattern of development in piglet enterocytes (M'Rabet-Touil et al. 1993). Similarly, mesenteric and pulmonary artery endothelium production of NO has been shown to undergo a developmentally regulated increase in basal and stimulated levels in fetal and newborn animals (Shaul et al. 1993, Brown and Tepperman 1997). If constitutive production of NO undergoes an ontogenically determined pattern of development, cytoprotection and clearing mechanisms of fasting motor activity,

associated with increasing basal levels of NO, will render the gut less susceptible to damage by intraluminal substrate. We have further investigated this notion in premature piglets (section 3.2).

The data presented here point to the potential use of the amino acid L-arginine as a therapeutic modality in the treatment of NEC. Further studies using NO-donating drugs and the other product of the NO synthetic pathway L-citrulline, which newborn piglet enterocytes can convert to L-arginine (Blachier et al. 1993), will help characterize the the role of NO in the pathogenesis of NEC.

3.2 INTRAVENOUS L-ARGININE PROPHYLAXIS FOR NEC: THERAPEUTIC EVALUATION IN A PREMATURE PIGLET MODEL

Subsequent work was then carried out on premature animals using the same expertimental protocol. An additional group was added where the NO donor, sodium nitroprusside (SNP), was administered at known human therapeutic doses (3-10 μg/kg/min) and titrated to maintain blood pressure within normal limits. SNP was tested in order to determine whether the effects mediated by L-arginine were actually through NO production.

In addition, varying doses of L-arginine were tested prior to and immediately after NEC induction in order to ascertain the lowest effective dose and the appropriate timing of administration.

3.2.1 Methods

3.2.1.i *Model*

Briefly, under general anesthesia, neonatal piglets born 5 days prematurely were laparotomized and closed loops created from terminal ileum to proximal colon were injected with acidified casein, separated by saline injected control loops for a period of 3 or 6 hours. See Chapter 2, section 2.4 for details.

3.2.1.ii *Treatment and doses*

A continuous intravenous infusion of L-arginine, 20-600 mg/kg/hr (n=6), D-arginine, 600 mg/kr/hr (n=2), D (n=2) or L-NAME, an non-specific NO synthase inhibitor, 20 mg/kg/hr (n=4) or sodium nitroprusside (SNP), an NO donor, 3-10 μg/kg/min (n=6) was administered over a 3 hour period.

3.2.1.iii *Timing of treatment*

The above named drugs were then begun 3 hours prior to or 3 hours after NEC induction for a total experimental duration of 6 hours (L-arginine n=16, L-NAME n=6, SNP n=8). Continuous blood pressure monitoring was carried out via carotid artery cannulation.

Gut loops were then excised and processed for standard hematoxylin and eosin staining and graded microscopically as previously described: 0 = intact villi to 4 = transmural necrosis. The animals were euthanized with an intra-cardiac injection of pentobarbital.

Fig.3.3 Micrographs of NEC-induced damage in terminal ileum and colon in premature piglet after various treatments.

A. control, saline injected ileal loop, showing preservation of mucosal architecture

B. acidified casein-induced (NEC) damage in the terminal ileum. Note necrosis of the villus tips (arrows) extending to the submucosa

C. acidified casein-induced (NEC) damage in the terminal ileum after i.v. L-NAME.
Note the hemorrhagic necrosis of the mucosa which extends to the submucosa.

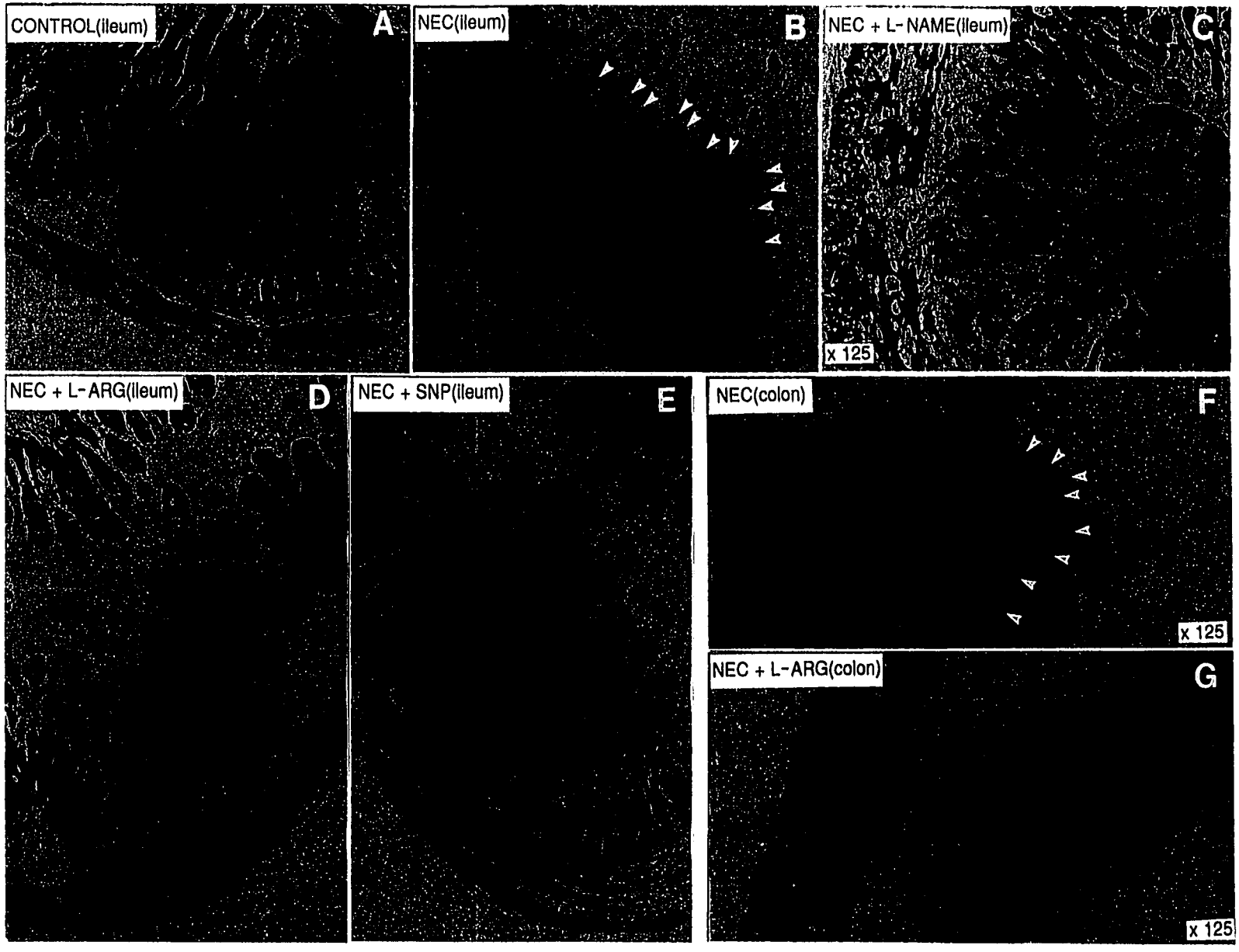
D. acidified casein-induced (NEC) damage in the terminal ileum after i.v. L-Arginine.
Note the attenuation of mucosal damage, with necrosis limited to the villus tips.

E. acidified casein-induced (NEC) damage in the terminal ileum after i.v. sodium nitroprusside.
Note that the extent of attenuation of damage is similar to the L-arginine treated animal.

F. acidified casein-induced (NEC) damage in the proximal colon. Note necrosis of the villus tips (arrows) extending to the submucosa

G. acidified casein-induced (NEC) damage in the proximal colon after i.v. L-Arginine.
Note the attenuation of mucosal damage, with necrosis limited to the villus tips.

Magnification in **A, B, D, E**: x75; in **C, F, G**: x125


CONTROL(ileum)
A
NEC(ileum)
B
NEC + L-NAME(ileum)
C
x 125
NEC + L-ARG(ileum)
D
NEC + SNP(ileum)
E
NEC(colon)
F
x 125
NEC + L-ARG(colon)
G
x 125

3.2.2 Results

All animals survived the experimental protocols without adverse systemic effects as documented by maintenance of normal vital signs (blood pressure, heart rate, temperature and respiratory rate). All test loops demonstrated macroscopic evidence of injury, the extent of which was easily assessed.

3.2.2.i *NEC induction group*

Histological damage in the untreated acidified casein loops included areas of necrosis (average grade = 2.6±.3), submucosal edema and mild inflammatory infiltrate, demonstrating no regional variability in the terminal gut (Fig.3.3 B&F).

3.2.2.ii *L-NAME treatment group*

Continuous i.v. infusion of L-NAME caused a mild sustained increase (approximately 5 mmHg) in systolic and diastolic blood pressures. Intestinal damage (average grade 2.6±.2) was similar to the untreated NEC group, but was characterized by marked hemorrhagic congestion (Fig.3.3 C red areas).

3.2.2.iii *L-Arginine treatment group*

Continuous i.v. infusion of L-arginine caused a sustained mild decrease (approximately 5 mmHg) in systolic and diastolic blood pressures. All L-arginine treated NEC-induced intestinal loops demonstrated greatly attenuated damage, with an average grade of 1.3±.15, compared to the untreated NEC loops. Necrosis was limited primarily to the villus tips (Fig.3.3 D&G).

Fig.3.4 Histogram of the effect of treatment doses of i.v. L-arginine (ranging from 20-600 mg/kg/hr) on graded depth of damage (0=intact villi to 4=transmural necrosis) in the 3 intestinal test regions when administered from the time of NEC induction over a 3 hour period. All doses were effective in attenuating damage.

ILE 30: distal ileum measured at 30 cm from the ileo-caecal valve.

ILE 10: terminal ileum measured at 10 cm from the ileo-caecal valve.

* = $p < 0.05$

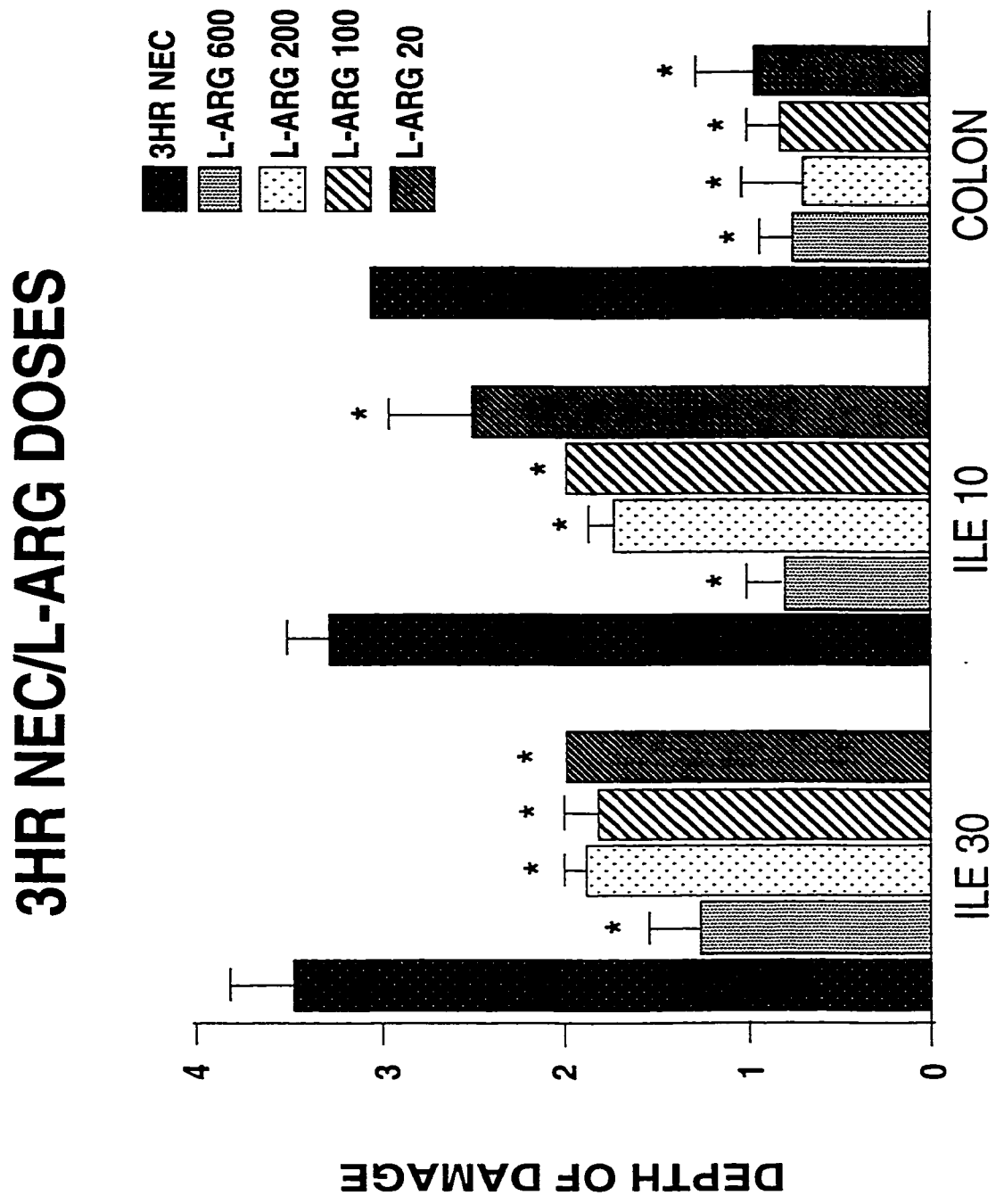
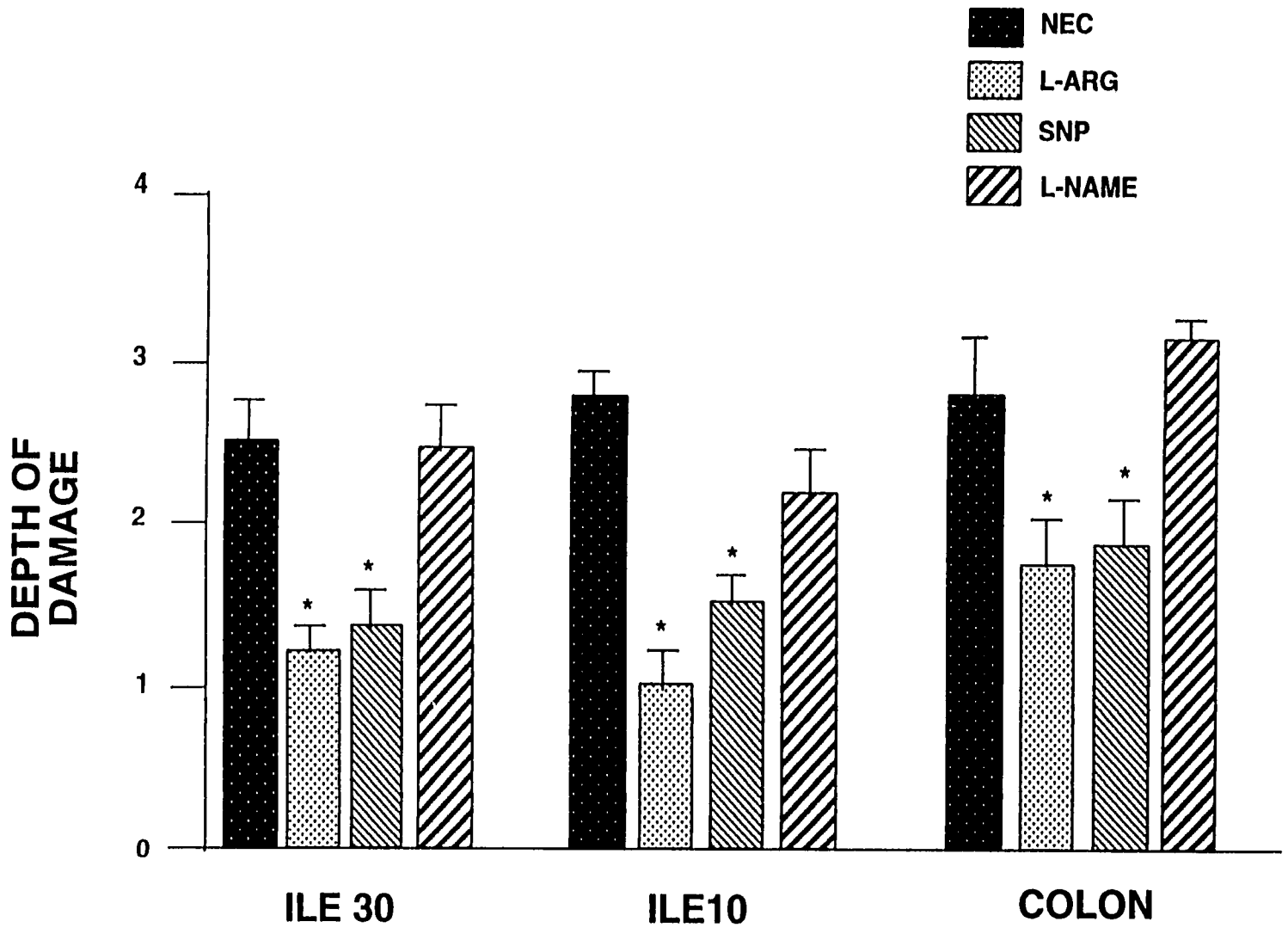

3HR NEC/L-ARG DOSES
3HR NEC
L-ARG 600
L-ARG 200
L-ARG 100
L-ARG 20
DEPTH OF DAMAGE
0
1
2
3
4
ILE 30
ILE 10
COLON
*

Fig.3.5 Histogram of the effect of different i.v. treatment regimens administered at the time of NEC induction and continued over the 3 hour test period as measured by graded depth of intestinal damage in the 3 test loops (see legend Fig.3.4).

PREMATURE / 3HR NEC VS TREATMENTS



3.2.2.iv *Sodium Nitroprusside (SNP) treatment group*

Continuous i.v. infusion of SNP was titrated to maintain blood pressure above 40 mmHg systolic and above 25 mmHg diastolic (3-10μg/kg/min). Attenuation of NEC-induced gut damage was similar to the L-arginine treated group with an average grade of 1.6±.2 (Fig.3.3 E).

Regardless of treatment, saline injected loops revealed no damage (Fig.3.3 A). As in term neonatal piglets, D-arginine and D-NAME treatments had no effect on NEC-induced damage in these prematures. The average grade of injury was 2.5±.4 and 2.75±.2 respectively.

In analysing the effects of the different treatments on the 3 test regions (ileum at 30 and 10 cm from the ileo-caecal valve and proximal colon), a significant reduction in intestinal injury was observed with all doses of L-arginine administered (Fig.3.4) and with SNP. L-arginine was equieffective with SNP in the distal ileum (ILE30) and colon, however L-arginine was more effective in the terminal ileum (ILE10). Once again, inhibition of NO synthase with i.v. L-NAME caused hemorrhagic congestion of the gut wall and was ineffective in altering the depth of intestinal damage induced by intraluminal test solution (Fig.3.5).

3.2.2.v. *Timing and doses of L-arginine treatment*

The extent of injury, as per grading system described, was shown to be similar in after 3 or 6 hours of acidified casein-induced damage in premature piglets (Fig.3.6). The lowest dose of L-arginine used was 500 mg/kg/**day** or 20 mg/kg/hr. This is the clinically used dose described in the literature. When this treatment was

Fig. 3.6 Histogram of the depth of intestinal damage in premature piglets at 3 and 6 hours after NEC induction (NEC 3hr, NEC 6hr) compared to simultaneous administration of low i.v. dose of L-arginine during the 6 hour period of damage (NEC 6hr & L-arg).

* $p < 0.05$

PREM/DURATION OF NEC/L-ARG (500MG/KG/DAY)

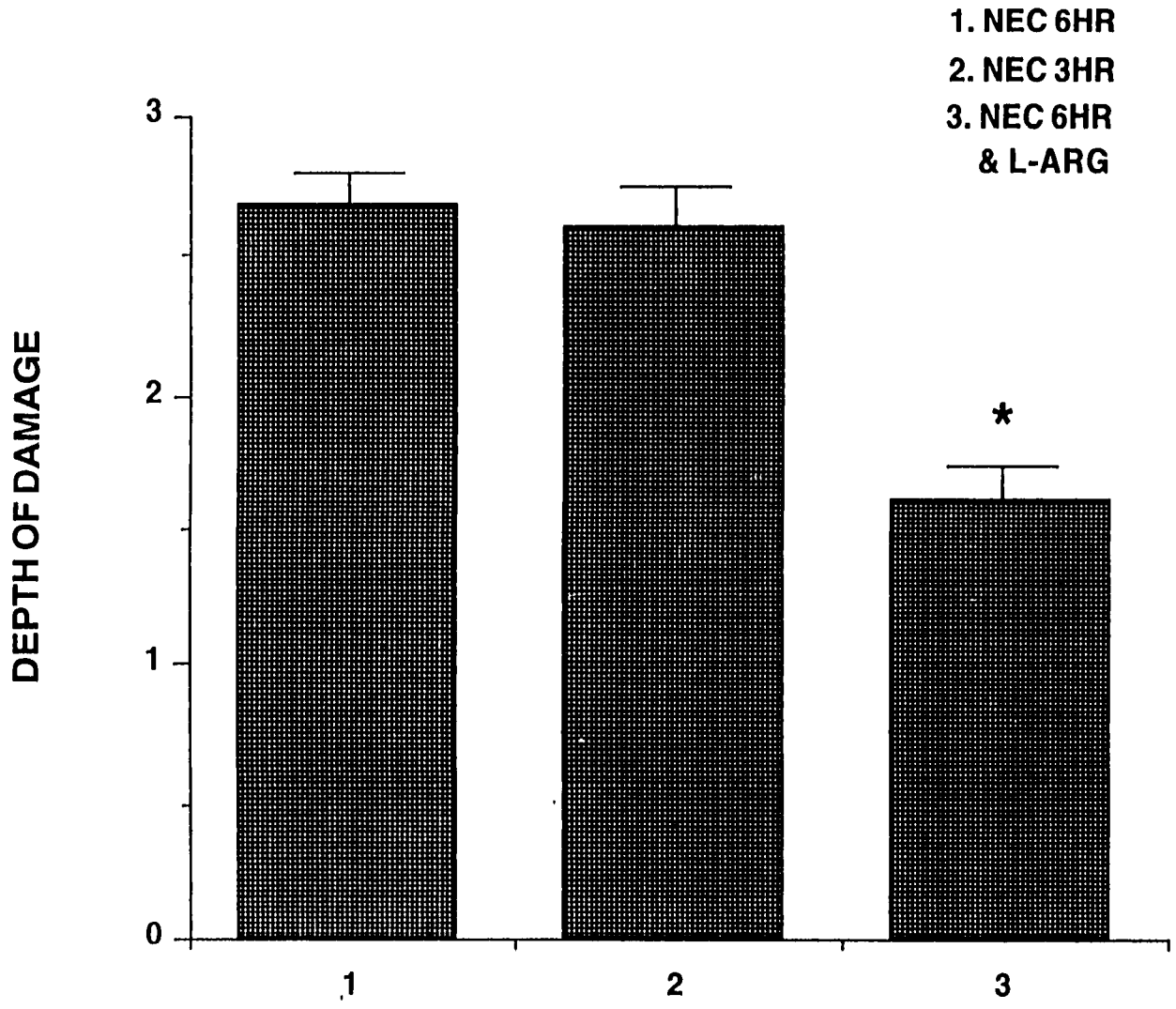


Fig. 3.7 Histogram of the depth of intestinal damage in premature piglets by varying the timing and doses of L-arginine administration.

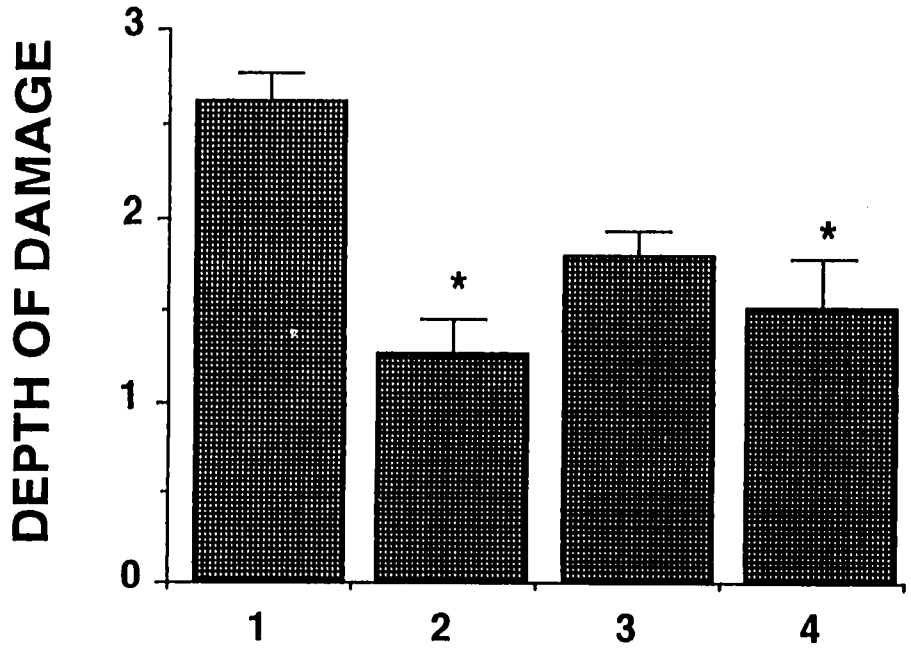
NEC 3HR: extent of damage at 3 hours

3HR L-ARG 500/ 3HR NEC: 3 hours of low dose L-arg followed by 3 hours of NEC. *= $p<0.05$

3HR NEC/ 3HR L-ARG 500: 6 hours of NEC with low dose L-arg over the last 3 hours

3HR NEC/ 3HR L-ARG **600**: 6 hours of NEC with **high** dose L-arg over the last 3 hours. *= $p<0.05$

C-PREM/NEC/TIME/DOSE



1. NEC 3HR
2. 3HR L-ARG 500/3HR NEC
3. 3HR NEC/3HR L-ARG 500
4. 3HR NEC/3HR L-ARG 6OO

administered 3 hours after NEC induction, over a 3 hour period, we observed no effect on the extent of gut lesions. However, if the higher experimenetal dose commonly used in the scientific literature (600mg/kg/hr) was given during the same time frame, a significant attenuation of damage was seen (Fig.3.7). Of great importance to this work is that the continuous prophylactic i.v. administration of the lower clinical dose of L-arginine (20mg/kg/hr) over 3 hours prior to NEC-induced damage was effective in attenuating gut injury as observed after the 3 hour injury period (Fig.3.7). In addition, the concommitant administration of this clinically used dose of L-arginine at the time of intestinal injury was also effective in decreasing the depth of damage (Fig.3.6).

3.3 DISCUSSION

In neonatal premature piglets, intestinal injury induced by this intraluminal model of NEC is similar to that which we described in term animals. Because of the fragile nature of these animals, all necessary precautions were taken in order to insure that any adverse contributory factors, such as hypothermia and hypoxia, were avoided. We also found that the duration of insult, whether 3 or 6 hours, had no significant effect on the depth of gut damage. This may be explained by the time course of induction of NO synthesis which does not seem to occur during the first 180-240 minutes of intestinal injury as documented in an ischemia/reperfusion feline model (Kanwar et al. 1994). Thus, the absence of excess NO production during the 2 experimental time frames may have affected the anticipated extent of gut damage in the 6 hour group.

Histological assessment of damage was again typical of human NEC, and interestingly, inflammatory infiltration was very mild or absent due to the acute nature of

the insult. This is also in keeping with human NEC histology (Kleinhaus, Weinberg et al. 1992). Furthermore, myeloperoxidase activity, which is an index of inflammatory infiltration, was not elevated in these gut specimens (see Appendix VI).

The marked hemorrhagic congestion associated with L-NAME treatment (Fig.3.3 C) has also been described in a rat model of LPS-induced intestinal damage (Wang et al. 1994). The early clinical manifestation of heme-positive stools in infants with suspected or proven NEC may thus be a reflection of inhibition of NO release. L-NAME is a non-selective NO synthase inhibitor which had no effect on acidified casein induced gut damage. Despite the mild increase in blood pressure observed with i.v. L-NAME, intestinal vasoconstriction has been shown to persist, thus abrogating any potential benefit of the improved cardiac output (Spain et al. 1994). Interestingly, Kubes (1993) had shown that feline superior mesenteric blood flow is decreased by L-NAME which also causes an increase in gut microvascular permeability. Inhibition of NO production by L-NAME increases gut epithelial cell permeability which is leukocyte independent (Kubes 1993; Granger and Kubes 1996). Exogenous sources of NO were then shown to reduce mucosal barrier dysfunction independent of alterations in intestinal blood flow (Payne and Kubes 1993).

L-arginine or SNP treatment at the time of NEC induction significantly decreased the extent of gut damage in all 3 test regions, however, L-arginine was more effective than SNP in the terminal ileum. In the piglet, as in the human, this gut region is the site of lymphatic Peyer's patches. In the rat, intestinal lymphatic vessels have been shown to release endothelial-dependent vasodilators which dilate nearby arterioles and cause spontaneously active lymphatic vessels to constrict (Bohlen and Lash 1992), thus

possibly decreasing bacterial translocation and protecting intestinal barrier function. More recently, L-NAME has been shown to accelerate the appearance of lymphocytes in these lymphatics (Hokari et al. 1998). This may explain why L-arginine was more effective in the terminal ileum of our piglet model.

The highest levels of cNOS and iNOS activities have been described in intestinal villus cells and lowest in the crypts. Induction of NO synthase activity has been associated with a decrease in cellular viability (Tepperman, Brown et al. 1993). Therefore, because acidified casein-induced damage is initiated in the villus tips, we believe that the resulting decrease in NO availability is detrimental to the gut. The protective effect of L-arginine may thus be explained. In investigating the ontogenic variations in arginine metabolizing enzymes, proximal gut loops of the fetal rat contained higher concentrations than the more distal loops. This difference was no longer evident after birth (De Jonge et al. 1998). These findings may contribute, in part, to the regional predilection of the terminal ileum to NEC.

The sepsis resulting from altered gut barrier function (bacterial translocation) has been shown to alter intraluminal absorption of amino acids, particularly L-arginine (Sodeyama et al. 1993). Thus our systemic route of administration assures L-arginine availability by circumventing enteral absorption. Constitutive NO has been shown to counteract intestinal mucosal injury in acute endotoxemia (Laszlo and Whittle 1994) and in hypoxia-induced gut injury (Caplan et al. 1992). Inhibition of NO synthesis in a canine model of endotoxemia improved arterial blood pressure without improving intestinal microvascular O_2 extraction (Schumacker et al. 1995). Endogenous NO was later shown to attenuate sympathetic evoked constriction in intestinal microvasculaure in

the rat (Nase and Boegehold 1996). Nowicki and co-workers (1992) showed that in the neonatal piglet, the NO-cGMP axis is of greater importance in the regulation of intestinal vascular resistance in the early post natal period (3-day versus 35-day old piglets). Thus, ontogenic variations in the control of intestinal blood flow in the piglet render the gut more susceptible to variations in NO availability in the early neonatal period. Later work from this group demonstrated that the early post-natal intestinal circulation constricts in response to sustained low blood flow, and occurs in the 3-day but not 35-day old piglets (Nowicki 1998). This was postulated to result from a decrease of NO production and was subsequently confirmed through the demonstration that in newborn intestine, sustained reduction in intestinal blood flow decreases constitutive NO production, thus enhancing vasoconstrictive efficacy (Nowicki 1999). In the mid-gestational fetal lamb, NO has also been shown to play a more important role in regulation of blood flow and vascular tone in the gut than later in development (Fan et al. 1998).

In support of our findings regarding the beneficial effects of i.v. L-arginine on NEC-induced intestinal injury, recent analysis of plasma arginine concentrations in premature infants with NEC were found to be decreased when compared to control prematures (Zamora et al. 1997). Prophylactic adminstration of L-arginine had also been shown to improve gut barrier function after mesenteric ischemia in the rat (Schleiffer and Raul 1996) and after small bowel transplantation (de Oca et al. 1997). We have, in addition, documented the beneficial effects of low dose L-arginine prior to induction of acidified casein-induced gut damage in the premature piglet.

In trauma victims, the early administration of L-arginine at the onset of fluid resuscitation improved cardiovascular function (Angele et al. 1998).

Hyperlacticacidemia characteristic of hypoperfusion has been shown to decrease intestinal synthesis of arginine and citrulline in pig enterocytes, further affecting NO availability (Dillon et al. 1999).

In conclusion, we have adapted an intraluminal model of NEC which resembles the human condition, both macroscopicaly and histologically, to the premature piglet. We have also shown that manipulation of the nitrergic system, by providing the NO synthase substrate, L-arginine, or the NO donor, sodium nitroprusside, attenuates NEC-induced intestinal damage without any systemic effects. Furthermore, prophylactic i.v. administration of clinically used doses of L-arginine is also effective in attenuating this damage. Supporting evidence for a developmental predisposition to NEC has been presented as well as its potential relationship to constitutively derived NO. The potential large-scale therapeutic application of these findings in the prophylactic treatment of NEC as well as possible physiological mechanisms as they relate to the ontogeny of enteric nitrergic innervation and enzyme activities are described in the forthcoming chapters.

CHAPTER 4

ONTOGENY OF THE PIGLET ENTERIC NERVOUS SYSTEM: NITRIC OXIDE, MOTILITY AND NEC

4.1 THE ENTERIC NERVOUS SYSTEM

The Enteric Nervous System (ENS) in reference to the fine nerve networks intrinsic to the gastrointestinal tract (Langley 1921) controls gut reflex behaviour and responses to stimuli. Included among them are intestinal motility, blood flow, ion transport, acid secretion and release of chemicals. These behaviours, although dependent upon the enteric nerve networks, can be modulated by the CNS via sympathetic and parasympathetic innervation of the gut wall. Knowledge of the structural organization of the gut wall and its neural elements along with the ontogeny of these constituents is necessary to the understanding of potential pathophysiological mechanisms affecting gut development and its susceptibility to NEC.

4.1.1 Anatomy of the gut wall

The gut wall consists of a planar longitudinal arrangement of various layers of neural and non-neural elements. The outermost layer is the serosa, and the innermost is the mucosa. From serosal to mucosal surfaces are the following principal layers: *longitudinal smooth muscle, myenteric nerve plexus, circular smooth muscle,* the *sub mucosa* consisting of Henle's nerve plexus, the vascular plexus and Meissner's nerve plexus, and the *muscularis mucosae* (fig. 4.1).

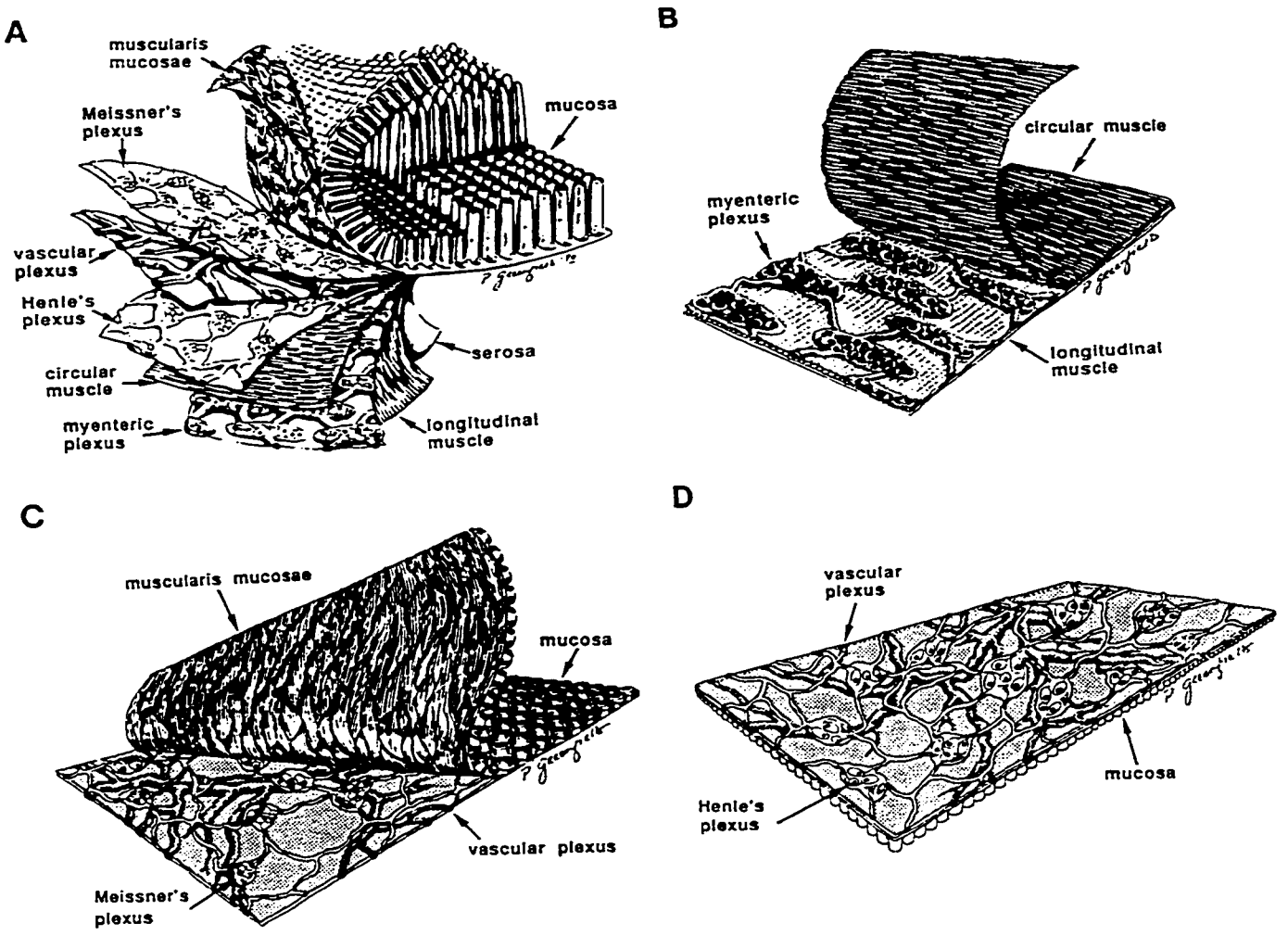


Figure 4.1 Schematic diagrams of intestinal wall. (A) Illustration of the intestinal wall with the laminae partially dissected to display the organization of the neural and vascular elements. B, C and D display those preparations taken from the piglet intestine. (B) A laminar preparation of the myenteric plexus with the circular muscle peeled back. (C) A laminar preparation "mucosa up" showing the muscularis mucosae and mucosa partly separated to reveal Meissner's plexus and the underlying nerve networks. (D) A laminar preparation oriented "mucosa down" with Henle's plexus uppermost.

4.1.2. Enteric neurons

Despite the complexity of its roles, the ENS is a phylogenetically old system whose function is based almost exclusively on reflex activities. Enteric neurons have been classified into various types, based on morphology, neurochemistry and function. Dogiel (1899) proposed the first classification of enteric neurons which consisted of 3 types of morphologically distinct nerve cells. Type I cells were flattened and angular with several short dendrites and a long axon coursing through several ganglia before ramifying within the circular muscle. Type II cells were elongated and angular, with fewer dendrites and axons arising from the conical part of the cell and projecting to adjacent ganglia. Type III cells were similar to Type II cells but with shorter dendrites and axons confined to the ganglia of origin or adjacent ganglia. In addition, Dogiel proposed that neuron morphology was related to function. Motor neurons were Type I cells because their axons go to muscle, and Type II cells were sensory since some were seen to project to mucosa. Later, other cell types were identified which did not fit Dogiel's initial description. This morphological classification of neurons was later expanded to included a total of 8 types (Stach 1989).

Pharmacological and electrophysiological investigations have revealed thus far three functional groups of enteric neurons (Costa et al. 1992). Motor neurons affect intestinal activity by evoking muscular contraction or relaxation, vasoconstriction or dilation, or mucosal transport of water and electrolytes. Sensory neurons monitor the chemical composition of intestinal contents or transmural tension. Interneurons form connections between enteric neurons and relay information from sensory to motor neurons.

4.1.3. Enteric neurotransmitters

The classification of enteric neurons, through the correlation of functional and neurochemical status, is an ongoing process. Over 30 functional types of neurons are described, using several of the approximately 25 different neurotransmitters identified. Several neurotransmitters are utilized by most neurons, with one as a primary transmitter and others as neuromodulators. The primary transmitter is responsible for acute changes in excitability of the innervated cell. These primary transmitters are conserved in functionally equivalent neurons in different regions of the gastrointestinal tract in different species. However, neuromodulators of equivalent neurons are not necessarily the same.

Only 7 of the enteric neurotransmitters are thought to be primary transmitters. Acethylcholine (ACh) is the primary transmitter of enteric interneurons, excitatory motor and secretomotor neurons. Tachykinins are co-primary transmitters of a subpopulation of cholinergic motor neurons. Tachykinins are proposed to be primary transmitters of enteric sensory neurons at neuro-neuronal synapses. Serotonin is a primary transmitter of enteric interneurons. Nitric oxide (NO) is a primary transmitter of enteric inhibitory motor neurons and has recently been identified as a transmitter of interneurons. Vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP) are neuromodulators of enteric neurons, but are also primary transmitters within separate populations of inhibitory motor neurons. For example, VIP is the primary transmitter of non-cholinergic secretomotor neurons. Motor neurons to gastrin cells use gastrin releasing peptide as their primary transmitter (reviewed in McConalogue and Furness 1994).

4.1.4. Nitric Oxide (NO)

As a neurotransmitter, NO was initially discovered in the brain, where its synthesis is stimulated by Ca^{++} (Garthwaite 1991). NO then diffuses to neighbouring cells, interacts with the ferrous heme of soluble guanylate cyclase and stimulates cyclic GMP formation. Moncada et al (1991) have observed this process in inter neuron signalling and between neurons and glial cells.

Synthesis of NO by neuronal NO synthase may occur when local calcium concentrations exceed 500 nM (Schmidt, Smith et al. 1992). Electron microscopy of enteric neurons indicate that NO synthase may be localized beneath the plasma membrane where it would be directly exposed to entering calcium (Llewellyn-Smith et al. 1992). Thus, it is likely that the classical concept of prestorage and release of transmitter by vesicular exocytosis is not applicable to NO neurons, but rather that release occurs on demand (Sanders and Ward 1992). Moncada's group reported visualization of NO release, through the generation of photons produced by its reaction with luminol and hydrogen peroxide, from electrically stimulated myenteric plexus and hypogastric nerve (Wiklund et al. 1993). The emitted light was observed at nerve terminals and at the axon and soma, indicating that NO released form the nerve cell may affect targets surrounding all parts of the nitrergic neurons. NO thus possesses a unique mode of synaptic and non-synaptic communication.

The fact that NO synthase displays reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) activity in fixed tissue by reducing a tetrazolium dye to a visible blue formazan precipitate in an NADPH-dependent manner (Hope et al. 1991) has been used to investigate the anatomy of nitrergic enteric

innervation. Corroborating data exist indicating that sites of NO synthase activity can be detected by NADPHd histochemistry. For example, in the ENS, NADPHd positive neurons are also NO synthase immuno-reactive (Schmidt et al. 1992; Timmermans et al. 1994; Young et al. 1997; Young and Ciampoli 1998; Belai and Burnstock 1999). NO synthase is the only enzyme in the ENS known to retain NADPHd staining after aldehyde fixation of tissues (Huang et al. 1993). Calcium chelators and NO synthase inhibitors do not affect the level of staining indicating that arginine and NADPH interact at separate sites of the NO synthase molecule. The diaphorase activity of NO synthase is due to the reduction by NADPH of a flavin associated with NO synthase which directly reduces the tetrazolium dye, since it has been shown that inhibition of diaphorase staining occurs with binding of the flavin moiety of NO synthase with diphenyleneiodonium (DPI) (Griffith and Stuehr 1995). NADPHd histochemistry is thus a simple and economical method for localizing NO synthase activity.

Historically, the first direct evidence of NO production from guinea-pig myenteric plexus enteric ganglia was provided by Grider and Jin (1993). NOS-immunoreactive neurons were demonstrated in all 3 plexuses of the porcine small intestine, with very few positive nerve cells in the internal submucosal plexus (Krammer 1993). Similar findings were also confirmed in the rat using NADPH diaphorase histochemistry by investigators in this laboratory and others (Aimi et al. 1993; Nichols, et al. 1993b; Nichols et al. 1994a). Concomitant work from this laboratory investigated vascular and neuronal NO synthase activity in the human gut (Nichols et al. 1993a; Nichols et al. 1994b).

Histochemical and immunohistochemical techniques were subsequently used to classify enteric nerve cells in the porcine small intestine. Using acetylcholinesterase (AChE), nicotinamide adenine dinucleotide diaphorase (NADHd) and nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) enzyme histochemistry, De Ridder and Weyns (1994) investigated the ganglionated plexuses of the porcine small intestine ENS. They demonstrated that postnatal porcine enteric small bowel neurons can be subdivided into AChE-NADHd and NADPHd populations. Since neuronal NO synthase enzyme colocalizes with NADPHd (Hope et al. 1991), they suggested a further subdivision of these enteric neurons into AChE-NADHd and NOS-NADHd types. Interestingly, they also noted that the number of NADPHd neurons seemed to be less in the fetal specimens. Barbiers and co-workers published similar findings in the porcine large intestine (Barbiers et al. 1994).

Timmermans and co-workers (1994) investigated the presence of NO in the developing human enteric nervous system, once again confirming animal findings. However, they also observed a decrease in the density of NOS containing neurons in the outer (Henle's) submucosal plexus and myenteric plexus with increasing post conceptional age, in addition to their variable morphological appearance with development. Later work on the ontogeny of the ENS demonstrated, in duodenum from fetal, newborn and weanling pigs, that nitrergic neurons as identified and counted using NADPHd histochemistry doubled postnatally in the myenteric plexus, whereas those in the inner submucosal plexus increased 50-fold during gestation, to fall to 10% of the prenatal value at birth and return to prenatal values with development (Van Ginneken et al. 1998).

In a more detailed study of the ontogeny of nitrergic innervation of the developing human gut, Brandt et al (1996) using fetuses ranging in gestational age from 12 to 23 weeks, showed that by 12 weeks, nitrergic neurons were present in the myenteric plexus at all gut levels. Nitrergic innervation of the submucosal plexus became evident at 14 weeks, with a mature postnatal pattern being present at 23 weeks gestation. These authors confirmed that the onset and pace of development of nitrergic innervation of the human gut are similar to adrenergic and cholinergic innervation, and occur prior to peptidergic innervation.

More pertinent to our work is recently published data by Sigge et al. (1998) presenting their findings regarding the morphological alterations of the ENS in human NEC. NANC inhibitory innervation damage, as characterized by loss of NO and VIP immunoreactivity in the submucosal plexuses, was observed in even the early stages of NEC. Speculation is that these early neuropathological lesions induced by NEC may cause futher intestinal injury through dysmotility with the resultant functional obstruction leading to intraluminal bacterial overgrowth and translocation. These findings, however, are not likely to be specific to NEC-induced gut damage, since any pathophysiological process causing intestinal necrosis can affect the viability of different components of the gut wall, including the various nerve plexuses.

NO is thus a mediator of intrinsic inhibitory innervation of the gastrointestinal tract resulting in intestinal smooth muscle relaxation. The status of this innervation can profoundly influence gut motility and function. However, little is known about this innervation in the *developing* gut. We therefore investigated the distribution of

NO neurons in myenteric plexus (MP) and in Henle's plexus (HP) in developing piglet intestine using NADPH diaphorase histochemistry of gut laminar preparations.

4.1.4.i *Materials and Methods*

Piglets were obtained from Agriculture Canada, Ottawa and delivered to the animal care facility at the University of Ottawa on the day of the experiment. Premature piglets were born 9 days preterm by cesarian section and transported in heated trucks and wrapped in warming blankets to minimize the effects of hypothermia. These animal were unfed prior to sacrifice. The older animals were born at term by spontaneous vaginal delivery and had been suckling. All animals were sacrificed using intracardiac injection of sodium pentobarbital (65mg/ml). A midline laparotomy incision was used to harvest intestinal segments 2.5 to 5 cm in length. Duodenal segments were harvested distal to the ampulla of Vater. Small bowel segments were harvested in the mid-jejunum and distal ileum, followed by the caecum and the mid colon. Intestinal contents were cleared and the segments were immediately washed in pre-chilled 0.1 M sodium phosphate buffered saline (PBS), pH 7.2. Specimens were then incised along their mesenteric border opened and pinned to styrofoam with the mucosal side face up and incubated for 2 hours at 4°C in 4% paraformaldehyde / 0.4% picric acid in 0.16 M sodium phosphate buffer (PB), pH 7.0. The segments were then rinsed 3 times in pre-chilled 0.1 M PBS, pH 7.2 and stored at 4°C in fresh PBS.

The different layers of the gut wall were dissected using a modification of the method described by Nichols, Staines and Krantis (1993b). Intestinal segments were removed from the styrofoam supports and stretched and pinned to the bottom of a 0.1 M

PBS-filled container (pH 7.2) with serosal side up, under a dissecting microscope at 3-5X magnification. Laminar preparations of the myenteric plexus were obtained by incising along one edge of the specimen to a depth allowing the separation of serosa, longitudinal muscle and myenteric plexus together from the underlying layers using fine forceps. This laminar preparation was positioned with the myenteric plexus up and stripped of any adherent circular muscle fibers. The remaining layer of tissue was further dissected to expose the outer submucosal plexus (Henle's plexus) layer by stripping off the circular muscle. The inner submucosal layer (Meissner's plexus) is exposed by turning this specimen over and dissecting off the overlying mucosa. These laminar preparation of myenteric plexus, Henle's plexus (mucosa down) and Meissner's plexus (mucosa up) were then stretched onto electrostatic glass slides, allowing the outer edges of the preparations to dry and adhere. The air-dried laminar preparations were then incubated overnight at 4°C in 3% Triton x-100 0.1 M PBS pH 7.2 in order to enhance penetration of the histochemical stain.

Laminar preparations were then processed for histochemical localization of nitrergic neural elements after the method of Nichols, Staines and Krantis (1993b). Specimens were washed in several changes of phosphate buffer (10mM PB, pH 8.0) and incubated for 1 hour in a dark moist chamber at 37°C with a reaction medium for NADPH diaphorase activity consisting of 1mM NADPH, 0.5 m M nitro blue tetrazolium (NBT), and 0.3% Tritonx-100 in 10mM sodium phosphate buffer, pH 8.0 laminar preparations were then given a final wash with 10mM PB, pH 8.0, air dried, coverslipped with permount and analysed under light microscopy.

Laminar preparation of MP and HP of small and large intestine at different stages of development from piglets 9 days premature (n = 4), < 72 hours old (n = 4) and 2 weeks old (n = 4) were evaluated for the frequency of NO neurons in the duodenum, jejunum, ileum, caecum and colon. The average frequency of NADPH diaphorase positive labelled cells per ganglion was calculated from a sample population of 50 ganglia in the myenteric plexus and in Henle's plexus in each of the 5 anatomical regions of the 12 piglets. Only ganglia from which intact primary fasciculi could be identified were counted. Ganglia at the edges of slides were excluded. Extraganglionic cells were not counted.

Chemicals

Nitro blue tetrazolium (NBT), β-nicotinamide adenine dinucleotide phosphate, and its reduced form (NADPH) were purchased from Sigma, St.Louis, Missouri, U.S.A. Remaining reagents were purchased from BDH, Canada.

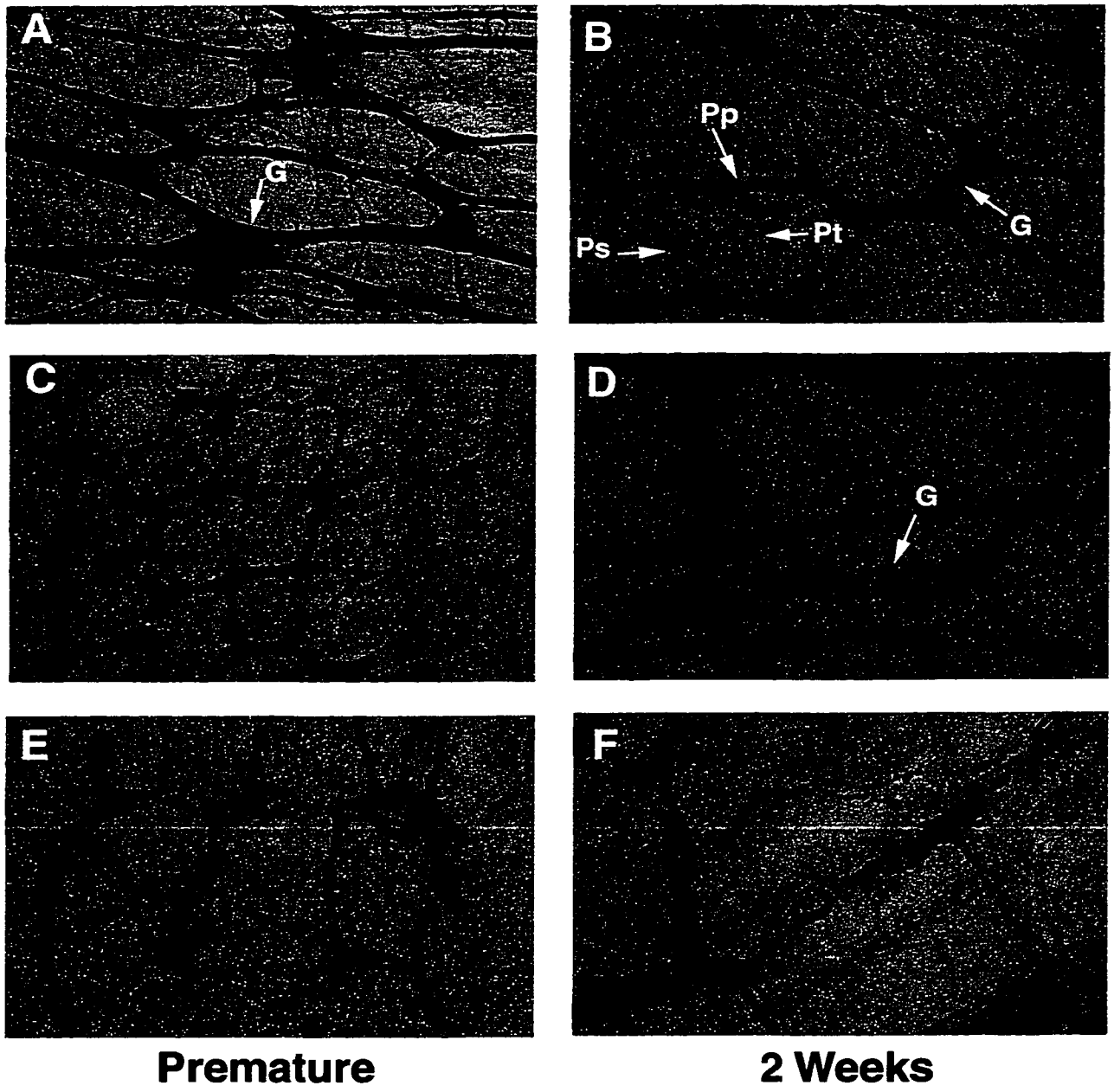
Statistical Analysis

The Instat computer package was used. ANOVA and Bonferroni post test was done to compare the differences in frequency of NADPH diaphorase positive neurons in the different layers of representative regions of the gut wall at different stages of development. P value of < 0.05 was considered significant.

Fig. 4.2 Light microscopic micrographs of laminae of the myenteric plexus from the intestine of piglets (9-day premature and 2-week old) treated for histochemical localization of NO synthase-related diaphorase activity.

A, B = Duodenum. C,D = Caecum. E, F = Colon. Intensely stained nerve cells and their processes present in the ganglia (G). Intensely stained fibres can be seen within the ganglia and interconnecting primary (Pp) and secondary (Ps) and tertiary (Pt) meshworks of this nerve network.

Magnification: x 125


A
G
B
Pp
Ps
Pt
G
C
D
G
E
F
Premature
2 Weeks

4.1.4.ii *Results*

Morphology

The morphologies of labelled neurons and nature of the fibre distribution was indistinguishable between the premature, neonatal and adult piglet intestine. For purposes of comparison, only micrographs of stained tissue from premature and 2week old piglets is presented here.

Laminar preparations of the myenteric plexus and submucosa taken from the piglet (9days premature, less than 3days old and 2weeks old) intestine and histochemically treated for NO synthase-related NADPH diaphorase activity showed all myenteric and Henle's nerve plexus ganglia to contain positively labeled cells (Fig.4.2). Intensely stained axonal processes can be followed from individual neurons as they course through the ganglion and on into the interconnecting fibre bundles. Intensely stained fibres can also be seen above the plane of the nerve networks at the level of the interface between the circular muscle layer and the submucosa typical of the fine network of nerve fibres located at this interface. Preparations of the myenteric plexus where overlying circular muscle has not been completely removed reveals NO synthase reactive fibers (Fig.4.3). The primary, secondary and tertiary meshwork fibre bundles of the myenteric plexus are clearly discernible (Fig.4.2B). Frequently, NO synthase related extraganglionic cells could be found in the interconnecting fasciculi of the primary meshworks.

There was no pattern to the projection of labeled fibres in the myenteric meshworks, projecting as they did, circumferentially, radially, aborally and orally. In many instances these labeled fibres were lost from view before any termination could be

Fig 4.3 A,B High magnification micrographs of a myenteric ganglion (G) from the colon of a 2-week old piglet, showing intensely stained cells and fibres. In micrograph A, the focal plane is centred upon stained fibres (arrow). Intensely stained fibres can be seen coursing in the plane of the circular muscle layer (cm) still adherent to the underlying submucosal laminae. **C**. High magnification micrograph of a submucosal arteriole (Va), showing intensely stained puncta in the endothelium. **D**. High magnification micrograph of a ganglion in the Meissner's plexus photographed with the laminar preparation oriented with the mucosal side uppermost. The ganglia displaying a typically rich innervation of stained fibres forming networks around clearly unlabelled cells. The underlying vascular plexus is evident by the presence of an arteriole.

Scale bar=50μm

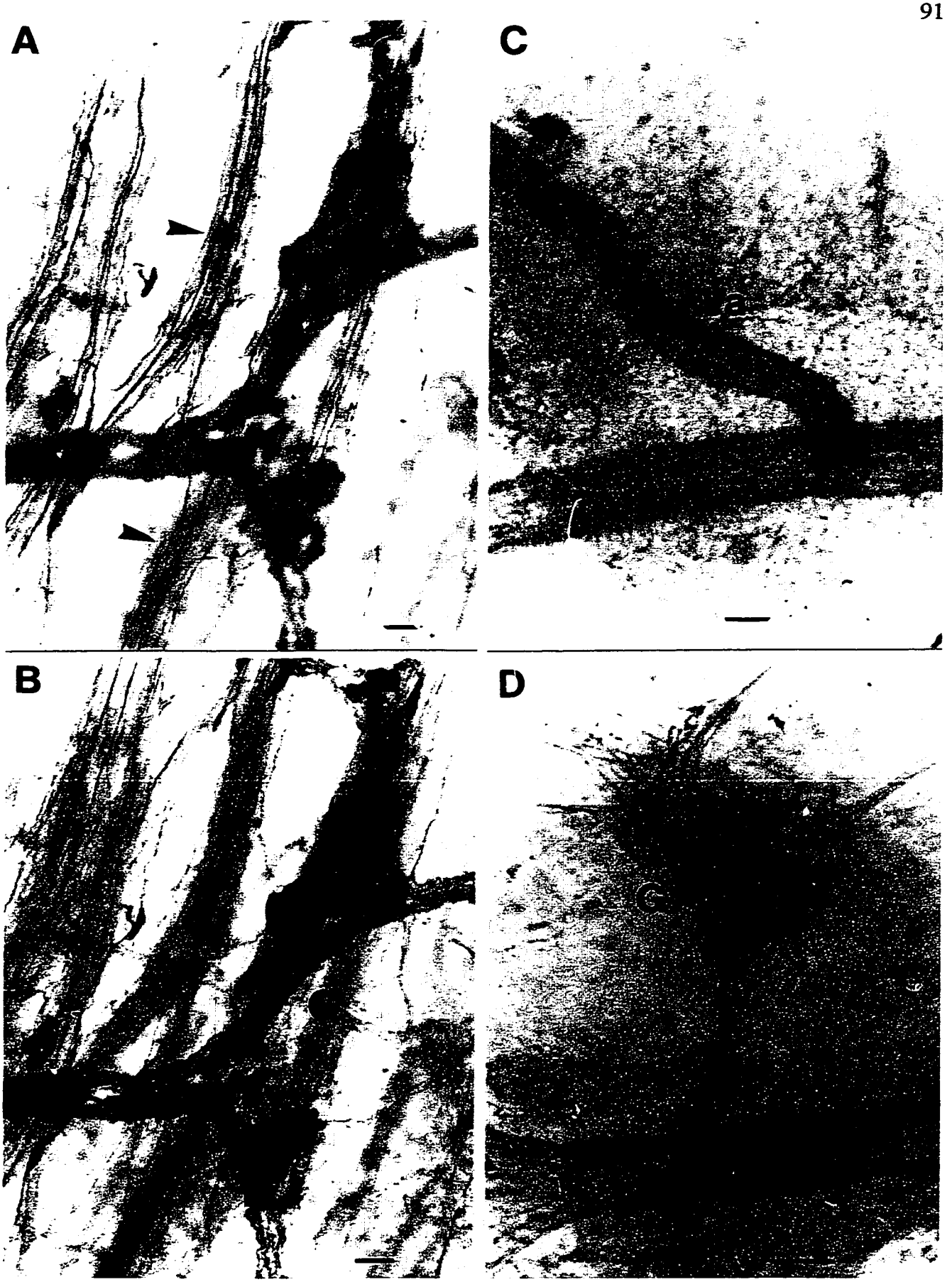
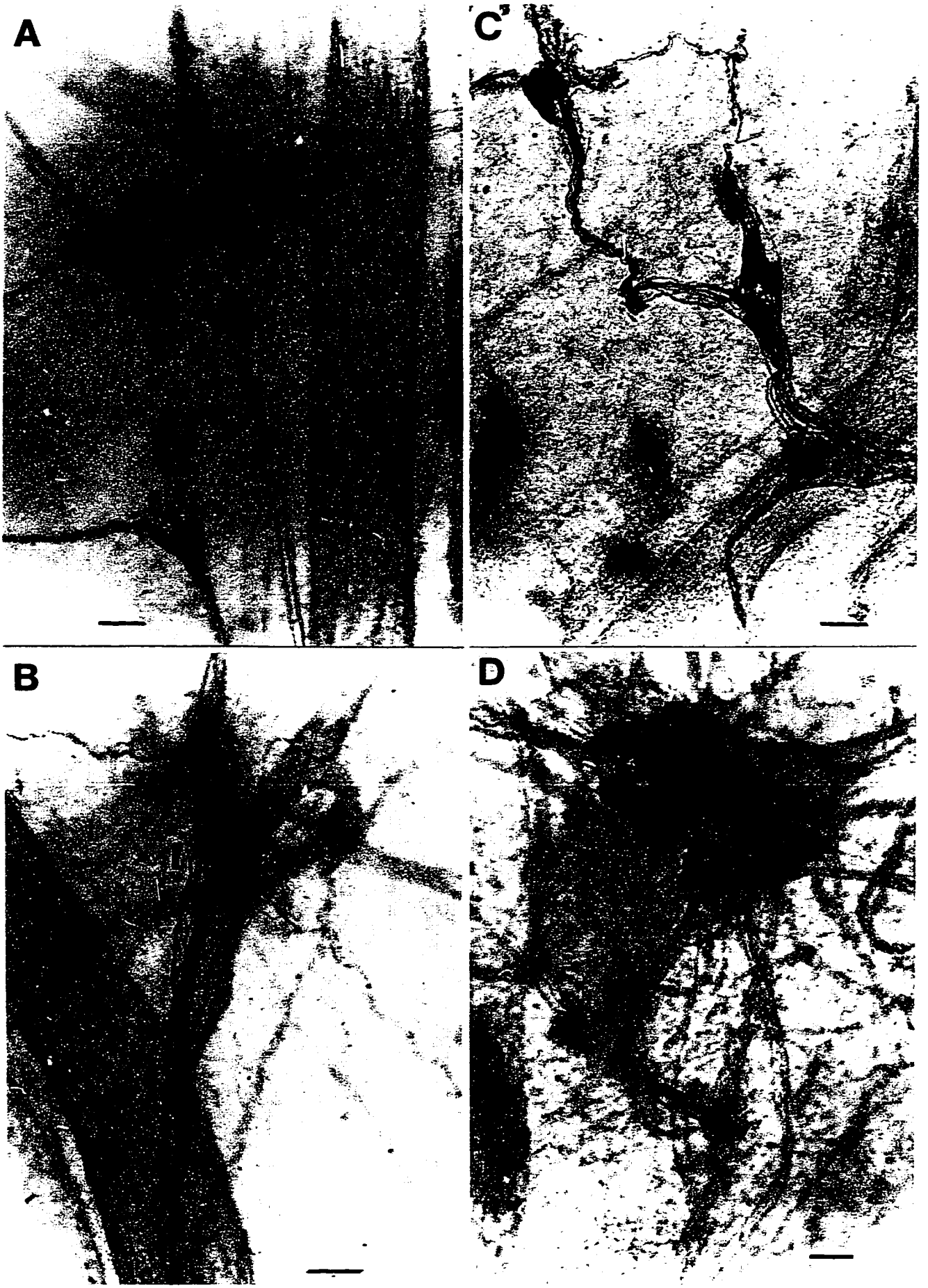

A
C
B
D

Fig.4.4 High magnification micrographs of laminar preparations of the submucosa positioned with the mucosa down. Each micrograph shows ganglia from the Henle's plexus containing intensely stained neurons and nerve fibres. These fibres could be seen to either i) emanate from clearly identifiable neurons and project outside the ganglion into the interconnecting fibre bundles (C), ii) enter the ganglion and ramify within the ganglion (A) or else, iii) form en passant fibres (D). In B an intensely stained nerve cell typical of a Type I neuron is evident.

Scale bar=50μm.


A
C
B
D

seen, suggesting that many of the labeled fibres projected considerably further than could be discerned here. Most of the labeled fibres within a ganglion appeared to originate from outside the ganglion some of which bore varicosities en passant. Others ramified within the ganglion around clearly unlabeled cells.

Variations in morphological type and staining intensity of NO labeled neurons were apparent and indicative of more than one class of neuron being associated with this enteric neurotransmitter in the piglet intestine. As in the rodent and human intestine, the histochemical reaction within labeled neurons, labeled the cells' cytoplasm and associated efferent fibres and proximal dendrites which afforded a suitable definition of cell shape to allow morphological classification. The labeled ganglion cells included Type I through Type VI morphologically classified neurons as described by Stach (1989) (Fig.4.5). Of these, intensely stained neurons corresponding to type II were most common. In addition, the longest emergent processes were always associated with this morphological type. The perikarya of these cells were most often located at the poles of the ganglia with the large emergent process projecting out into the interconnecting fibre bundles. These processes could often be traced considerable distances coursing through up to 4 ganglia of the primary meshwork on into the fine tertiary meshwork that innervates the circular muscle and connects with the submucosal nerve networks.

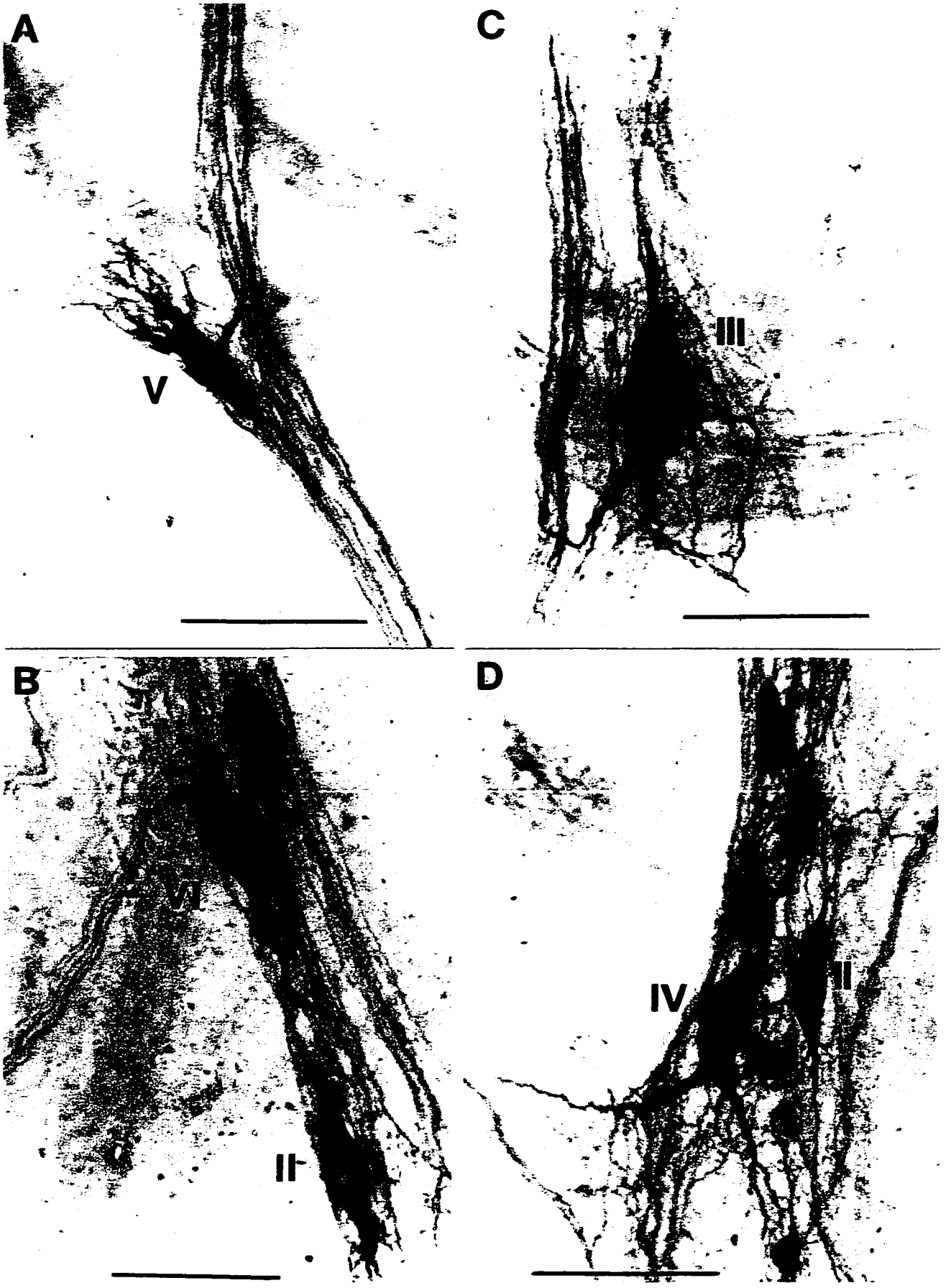
In the submucosa, only the Henle's plexus displayed labelled neurons, and like the myenteric plexus, these encompassed Types I through VI. However, both the Henle's plexus and Meissner's plexus displayed a profuse network of intensely stained varicose fibres within individual ganglia and throughout the interconnecting fibre bundles (Fig.4.4). This fibre network appeared to be more profuse than in the myenteric plexus

Fig.4.5 High magnification micrographs of ganglia from Henle'e plexus showing morphologically distinct nerve types stained intensely for NADPH diaphorase histochemistry.

A,C = Duodenum.

B,D = Colon

Scale bar = 50μm


A
V
C
III
B
II
D
IV
II

and could often be traced long distances (up to 1mm). Within the ganglia, stained varicose fibres could be seen to ramify around clearly unlabeled cells. Almost all Henle's ganglia contained labeled cells (Fig.4.5). The maximum number of labeled cells/ganglia was found to be 5.

Underlying the Henle's plexus an extensive vascular network is visible (Fig.4.3 C). The blood vessels and associated paravascular nerve bundles were in close contact with and often connected to fibres of Henle's plexus. Arterioles (Va) displayed an extensive punctate labeling comprised of uniformly large puncta distributed relatively evenly over the visible surface of the blood vessels (Fig.4.3C). These puncta appeared typical of patches of NO synthase activity within the endothelial cells seen in the rodent and human enteric vascular networks. The venules were not labeled and there was no obvious perivascular labeling.

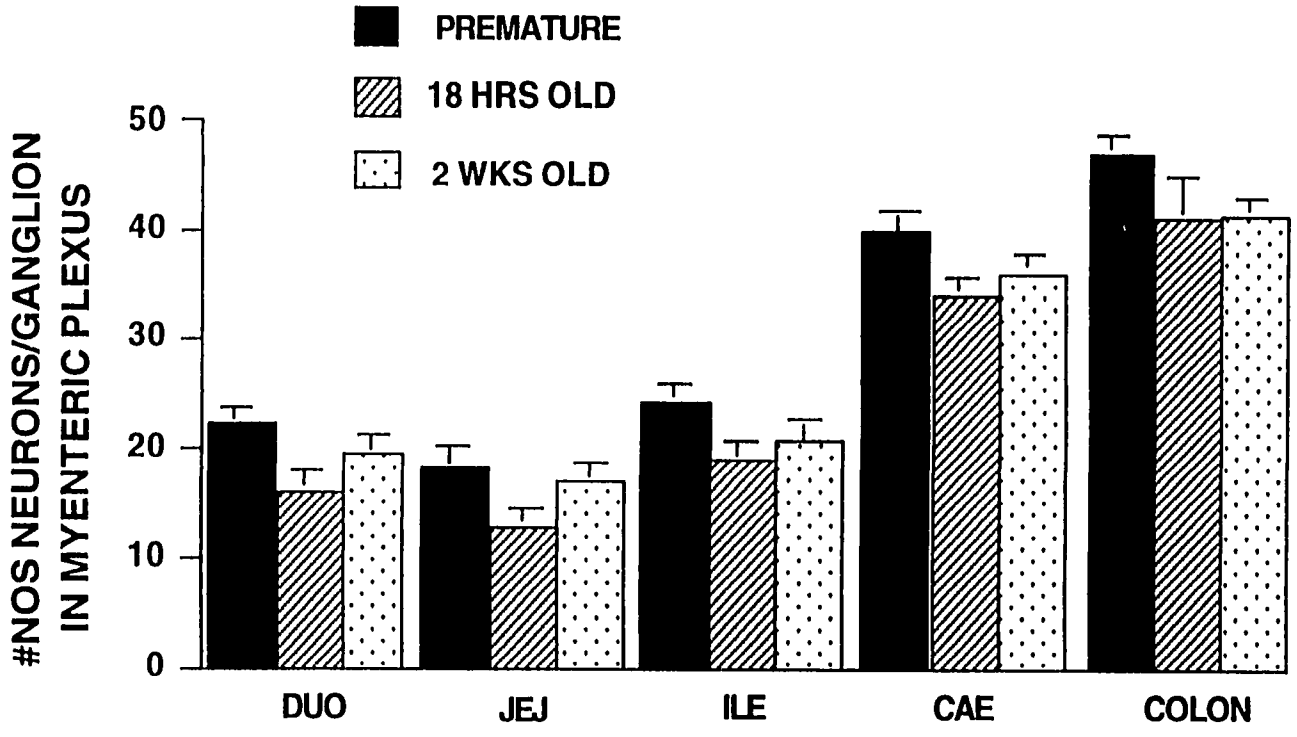
Frequency

NADPH diaphorase histochemistry revealed that in the myenteric plexus, although not reaching statistical significance, all 3 stages of development showed an aboral increase in frequency of positive neurons per ganglion from duodenum to colon. Premature animals showed the highest frequency of NO neurons in all regions. This number is lowest in term newborn piglets and increases at 2 weeks, but never attains premature levels. The caecum and colon of all age groups showed the highest frequency of NO neurons. However, we observed a decreasing trend in frequency of positive myenteric plexus neurons with increasing post-conceptional age in all regions of the

Fig.4.6 Histograms of the frequency of NADPH diaphorase positive neurons (NOS neurons) per ganglion in the developing porcine myenteric plexus in premature, 18-hour old and 2-week old neonatal piglets.

Although not statistically significant, note the increasing cranio-caudal (duodenum to colon) trend in all developmental stages and the higher frequency in all regions in premature animals.

#NOS NEURONS/GANGLION IN MYENTERIC PLEXUS /REGION/AGE



small bowel. This decrease was also evident for the large bowel, with maximal decrease already evident in the < 72 hour group (see Fig. 4.6).

A similar trend towards an increase in frequency of NO neurons in the cranio-caudal direction was demonstrated in Henle's plexus. Unlike our findings in the MP, premature animals did not have the highest frequency of NO neurons. This was highest in the proximal small bowel of 2 week olds. However, numbers were also higher in premature large bowel, and decreased with development in the distal intestinal tract (see Fig. 4.7)

4.1.4.iii Conclusions

Large numbers of NO neurons were present in all ganglia of the myenteric plexus and in Henle's nerve plexus at all developmental stages. Although rich in NO nerve fibres, the Miessner's nerve plexus, located close to the muscularis mucosae, contained few NO nerve cell soma. In the myenteric plexus, this frequency increases in the cranio-caudal direction. There is an overall decrease in the number of neurons with increasing post-conceptional age. However, in Henle's plexus, there is an opposite trend, with the highest number of NO positive neurons in proximal small bowel of older developing piglets.

4.1.4.iv Discussion

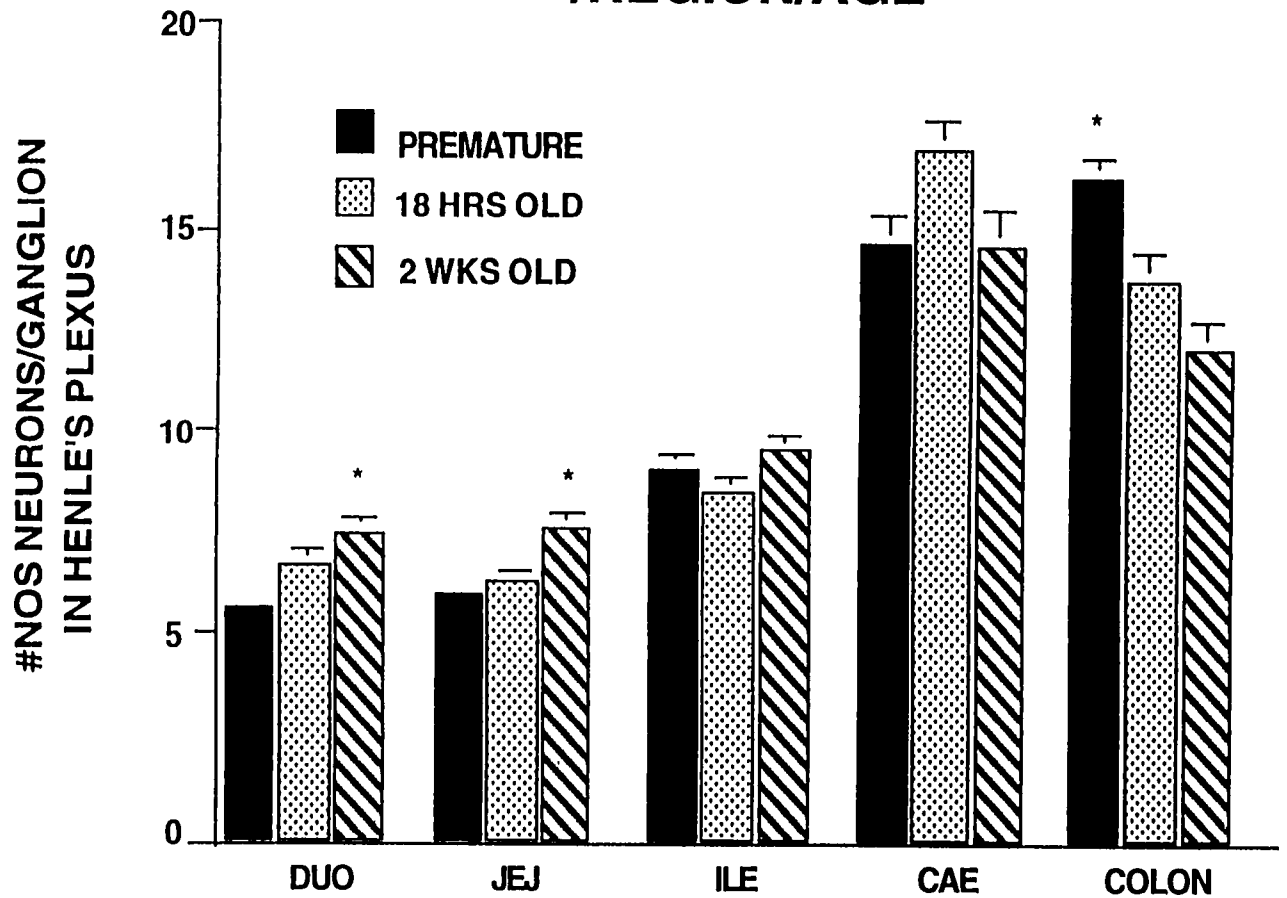
Our data correlate with recent evidence in the developing human gut (Timmermans et al. 1994). Opposite findings have been reported by Van Ginneken and co-workers (1998) in their analysis of fetal and postnatal pig duodenum, where the

Fig. 4.7 Histograms of the frequency of NADPH diaphorase positive neurons (NOS neurons) per ganglion in developing porcine Henle's plexus in premature, 18-hour old and 2-week old neonatal piglets.

Note the significant increase in frequency in the proximal small bowel of 2 week olds compared to premature piglets (duodenum and jejunum). However, this frequency is still significantly higher in the colon of prematures compared to 2 week olds.

*= $p<0.05$

#NOS NEURONS/GANGLION IN HENLE'S PLEXUS /REGION/AGE



number of NO neurons in the myenteric plexus were found to double post natally, and these numbers were greater in the oral duodenal segment than in the aboral one. In Henle's plexus, numbers increased during gestation and fell after birth, to return to prenatal values. However these investigators used whole mount preparations and confined their observations to regions of the duodenum only. The accuracy of our findings are confirmed through our biochemical NO synthase assays and motility findings (see below and Chapter 5).

A striking feature of the nerve layers for all animals examined was the wide variety of morphological cell types displaying NO synthase activity. Intensely stained Types I through IV neurons were found in both myenteric and submucosal ganglia. In addition, occasional extraganglionic neurons within the nerve neetworks were also stained and these always displayed a Type II morphology. This is consistent with the findings of Timmermans et al (1994) who reported the distribution pattern and neurochemical features of nitrergic neurons in the small intestine from 5-6 week old domestic pigs. However, the findings of this study reveal that all of the relevant morphological cell types are already present and displaying NO synthase activity in the gut wall of 9 day premature piglets. The enteric nerve networks of these premature animals also displayed a typically rich innervation of NO nerve fibres. Although we cannot here determine the functional status of these neurons, it is nevetheless noteworthy that the NO neural network apears to be both extensive and profuse in prematures. Our findings likely reflect the developmental variation among the different sources of NO and the change in regulatory potential of the enteric nervous system with age.

4.2 INTESTINAL MOTILITY

The patterns and ontogenic development of fasting enteric motor activity have been described in detail in Chapter 1. Given the evidence of a developmental component to fasting intestinal motor activity and to nitrergic innervation of the gut wall, we undertook the evaluation of intestinal motility *in vivo* in the anesthetized piglet at two stages of development, and documented the effects of pharmacological manipulation of the nitrergic system on this motility.

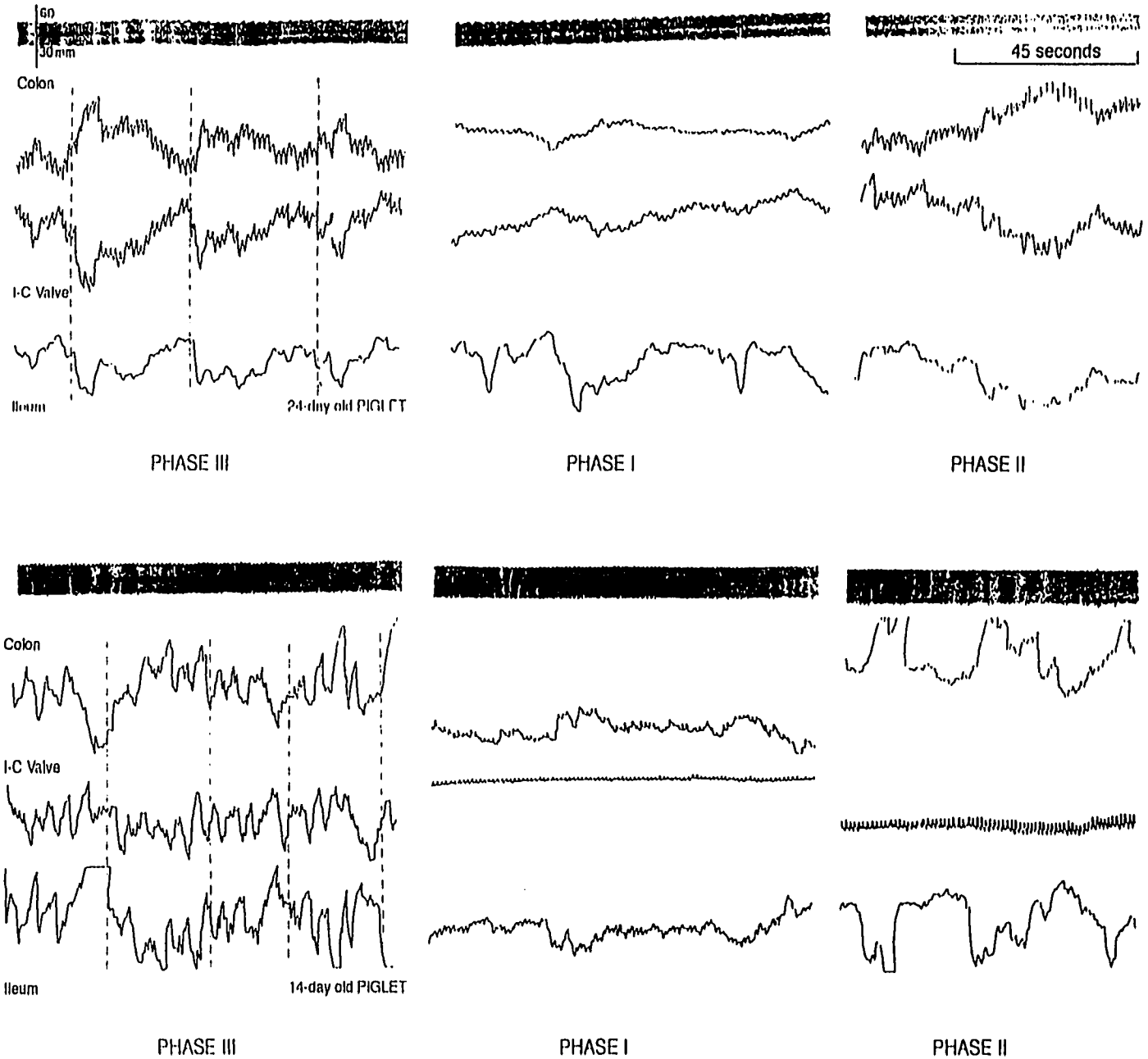
4.2.1. Materials and Methods

Methods

Under general Halothane anesthesia and endotracheal intubation, 10 fasted neonatal suckling piglets underwent carotid artery cannulation and mid-line laparotomy. The animals were then prepared for the assessment of motility in vivo.after the method of Krantis et al (1996). This approach involves the use of extraluminal foil strain gauges (Showa type N11-FA-2-120-11, Durham Instuments, Pickering ON) attached using Vet Bond glue (#1469, 3M Company), onto the outer serosal layer of the gut. To prepare for implantation, their terminal leads were soldered to 20 cm long, insulated wire leads (Alpha Wire Co. 32AWG). The clear side of the gauge was coated with a thin layer of Gagekote #8 acrylic polish (Durham Instruments, Pickering, ON), which was peeled off after the experiments and reapplied, thus allowing for the repeated usage of the strain gauges (up to 3 times).

With minimal manipulation, the distal small bowel and proximal colon were identified and strain gauges were glued to the anti-mesenteric serosal surface of the

Fig.4.8 Tracing 1 Sample motility tracings in 24 day old (a) and 14 day old (b) piglets showing phasic Phase III intestinal motor activity, Phase I quiescent activity and Phase II irregular activity.

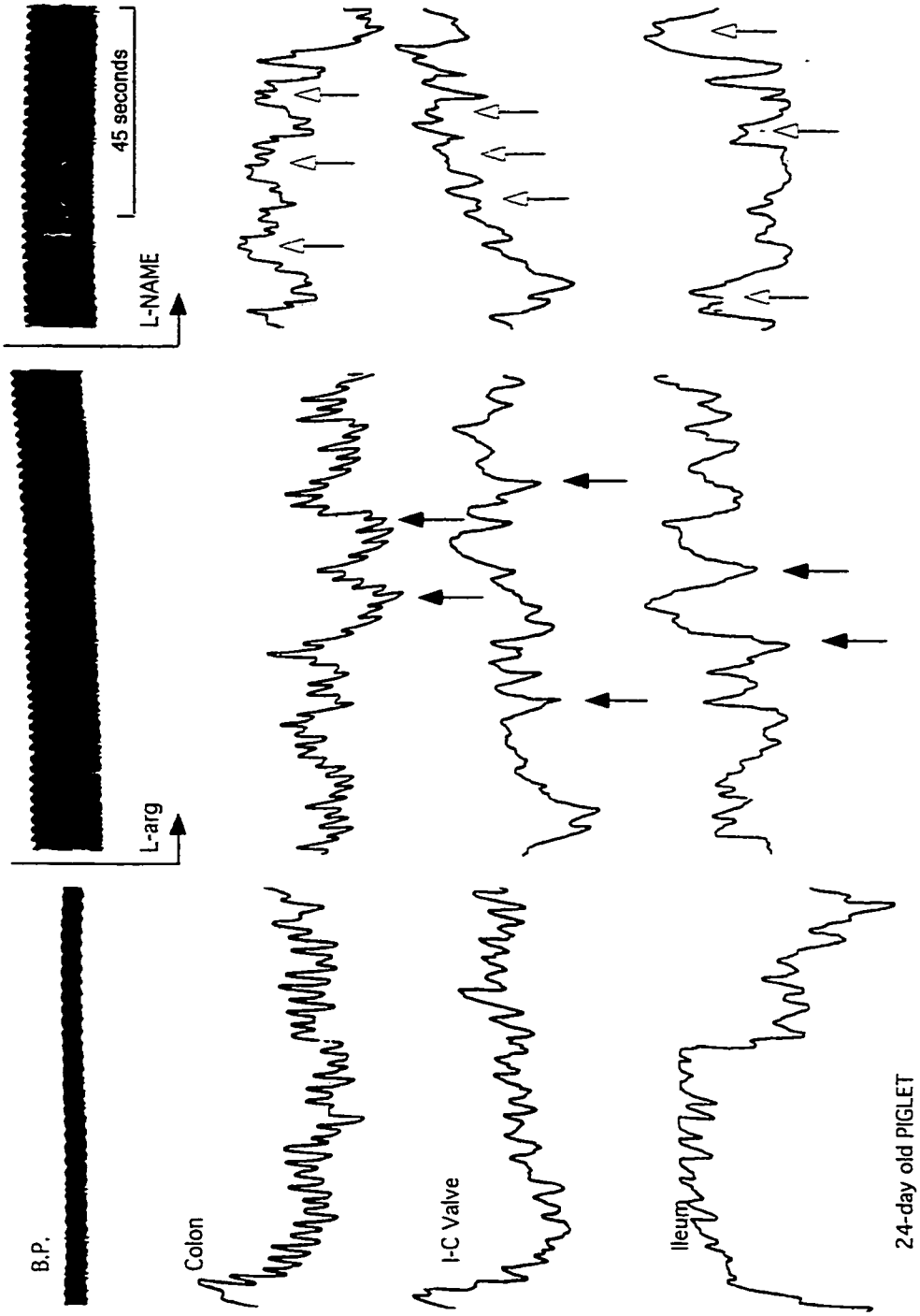

60
30mm
Colon
I-C Valve
Ileum
24-day old PIGLET
45 seconds
PHASE III
PHASE I
PHASE II
Colon
I-C Valve
Ileum
14-day old PIGLET
PHASE III
PHASE I
PHASE II

distal ileum, 10 cm from the ileo-caecal valve, at the ileo-caecal valve, and to the proximal colon, 5 cm from the ileo-caecal valve. The abdomen was closed with the wire leads exteriorized for attachment via a 3-channel interface box to a Grass force transducer (model FTO3C) coupled to a Grass polygraph (model 7) exiting from the distal extremity of the incision.

Blood pressure was also recorded for the duration of the experiment as well as vital signs. Intestinal motility was recorded from these 3 regions for periods of 4 to 6 hours. Control animals were divided into 2 age groups : 2 week olds (n=3), and 4 week olds (n=3), and underwent 4 hours of continuous motility recordings. Test animals, 2 week olds (n=2) and 4 week olds (n=2) underwent bolus i.v. administration of either L-NAME (10 mg/kg) or L- arginine (300 mg/kg) after an initial 2 hour stabilization period. Drug effects on blood pressure and motility tracings were noted as well as their duration. Upon termination of the recording, the abdomen was re-opened for verification of strain gauge position and the piglets were sacrificed using an intracardiac injection of 5 ml of Euthanyl. All surgical and experimental protocols (here and described elsewhere in this body of work) were carried out according to the Canadian Council on Animal Care guidelines administered by the Animal Care Committee at the University of Ottawa.

Polygraph tracings were observed for recognizable patterns of intestinal motor activity. Upward deflections were identified as contractions and downward ones as relaxations.

Fig.4.9 Tracing 2 Motility tracing in 24 day old piglet showing response to i.v. bolus of L-arginine, or L-NAME. Note the downward deflections indicating augmented relaxation with L-arginine (black arrows), and upward deflections indicating augmented contractions with L-NAME (white arrows) compared to control tracing (no arrows).


B.P.
L-arg
L-NAME
45 seconds
Colon
I-C Valve
Ileum
24-day old PIGLET

Materials

All drugs were dissolved in NaCl 0.9%. D and L-NAME, D and L-arginine were purchased from Sigma, Toronto ON. Anaesthetics were provided by the Animal Care Facility of the University of Ottawa.

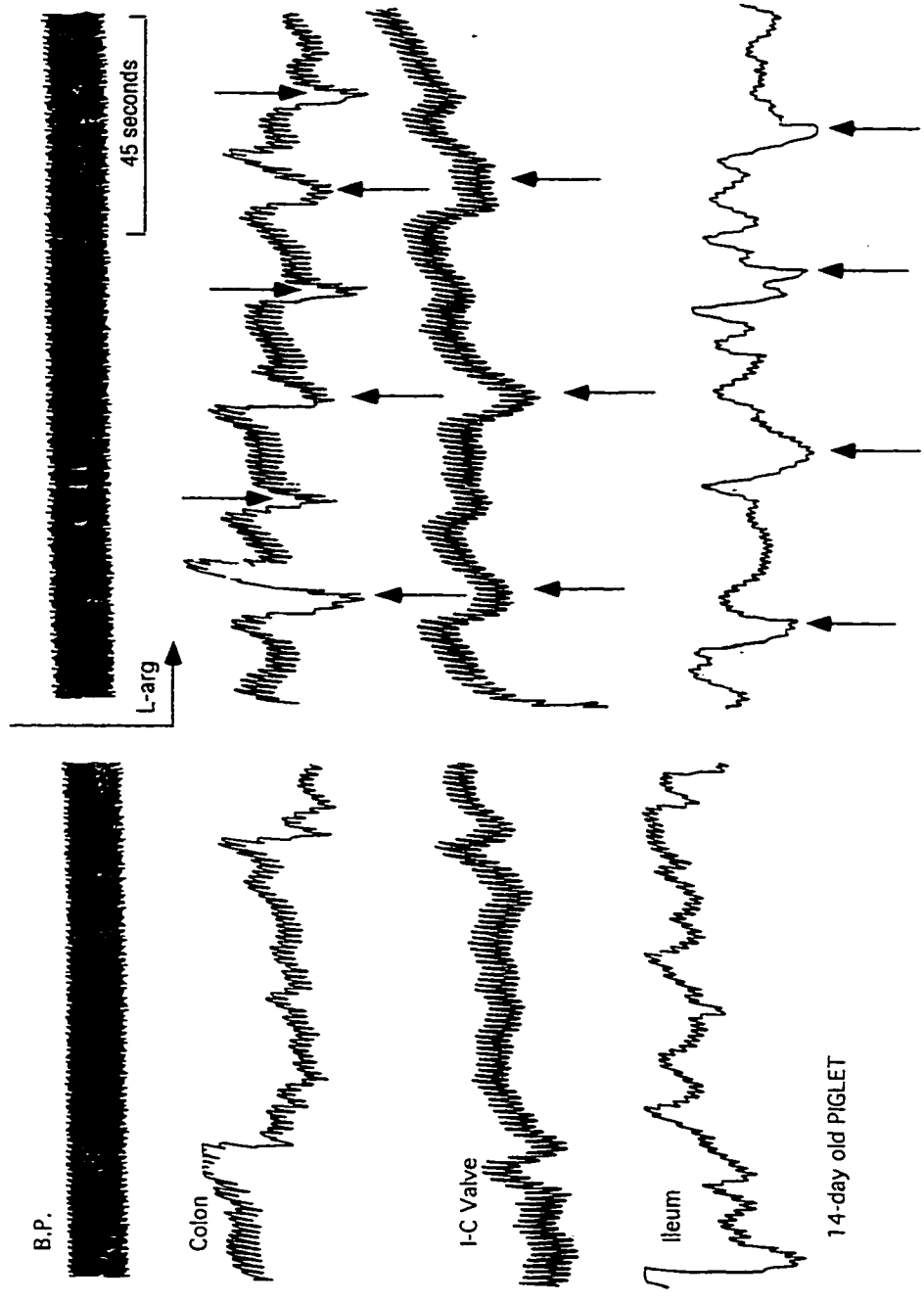
4.2.2. Results

The following are purely descriptive results and are meant in no way to quantify aspects of intestinal motor activity in the developing piglet, but simply to document evidence confirming the maturational aspect of fasting motor activity and the intestinal response to nitrergic manipulation in our animal model

In the aproximately 4 week old control animals, three patterns of activity were identified; these cycled every 35 to 60 minutes (Fig.4.8, Tracing I: top). This activity was characterized by a period of sporadic contractions of variable force and frequency (phase II), followed by a series of intense aborally migrating contractions (phase III) which were followed by quiescence (phase I). In test animals, single dose i.v. L-arginine caused a transient decrease of approximately 5 mmHg in systemic blood pressure and increased the amplitude and duration of relaxation more markedly in the proximal colon lasting approximately 3 to 6 minutes (Fig.4.9,Tracing 2, black arrows). L-NAME caused a transient elevation in blood pressure of similar magnitude and duration, but increased the frequency and amplitude of contractions in all three regions (Fig.4.9,Tracing 2, white arrows).

In 2 week old controls, all three patterns of motor activity were observed, but were of lower amplitude and less coordinated, more particularly in the terminal ileum

Fig.4.10 Tracing 3 Motility tracing in 14 day old piglet showing response to i.v. bolus of L-arginine. Note the downward deflections indicating augmented relaxation with L-arginine compared to control tracing.


B.P.
45 seconds
L-arg
Colon
I-C Valve
Ileum
14-day old PIGLET

and at the ileo-caecal valve (Fig.4.8, Tracing 1: bottom). L-arginine effects were similar to the more developed animals but of greater magnitude and were evident in all three regions (Fig.4.10, Tracing 3). When various doses of L-NAME were used these young animals expired (n=2) from respiratory acidosis secondary to apnea and this, despite lowering the doses of Halothane administration and despite aggressive ressuscitation. Notably, during periods of instability, intestinal motor activity became irregular and heralded a rapid onset of deterioration and demise.

4.2.3. Conclusions

In the developing piglet, three patterns of fasting intestinal motor activity have been identified in the terminal small bowel and proximal colon, and are similar in sequence to those described in the human. In addition, younger animals displayed more uncoordinated motor activity which was of lower amplitude. Systemic i.v. boluses of L-arginine caused transient increase in the amplitude and frequency of relaxations which seemed more marked in the colon of older animals. However, this response was greater in all three gut regions of younger piglets. L-NAME increased amplitude and duration of contractions in all gut segments studied in the older animals. Documentation of L-NAME effects in the 2 week old piglets was not possible due to the high mortality rate upon its administration in this age group.

4.2.4 Discussion

The preceding observations in the neonatal piglet confirm the presence of intestinal motor activity which is comparable to the human (Berseth 1994). In addition,

the developmental aspect of such motor function has been documented through the observation of more erratic activity within the same patterns in the more immature animals.

Enteric responses to nitrergic manipulation have been shown here to be more evident in the terminal small bowel and colon of younger animals, which closely correlate with the increasing numbers of NO neurons in the myenteric plexus of these regions in more immature animals as documented by our histochemical data. This data may thus reflect an increased regional and developmental predispostion to the injurious effects of decreased NO availability (see Chapter 3)

Many in vivo studies have documented endogenous NO's role in the modulation of intestinal motility in the rat, including those published from this laboratory (Calignano et al. 1992; Glasgow et al. 1998; Krantis et al. 1998). However, NO-containing NANC neurons are not alone in regulating fasting motor activity. Phase I activity has been shown to persist during inhibition of NO synthase by L-NAME (Sarna et al. 1993). Later investigators reported conflicting evidence where inhibition of NO synthesis seemed to induce a fasting motor pattern and that NO donors mediated a fed pattern of small intestinal motor activity in the rat and chicken (Rodriguez-Membrilla et al. 1995). NO was also shown to inhibit MMC's possibly through VIP (Hellstrom and Ljung 1996). LPS was later shown by this group to inhibit MMC's and was accompanied by rapid transit. This effect was abolished by NO inhibition (Hellstrom et al. 1997). Later still, inhibition of fasting small bowel motility was again shown to be NO dependent (Tolessa et al. 1998). All these studies, however, were carried out in the rat.

Regarding the intensity of response to L-arginine in the younger animals, there is evidence supporting the fact that nitrergic contribution to gut relaxation decreases with age (Smits and Lefebvre 1996). In fact we have demonstrated a quantitative, ontogenically determined, decrease in the number of NO neurons in the myenteric plexus of the developing piglet.

The deleterious effects of L-NAME on infant piglet intestinal blood flow has been described. Schleien et al (Schleien et al. 1998) reported that i.v. L-NAME decreased intestinal blood flow while increasing vascular pressures and cardiac output. These findings may explain our results regarding the adverse effects of L-NAME administration on intestinal motility. In addition, L-NAME has been shown to lower the threshold for Halothane anesthesia, and may have led to hypoxia in the more susceptible younger animals, with the resultant effect on gut motor activity.

These *in vivo* studies in the neonatal piglet are consistent with the presence of an ontogenically determined pattern of fasting motor activity in the maturing piglet whose behaviour is differentially affected by nitrergic manipulation based on developmental status. As such, these findings contribute to the validity of this animal model in our ongoing investigation of potential pathophysiological mechanisms leading to NEC.

CHAPTER 5

NOS ISOENZYME ACTIVITIES OF THE DEVELOPING PIGLET GUT WALL: RELEVANCE TO THE PATHOGENESIS OF NEC

Grider and co-workers (1993) provided the first direct evidence of NO production from enteric ganglia. NO, as measured by L-[3H] citrulline production, was investigated in isolated ganglia from the myenteric plexus of the guinea-pig intestine. Other investigators (North et al. 1994) have demonstrated a developmental regulation of endothelial and neuronal NOS gene expression in the rat lung. Endothelial NOS expression was increased with gestation, whereas bronchial epithelial neuronal NOS decreased late in gestation and subsequently rose postnatally. Similar findings of increasing basal NO production in the fetal lamb have also been reported (Shaul et al. 1993). Thus, this ontogenic variation in NOS activity has been observed in different animal species and in different organs.

The ontogeny of intestinal clearing mechanisms (dysmotility associated with prematurity) and mucosal protection (damage by intraluminal substrate) related to NEC require further elucidation. In this regard, and because of NO's dual role in neural control of intestinal motility and mucosal protection, we measured constitutive (cNOS) and inducible (iNOS) NO synthase activity in the wall, and then in the muscularis and the mucosal layers of premature and newborn piglet intestine both in the intact (Study A) and in the NEC-induced (Study B) state.

5.1. MEASUREMENT OF NO SYNTHASE ACTIVITY

5.1.1 Methods

After sacrifice with intra-cardiac injection of sodium pentobarbital (65 mg/ml), small and large bowel were harvested from Yorkshire piglets (9 days premature, n=6 and 1 week old term piglets, n=6), cleared of their contents and placed in phosphate buffer solution. The sero-muscular layer and the mucosal layers from representative samples of duodenum, jejunum, ileum, caecum and colon were separated under the dissecting microscope and each assayed for NOS activity using the conversion of C14 L-arginine to C14 L-citrulline (Salter et al 1991).

The method of NEC induction was that described in Chapter 2, and the duration of injury was either 3, 6, or 16 hours, for each age group (premature delivered by cesarian section [C-prem], n=4 x2; premature delivered by maternal injection of prostaglandin, n=2 x2; 3 day old term, n=4 x2).

In order to compare NOS activities between the NEC damaged and intact gut, activity was measured in full-thickness specimens because injured gut layers could not be accurately dissected. Each animal served as its own control (saline injected gut loop alternating with acidified casein injected loop). Total NOS, cNOS and iNOS activities in ηmol/min/gr of protien were calculated in test and control gut loops and compared.

5.1.1.i *NOS assay*

NO synthase activity was assessed using the ^{14}C-L-arginine to ^{14}C-L-citrulline conversion assay (Salter et al. 1991). Briefly, frozen segments of duodenum

were thawed on ice and placed in ice cold Krebs buffer (pH 7.4) consisting of 115mM NaCl, 8mM KCl, 2mMKH_2PO_4, 25mM $NaHCO_3$, 1.3mM $CaCl_2$ and 2.4mM $MgCl_2$. Tissues weighing 50-100 mg were placed in 250 µl of a homogenization buffer (pH 7.4) at 4°C consisting of 10mM HEPES, 320mM sucrose, 1mM dithiothrietol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10µg/ml leupeptin and 2 µg/ml aprotinin. Using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario), tissues were homogenized for 20 sec. at high speed and centrifuged at 14 000 x g for 10 min. at 4°C. Subsequently, two separate 20 µl aliquots of the supernatant were added to:

1) 50 µl of incubation buffer at 37°C consisting of 975µM NADPH, 190µM $CaCl_2$, 948µM $MgCl_2$, 38mM KH_2PO_4, 6mM valine, 58µM L-arginine and 0.047 µCi ^{14}C-L-arginine;

2) incubation buffer also containing 977 µM Ca^{2+} chelator etlylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) to differentiate between the Ca^{2+}-independent inducible enzyme isoform. An internal control sample [to assess background (BG) activity] consisted of 50 µl of incubation buffer at 37°C with ^{14}C-L-arginine and 20 µl of filtered deionized water (ddH_2O). All mixtures were vortexed and placed in a metabolic shaking incubator for 10 min. at 37°C. Separation of ^{14}C-L-arginine from ^{14}C-L-citrulline was achieved using ion exchange chromatography. Resin (Na- form, 100-200 mesh, Bio-Rad) was mixed with concentrated NaOH and rinsed several times (≈ 20 times) with ddH_2O to attain a pH of 8.0. Samples were loaded onto Dowex 50W-X8 ion exchange columns (1 ml volume) and eluted with 2 ml ddH_2O. 1 ml aliquots of eluent were mixed with 14 ml of liquid scintillation fluid, vortexed and placed in a Beckman LS-9000 scintillation counter for radioactive counts. NO synthase

activity is expressed as ηmol/min/g tissue. The activity of the different isoforms of NO synthase were then calculated. Those samples which did not contain EGTA (a calcium chelator) in the reaction mixture, reflected total NO synthase activity, while those containing EGTA reflected NO synthase activity of the inducible isoform. The activity of constitutive NO synthase was calculated from the difference between total and inducible NO synthase activities. The following is a sample calculation of the amount of ^{4}C-L-arginine converted to ^{14}C-L-citrulline or NO synthase activity. [(2 x sample CPM) – (2 x BG CPM)] x dilution factor x [^{14}C activity in incubation buffer / total CPM] x [mol cold arginine in incubation buffer / mols of hot arginine in incubation buffer] / tissue weight x 1000 mg / incubation time = ηmol NO / min. / g tissue.

Chemicals:

All drugs were directly dissolved in 0.9% saline. L-arginine, L-NAME, EDTA, β-aminoethyl ether EGTA, NADPH, soybean trypsin inhibitor, leupeptin, aprotinin and valine were purchased from Sigma (St. Louis, Missouri). NaCl, $CaCl_2$, KCl, MgCl, NaOH, $NaHCO_3$, KH_2PO_4, HEPES and dithiothreitol were obtained from NEN Dupont (Mississauga, Ontario). Dowex 50W-X8 resin (Na-form, 100-200 mesh) and the chromatography columns were purchased from Bio-Rad Laboratories (Richmond, California).

Statistical analysis:

The mean and standard error of the mean was calculated for each specimen. Statistical analysis was undertaken using the Instat statistical computer program. The group means were used to compare the two ages regarding region and gut

Fig.5.1 Histogram of the **total NO synthase** activity (measured in ηmol/minute/g of tissue) in the **muscularis** of premature and and 1-week old piglets in different gut regions.

DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE=Caecum

* denotes $p<0.05$

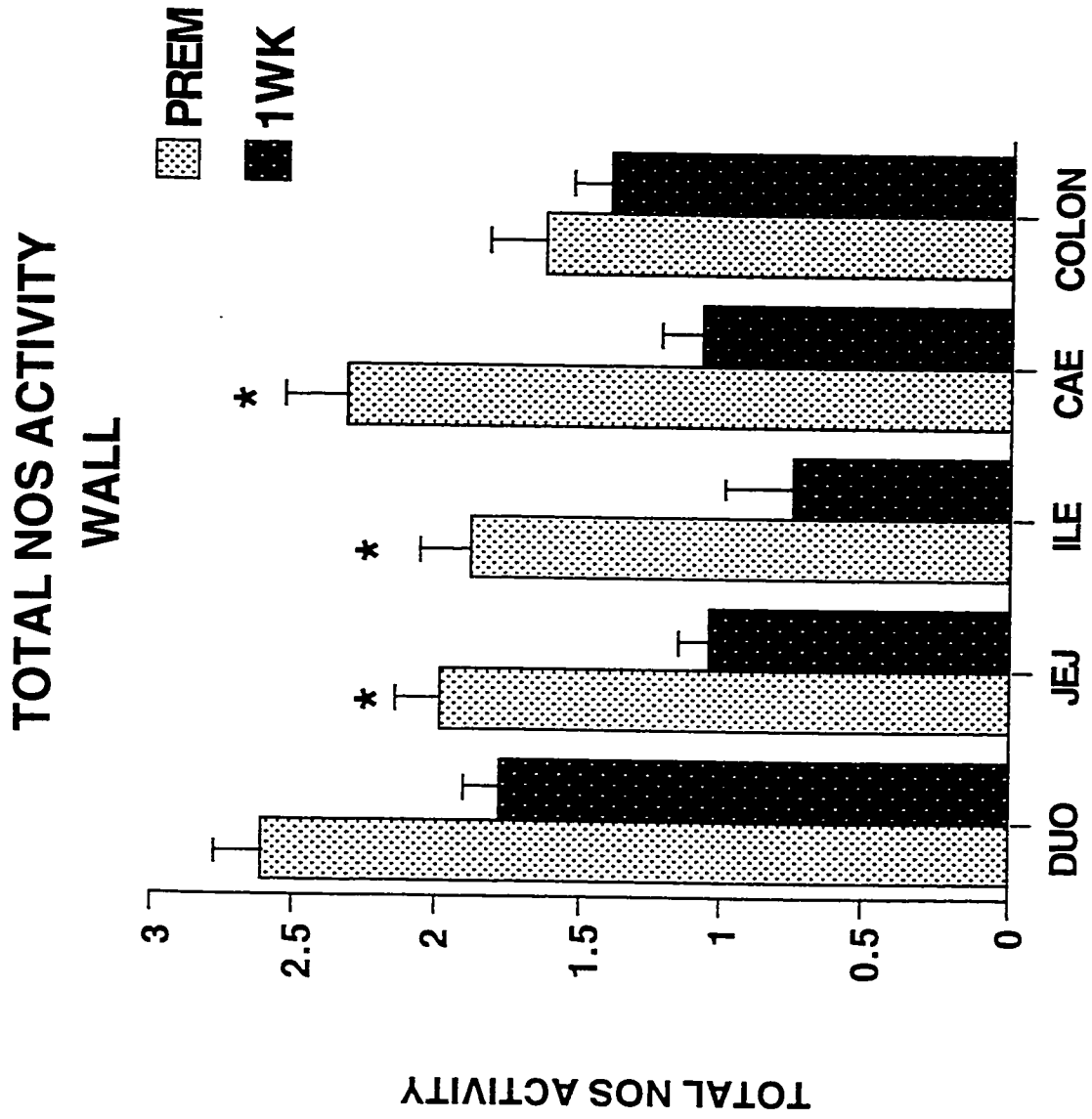

TOTAL NOS ACTIVITY
WALL
PREM
1WK
*
*
*
3
2.5
2
1.5
1
0.5
0
DUO
JEJ
ILE
CAE
COLON
TOTAL NOS ACTIVITY

Fig.5.2 Histogram of the **total NO synthase** activity (measured in ηmol/minute/g of tissue) in the **mucosa** of premature and and 1-week old piglets in different gut regions.

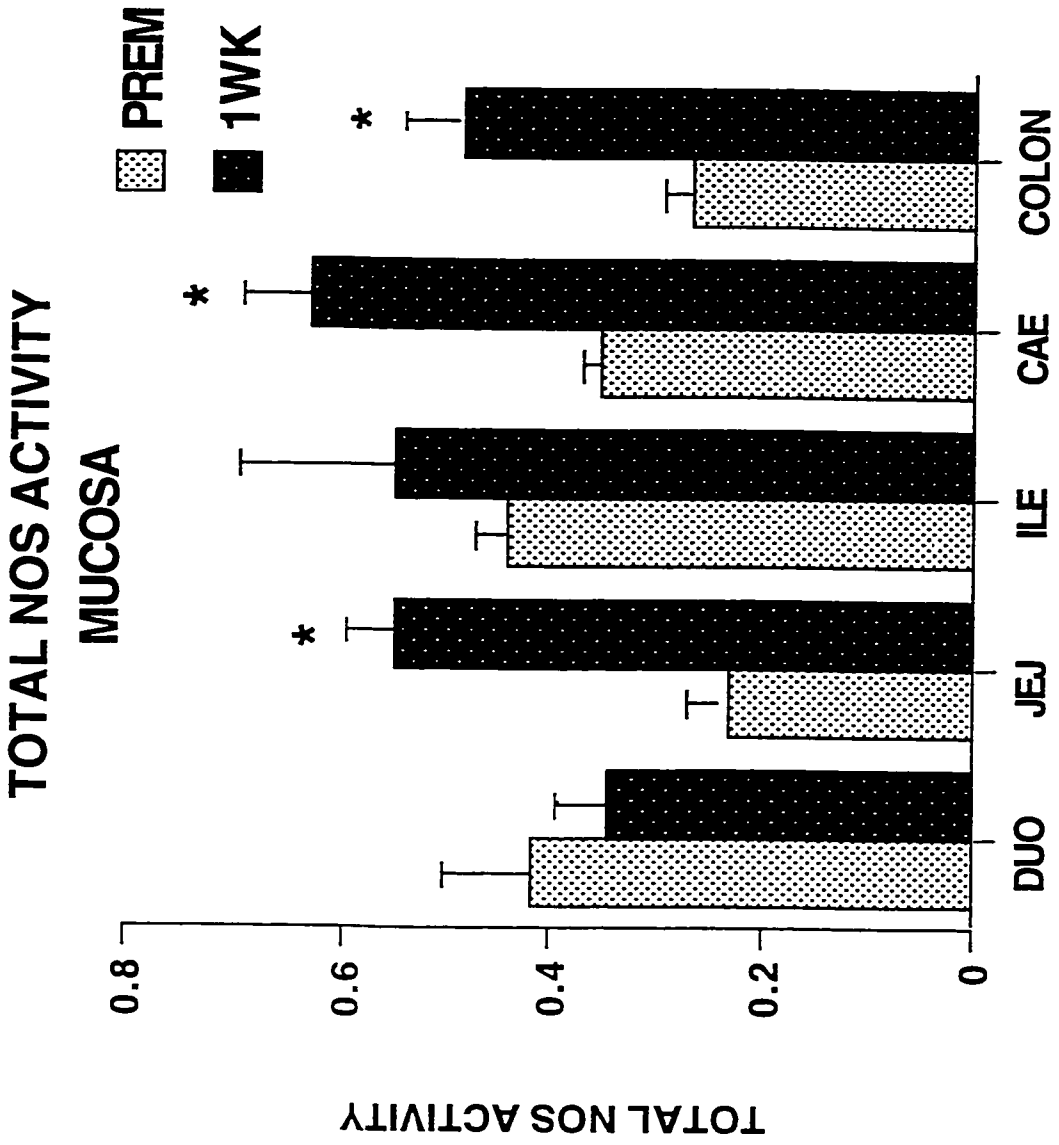
DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE= Caecum

* denotes $p<0.05$


TOTAL NOS ACTIVITY
MUCOSA
PREM
1WK
*
*
*
0.8
0.6
0.4
0.2
0
DUO
JEJ
ILE
CAE
COLON
TOTAL NOS ACTIVITY

layers. One way analysis of variance (ANOVA) and Bonferroni post test were used to compare differences among groups. P value of <0.05 was considered significant.

5.1.2 Results (Study A)

NOS ontogeny in the gut layers:

In the age groups studied, total NOS activity was higher ($p<0.05$) in the muscularis of premature piglets (Fig.5.1) and subsequently peaked in one week old piglet mucosa (Fig.5.2). This trend in increased activity, though not always statistically significant, was consistent across most gut regions.

cNOS was predominant in the muscularis of prematures (Fig.5.3), whereas iNOS predominated in one week old mucosa (Fig.5.5) and muscularis (Fig.5.6).

Although mucosal cNOS lacked regional variability within each group (Fig.5.4), premature mucosal cNOS activity was significantly higher in proximal small bowel compared to one week olds.

Muscularis iNOS activity was significantly higher in the colon of one week olds compared to premature colon (Fig.5.6).

Thus, in piglet gut:

1) total NOS activity is highest in the muscularis of prematures and subsequently shifts during development and, possibly, due to feeding, to the mucosa (the one week olds had been enterally fed, whereas the prematures were sacrificed prior to ever having been fed).

2) Constitutive NOS activity is highest in the muscularis of premature gut.

Fig.5.3 Histogram of the **constitutive NO synthase** activity (measured in ηmol/minute/g of tissue) in the **muscularis** of premature and and 1-week old piglets in different gut regions.

DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE= Caecum

* denotes $p<0.05$

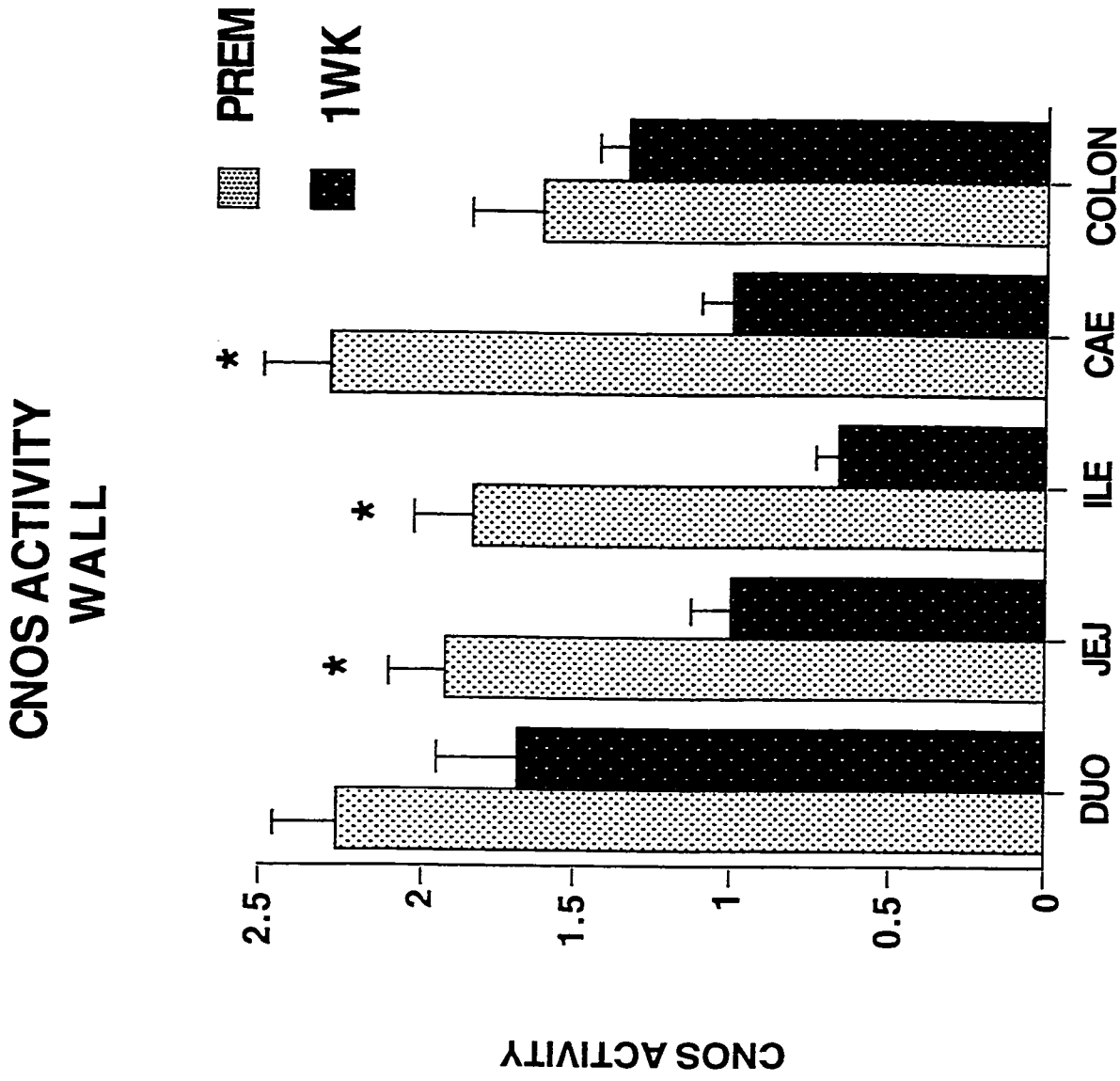

CNOS ACTIVITY
WALL
PREM
1WK
*
*
*
DUO
JEJ
ILE
CAE
COLON
2.5
2
1.5
1
0.5
0
CNOS ACTIVITY

Fig.5.4 Histogram of the **constitutive NO synthase** activity (measured in ηmol/minute/g of tissue) in the **mucosa** of premature and and 1-week old piglets in different gut regions.

DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE= Caecum

* denotes $p<0.05$

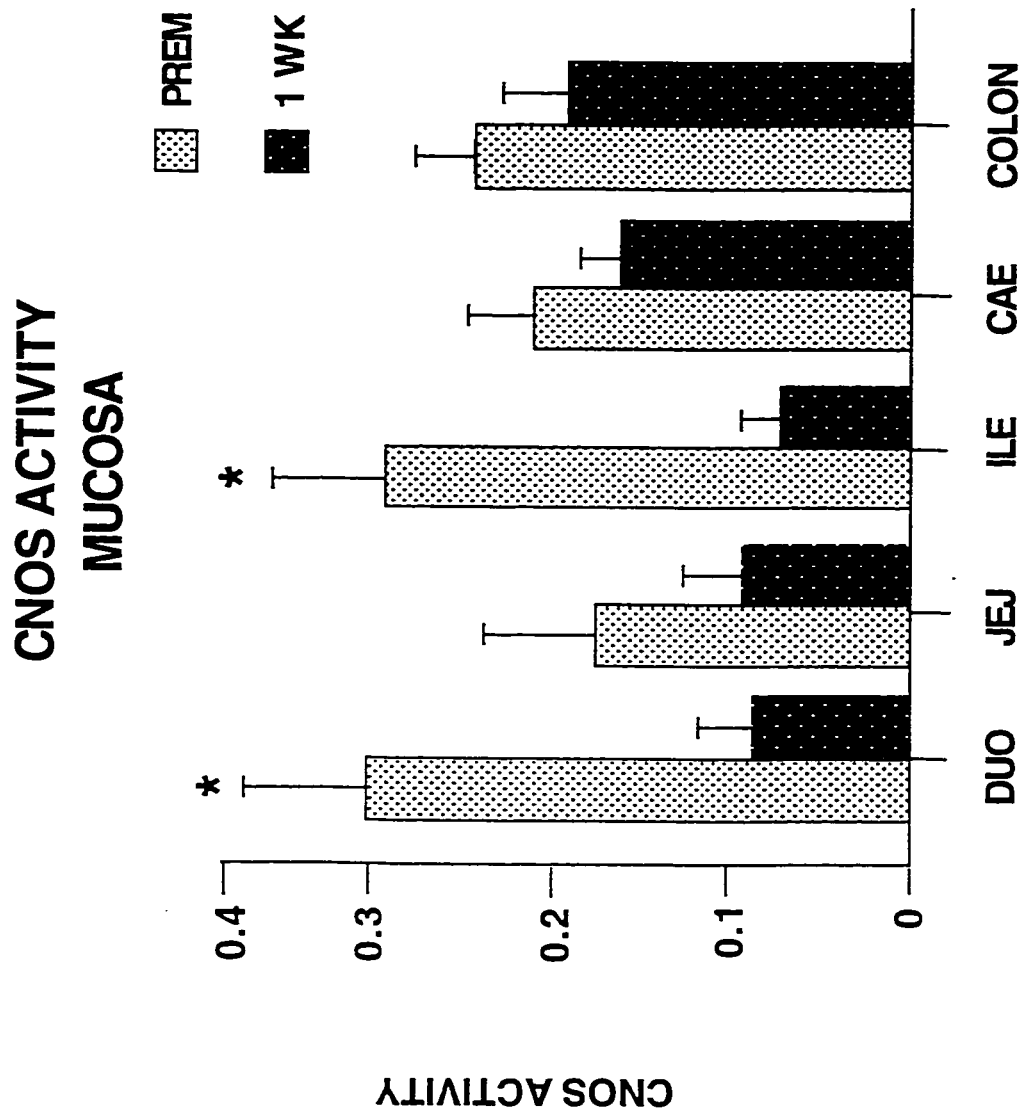

CNOS ACTIVITY
MUCOSA
PREM
1 WK
*
*
DUO
JEJ
ILE
CAE
COLON
0.4
0.3
0.2
0.1
0
CNOS ACTIVITY

Fig.5.5 Histogram of the **inducible NO synthase** activity (measured in ηmol/minute/g of tissue) in the **mucosa** of premature and and 1-week old piglets in different gut regions.

DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE= Caecum

* denotes $p<0.05$

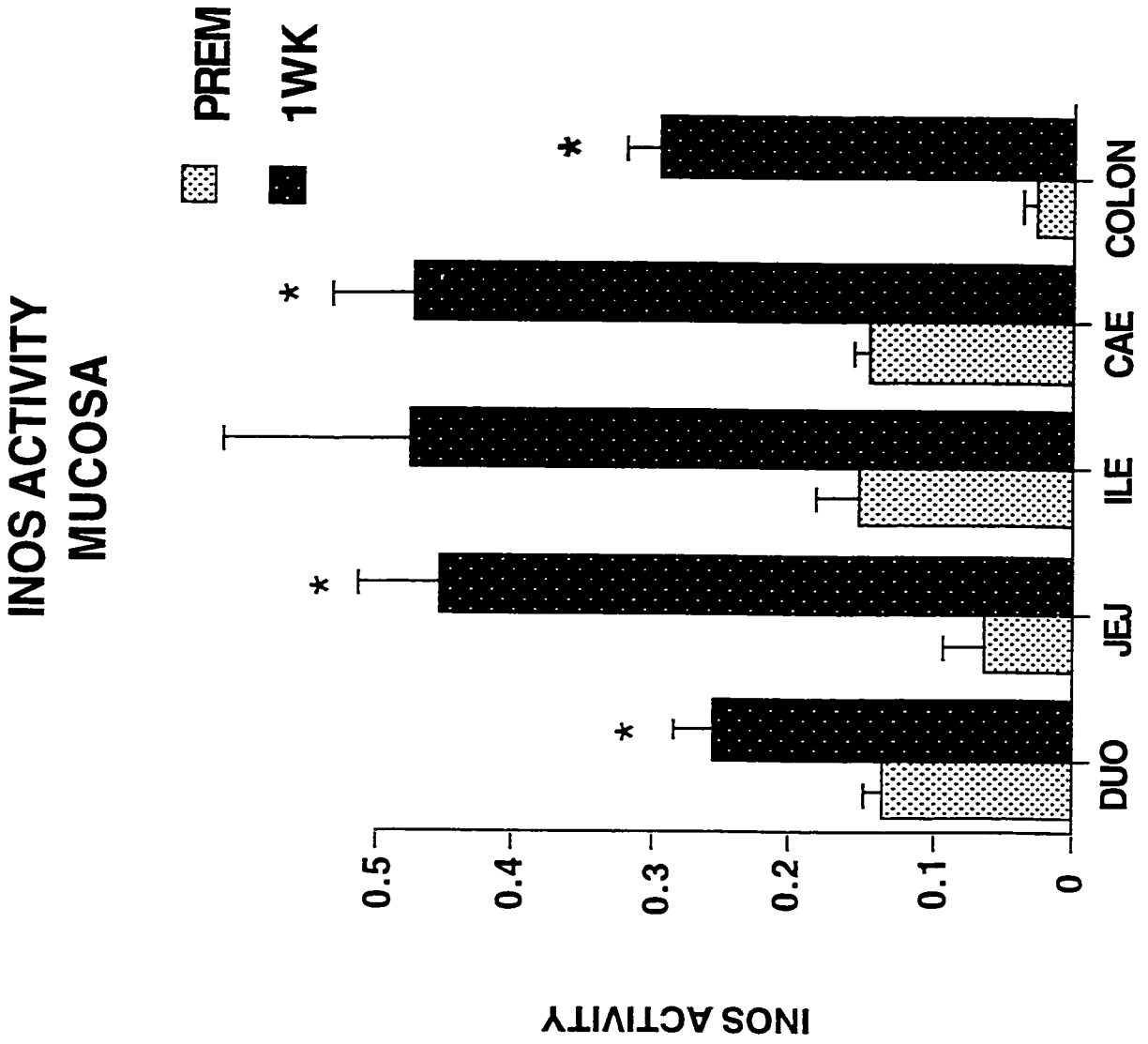

INOS ACTIVITY
MUCOSA
PREM
1WK
INOS ACTIVITY
0
0.1
0.2
0.3
0.4
0.5
DUO
JEJ
ILE
CAE
COLON
*
*
*
*

Fig.5.6 Histogram of the **inducible NO synthase** activity (measured in ηmol/minute/g of tissue) in the **muscularis** of premature and and 1-week old piglets in different gut regions.

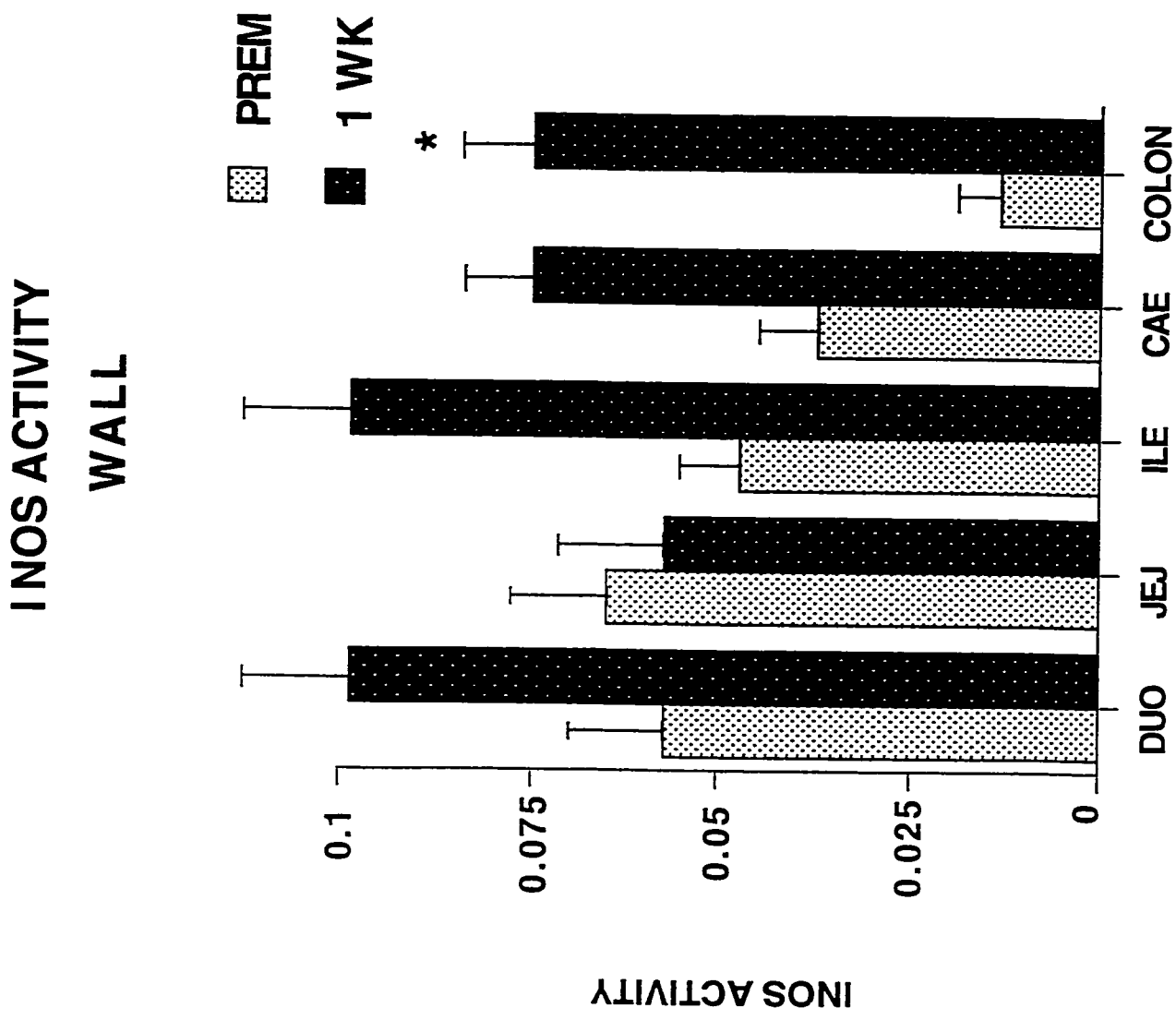
DUO= Duodenum

JEJ= Jejunum

ILE= Ileum

CAE= Caecum

* denotes $p<0.05$


INOS ACTIVITY
WALL
PREM
1 WK
*
0.1
0.075
0.05
0.025
0
INOS ACTIVITY
DUO
JEJ
ILE
CAE
COLON

3) High mucosal iNOS may be a result of induction by intraluminal substrate.

5.1.3 Results (Study B)

NOS activity in response to NEC-induced gut injury:

After 3 hours of NEC induction, iNOS levels were significantly higher in the test loops of C-prem compared to the 3 day old group (Fig.5.7, p=0.0380), whereas control loop iNOS did not differ between the 2 age groups (Fig.5.8). Surprisingly, control and test loop cNOS levels did not differ in comparing the C-prem group to the 3-day old group (Fig.5.9 & 5.10). Control loop cNOS levels were found to be highest in prematures (Fig.5.9).

We then compared NOS activity in C-prem animals at 3 and 6 hours of NEC-induced gut damage. Test loop iNOS levels were significantly higher after 6 hours (Fig.5.11, p=0.0241). Control loop iNOS did not vary with duration of injury (Fig.5.8). Test loop cNOS activity did not vary with time (Fig.5.9). Interestingly, the prematures delivered by maternal injection of prostaglandin failed to demonstrate any difference in iNOS activities in test loops between the 3 and 6 hour group (Fig.5.11). It would seem that increased iNOS production in injured gut over time was blunted in this group.

In 3 day old term piglets, test and control loop iNOS increased with duration of gut injury. In this group we were able to demonstrate a marked rise in iNOS at 16 hours of NEC (Fig.5.7 and 5.8), however cNOS levels remained stable (Fig.5.9 and 5.10).

Fig.5.7 Inducible NO synthase activity (measured in ηmol/min./g of tissue) in NEC induced test loops of intestine at 3, 6 and 16 hours post induction of injury, in prematures (C-prem), prematures born by maternal prostaglandin-induced delivery (prem) and 3 day old term newborn piglets.

The 3 hour iNOS activity in C-prems is significantly higher than in 3 day olds. In both these groups, iNOS activity increases with ongoing duration of damage (3 and 16 hours of NEC). The prem group failed to show any increase in activity.

*= $p<0.05$

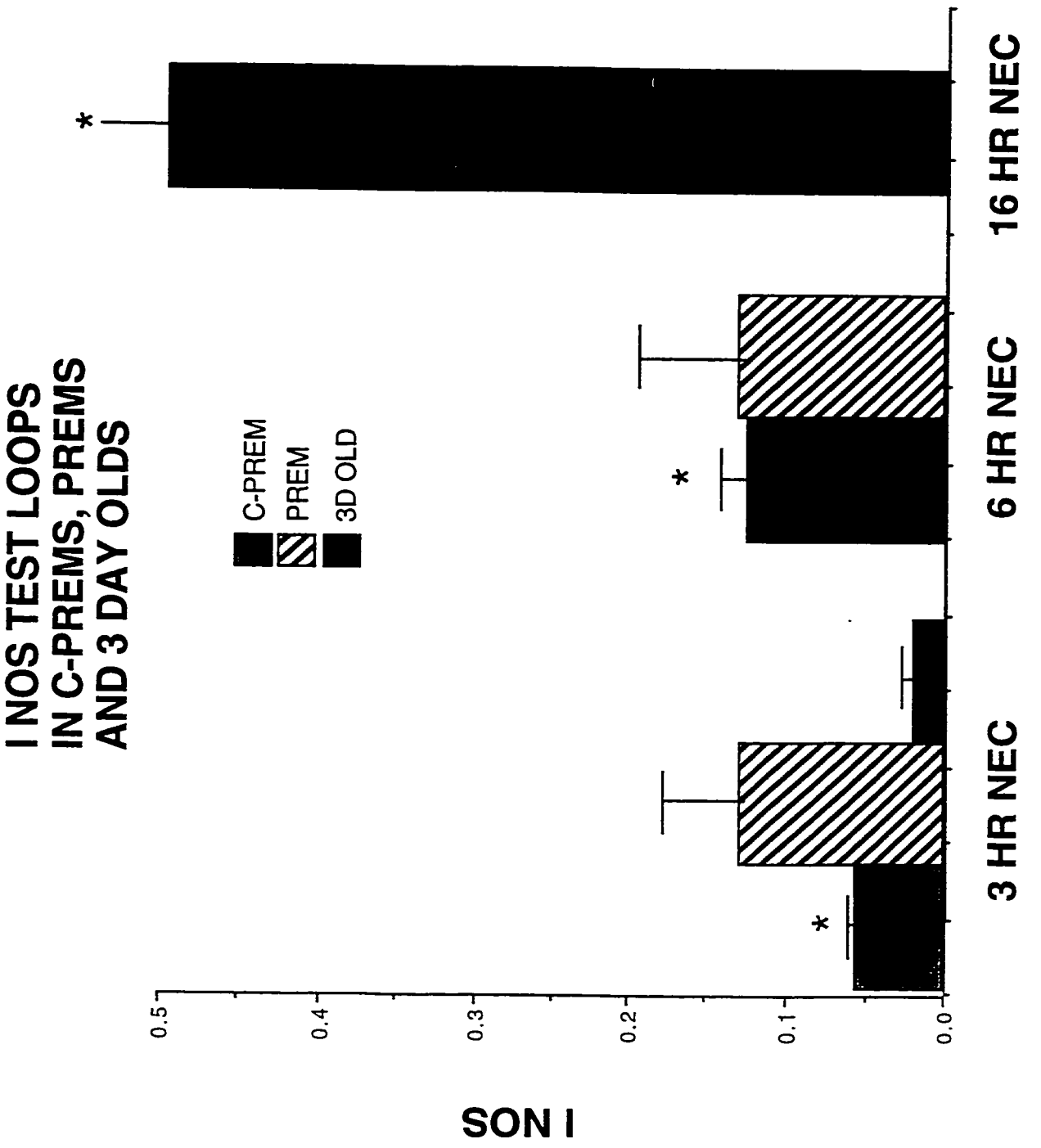

I NOS TEST LOOPS
IN C-PREMS, PREMS
AND 3 DAY OLDS
C-PREM
PREM
3D OLD
SON I
0.5
0.4
0.3
0.2
0.1
0.0
3 HR NEC
6 HR NEC
16 HR NEC
*
*
*

Fig.5.8 Inducible NO synthase activity (measured in ηmol/min./g of tissue) in control loops of intestine at 3, 6 and 16 hours post induction of NEC in prematures (C-prem), prematures born by maternal prostaglandin-induced delivery (prem) and 3 day old term newborn piglets.

iNOS activity is significantly higher in 3 day old control loops at 16 hours compared to 3 hours post NEC induction.

*= $p<0.05$

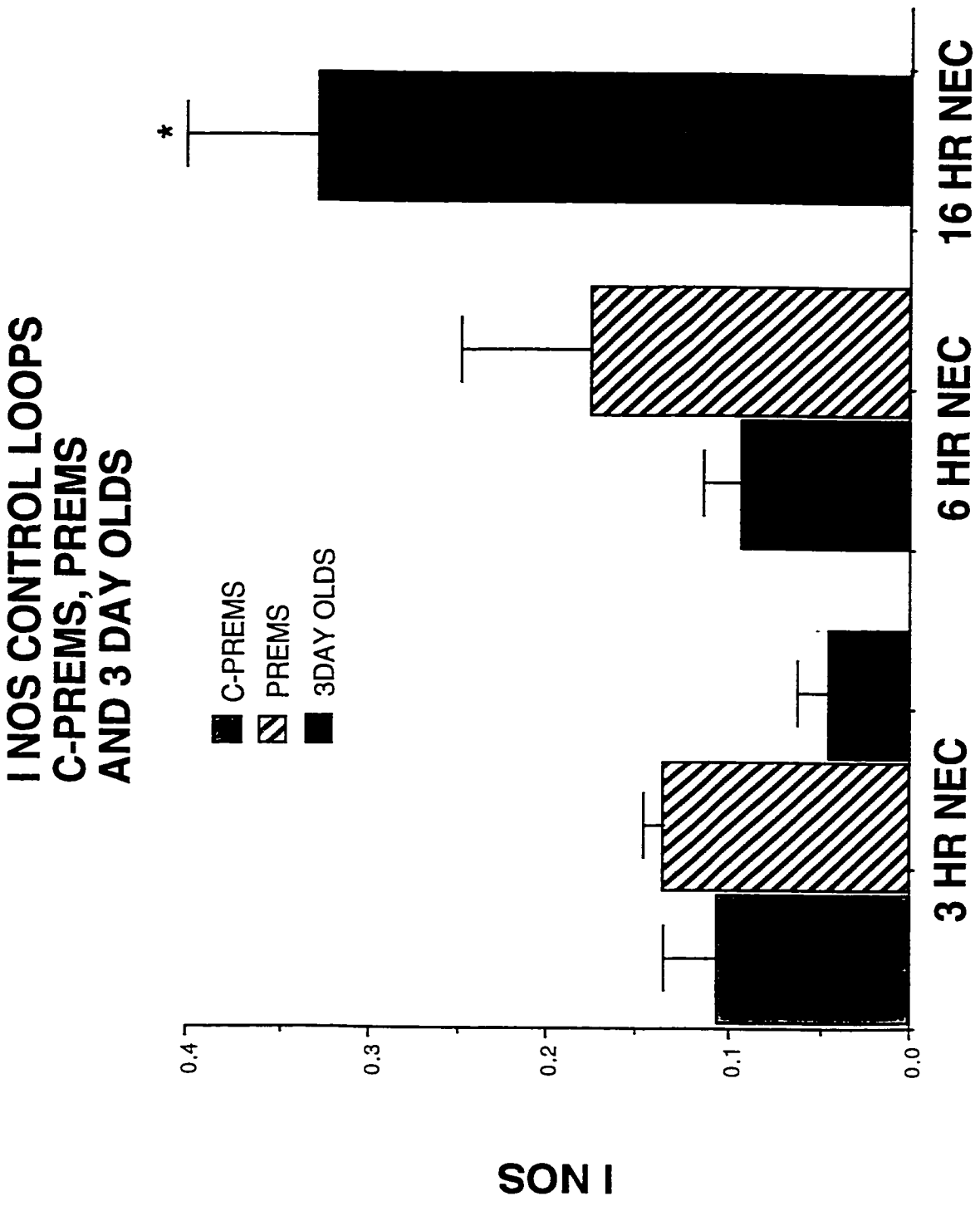

I NOS CONTROL LOOPS
C-PREMS, PREMS
AND 3 DAY OLDS
C-PREMS
PREMS
3DAY OLDS
*
0.4
0.3
0.2
0.1
0.0
SON I
3 HR NEC
6 HR NEC
16 HR NEC

Fig.5.9 Constitutive NO synthase activity (measured in ηmol/min./g of tissue) in control loops of intestine at 3, 6 and 16 hours post induction of NEC in prematures (C-prem), prematures born by maternal prostaglandin-induced delivery (prem) and 3 day old term newborn piglets.

cNOS activity is significantly higher in the prem group compared to the C-prems and 3 day olds at 3 hours of NEC.

cNOS activity is significantly higher in the C-prem group at 6 hours of NEC compared to the C-prems at 3 hours.

*= $p<0.05$

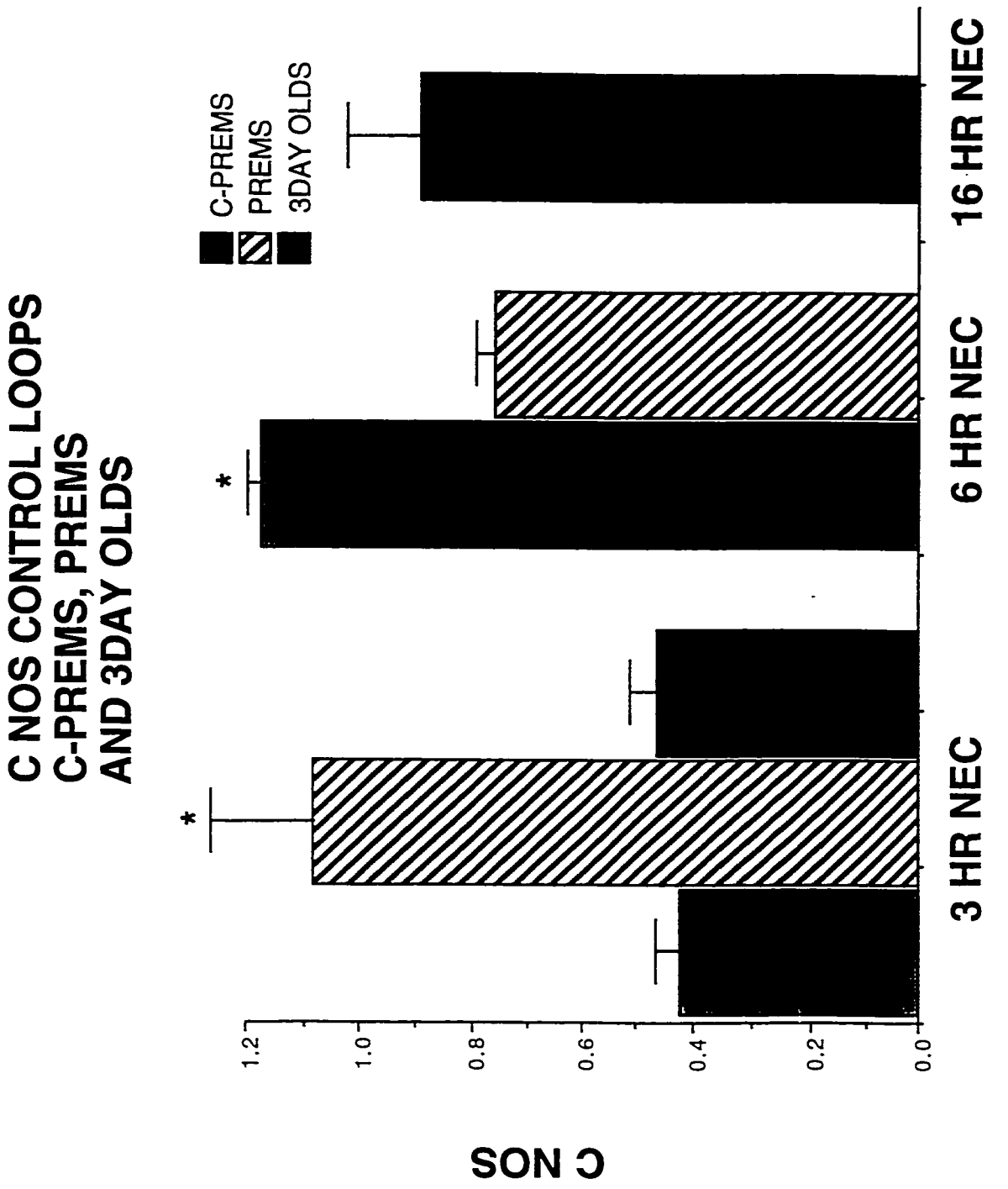
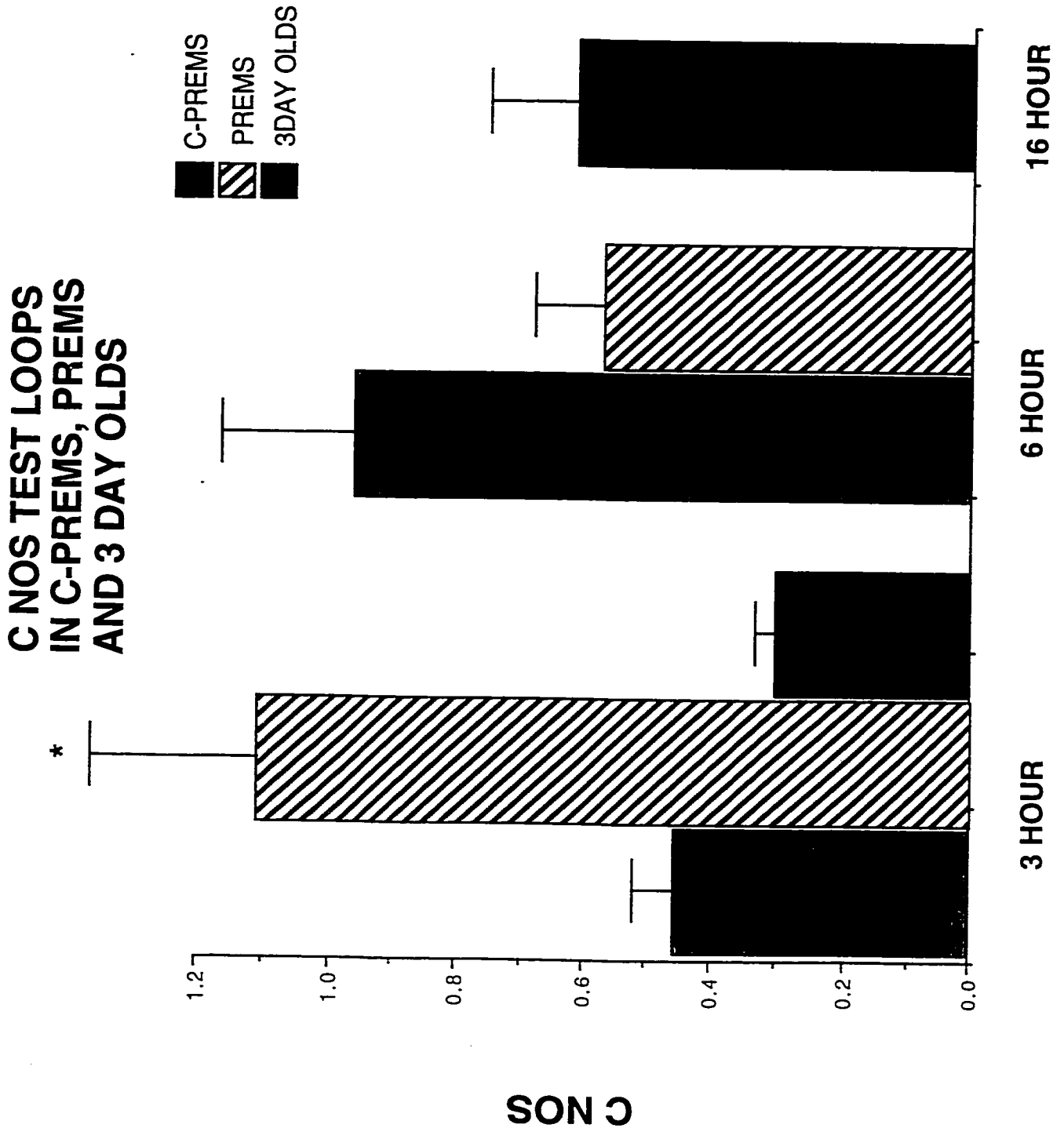

C NOS CONTROL LOOPS
C-PREMS, PREMS
AND 3DAY OLDS
C-PREMS
PREMS
3DAY OLDS
C NOS
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
3 HR NEC
6 HR NEC
16 HR NEC

Fig.5.10 Constitutive NO synthase activity (measured in ηmol/min./g of tissue) in NEC induced test loops of intestine at 3, 6 and 16 hours post induction of injury, in prematures (C-prem), prematures born by maternal prostaglandin-induced delivery (prem) and 3 day old term newborn piglets.

At 3 hours of NEC, cNOS activity is significantly higher in the prems compared

to the C-prems and 3 day olds.

*=$p<0.05$


C NOS TEST LOOPS
IN C-PREMS, PREMS
AND 3 DAY OLDS
C-PREMS
PREMS
3DAY OLDS
*
3 HOUR
6 HOUR
16 HOUR
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C NOS

5.2 DISCUSSION

The findings in study A correlate with our morphological data (see Chapter 4) where NO neurons in the myenteric plexus decrease (corroborating human data) while those in the submucosal plexus increase with age. Our data seem to disprove those previously reported by Furness's group indicating that the presence of NADPH diaphorase staining does not reliably or specifically indicate the presence of one or more NOS isoforms. However these findings were described in formaldehyde-fixed tissue and were found to be true in kidney and adrenal cortex only. Vascular endothelial cells and enteric neurons showed a correlation between NADPH diaphorase staining and NOS (Young et al. 1997).

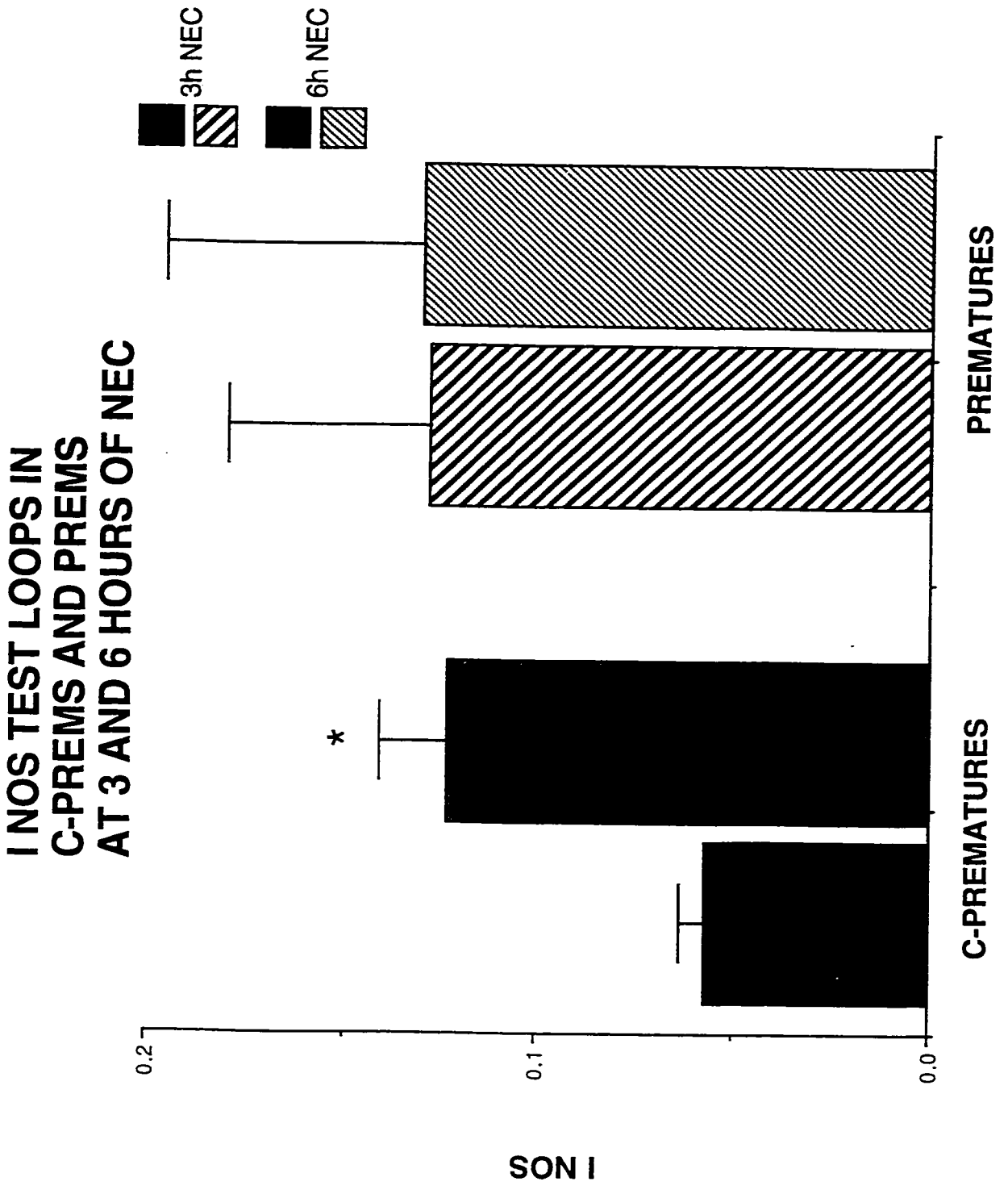
Study B findings are somewhat more difficult to interpret. It is unclear, at this time why control loops in the C-prem group show an increase in cNOS production at 6 hours (Fig.5.9). Nevertheless, it is clear that after 3 hours of NEC-induced gut damage iNOS production is increased in the C-prem piglet with a time course which is similar to that described in the literature (Fig.5.11) (Bune et al. 1996). These findings are in agreement with human data indicating increased expression of iNOS in the intestine of infants with NEC (Ford, Watkins et al. 1997). The blunting of this response in the second group may be a consequence of antenatal maternal administration of prostaglandin to induce labour. Evidence using a rat model of gastric ulceration points to the contribution of prostaglandins to mucosal defense (Ehrlich et al. 1998). Placental transfer of maternally administered prostaglandin to piglets at birth may explain our findings. A biochemical link between NO and prostaglandin biosythesis through superoxide anion had been described (Landino et al. 1996; Salvemini 1997). In addition,

Fig.5.11 Inducible NO synthase activity (measured in ηmol/min./g of tissue) in NEC induced test loops of intestine at 3 and 6 hours post induction of injury, in prematures (C-prem) and premature piglets born by maternal prostaglandin-induced delivery (prem).

Test loop iNOS activity rose significantly in the C-prems with duration of injury;

no increase was observed in the prems during the same time periods.

*= $p<0.05$


I NOS TEST LOOPS IN
C-PREMS AND PREMS
AT 3 AND 6 HOURS OF NEC
3h NEC
6h NEC
*
C-PREMATURES
PREMATURES
0.2
0.1
0.0
SON I

previous work on LPS-activated macrophages has shown that prostaglandins are involved in feedback modulation of inducible NO production (Pang and Hoult 1996).

Notably, our observations show that in both the premature and the fully developed newborn piglet, NEC-induced intestinal injury is associated with the expected increases in iNOS activity over time. How this relates to the susceptibility of premature animals to NEC is unclear, but may be related to other factors involving the enteric nervous system.

We propose that the observed variations in NOS isoenzyme activities with age and duration of NEC-induced intestinal damage reflect an everchanging balance between intestinal motility and mucosal protection which is intrinsic to the pathogenesis of NEC.

CHAPTER 6

L-ARGININE:
TOXICOLOGY AND DOSAGE STUDIES IN THE PREMATURE PIGLET

We hypothesize that in NEC-susceptible infants, decreased L-arginine levels cause lower whole body NO synthesis. In support of this, Castillo et al (1995) demonstrated decreased plasma arginine utilization during the acute vasoconstrictive state of persistent pulmonary hypertension of the newborn infant, suggesting that arginine availability may become an important factor in NO production. Oral L-arginine improves endothelial dependent dilatation in hypercholesterolemic young adults (Clarkson et al. 1996). Acute hypoxia increases intracellular L-arginine in cultured porcine pulmonary artery endothelial cells, suggesting that hypoxia can modulate the availability of free intracellular L-arginine (precursor of NO and primary substrate of NO synthase) by affecting the synthesis and degradation of cell proteins (Su and Block 1996). In addition, NO has been shown to enhance oxygen transfer from erythrocytes to tissues (Kosaka and Seiyama 1996).

Given the multi-faceted relevance of the L-arginine-NO pathway in biological systems, *in vivo* investigation of the quantitative aspects of arginine metabolism in premature and term neonates is important. This study sought to determine the parameters for eventual testing of our discovery in premature human neonates through the evaluation of the toxicological aspects of continuous L-arginine administration in premature piglets, and the effects of timing and doses of administration on NEC.

6.1 L-ARGININE

6.1.1 L-arginine synthesis

Circulating arginine is mostly produced in the kidney of the adult mammal, however, a large proportion of citrulline to arginine conversion occurs outside the kidneys in gut, liver and splanchnic region (Hurwitz and Kretchmer 1986; Riby et al. 1990; Wakabayashi et al. 1995; Yu et al. 1996). In the rat, citrulline absorption occurs in the distal ileum (Vadgama and Evered 1992), thus arginine becomes an essential amino acid after small intestinal resection in these animals (Wakabayashi et al. 1994). In addition, arginine synthesis from glutamate has been described in the human small intestine (Wakabayashi et al. 1991) and glutamine oxidation by mucosa, in addition to providing nitrogen precursors for mucosal anabolic pathways to maintain intestinal sturcture and function, provides citrulline and thus arginine for the whole organism (Plauth et al. 1999).

6.1.2 L-arginine and the intestine

In both animals and humans, small intestinal mucosa plays a major role in degrading arginine and other amino acids, such that 30-50% of these dietary amino acids are not available to extraintestinal tissues. Dietary amino acids serve as the fuel for small intestinal mucosa and are essential precursors for intestinal synthesis of, among other substances, citrulline and NO. Thus, intestinal amino acid catabolism plays an important role in modulating dietary amino acid availability to extraintestinal tissues (Stoll et al. 1998; Wu 1998). Pathophysiological insult to the gut can therefore have profound and far-reaching effects on this availability, leading to ongoing local and systemic

repercussions. Indeed, supplemental dietary arginine has been shown to accelerate mucosal regeneration and to enhance bacterial clearance from mesenteric lymph nodes following radiation enteritis in rats (Gurbuz et al. 1998). Furthermore, lactate causes a decrease in intestinal synthesis of ornithine, citrulline and arginine by piglet enterocytes (Dillon, et al. 1999). Pathological conditions leading to increased lactate production, such as in systemic hypoperfusion or sepsis, may thus limit the availability of circulating citrulline and arginine in neonates.

Local factors such as acid-base balance and sepsis affect intestinal amino acid absorption with maximal absorption of arginine occuring at pH 6.0 (Riley and Austic 1989). Arginine absorption has been shown to be impaired in systemic models of sepsis (Sodeyama, Gardiner et al. 1993), however survival from sepsis has been shown to be improved with arginine-supplemented diets (Gianotti et al. 1993).

Approximately 50% of small bowel and 80% of large bowel nourishment comes from the lumen. Thus, even in the presence of parenteral nutrition, mucosal atrophy is observed, promoting bacterial translocation. Gastrointestinal surface protection has been shown to be afforded by L-arginine rich diets (Bengmark and Jeppsson 1995). In situations of surgical stress and malnutrition (potential clinical settings for the premature infant susceptible to NEC), intestinal weight and mucosal mass were greatest in a rat model receiving an arginine-rich diet (Vazquez et al. 1996). In addition, L-arginine added to oral rehydration solution stimulates water and electrolyte absorption by the rat small intestine (Wapnir et al. 1997). Finally, oral supplementation of the glutamine and arginine precursor, ornithine-alpha-ketoglutarate, protects intestinal barrier function after orthotopic small bowel transplantation (de Oca et al. 1997).

6.1.3 Ontogeny

The ontogeny of amino acid metabolizing enzymes has been studied mostly in rodent models, however, extrapolation of these findings to humans is questionable. Human and ungulate embryos are able to catabolize amino acids, whereas, rodent embryos cannot. During development, amino acid and ammonia metabolizing enzymes first appear in the liver, then in the kidney and finally in the intestine of human embryos. The early appearance of these enzymes is not seen in the rat liver. The expression of enzymes involved in the ornithine cycle in the developing human kidney and gut indicates that these organs are involved in ornithine cycle intermediates auch as arginine or citrulline (Dingemanse and Lamers 1994).

6.1.4 Metabolic regulation

Despite the biochemical importance of arginine as the precursor for NO, little is known about the in vivo regulation of L-arginine metabolism or how it varies in pathophysiological states. Although plasma arginine is made available during one complete turn of the urea cycle, vascular endothelial cells, when stimulated, can exhibit a direct metabolic pathway which converts the citrulline produced by the action of endothelial NOS to arginine via argininosuccinate synthase and argininosuccinate lyase. (Brosnan et al. 1992)

Co-induction of iNOS, argininosuccinate synthase and argininosuccinate lyase have been demonstrated in rat tissue. Therefore citrulline-arginine recycling is important in NO synthesis, especially under stimulation by lipopolysaccharides (LPS) (Nagasaki et al. 1996).

Impaired NO and citrulline production may limit intracellular arginine availability, leading to greater dependence on plasma arginine and/or exogenous sources. Under pathological conditions such as pulmonary hypertension, hypoxia, and hypercholesterolemia, animal and human studies have shown that the administration of L-arginine may reverse or attenuate vasoconstriction, *suggesting that arginine availability may become a rate-limiting step in NO formation* (Drexler et al. 1991; Daghigh et al. 1994; Clarkson et al. 1996). However, the factors that affect arginine utilization have not yet been determined. The compartmentation of arginine metabolism is likely to influence arginine availability at different levels. It has been shown that altered extracellular concentrations of L-lysine and L-ornithine competitively inhibit arginine transport thus regulating cellular NO synthesis (Noeh et al. 1996).

Plasma and tissue levels of L-arginine vary, and are tissue specific (Noeh et al. 1996). Arginine injection has also been shown to affect amino acids and other compounds in rat plasma and tissues, notably ornithine, isoleucine, phosphoserine, leucine and ethanolamine. In rat liver, arginase has been shown to be inhibited by an intermediate in the biosynthesis of NO from L-arginine (NG-hydroxy-L-arg), thus favouring the utilization of arginine as a substrate for endothelial NOS (Daghigh et al. 1994).

Nutritionally, arginine is not an essential amino acid for the maintenance of nitrogen balance in healthy adults of most mammalian species (Rose 1937; Borman et al. 1946). Under certain circumstances, however, such as immaturity and severe stress (sepsis, trauma, nitrogen overload), arginine becomes essential for adequate nitrogen balance and physiologic function (Ha et al. 1978; Morris and Rogers 1978; Iyamu and

Adamson 1982). In humans, infusion of arginine-free amino acid solutions causes hyperammonemia and even coma. Decreased dietary arginine in infants with rare urea cycle enzyme deficiencies also leads to hyperammonemia requiring arginine supplementation for normal growth and development (Brusilow and Batshaw 1979; Batshaw and Brusilow 1980) L-arginine administration has been shown to increase survival in peritonitis (Madden et al. 1988) and to enhance wound healing and lymphocyte immune responses in humans (Efron et al. 1991). Therapeutic oral doses of up to 30g/day are safely tolerated in the adult human. Normal hematologic parameters, blood urea nitrogen (BUN), creatinine, mild hyperchloremic acidosis with decreased K+, Ca++ and bicarbonate were observed. Chronic L-arginine administration (20g/day for 28 days) has been shown to elevate levels of liver enzymes and urea in humans (Chin-Dusting et al 1996). The safe pharmacological dose of intravenous L-arginine is 500mg/kg/day (Sabex, Boucherville, Québec).

6.1.5 Methods

Under general Halothane anesthesia and endotracheal intubation, neonatal piglets born 5 days prematurely (n=13) underwent external jugular vein cannulation via a cervical skin incision. The intravenous tubing was then exteriorized subcutanoeusly in the interscapular region and sutured securely to the skin. Prior to skin incision, a urine sample was obtained by percutanous bladder puncture. A baseline blood sample was obtained at the time of venous cannulation. Upon recovery from anesthesia venous blood gas analysis was performed. A continuous intravenous solution consisting of either NaCl 0.9% with L-arginine 600mg/kg/hr (high dose group) or 5% dextrose in water with NaCl

0.2% and L-arginine 20mg/kg/hr (clinical dose group) was administered for a period of 96 hours. The piglets were kept warm and fed formula, and carefully observed in the animal care facility during the test period. Daily venous blood gases were obtained as well as blood samples for biochemical analysis (liver, renal and pancreatic function and serum electrolytes). All biochemical analyses were done by the Department of Biochemistry, Childern's Hospital of Eastern Ontario (sample biochemical analysis appended). At the time of sacrifice (5ml of intracardiac Euthanyl), a second urine sample was obtained.

Chemicals

L-arginine was purchased from Sabex (Boucherville, Quebec).

5% dextrose in water, NaCl 0.9%, NaCl 0.2%, from Baxter.

6.1.6 Results

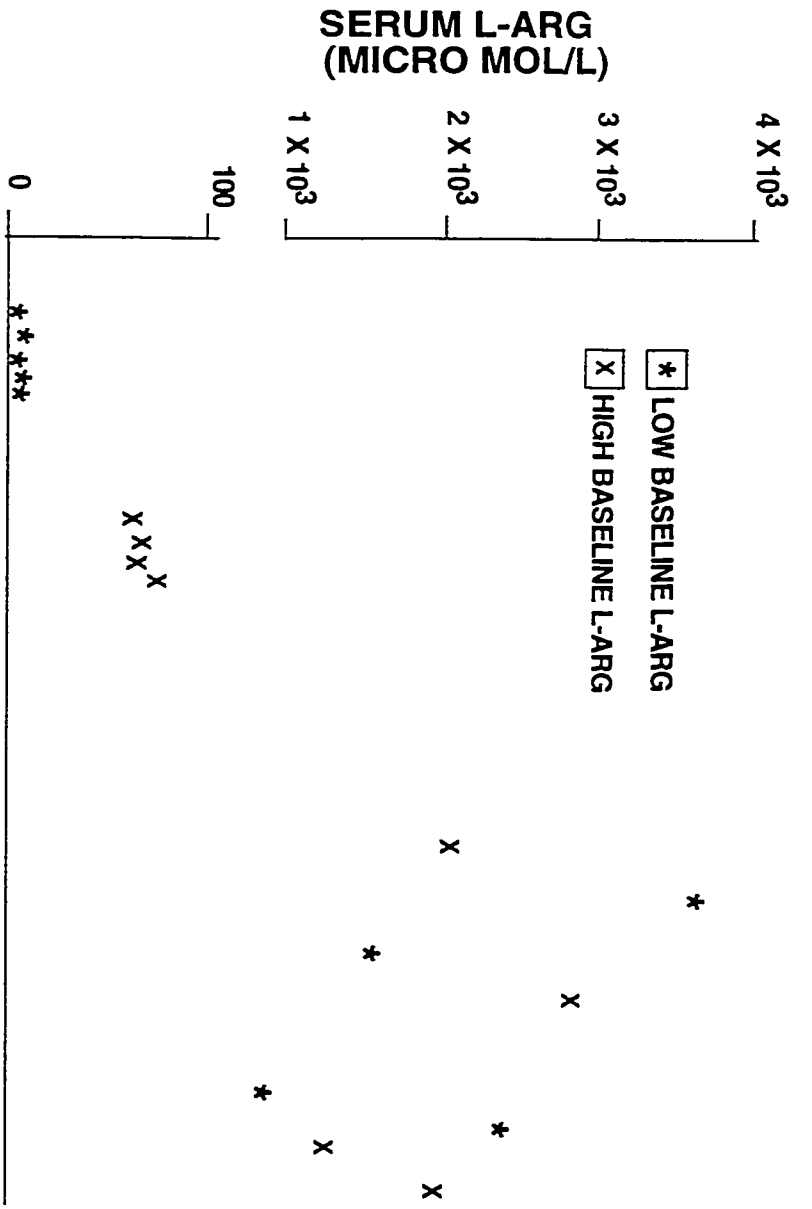
All 4 premature piglets receiving high dose L-arginine in NaCl 0.9% became lethargic, ataxic and developed severe metabolic acidosis and were sacrificed within 24 hours. Necropsy revealed gross cerebral edema and hemorrhagic congestion. The 9 animals receiving the lower clinically used dose of L-arginine in NaCl 0.2% survived the 96 hour infusion without any adverse effects. Gross anatomy was normal at necropsy. These piglets' baseline L-arginine levels showed a bi-modal distribution (2.67-6.74 μMol/l , n=5 and 63.05-73.77μMol/l n=4). L-arginine levels rose at 24 hours after onset of intravenous administration and continued to do so over time (maximal range was 676.25-4,825.47 μMol/l). There was no correlation between baseline and final L-

Fig. 6.1 L-arginine levels in premature piglets during 96 hour intravenous infusion of 20mg/kg/hr.

Day 0: baseline L-arginine levels show a bi-modal distribution.

Day 4: L-arginine levels rose over the test period to levels which did not correlate with baseline values.

L-ARGININE LEVELS IN PREMATURE PIGLETS DURING 96-HR I.V. INFUSION



arginine levels (see Fig.6.1). All other biochemical parameters remained within normal limits in survivours. Nitrites and nitrates were absent from urine samples of piglets in this group.

6.1.7 Discussion

This study sought to reproduce all aspects of the eventual human application of continuous intravenous L-arginine administration. Therapeutic doses of up to 30g/day of L-arginine per os are safely tolerated in the adult human. Normal hematologic parameters, blood urea nitrogen (BUN), creatinine, mild hyperchloremic acidosis with decreased potassium, calcium and bicarbonate have been observed. Chronic L-arginine administration (20g/day for 28 days)has been shown to elevate liver function enzymes and urea in humans (Chin-Dusting et al. 1996). The safe pharmacological dose of intravenous L-arg is 500mg/kg/day (Sabex). This dose was chosen because its use over time has been determined to be safe. The duration of infusion was chosen to be 4 days which is the average time bowel anastomoses take to heal. Thus any beneficial effect of intravenous L-arginine treatment on intestinal viability was hypothesized to be evident after this time period.

Of major interest are our findings which identify 2 populations of animals with either low or high baseline L-arginine serum levels. In a human study of NEC published subsequently, Zamora et al (Zamora, Amin et al. 1997) showed that plasma arginine levels were lower in premature infants with NEC than in unaffected infants. It is possible that individual susceptibility to NEC may be related to lower baseline plasma

concentrations of L-arginine. These findings further corroborate our own experimental data.

Reported adverse reactons following iv administraton of amino acids, especialy if administered too rapidly include: nausea, vomiting, numbness, headache, and venous irritation. Nevertheless, L-arginine hydrochloride is particularly well tolerated and adverse effects were not observed in our premature piglet population. Leakage of i.v. solutions of L-arginine hydrochloride into the surrounding tissue may cause necrosis and superficial phlebitis. This also did not occur due to the meticulous care taken to perform the venous cannulation and the surveillance which followed during the period of L-arginine administration (see appended photograph). Piglets were free to move in crates especially constructed by our Animal Care department but were unable to chew off the intravenous tubing. This situation is analogous to treating premature infants whose collaboration is obviously not to be counted upon and who must also undergo analogous methods of restraint.

L-arginine reported drug interactions include the following. Arginine increases blood glucose concentation. This effect may be direct, with glucose released from the liver or indirect, via stimulation of glycogenolysis and gluconeogenesis by glucagon release. Plasma insulin concentrations following arginine stimulation may be further elevated by thiazide diuretics and aminophylline. In one study, phenytoin reduced the plasma insulin response to arginine when glucose intolerant patients were given a glucose load. Severe, potentially fatal, hyperkalemia has occurred following L-arginine hydrochloride therapy for metabolic alkalosis in several patients with severe hepatic disease who had recently received spironolactone. Severe hyperkalemia in these patients

probably resulted from an arginine-induced extracellular shift of potassium from cells, impaired hepatic metabolism of arginine, and/or a spironolactone-induced decrease in renal excretion of the ion; spironolactone's effect on potassium persists for several days following cessation of the drug. Patients receiving a potassium-sparing diuretic are at increased risk of arginine-induced hyperkalemia.

6.2 L-ARGININE TREATMENT

6.2.1 Doses

Having determined the safety of continuous i.v. administration of the clinically used dose of L-arginine, we now sought to determine the effects of various doses, ranging from the above tested clinical dose (20mg/kg/hr) to the very high dose used in published animal studies, on NEC-damaged gut in the premature piglet. In addition, the effects of timing of treatment were investigated, as they relate to either prophylaxis or treatment of established NEC lesions (for ease of comparison with the other study groups, this data was shown in Chapter 3 [sections 3.2.1. and 3.2.2.]).

6.2.2 Methods

A continuous i.v. infusion of L-arginine of 20 mg/kg/hr (clinical dose, n=4), 100 mg/kg/hr (n=4), 200 mg/kg/hr (n=4), or 600 mg/kg/h (experimental dose reported in animal sudies, n=4) was administered at the time of NEC induction for a 3 hour period (see Chapter 3 [section 3.2.1] for detailed description). Gut loops were then excised and processed, hematoxylin and eosin stained, and graded macroscopically, from 0 for intact villi to 4 for transmural necrosis, as per protocol described in Chapter 2.

6.2.3 Results

All animals survived the experimental protocol. The continuous intravenous 3 hour administration of all doses of L-ariginine at the time of NEC induction in the premature piglets significantly reduced intestinal injury in the test loops compared to the untreated NEC group (Fig. 3.4), and this without any significant hemodynamic effects (see Chapter 3 section 3.2.2.).

6.3 TIMING OF L-ARGININE ADMINISTRATION

6.3.1 Methods

In order to evaluate the prophylactic potential of i.v. administration of L-arginine, anesthetized piglets received a 3 hour infusion of 20mg/kg/hr prior to NEC induction which was continued subsequently for 3 hours (n=4). These results were compared to the untreated NEC group of 3 hour duration. In addition, low (20mg/kg/hr) or high dose (600mg/kg/hr) L-arginine was infused for a 3 hour period, 3 hours after NEC induction (n=4 each). Gut loops were harvested, processed, graded and statistical analysis done as previously described in Chapter 3 (section 3.2.1.).

6.3.2 Results

The prophylactic i.v. administration of the clinical dose of L-arginine, for 3 hours prior to NEC induction significantly attenuated intestinal damage in test loops, compared to the untreated NEC group. However, this dose failed to significantly reduce intestinal damage when administered 3 hours post NEC induced injury. High dose L-

arginine was effective even when administered 3 hours post NEC induction (Fig.3.7). For details see Chapter 3 section 3.2.2.

6.4 Discussion

In these studies, we have successfully documented the potential for NEC prophylaxis of continuous intravenous L-arginine administration in premature neonatal piglets. Pre-treatment with a clinically used dose of L-arginine is as effective in attenuating NEC-induced intestinal injury as very high doses given post NEC induction.

Much evidence has been presented from work published after our studies which substantiates the fact that the NO precursor, L-arginine, is involved in intestinal mucosal protection, and that low levels of this amino acid, for whatever reasons, be they developmental or pathological, are associated with an increased susceptibility to intestinal injury. In fact, we have documented 2 distinct populations of premature neonatal piglets; one with low baseline L-arginine blood levels, and one with high levels. As mentioned, this may be a reflection, if extrapolated to the human, of individual susceptibilities to NEC. Indeed, measurements of L-arginine blood levels in premature infants have shown it to be decreased in infants developing NEC. In addition neonates with congenital diaphragmatic hernia who develop persistant pulmonary hypertension were also found to to have lower L-arginine blood levels when compared to non-affected matched controls.

In preparation for the potential clinical evaluation of continuous i.v. L-arginine as a therapeutic modality in the prophylaxis of NEC, we further documented the safety of chronic i.v. L-arginine administration over a 4 day period. This, again, was

achieved by meticulous monitoring of premature piglets' acid-base balance, and the potential effects of such chronic treatment on renal, hepatic and pancreatic function, while taking into account the possible neuro-toxicity of metabolic acidosis in piglets. Toxicological evaluation of this treatment regimen in the premature piglet has failed to demonstrate any averse side effects.

Finally, a Phase I clinical trial to evaluate the safety of continuous intravenous L-arginine infusion in premature infants is close to completion. Thus far, the trial has failed to reveal any adverse effects related to this regimen.

CHAPTER 7

PHASE I CLINICAL TRIAL TO EVALUATE THE SAFETY OF CONTINUOUS INTRAVENOUS L-ARGININE INFUSION IN PREMATURE INFANTS

7.1 BACKGROUND

We have obtained significant preliminary expermental evidence suggesting that either prophylactic or post injury intravenous infusion of non-toxic doses of the NO synthase substrate, L-arginine, attenuates intestinal damage that occurs with our premature neonatal piglet model of NEC. On this basis, we envisage the prophylactic use of intravenously administered L-arginine in addition to total parenteral nutrition (TPN), in all sick premature infants.

This pilot clinical trial seeks to determine the possible toxic effects of continuous intravenous administration of a clinically used dose of L-arginine in addition to TPN on premature infants in a neonatal intensive care setting.

The clinical and economic impacts of this potential therapeutic modality are wide ranging in that L-arginine-TPN therapy would be easy to implement, inexpensive, and if used on a routine basis in the neonatal intensive care setting, should significantly reduce infant morbidity and mortality.

7.1.1 L-Arginine

The amino acid requirements of human neonates, especially those born preterm and receiving total parenteral nutrition (TPN) are ill-defined. Wykes et al.

(1992) have shown that amino acid kinetics in low-birthweight infants (LBW) can differ depending on the route of delivery. Preterm neonates on TPN also exhibit marked reductions in whole body protein turnover (Duffy and Pencharz 1986) possibly due to intestinal mucosal atrophy and resultant decrease in gastrointestinal protein turnover. These differences in amino acid kinetics and physiological response to TPN in the neonate may thus affect the pattern of amino acid required. More specifically then, with regards to L-arginine, low birth weight (LBW) neonates given an amino acid solution (2.5g/kg/day) with the highest available L-arginine concentration (12.2g/100g amino acids; 4.6g/100g amio acids in human milk) had weight gain and nitrogen retention similar to their in utero counterparts. All plasma amino acids except phenylalanine were within 95% confidence limits of the plasma concentration goals (Heird et al. 1988).

We may thus hypothesize that in NEC-susceptible infants, L-arginine utilization for whole body NO synthesis is decreased. Similar conclusions have been drawn by Castillo et al (1995) who demonstrated decreased plasma arginine utilization during the acute vasoconstrictive state of persistent pulmonary hypertension of the newborn infant, suggesting that arginine availability may become an important factor in NO production.

7.1.2. Experimental models of NEC

The study of premature infant gut is in the first instance problematic, and although we routinely obtain resected specimens of neonatal and infant colon for morphologic, biochemical and in vitro pharmacologic study of healthy and Hirschsprung's disease tissue, the supply of tissue from NEC patients would not be

adequate to complete a timely study. On this basis we investigated the efficacy of various animal models for NEC, which would provide much of the information necessary to test our hypothesis. Human neonates and piglets are similar anatomically and physiologically including maturation and function of the kidney, GI tract, body composition and aspects of brain development (House 1995). With respect to protein and amino acid metabolism, ethical concerns of taking blood samples from small and compromized subjects may limit the power of studies designed to estimate amino acid metabolism. In addition, the amino acid profile of human and swine milk and tissue protein is similar. Thus the piglet is considered an appropriate model for questions related to human nutrition.

Our most significant finding was the dramatic reduction in NEC-related mucosal damage with concurrent intravenous infusion of NOS substrate (L-arginine) or NO donor (sodium nitroprusside). NOS inhibition did not affect the development of NEC. We concluded that NO is a protective agent against NEC-induced intestinal damage. ***The results of the study raised the prospect that increasing the availability of NO in premature infants may be a simple yet efficacious therapy for NEC.***

On this basis our follow-on studies focussed on the clarification of the effectiveness of different doses of NOS substrate, L-arginine, the optimal timing of substrate administration, and the potential toxic effects associated with the administration of high doses of NOS substrate in our piglet NEC model.

The effectiveness of *prophylactic* administration of a clinically relevant dose of L-arginine in attenuating the severity of intestinal injury associated with NEC, strongly suggested that chronic i.v. administration of L-arginine with TPN would be

effective prophylaxis for this disease. Toward this hypothesis, we sought to determine the toxicology of L-arginine infusion over the period of time generally accepted to be necessary for anastomosed bowel ends to heal (the surgically equivalent end point).

Continuous iv administration of a solution consisting of 5% dextrose, NaCl 0.2% and L-arginine(20mg/kg/hr) over a 4 day period is safely tolerated in 5day *premature enterally fed neonatal piglets*. Liver, kidney and pancreatic function based on daily biochemical analysis were within normal limits. Daily venous blood gases were also within normal range.

7.2 RATIONALE FOR CLINICAL DEVELOPEMENT

Although more data are needed, avoidance of aggressive early enteral feeding in LBW infants might decrease the incidence of NEC. Thus these high risk infants will require extended nutritional support provided by TPN. When an infant develops NEC, TPN is an integral component of the medical management regimen. Not only will concurrent TPN be necessary to maintain nutritional status, but it is likely that the use of L-arginine supplemented TPN may become an effective therapy for NEC. In addition, routine use of L-arginine-supplemented TPN in NEC-susceptible infants, may decrease the incidence and severity of this disease.

7.3 AIM

The aim of this Phase I pharmacology/toxicity clinical trial is ***to determine if premature infants can safely tolerate a continuous intravenous infusion of L-arginine over a period of four days.***

7.4 STUDY DESIGN

This pilot study is a prospective controlled phase I clinical trial. Patients who satisfy the inclusion/exlusion criteria will be entered into the study. Subjects will be randomly allocated to one of two study groups by pre-assigned sealed envelopes to be picked by the bedside nurse. The control (CON) group will receive a TPN solution without additional L-arginine. The study (L-ARG) group will receive L-arginine supplemented TPN. During this study period, blood and urine samples will be collected for biochemical analyses. Physical exam findings, demographic data, and biochemical data will be extracted from patients' clinical charts.

7.4.1 Study Population

Subjects will be recruited from the newborn intensive care units of two Canadian centers (Children's Hospital of Eastern Ontario, Ottawa General Hospital).

Suitable subjects will be recruited for the study only if parental/guardian written consent is obtained.

7.4.2. Inclusion Criteria

Patients will be eligible to enter if all of the following criteria apply:

a) Males or females born at less than or equal to 32 weeks gestational age

b) Patients are receiving TPN with a minimum of 3 g/kg/day of amino acids

7.4.3. Exclusion Criteria

a) Oral intake of expressed breast milk or infant formula of greater than 4 ml per day

b) Suspected or confirmed inborn errors of metabolism

c) Suspected or confirmed chromosomal anomaly or congenital syndrome

d) Biochemical evidence of liver failure

e) Perinatal asphyxia with evidence of multiorgan failure

- renal failure - urine output less than 1 ml/kg/hour

- liver failure - elevated liver enzymes

f) Culture proven septicemia or meningitis

g) Family history of severe allergic reactions (exclude non-urticarial rash)

h) Confirmed or suspected congenital infection

i) Participation in a concurrent clinical trial which requires blood sampling during the period of L-arginine infusion (to avoid excessive iatrogenic blood loss).

7.5 STUDY TREATMENT

Of the TPN amino acid solutions currently used in the premature infant population , the following contain the highest concentration of L-arginine: Trophamine (McGraw Laboratories, Irvine, CA) 12.2 g/100 g amino acids and Aminosyn (Abbott Laboratories, Chicago, IL) 12.3 g/100 g amino acids. Based on the L-arginine content of these solutions and a protein intake from TPN of 3 g/kg body weight/24 hours, an infant would receive 369 mg L-arginine/kg/24 hours. The amino acid solution currently used at

HEO and OGH is Vamin which contains 4.7 g L-arginine/100 g amino acids would provide 141 mg L-arginine/kg/24 hours.

Based on our work involving enterally fed premature neonatal piglets, an additional intravenous supplement of 500 mg/kg/24 hours L-arginine is safely tolerated. Furthermore, this dose of L-arginine is in current clinical use for growth hormone stimulation testing in pediatric patients (maximum total dose of 30 grams /24 hours). We therefore estimate that a total intake of L-arginine of 1g/kg/24hours is safely tolerated.

Since the intake of L-arginine will vary according to the specific amino acid solution used in TPN, the supplemental dose of L-arginine (Sabex Inc., Boucherville, Que.) (250 mg/ml) will be calculated to provide a total of 1 g L-arginine/kg body weight/24 hours. L-arginine will be added directly into the amino acid solution. We have determined that the addition of L-arginine had no effect on pH and turbidity, as shown in Table 7.1.

Supplemental L-arginine will be continuously infused over a period of 96 hours. This time period was chosen based on the average time for bowel anastomoses to heal completely. It is likely that should L-arginine be used in the prophylaxis or treatment of NEC, maximal healing would have occurred by 4 days.

7.5.1 Infusion rate of solution

L-arginine will be given as a continuous infusion. As per Neonatal Intensive Care Unit routine care, i.v. sites are assessed routinely (every 2-3 hours) and any interstitial i.v. will be discontinued. Blood glucose levels are monitored every 2-4 hours by heel prick Dextrostix as part of routine care.

pH and turbidity of Vamin amino acid solution with added L-arginine

SOLUTION	pH_1	pH_2	ABSORBANCE @ 520 nm (1)	ABSORBANCE @ 520 nm (2)
TPN	5.60	5.60	0.0130	0.0160
TPN + L-arginine	5.64	5.59	0.0130	0.0140
TPN + L-arginine @ 37°C for 30 minutes	5.55	5.56	0.0200	0.0220
TPN + L-arginine @ RT for 24 hours	5.58	5.59	0.0120	0.0180
TPN + L-arginine @ 37°C for 30 minutes, RT for 24 hours	5.54	5.55	0.0230	0.0190
TPN + L-arginine @ 4°C for 24 hours	5.68	5.65	0.0130	0.0160
TPN + L-arginine @ RT for 24 hours, 37°C for 30 minutes	5.54	5.56	0.0250	0.0270

TPN = Vamin, RT = room temperature, pH_1 pH_2 = repeat pH measurements, absorbance $\geq$ 1 = precipitate present

Table 7.1 Absorbance studies done to verify the potential turbidity of L-arginine-supplemented TPN, reflecting all potential clinical scenarios during i.v. administration. Note the low absorbance values indicating the absence of precipitation.

L-arginine infusion will be discontinued in any infant who develops: **1)** hyperglycemia (blood glucose levels > 7 mmol/L and glucosuria, positive for glucose on urine dipstick), **2)** hypoglycemia (blood glucose ≤ 2 mmol/L in those infants receiving thiazide diuretics and/or aminophylline) **3)** hyperkalemia (> 6 mmol/L) **4)** liver failure (enzyme elevation) **5)** renal failure (urine output < 1.0 ml/kg body weight/hour) **6)** septicemia and/or meningitis (culture proven) **7)** an allergic reaction (urticarial rash, respiratory distress, edema) **8)** tolerance to oral feeds of > 4 ml/day of formula and/or breast milk **9)** elevated serum and urine guanidino compounds relative to control patients (no normative data available) **10)** seizures (generalized, focal, subtle).

Any adverse effects resulting from the intravenous administration of L-arginine will be reported immediately to each of the Research Ethics Committees by the site coordinator.

7.6. RISK FACTORS

(as per SABEX Inc. drug monograph)

Reported adverse reactons: following iv administraton of amino acids, especially if administered too rapidly include: nausea, vomiting, numbness, headache, and venous irritation. Nevertheless, L-arginine hydrochloride is particularly well tolerated and the above-mentioned adverse effects are infrequent. Leakage of iv solutions of L-arginine hydrochloride into the surrounding tissue may cause necrosis and superficial phlebitis. Arginine increases blood glucose concentation. This effect may be direct with glucose released from the liver or indirect via stimulation of glycogenolysis and gluconeogenesis by glucagon release.

L-arginine reported drug interactions: Plasma insulin concentrations following arginine stimulation may be further elevated by thiazide diuretics and aminophylline. Phenytoin reduced the plasma insulin respnse to arginine when glucose intolerant patients were given a glucose load. Severe, potentially fatal, hyperkalemia has occurred following L-arginine hydrochloride therapy for metabolic alkalosis in several patients with severe hepatic disease who had recently received spironolactone. Severe hyperkalemia in these patients probably resulted from an arginine-induced extracellular shift of potassium from cells, impaired hepatic metabolism of arginine, and/or a spironolactone-induced decrease in renal excretion of the ion; spironolactone's effect on potasium persists for several days following cessation of the drug. Patients receiving a potassium-sparing diuretic are at increased risk of arginine-induced hyperkalemia.

Contraindication: Should not be used in patients with allergic tendencies.

Precautions: L-arginine hydrochloride should be administered with caution in patients with hyperchloremic acidosis or with renal failure.

The proximal convoluted tubule of the kidney is a site of arginine production in mammals . L-arginine has been shown to be required for the synthesis of neuroactive guanidino compounds (GC) (D'Hooge et al. 1994; Watanabe et al. 1994). Guanidinosuccinate (GSA) and methylguanidine (MG) are endogenous convulsant GC's which have been shown to be greatly increased in uremic patients (Marescau et al. 1995) more specifically, urea is a precursor of GSA (Meert et al. 1993). Furthermore, there seems to exist an age dependent sensitivity to the convulsive effects of GC's, in that the mean latency for the appearance of clonic convulsions increases with increasing age in mice (D'Hooge, Pei et al. 1994). Uremia modifies GC metabolism, especially those

synthesized from arginine. Since renal function in the premature infant is not fully developed, any clinical evidence of possible neurotoxicity and/or elevation of serum or urinary GC's will necessitate the cessation of supplemental intravenous L-arginine.

7.7. STUDY PROCEDURES

Written Informed Consent

All study-related procedures must be fully explained to the patient and parent/guardian. Written consent must be obtained by a qualified person involved with this study (excluding principle investigators) prior to performing any assessments.

Medical History

The following information will be extracted from the patients' clinical chart: gender, date of birth, gestational age based on first trimester, ultrasound dating or postnatal Dubowitz scoring, birthweight, postnatal age, urine output (ml/kg/hour), blood urea nitrogen, serum creatinine, liver function tests (AST, ALT, γGT, bilirubin total and direct), electrolytes, acid/base status, glycemia, amylasemia.

Physical Examination

A complete physical exam will be performed daily and will also include vital signs (heart rate, blood pressure, respiratory rate) and weight.

Sampling of Specimens

Pain control: Topical anesthesia will be offered but not tested in premature infants.

Blood:

Times: study entry, daily for 4 days

Amount blood: Total per day: 1 ml (in addition to routine TPN surveillance bloodwork)

Method of sampling: venous for all except blood gas (capillary)

Urine:

Times: study entry and day 4

Method of sampling: spot bag collection

Biochemical Assessment

Analysis done by Biochemisty laboratory at CHEO. Involvement of the Dept. of Biochemistry for the analysis of our animal experiments will continue for this Phase I Trial.

Daily:

-acid/base: blood gas (capillary or arterial) with specific attention to metabolic acidosis

-electrolytes: with specific attention to hyperchloremia

-pancreatic function: amylase, glucose (hyperglycemia)

-renal function: BUN, creatinine

-index of increased NO production: urine nitrites/nitrates

-hepatic function: AST, ALT, γGT, bilirubin total and direct

-neurotoxicity: serum and urine guanidino compounds

At times 0, 24 and 96 hours:

-amino acid metabolism: serum amino acid levels

Clinical Assessment

Signs of seizure activity

Withdrawal of Patients from Study

Signs of convulsion

Increased serum and urinary guanidino compounds

Data Recording

Case Report Form will be provided for each patient recruited.

7.8. STATISTICS

Due to the nature of this Phase1 trial, all analyses will involve comparison of control versus L-arginine-supplemented patients. Interim analysis of urine and plasma guanidino compounds will allow definition of adequacy of sample size. We anticipate that a sample size of 20 in each group should yield useful results regarding monitoring for biochemical indices of potential adverse effects of L-arginine supplementation.

Analysis of continuous variables (biochemical and hematologic indices) will be done by analysis of variance with post Student's t-test. Incomplete data sets (< 4

completed days of L-arginine infusion) will be included in the data analysis. The Chi squared test will be used for categorical data. The level of significance will be $p<0.05$.

7.9. EXPECTED OUTCOMES

- elevated serum L-arginine
- mild hyperchloremic metabolic acidosis

7.10. ANTICIPATED PROBLEMS

- low recruitment rate due to concurrent ongoing studies

7.11 PRELIMINARY RESULTS

No adverse effects have been observed to date in 10 study patients.

CHAPTER 8

CONCLUSIONS

NEC is a heretofore common disease of unclear pathogenesis which will potentially affect more and more premature infants as technological advances increasingly allow for the recovery of immature lung function in the postnatal period. To date, specific treatment of this disease based on clear pathogenetic mechanisms has eluded us, such that it is precisely those infants whose initial survival has been assured by improvements in respiratory and nutritional support of the premature, who will succumb to the morbidity and mortality associated with NEC. Little knowledge has been gained over the past few decades regarding the treatment and prevention of this disease and thus, because of its unpredictable nature, outcomes have remained arbitrary. It is thus of paramount importance that research directed at the specific etiology and hence, the treatment of NEC continue.

This body of work has sought to determine, through analysis of the two most common risk factors, prematurity and enteral feeding, potential pathogenetic mechanisms and therapeutic modalities for NEC. Through the use of a premature and neonatal piglet model where NEC lesions are induced via the intraluminal injection of a solution (acidified casein) resembling the intestinal contents of NEC babies at the time of surgery, we were able to reproduce, as accurately as possible, the human condition. The

intestinal dysmotility associated with prematurity was reproduced by the creation of closed intestinal loops at laparotomy.

Analysis of the literature, at the time of undertaking this research, revealed that NO had been shown to be involved in gut motility as well as in mucosal protection of the mammalian gastrointestinal tract. Thus, NO seemed a likely candidate for investigation as a factor in the development of NEC. Our initial demonstration that increasing NO availability, through the intravenous administration of the NO synthase substrate, L-arginine, significantly attenuated NEC-induced intestinal injury in the neonatal piglet was the first publication of its kind in the literature. Our subsequent studies in *premature* piglets using the NO donor, SNP, or L-arginine confirmed these findings. Inhibition of NO synthesis with L-NAME did not affect outcome in either developmental category.

In order to determine the clinical applicability of these findings, we then sought to determine the efficacy of prophylactic intravenous administration of L-arginine. This was done in order to circumvent the obviously unpredictable nature of NEC, and with the universal empirical administration of this amino acid in the intravenous alimentation of all premature infants and those term infants deemed to be at risk, in mind. Such infants are routinely fed with intravenous amino acid solution containing variable amounts of L-arginine. The feasibility of supplementing TPN with L-arginine was evident. We showed that continuous prophylactic intravenous administration of a clinically used dose (20mg/kg/hr) of L-arginine was effective in attenuating NEC-induced gut damage in the premature piglet. Furthermore, toxicological investigation of

the chronic intravenous administration of this dose of L-arginine over a four-day period showed that L-arginine failed to cause any adverse effects in these premature piglets.

Concomitant work was also undertaken to investigate the ontogeny of enteric nitrergic innervation in the piglet. Through histochemical staining using NADPH diaphorase, NO neural elements in the the myenteric and submucosal plexuses of the developing piglet gut were quantified in laminar preparations of the small and large intestine. These findings revealed that although the morphology and distribution of NO neurons remained consistent with age, there was an ontogenically determined pattern of enteric nitrergic neuronal development whereby the frequency of NO neurons decreases with maturity in the myenteric plexus and subsequently increases postnatally, never to achieve early premature levels. Biochemical analysis of NO synthase activity parallels these findings, with the additional finding that iNOS activity is high in the mucosa of fed piglets. This may be of pathogenetic relevance, since iNOS is associated with increased intestinal inflammation and its activity may possibly be induced by intraluminal substrate. Notably, we have documented higher iNOS levels in acidified casein-damaged gut loops in premature animals than in term piglets. These levels increased with duration of insult in both groups. Higher cNOS levels found in premature piglet control loops remain to be explained, but correlate with the high frequency of NO neurons in control prematures.

Our *in vivo* motility studies confirmed the presence of ontogenically determined patterns of fasting intestinal motor activity in the developing piglet which can be modified by manipulation of the nitrergic system. Younger piglets demonstrated a less coordinated pattern of motor activity in the terminal gut which showed a greater

response to L-arginine and L-NAME (increased frequency and amplitude of relaxations and contractions respectively) in the colon. These documented physiological observations correlate with our histochemical data in that we have shown a higher frequency of NO neurons in colon myenteric plexus of less developed piglets.

Future research endeavours will most likely revolve around the elucidation of molecular mechanisms involved in the protective effects of NO on NEC-induced intestinal damage in particular and on acute inflammation in general. The potential role of NO in sepsis, which is closely related to NEC, is very controversial, with some findings pointing to a beneficial role for NO and others to a detrimental one, and as such, merits further investigation.

Molecular biological probes of surgical specimens have shown an increase in iNOS activity in enterocytes of the intestinal wall of infants with NEC. Furthermore, the only documented cytokine which has been shown to be *consistently* upregulated in NEC infants, but not in control patients, was interferon gamma (IFNγ which is an inducer of NO production. Thus, loss of mucosal integrity leads to bacterial invasion and release of LPS and IFNγ mediating over-expression of NO by enterocytes and causing tissue destruction.

Inefficient intestinal clearance of gut contents in premature infants is certainly contributory to bacterial proliferation, whereby changes in local intestinal environment through bacterial metabolisation of excess intraluminal carbohydrate facilitate villus damage and favour bacterial invasion. Here again, the role of NO in the ontogeny of enteric neurotransmission and motility must also continue to be probed.

Intestinal barrier failure in the gut-origin of sepsis and NEC allows for bacterial translocation. However, the mechanism of this translocation is unclear. Recent work seems to point to extrusion zones created by desquamated enterocytes in intestinal villus tips allowing for intact bacterial passage to the submucosa and their translocation to mesenteric lymph nodes. Thus, NO and its role in bacterial translocation requires further molecular elucidation.

Potential Pathogenetic Mechanisms Leading to NEC

The question, however, as to why constitutive NO production is beneficial to NEC and increased NO production via inducible NOS is detrimental, remains, as of yet, to be answered.

Based on our findings and a review of the pertinent literature on NEC, we postulate a series of potential pathogenetic mechanisms leading to NEC: *Bacteria and enteral feeding* initiate a cascade of events which injure the *immature gut* leading to *intestinal damage* known as NEC. We will present here a summary of these key factors, and highlight our contributions.

Formula, high in carbohydrate content, remains undigested in the terminal neonatal ileum. Developmentally low levels of lactase in this region, coupled with ***intestinal dysmotility*** characteristic of premature infants, favouring bacterial proliferation, allow for the fermentation of excess carbohydrate to short-chain fatty acids which decrease intraluminal pH and initiate mucosal damage. Because L-arginine metabolism has been shown to be localized to the villus tips and matures in a cranio-

caudal direction, initial damage would affect L-arginine availability and thus constitutive NO levels in these infants.

The ***higher frequency of NO neurons, and NO synthase activity in the more immature distal intestine*** make this region more susceptible to variations in NO availability. Indeed, ***we have demonstrated an increased intestinal motility response to manipulation of NO availability in younger piglets.*** In addition, O_2 demands are higher in immature gut, and as such, there is a sustained vasodilator response to nerve stimulation but not to sustained hypotension or hypoxia in newborn intestine. Interestingly, basal intestinal vascular endothelial NO production is higher in newborns and is age dependent.

Thus, limited L-arginine availability, due to altered intestinal metabolism, leads to decreased basal NO production altering its protective gut barrier function and along with damaged enterocytes, facilitate bacterial translocation and cytokine production, in turn leading to stimulation of production of biological mediators (ex. PAF), ***induction of NO synthesis and increasing gut damage*** through peroxynitrite formation and lipid peroxydation, and inflammation through increased leukocyte adhesion. Indeed systemic bacteremia causes hypotension and intestinal vasoconstriction and hypoxia, which exacerbate these effects. Also, clinically advanced NEC is characterized by leukocytopenia. ***Individual variability in baseline L-arginine levels*** may thus influence susceptibility to intestinal damage. These findings may serve to explain the developmental susceptibility to NEC and why ***increasing systemic NO availability may be prophylactic for NEC.***

This research has thus contributed to our understanding of certain aspects of the devlopment of enteric nitrergic innervation in the piglet gut and how NO, through its roles in intestinal motility and mucosal protection, may be involved in the pathogenesis of NEC. In addition, the data point to the potential therapeutic application of the NO substrate, L-arginine, for the treatment of NEC. More exciting, is the simplicity and efficacy of L-arginine administration at known clinical doses, the lack of adverse effects, and how it targets injured intestinal segments.

To further this specific therapeutic end, a clinical toxicology trial has been undertaken, which, to date, has failed to demonstrate any adverse effects related to the chronic intravenous administration of L-arginine in selected premature infants. Subsequent to the completion of this Phase I trial, we will undertake a multicentre Phase III trial, whereby the therapeutic efficacy of this treatment regimen will be investigated in infants at risk for developing NEC.

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APPENDIX I

An Intraluminal Model of Necrotizing Enterocolitis in the Developing Neonatal Piglet

An Intraluminal Model of Necrotizing Enterocolitis in the Developing Neonatal Piglet

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● **The most common risk factors for the development of necrotizing enterocolitis (NEC) are prematurity and enteral feeding. Most models of NEC involve ischemic insult resulting in generalized necrosis, different from the classical ileocecal predilection of NEC. This anatomic predisposition is explained by dysmotility of immature gut, leading to bacterial overgrowth in the terminal ileum and colon. Infant formula containing lactose as the sole carbohydrate source overwhelms partially developed lactase activity, allowing enteric bacteria to ferment excess carbohydrate to short-chain fatty acids, decreasing intraluminal gut pH and predisposing to mucosal injury. Impaired clearance of intraluminal contents exacerbates this effect. In the present study the authors used a model of NEC, originally developed in rabbits and based on analysis of intestinal contents of NEC babies, modified and adapted here to neonatal piglets, the gastrointestinal tract of which more closely resembles the human neonate. *Method:* Piglets <3 days old and 2 weeks old were laparotomized. Loops created from the distal ileum to the proximal colon were injected with isoosmolar acidified casein solution or 0.9% saline. Segments were harvested 3 hours later, sectioned for H&E, and graded from 0 (intact villi) to 4 (transmural necrosis). *Results:* Acidified casein-induced damage included areas of necrosis, submucosal edema, inflammatory cell infiltrate, and lymphatic distension. In younger animals, lesions were more pronounced (3.25 ± 0.13 for the <3-day-old *v* 2.43 ± 0.14 for the 2-week-old piglets; $P < .005$). *Conclusion:* The authors believe that this piglet NEC model most closely approximates human NEC because it incorporates two of the most common risk factors: dysmotility (by creating intestinal loops) and enteral feeding (by intraluminal injection of acidified casein).**

INDEX WORDS: Necrotizing enterocolitis, developing piglet, intraluminal model.

NECROTIZING enterocolitis (NEC) is the most common acquired, life-threatening gastrointestinal disease in the neonatal period.[1] In addition, it is the most common acquired surgical emergency in this age group.[2] To date, NEC remains a disease of uncertain etiology and pathogenesis. Various risk factors have been postulated to play a role in the development of NEC, among them prematurity, ischemia, intestinal bacteria, and feeding. Of these, the only consistently identifiable risk factors for NEC have been shown to be prematurity and enteral feeding.[3] Because more infants are surviving prematurity, the prevalence of NEC can be expected to increase, but despite great interest in this disease, clinical progress has been stagnant.

Most animal models of NEC involve some form of ischemic insult that results in more generalized intestinal necrosis,[1,4,5] which differs from the classical anatomic predilection of NEC in the terminal ileum and colon. This anatomic predisposition can be tentatively explained by considering the aforementioned risk factors for NEC, in the following manner: the immature human gastrointestinal tract has been shown to be dysmotile, with a gestationally timed pattern of development of intestinal motor activity, resulting in a normal cycling pattern of fasting motor activity by approximately 37 weeks' gestation.[6,7] Thus, the resulting lack of fully matured clearance mechanisms in the premature gastrointestinal tract may lead to bacterial overgrowth in the terminal ileum and colon. In addition, full lactase activity is only partially developed in the premature infant.[8,9] Infant formula, containing lactose as the sole carbohydrate source, overwhelms this partially developed lactase activity, allowing enteric bacteria to ferment excess carbohydrate to short-chain fatty acids, thus decreasing intraluminal pH in the terminal ileum and colon, predisposing to mucosal injury. Impaired clearance of intraluminal contents exacerbates this effect.

Herein we present an intraluminal model of NEC, originaly described in rabbits and based on analysis of intestinal contents of NEC infants,[10] which we have adapted to the developing piglet and which we believe most closely resembles the human condition.

MATERIALS AND METHODS

NEC Model

A modification of the model described in the rabbit by Clark et al[10] was adapted to the pig. The experimental protocol was approved by the Animal Care Committee of the University of Ottawa, Canada. Newborn Yorkshire suckling piglets (n = 8, supplied by the Center for Food and Animal Research, Agriculture Canada, Ottawa) were divided into two age groups: group 1, <3 days old; group 2, 2 weeks old. The animals were anesthetized using midazolam (0.1 mg/kg intramuscularly) and a halothane (1%

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Presented at the 26th Annual Meeting of the Canadian Association of Paediatric Surgeons, Toronto, Ontario, September 19-21, 1994.

M. Di Lorenzo is supported by a grant from Les Fonds de la Recherche en Santé du Quebec, Canada.

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0022-3468/95/3008-0007$03.00/0

to 2.5%) and oxygen (97.5% to 99%) mixture via an endotracheal tube. A midline laparotomy was performed. Without causing ischemia, a ligature was placed at the proximal ileum, 40 cm from the ileocecal valve, and was flushed distally by transmural injection of warm sterile 0.9% saline, using a 26-gauge needle. Six intestinal loops were created (4 ileal, 1 cecal, 1 colonic) by placing ligatures at consecutive 10-cm intervals. The loops were injected transmurally with sterile 0.9% saline (control) or with test isoosmolar solution consisting of bovine casein (10 mg/mL) and calcium gluconate (50 mg/mL) acidified to pH 4.0 with propionic acid, using a 24-gauge needle. During injections, care was taken to avoid overdistension of bowel loops, eliminating a possible ischemic component to the induced damage. The abdominal cavity was then closed. During the experiment, all animals received 5 mL/kg/h of 0.9% saline intravenously or subcutaneously. Body temperature was maintained at 37°C using a heated mattress and warming lamp. After 3 hours the intestinal segments were inspected macroscopically for lesions and were harvested. The animals were then killed with intracardiac sodium pentobarbital (65 mg/mL).

All tissues were prepared for microscopic analysis by clearing the lumen of material with gentle perfusion of 0.9% saline solution, followed by immersion in 10% formalin. Transverse frozen sections of treated and control intestine were cut and then stained with H & E according to standard procedures.

Intestinal segments harvested from the piglets (group 1 [<3 days old], n = 24 loops; group 2 [2 weeks old], n = 24 loops) were sectioned and evaluated microscopically. The degree of necrosis was scored using a scale of 0 to 4, according to the method of Clark et al[11]: 0, villi completely intact; 1, villus tip necrosis with preservation of villus crypts; 2, necrosis of villus tips and crypts with loss of mucosal and submucosal architecture; 3, necrosis extending to the muscularis; and 4, transmural necrosis. The presence of hemorrhage, inflammatory cell infiltrate, and lymphatic distension was noted.

Statistical Analysis

The mean and standard error of the mean were calculated for each gut loop after grading for damage. Statistical analysis was performed using the Instat statistical computer program (Graph Pad Software, San Diego, CA). The unpaired, nonparametric Mann-Whitney Two Sample two-tailed *t* test was used to compare control or test loops between the two age groups. One-way analysis of variance (ANOVA) and the Bonferroni posttest were used to compare differences among the control or test loops within the same group. A *P* value of less than .05 was considered significant.

Chemicals

The chemicals used were bovine casein (Sigma, St Louis, MO); calcium gluconate and propionic acid (BDH Inc, Montreal, Quebec); midazolam (Hoffman-LaRoche Ltd, Toronto, Ontario); halothane (Ayerst Laboratories, Toronto, Ontario); and sodium pentobarbital (MTC Pharmaceuticals, Toronto, Ontario).

RESULTS

All piglets remained stable for the duration of the experiments based on maintenance of vital signs within normal limits. Upon macroscopic inspection, necrotic damage was consistently induced in all loops of intestine injected with the acidified casein test solution. The damage was more apparent in the younger age group. More particularly, the test loops remained distended after the 3-hour period. This finding is indicative of mucosal damage with resultant loss of absorptive capacity. The control loops were collapsed. Upon inspection of the luminal aspect of the bowel wall, the observed lesions were patchy with areas of confluence characterized by mucosal hyperhemia or frank necrosis extending below the mucosa and in some cases through the muscle layers.

Microscopic evaluation of these segments showed areas of necrosis, submucosal edema, inflammatory cell infiltrate, and lymphatic distension (Fig 1B, D, E, and F). In the <3-day-old group, intestinal damage extended through the muscle layers (Fig 1D and F) and the average grade was 3.25 ± 0.13. In the 2 week olds, damage included the mucosa, with loss of submucosal architecture, generally sparing the muscle layers (Fig 1B and E), with an average grade of 2.43 ± 0.14.

Comparison of the extent of injury in the NEC-induced loops between the two age groups showed a significant ($P < .05$) difference (Fig 2). Anatomically matched NEC loops showed significant ($P < .05$) damage in the <3-day-old group in the terminal ileum, 10 cm from the ileocecal valve and in the colon (Fig 3). This difference in damage (although not significant [$P = .057$]) was also evident in the more proximal ileum, 30 cm from the ileocecal valve. There was no significant ($P > .05$) regional difference among the NEC-damaged loops of ileum and colon within the same age group. Similarly, no significant ($P > .05$) difference was evident among saline control loops (not shown) within and between groups.

DISCUSSION

The piglet NEC model used in this study involves intraluminal injection of a solution similar to the intestinal contents of NEC infants, and therefore more closely resembles the human condition. It is postulated that the organic acid (propionic acid) used in the test solution contributes to mucosal damage and facilitates transepithelial migration of casein, the main protein in cow's milk–based infant formula, causing recruitment of lymphocytes and release of inflammatory mediators.[12] In addition, casein has been shown to increase carbohydrate fermentation by enteric gram-negative bacteria to short-chain fatty acids, further lowering intraluminal pH.[13] Our results show the histological presence of pneumatosis intestinalis, the radiological hallmark for NEC, which is the result of hydrogen gas in lymphatics produced by bacterial fermentation of carbohydrates. This finding was not demonstrated in another previously reported study using piglets, in which NEC was induced using luminal perfusion of a variety of full-strength infant

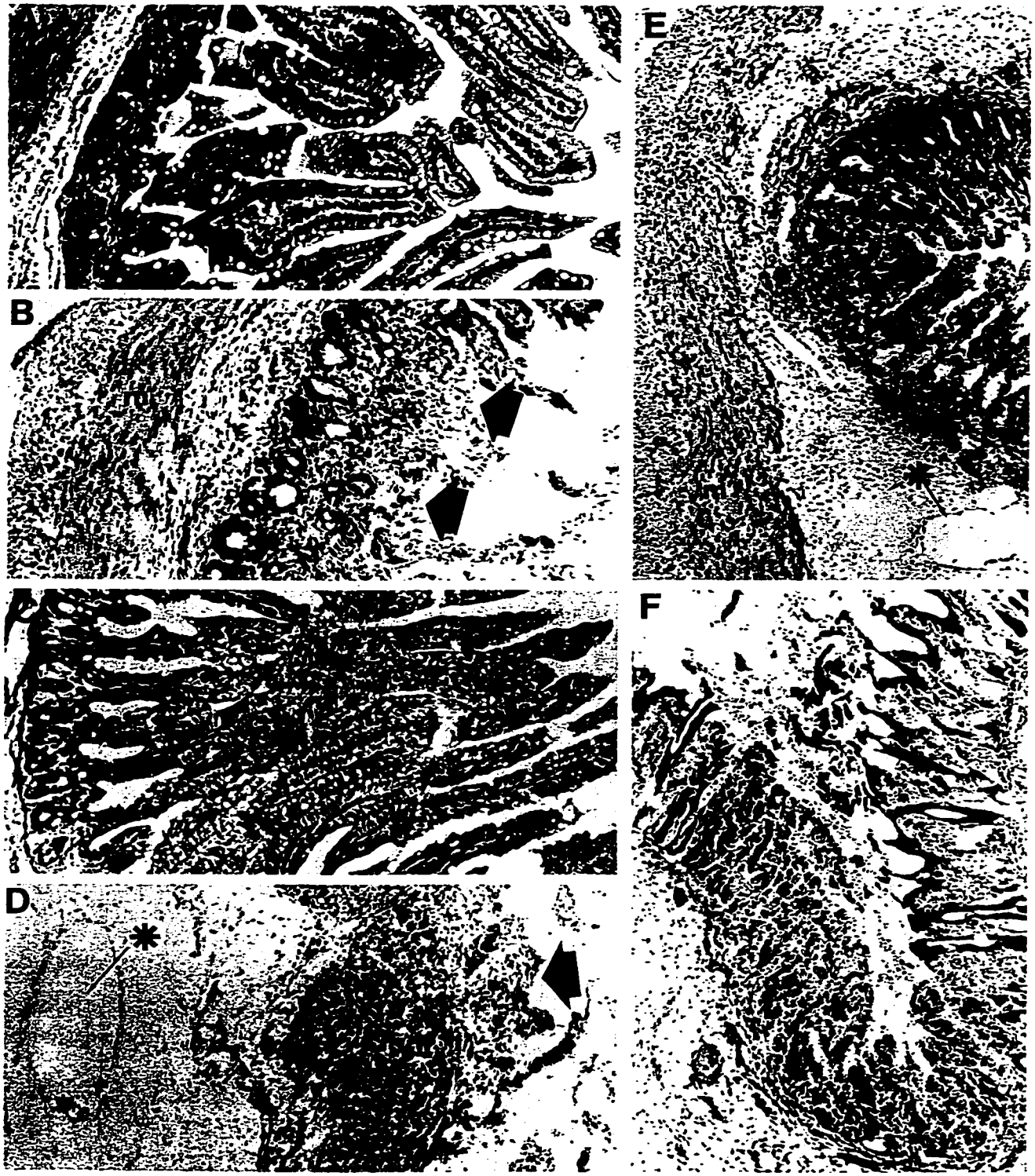

Fig 1. Photomicrographs of H&E-stained sections of piglet intestine. (A) Saline control loop of terminal ileum taken from a 2-week-old piglet. (B) Acidified casein-injected loop of terminal ileum from a 2-week-old piglet. (C) Saline control loop of terminal ileum taken from an 18-hour-old piglet. (D) Acidified casein-injected loop of terminal ileum from an 18-hour-old piglet. (E) Acidified casein-injected loop of colon from a 2-week-old piglet. (F) Acidified casein-injected loop of colon from an 18-hour-old piglet. Arrowheads indicate mucosal damage in B and damage extending through the submucosa in D. Asterisks denote lymphatic distension. m, muscularis.

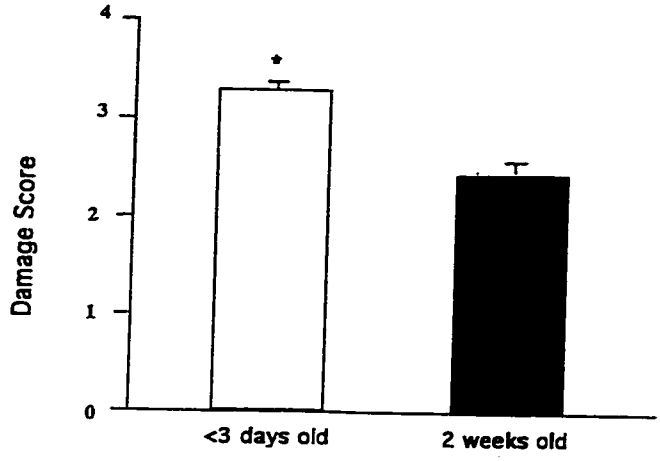

Fig 2. Intensity of acidified casein-induced damage assessed in H&E-stained sections of intestinal loops from <3-day-old (n = 12) versus 2-week-old piglets (n = 12). 0, villi completely intact; 1, villus tip necrosis with preservation of villus crypts; 2, necrosis of villus tips and crypts with loss of mucosal and submucosal architecture; 3, necrosis extending to the muscularis; 4, transmural necrosis. Asterisk denotes a statistically significant ($P < .05$) increase in damage between the groups.

formulas, together with ischemia, to produce necrosis.[14] The intraluminal infusate used in the present study is based on the intestinal contents of NEC infants[10] and is certainly more physiological. In addition, in their study, Crissinger et al[14] did not recover the piglets from surgery. The piglet model used in our study can be applied to conscious animals, because we have recovered neonatal piglets for up to 16 hours (unpublished data).

The use of closed intestinal loops in our piglet NEC model incorporates dysmotility, an important feature of the immature human gastrointestinal tract. Evidently, the resulting impaired clearance of luminal contents favors bacterial proliferation and translocation through organic acid-damaged mucosa. Furthermore, one of the biologically active fragments of bovine casein, β-casomorphin, has been shown to inhibit intestinal motility.[15,16]

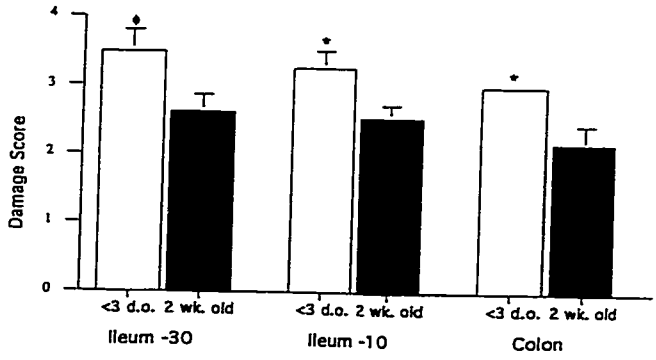

Fig 3. Graph of the depth of acidified casein-induced damage in H&E-stained sections of anatomically matched intestinal loops in <3-day-old versus 2-week-old piglets. Ileum-30, ileum 30 cm from the ileocecal valve; ileum-10, ileum 10 cm from the ileocecal valve. Asterisk denotes a statistically significant ($P < .05$) increase in the depth of damage in the younger animals. ϕ: Although not significant, the P value obtained was .057.

It is well known that NEC lesions show anatomic predilection for the terminal ileum and proximal colon. Within our piglet model we were able to choose this anatomic area and to subsequently demonstrate this increased susceptibility to damage in the younger animals.

In another study of NEC (by Gollin and Marks[17]), using a mature rat model, mucosal injury was mild and limited to villus blunting 3 hours after acidified casein injection. These findings contrast with those reported by Clark et al[10] in weanling rabbits infused with acidified casein solution and with our findings in neonatal piglets (described herein), where more necrosis was evident 3 hours after a single injection of identical test solution. This discrepancy in the extent of injury may be species related and, more particularly, developmentally related, as demonstrated by the results of this study. Preliminary unpublished findings in our laboratory, applying this model to premature piglets, further validate this notion. Using this intraluminal model to compare the outcome in two age groups of neonatal piglets, we have reproduced in a laboratory setting one consistent epidemiological finding: that the incidence of NEC is highest in the least mature infants.[1]

In summary, our in vivo model of NEC in the developing neonatal piglet incorporates two of the most common epidemiological risk factors for NEC: immaturity, with its resulting intestinal dysmotility, by the use of intestinal closed loops; and enteral feeding, via the use of intraluminal injection of a solution mimicking the intestinal contents of NEC infants. Because of its simplicity, reproducibility, consistency, applicability to conscious neonatal animals, and histological similarity to the human disease, we believe this model of NEC to be a valuable tool enabling in vivo pharmacological manipulation in the study of this disease. As such, this model will facilitate the study of a multitude of possible etiological mechanisms that in turn may lead to the potential development of therapeutic modalities for this common disease of uncertain pathogenesis.

ACKNOWLEDGMENT

The authors thank the Animal Care and Experimental Surgery Services of the University of Ottawa, Ontario, Canada, and the Centre for Food and Animal Research, Agriculture Canada. Particular thanks to Dr Julie Lebeau, Miza Bosc-Davie, Jirina Sistek, and Pierre Bradley for their expert advice and technical assistance.

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APPENDIX II

Use of L-arginine in the Treatment of Experimental Necrotizing Enterocolitis

Use of L-Arginine in the Treatment of Experimental Necrotizing Enterocolitis

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● **Although multifactorial in etiology, prematurity and feeding are two of the most common risk factors associated with necrotizing enterocolitis (NEC). To understand the pathogenesis of NEC, the complex interaction between intestinal contents and clearing mechanisms in the immature human gut must be elucidated. Nitric oxide (NO) is a proposed mediator of nonadrenergic noncholinergic neural inhibition, causing relaxation in the gut. In addition to its role as a neuroeffector substance, studies suggest that endogenous formation of NO maintains intestinal mucosal integrity, protecting the gut from blood-borne toxins and tissue-destructive mediators. Thus, NO has a dual role in both gut smooth muscle relaxation and mucosal protection. Because two of the primary risk factors in the development of NEC are prematurity (as it relates to gut dysmotility) and enteral feeding (as it relates to mucosal damage by intraluminal substrate), the authors chose to investigate the role of NO in the pathogenesis of NEC induced by intraluminal injection of acidified casein solution in neonatal piglets. *Methods:* Having confirmed the consistent induction of NEC both macroscopically and histologically with this model (n = 32), the following were undertaken. Neonatal piglets (<3 days old) were laparotomized, and intestinal loops were created from the terminal ileum to the proximal colon. The loops were injected with acidified casein solution and separated by saline-injected control loops. When the abdomen was closed, a continuous peripheral intravenous infusion of L-arginine, an NO synthase substrate (600 mg/kg/h [n = 6]), or N-omega-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor (20 mg/kg/h [n = 6]), was begun. Gut segments were harvested 3 hours later and processed for evaluation of the extent of necrosis. *Results:* Macroscopically, the L-NAME-treated group showed areas of hemorrhagic necrosis in the NEC-induced loops. The L-arginine-treated group had greatly diminished or virtually absent lesions. H&E-stained sections were graded microscopically, using a scale from 0 to 4, ranging from intact villi (grade 0) to transmural necrosis (grade 4). In the untreated NEC group, intestinal damage in the acidified casein loops was exhibited by areas of necrosis (extending, in some cases, transmurally), submucosal edema, and inflammatory cell infiltrate (average grade, 3.5). In the L-NAME-treated group, the intestinal damage was similar to that of the NEC-induced group (average grade, 3.5), but also presented with marked hemorrhagic congestion. In the L-arginine group, NEC-induced tissue damage was greatly attenuated, with necrosis limited primarily to the villus tips (average grade, 1). Nevertheless, inflammatory cell infiltrate and mild submucosal edema were still present. *Conclusion:* Continuous intravenous infusion with the NO synthase substrate L-arginine markedly attenuates intestinal injury in this neonatal piglet model of NEC. Intravenous administration of the NO synthase inhibitor L-NAME causes hemorrhagic congestion of the gut wall. Based on these findings, the authors propose that treatment with the amino acid L-arginine should be considered as a potential therapeutic modality for NEC.**

INDEX WORDS: Necrotizing enterocolitis, L-arginine, nitric oxide.

NECROTIZING ENTEROCOLITIS (NEC) is the most common surgical condition acquired in the neonatal period.[1] In North America, NEC develops in approximately 10% of premature babies, and 90% of the cases occur in those born at fewer than 36 weeks' gestation.[2] The disease usually develops during the first 2 weeks after birth. Gastrointestinal manifestations include abdominal distension and tenderness, emesis, and hemorrhage, progressing to peritonitis and intestinal perforation, with the terminal ileum and proximal colon being the most commonly affected areas.

Prematurity is the primary risk factor for NEC. It is well known that premature infants characteristically display intestinal dysmotility, which assumes a more normal pattern with the passage of time.[3] Successful postnatal adaptation in the very premature infant is frequently precluded by the immaturity of gastrointestinal function. By 28 weeks' gestation, absorptive[4] and digestive[5] functions are moderately developed. Adequate motor function lags many weeks behind, and thus can limit tolerance to enteral feeding. Studies of fasting motor patterns in preterm infants show a gestationally dependent process of maturation, with full activity appearing only between 37 and 42 weeks.[3] This change in fasting intestinal motor activity is likely related to the development of the enteric nerve networks.

The second most important risk factor for the development of NEC is enteral feeding. Ninety-five percent of affected neonates have been fed formula created from cow's milk,[6] and available data indicate that the ingestion of bovine casein is a potential etiologic factor.[7,8] Using a rabbit model of NEC,

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Presented at the 25th Annual Meeting of the American Pediatric Surgical Association, Tucson, Arizona, May 14-17, 1994.

M. Di Lorenzo is supported by a grant from Les Fonds de la Recherche en Santé du Quebec, Canada.

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0022-3468/95/3002-0015$03.00/0

Clark et al[7] found that intestinal loops injected with protein, especially casein combined with organic acid having a pH of less than 5, resulted in progression of intestinal damage, starting with fluid secretion and leading to villus necrosis, bowel wall edema, focal hemorrhage, and transmural necrosis, causing systemic effects and death. Thus, it is clear from the preceding, that to understand the pathogenesis of neonatal NEC, the complex interaction between intestinal contents and clearing mechanisms in the immature human gut must be analyzed.

L-arginine-supplemented diets recently have been shown to improve survival rates in cases of gut-derived sepsis and peritonitis.[9] This beneficial effect of L-arginine appears to be mediated by improvement of bactericidal capacity via the nitric oxide (NO) synthase-catalyzed arginine-NO pathway.[9] Second, even though the capacity of newborn piglet enterocytes for net L-arginine production has been demonstrated,[10] repeated stress has been shown to significantly decrease plasma arginine levels in young rats.[11] This may be of particular pertinence to the premature neonate.

There are two forms of NO synthase—a calcium-dependent constitutive synthase found in neurons and vascular endothelial cells, and an inducible form found in macrophages.[12] Calcium-dependent NO synthase activity has been localized in arterioles of the guinea pig, rat, and human submucosa,[13-15] and in neurons and fibers throughout the wall of the stomach and intestine.[13,14] Of particular interest to us is evidence that NO is a putative transmitter of enteric nonadrenergic noncholinergic (NANC) inhibitory motor nerves that mediate reflex peristalsis[16,17] and may have a role in regulation of gastrointestinal mucosal blood flow and mucosal protection.[18-23] Because of the proposed dual role of NO with regard to intestinal motility and mucosal protection, and because two of the primary risk factors for NEC are immaturity (as it relates to intestinal dysmotility) and intraluminal substrate (which predisposes to mucosal damage), we chose to look at the effect of the NO synthase substrate L-arginine on the development of NEC in neonatal piglets, using a modification of the intraluminal NEC model of Clark et al.[7] The porcine gastrointestinal system is histologically and physiologically very similar to the human system. The advantage of this acute model is that it mimics most closely the cases of NEC associated with feeding. In addition, the anatomic location can be controlled, contrary to other models of mesenteric ischemia in which vascular compromise leads to more diffuse gut necrosis.

MATERIALS AND METHODS

NEC Induction

A modification of the rat model of NEC previously described by Clark et al[7] was used in the pig. Thirty-two newborn Yorkshire piglets (supplied by the Center for Food and Animal Research, Agriculture, Ottawa, Canada) of low and normal birth weight, from 0 to 4 weeks of age (group 1), were anesthetized using midazolam (0.1 mg/kg intramuscularly) oxygen (97.5% to 99%), and halothane (1% to 2.5%) delivered via face mask. After preparation for surgery, laparotomy was performed via a midline incision. Without causing ischemia, a ligature was placed at the proximal ileum, 40 cm from the ileocecal valve, and was flushed distally with warm sterile 0.9% saline, using a transmural 26-gauge needle. A series of six intestinal loops was created by placing ligatures at 10-cm intervals up to and including the proximal colon. These loops were injected using a transmural 24-gauge needle that contained sterile 0.9% saline (control) or a test solution consisting of bovine casein (10 mg/mL) and calcium gluconate (50 mg/mL) acidified to pH 4.0 with propionic acid. This solution is iso-osmolar. While injecting solutions, care was taken to avoid overdistension of the bowel loop, thus eliminating the possible contribution of ischemia to the induced damage. The abdominal cavity was then closed. Three or 16 hours later, the bowel segments were inspected macroscopically for lesions and were harvested. The 16-hour group was allowed to recover. Buprenorphine (4 to 8 μg/kg intramuscularly) was administered every 8 to 12 hours for analgesia. The animals were euthanized with intracardiac sodium pentobarbital (65 mg/mL) after harvesting.

All tissues were prepared for microscopic analysis by clearing the lumen of material with gentle perfusion of 10% formalin and then immersing the tissue in 10% formalin. Transverse frozen sections of treated and control intestine were then cut, mounted onto subbed slides, and counterstained with H&E according to standard procedures.

These sections were evaluated microscopically, and the degree of necrosis was scored using a scale of 0 to 4, according to the method of Clark et al.[24] zero represents villi completely intact; 1 denotes villus tip necrosis, with preservation of villus crypts; 2 represents necrosis of villus tips and crypts, with loss of mucosal and submucosal architecture; 3 denotes necrosis extending to the muscularis; and 4 represents transmural necrosis. The presence of edema, lymphatic dilatation, hemorrhage, and immune/inflammatory cell infiltrate also was noted.

NEC Treatment

For the treatment groups, piglets younger than 72 hours of age were used. The left internal carotid artery was cannulated for continuous blood pressure monitoring. Upon closure of the abdomen, continuous peripheral intravenous (IV) infusions were begun. Group 2 ($n = 6$) received L-arginine, an NO synthase substrate, and group 3 ($n = 4$) received D-arginine, its inactive dextrorotatory isomer, both at the rate of 600 mg/kg/h. Group 4 ($n = 6$) received L-NAME, an NO synthase inhibitor, and group 5 ($n = 4$) received D-NAME, its inactive dextrorotatory isomer, both at the rate of 20 mg/kg/h. Gut segments were harvested and processed for evaluation 3 hours later.

Statistical Analysis

The mean and standard error of the mean were calculated for each gut loop in all groups after grading for damage. Statistical analysis was undertaken using the Instat statistical computer program (Graph PAD Software, San Diego, CA). One-way analysis of variance (ANOVA) with corrected P value was used to

analyze the differences among treatment groups. Repeated-measures ANOVA was used to compare casein loops within the same treatment group. A paired one-tailed t test was used to compare control and casein loops within the same treatment group. A one-sample two-tailed t test was used to compare saline control loops with normal gut. A P value of less than .05 was considered significant.

Chemicals

The chemicals employed were bovine casein, L-arginine, D-arginine, L-NAME, D-NAME (Sigma, St Louis, MO); calcium gluconate, propionic acid (BDH Inc, Toronto, Ontario); midazolam (Hoffman-LaRoche Ltd, Montreal, Quebec); halothane (Ayerst Laboratories, Montreal, Quebec); buprenorphine (Reckitt & Colman Pharmaceutical Division, London, England); and sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario).

RESULTS

NEC Induction

In the 0- to 4-week-old piglets (group 1), the acidified casein-injected intestinal loops showed macroscopic damage indistinguishable from neonatal NEC. Intestinal injury ranged from mucosal sloughing to full-thickness necrosis and perforation, with more extensive damage occurring at 16 hours and in the younger animals (data not shown). In the 0 to 3 day olds of group 1, 3 hours after NEC induction, H&E-stained gut sections from acidified casein-injected loops showed damage that extended to the muscularis. The average grade was 3.25 ± 0.13 and was characterized by patches of necrosis, submucosal edema, and inflammatory cell infiltrate (Figs 1a and 1b).

L-NAME Treatment

Continuous IV infusion of L-NAME caused a mild sustained increase (approximately 5 to 10 mm Hg) in systolic and diastolic blood pressure. After 3 hours, the L-NAME-treated 0- to 3-day-old piglets (group 4) had macroscopic hemorrhage in the acidified casein-injected loops. Intestinal damage (2.78 ± 0.15) was similar to that of the untreated NEC group ($P > .05$) but was characterized by marked hemorrhagic congestion (Fig 1c).

L-Arginine Treatment

Continuous IV infusion of L-arginine (group 2) caused a sustained mild decrease (approximately 5 mm Hg) in systolic and diastolic blood pressure. All L-arginine-treated animals had absence of macroscopic lesions in the NEC-induced intestinal loops. Histologically, damage was greatly attenuated (by 74% compared with the untreated NEC group of the same age; $P < .0001$), with the average grade being 0.86 ± 0.12. Necrosis was limited primarily to the villus tips. Inflammatory cell infiltrate and mild submucosal edema were still present (Fig 1d). These results are summarized in Fig 2.

Saline Control Segments

In all groups, the saline control intestinal loops showed an absence of macroscopic damage, with mean histological grades of necrosis being 0.17 ± 0.08 for the NEC group, 0.11 ± 0.10 for the L-NAME-treated group, and 0.06 ± 0.06 for the L-arginine-treated group ($P > .05$ for all comparisons). None of the saline control loops differed significantly ($P > .05$) from the corresponding normal gut (Fig 1a).

D-NAME and D-Arginine Treatment

In these animals (groups 3 and 5) the NEC-induced intestinal loops showed tissue damage similar to that of the untreated NEC group (data not shown).

DISCUSSION

Our results show that in neonatal Yorkshire piglets, the intraluminal injection of acidified casein, by mimicking the intraluminal substrate found in infants with NEC, causes tissue damage that is macroscopically and histologically similar to "classical" neonatal NEC. More importantly, we have shown that continuous intravenous infusion of L-arginine, an NO synthase substrate, over a 3-hour period significantly attenuates NEC-induced intestinal damage, without any significant hemodynamic effects. This particular finding in our acute intraluminal model of intestinal inflammation corroborates previous studies in which inhibition of NO synthesis in acute models of intestinal ischemia was shown to be associated with increased gut injury.[25] On the other hand, studies with chronic-condition animal models using long-term chemical administration to induce intestinal damage, have shown the beneficial effects of the NO synthase inhibitor L-NAME on intestinal cytoprotection.[26,27] This may attributable to the protective effects of basal constitutive NO production in the acute setting and to the deleterious effects of induction of NO synthesis in the chronic setting.[27]

Perinatal risk factors, many of which produce different degrees of mesenteric hypoxia and ischemia, are also implicated in the pathogenesis of NEC, but as coincidental events. For instance, "ischemic" NEC lesions differ in anatomic location and appearance from classical NEC lesions. The former result in generalized necrosis and "gun shot"-like perforations of the gut, limited to the given territory of mesenteric vascularization. The latter results in necrosis that is commonly localized to the terminal ileum and colon.[28] In this study, precautions were taken during the surgical procedure to eliminate the possibility of

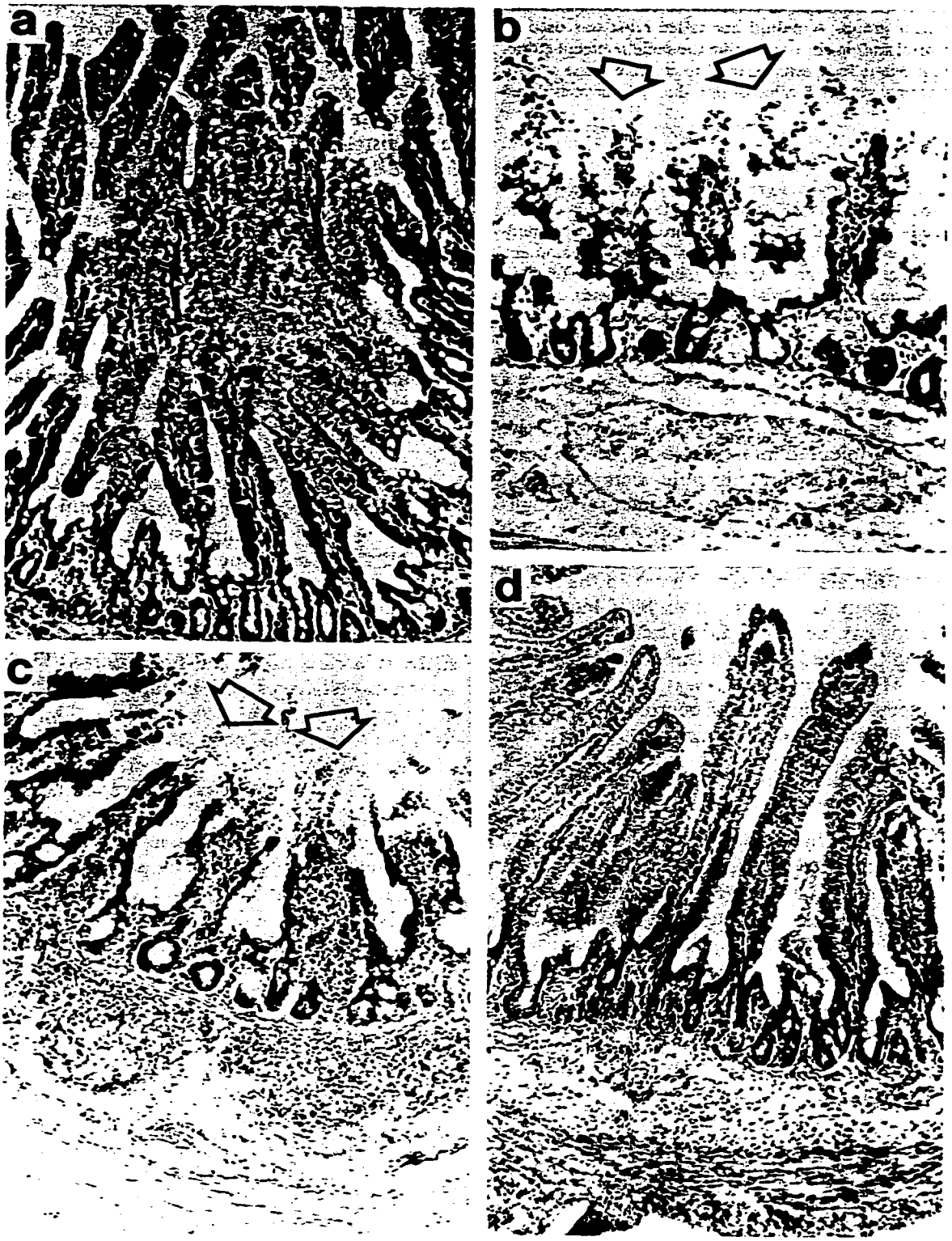

Fig 1. Photomicrograhs of H&E-stained frozen sections taken from the distal ileum of piglets (<72 hours of age). (a) Saline control. (b) Acidified casein-induced NEC. (c) Acidified casein-induced NEC in animals treated with IV L-NAME (20 mg/kg/h for 3 hours). (d) Acidified casein-induced NEC in animals treated with IV L-arginine (600 mg/kg/h for 3 hours). In b and c, mucosal damage (arrows) is extensive and extends to the villus crypts. In animals treated with L-arginine (d), the damage is attenuated, limited to the villus tips.

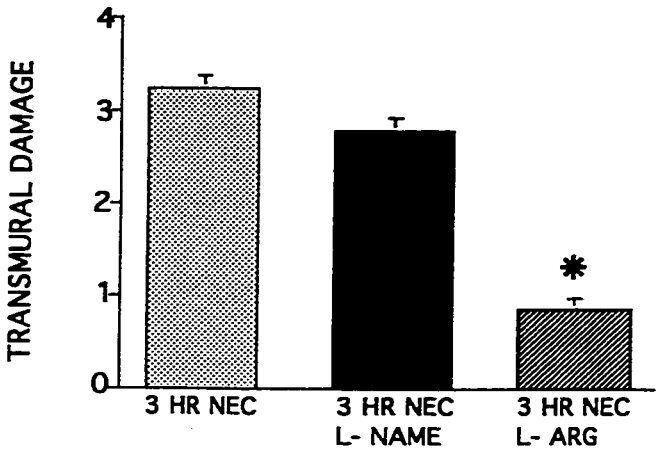

Fig 2. Bar graph shows transmural damage in piglets with NEC. The treatment groups are shown in the abscissa. Damage was graded from 0 to 4 for the H&E-stained frozen sections, with 0 representing completely intact villi; 1 representing villus tip necrosis, with preservation of villus crypts; 2 representing necrosis of villus tips and crypts, with loss of mucosal and submucosal architecture; 3 representing necrosis extending to the muscularis; and 4 representing transmural necrosis. L-NAME-treated animals had similar levels of damage. L-arginine treatment resulted in significant (*$P < .0001$) attenuation of lesions, with reduction of damage by 74%, compared with the untreated NEC group.

vascular compromise contributing to gut wall damage. First, gut loops were tied off without injury to mesenteric vessels, and second, the quantity of intraluminal fluid injected was insufficient to distend the bowel segment to the point of ischemia.

Inflammation is necessary for the repair of injured tissue and for the control of infection. Leukocyte adhesion is key to initiating this process. Uncontrolled leukocyte infiltration is associated with many inflammatory disorders and may contribute to gut damage associated with NEC, also known as acute neonatal inflammatory bowel disease. Recent evidence indicates that NO prevents such leukocyte adherence.[29] This may be one mechanism whereby NO production attenuates mucosal damage in our model.

It has been proposed that basal levels of constitutive NO production act to protect intestinal mucosa,[20,23] whereas induction of NO synthesis by bacterial endotoxin or cytokine(s) promotes intestinal damage.[30] Therefore, we hypothesize that in premature infants, levels of constitutive NO have not reached "full-term" levels, thus the gut is susceptible to damage. In support of this hypothesis is the finding that NO synthase activity undergoes an ontogenic pattern of development in piglet enterocytes.[31] Similarly, mesenteric and pulmonary artery endothelium production of NO has been shown to undergo a developmentally regulated increase in basal and stimulated levels in fetal and newborn lambs.[32] If constitutive production of NO undergoes an ontogenically determined pattern of development, cytoprotection and clearing mechanisms of fasting motor activity, associated with increasing basal levels of NO, will render the gut less susceptible to damage by the intraluminal substrate. We are investigating this notion in premature piglets.

The data presented here in indicate the potential use of the amino acid L-arginine as a therapeutic modality in the treatment of NEC. Further studies using NO-donating drugs and the other product of the NO synthetic pathway, L-citrulline, which newborn piglet enterocytes can convert to L-arginine,[10] will help characterize the role of NO in the pathogenesis of NEC.

ACKNOWLEDGMENT

The authors wish thank the Animal Care and Experimental Surgery Services of the University of Ottawa, Ontario, Canada, and the Centre for Food and Animal Research, Agriculture, Canada. The authors are also grateful to Dr Julie Lebeau, Miza Bosc-Davey, Jrina Sistek, and Pierre Bradley for their expert advice and technical assistance.

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Discussion

K. Oldham (Durham, NC): The concept is that inflammatory intestinal injury can be attenuated by the systemic administration of L-arginine, presumably through this NO pathway. We must first acknowledge that this and all other experimental models of NEC are contrived and that they do have important differences from the clinical problem. However, the importance of inflammatory tissue injury is enormous. Of significant interest in this preparation is the ability to demonstrate benefit while initiating treatment at the time of injury induction. Beneficial pretreatment regimens are historically common, as you know, but they are of limited clinical interest.

The results here appear quite clear and impressive, although it would be nice to have functional or corroborative data rather than simply histology. Along this line, the authors indicate that neutrophil sequestration is present within the gut of arginine-treated animals. This is not quantitated, although that would be easy to do with either a morphometric analysis or myeloperoxidase studies. Since NO-diminished neutrophil sequestration is likely a feature of this model, and because this type of bowel injury is neutrophil-dependent, one important question is whether the effect of NO is simply to decrease neutrophil sequestration and thereby attenuate the injury.

I have a couple additional questions and a comment. Given the multiple physiological roles that we heard about, I would like to ask what mechanism the authors believe is operative here. I have already mentioned the importance of polymorphonucleocyte (PMN) sequestration. In addition, we know that a primary effect of NO is to increase regional blood flow via its effect on vasoregulatory smooth muscle. Could the better outcome in the experimental group here simply be a result of increased intestinal blood flow? This could be easily measured. Conceivably, blood flow manipulations could be done in other ways that might be superior to NO-directed therapy. We do know from the recent literature that manipulation of the physiological NO balance can be detrimental or even lethal.

Do the authors believe that NO generation here is substrate-limited? The data suggest that it is, but other experimental data suggest alternatives (membrane transport or cofactor regulation). These are important issues as we talk about developing therapeutic strategies.

Your experiments are 3 hours in length. The induction of NO synthase requires a number of hours. I presume, therefore, that you are suggesting that NO regulation here is via substrate-limited constitutive NO synthase activity. I would like to ask whether you have tried to manipulate the inducible NO synthase system, because it is quantitatively more potent. For example, you mentioned cytokine treatment with gamma interferon. In a simplistic way, if some additional NO is good, is more better?

Last, my comment is that the clinical application of this has to be considered experimental. I have alluded to the fragility of the normal NO balance. There now are data for humans suggesting that manipulation of system may be disadvantageous.

M. Di Lorenzo (response): Our functional data are preliminary. Using a myeloperoxidase (MPO) assay as an index of inflammation, we have yet to arrive at any conclusive evidence that the mechanism involves polymorphonuclear sequestration. Our data appear to indicate that there is no difference between treated and untreated animals, but we have yet to assay the younger age groups.

We are currently undertaking NO synthase assays to quantitate calcium-dependent (constitutive) NO synthase activity in different-aged piglets. This will enable us to determine whether constitutive NO production acts to attenuate damage. As Dr Oldham mentioned, inducible NO production takes a much longer time, and we observe protective effects within 3 hours. Therefore we are assuming that this is because of constitutive NO production. Our hypothesis requires biochemical confirmation.

We cannot quite explain the mechanism of action yet. Our MPO data do not appear to indicate that leukocyte sequestration is involved. NO may act by scavenging oxygen-free radicals.

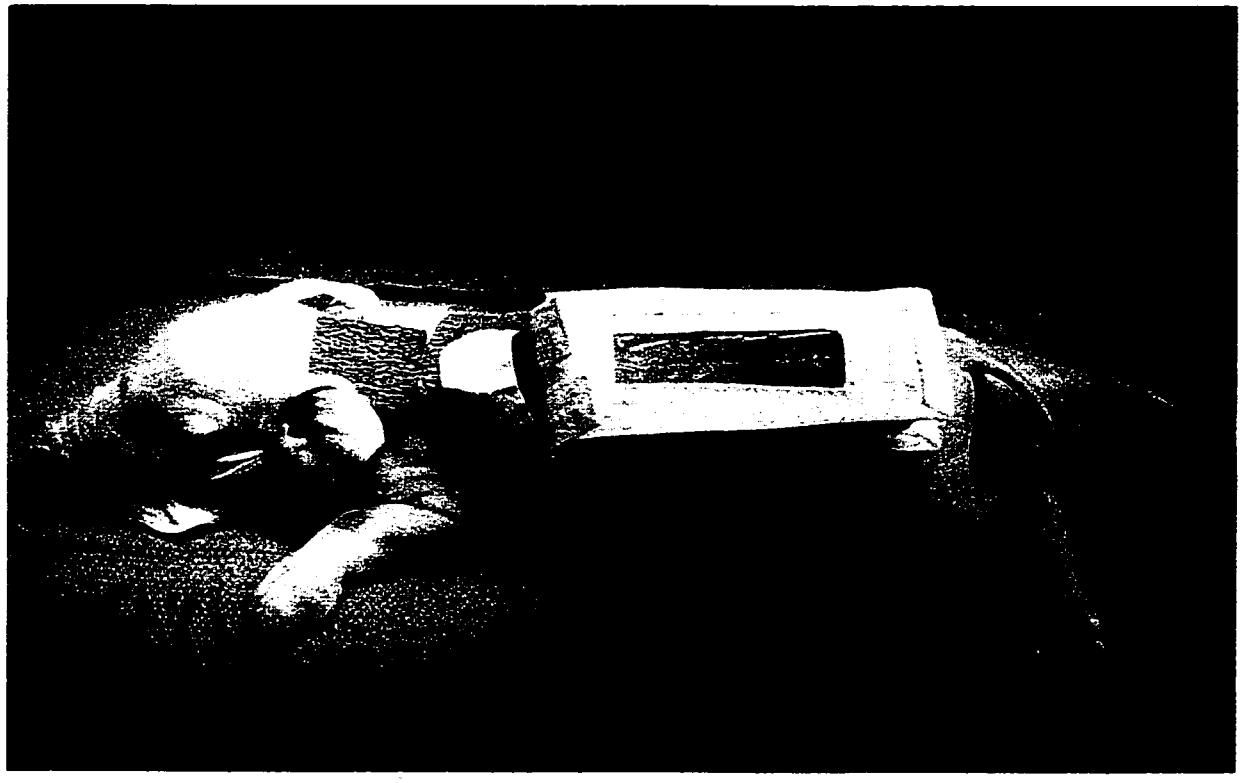
We believe that the mechanism is substrate-limited because of the protective effects of L-arginine. Interestingly, our preliminary studies using NO donor sodium nitroprusside have shown similar attenuation damage.

APPENDIX III

Operative photograph of strain gauges glued to serosal surface of piglet gut

APPENDIX IV

Photograph of recovering premature piglet with 24 hour infusion pump and jacket



APPENDIX V

Sample copy of C.H.E.O. Boichemistry Laboratory results for piglet toxicology study

LABORATORY MEDICINE
CHILDREN'S HOSPITAL OF EASTERN ONTARIO
401 SMYTH RD OTTAWA
BIRTHDATE: SEX:U
AGE:??
DRS: DR,NOT ON FILE (1:)

BIOCHEMISTRY LABORATORY
CHART REPORT (NEW WORK)

PIG,46

PRINT:MAY29/1996 14:03

LOC: R&D KRANTIS

R&D-L/721/1

********** BIOCHEMISTRY LABORATORY REPORT **********

TEST LAB NO.	REF. RANGE	UNITS	* May13/96 10:49H B61873
GLU (R)		mmol/L	10.7
UREA		mmol/L	17.7
NA	135-145	mmol/L	145
K	3.5-5.0	mmol/L	5.0
CL		mmol/L	139*
CREAT		umol/L	90
T.BIL	2-20	umol/L	<2*
D.BIL		umol/L	0
ALT		U/L	47
ALK.P		U/L	848
AMYL	25-100	U/L	662*

********** BIOCHEMISTRY SPECIMEN COMMENTS **********

TEST LAB NO.	* May13/96 10:49H B61873
SPEC.COMMENT	DAY 4

LABORATORY MEDICINE
CHILDREN'S HOSPITAL OF EASTERN ONTARIO
401 SMYTH RD OTTAWA
BIRTHDATE: SEX:U
AGE:??
DRS: DR,NOT ON FILE (1:)

BIOCHEMISTRY LABORATORY
CHART REPORT (NEW WORK)

PIG,46

PRINT:MAY29/1996 14:03

LOC: R&D KRANTIS

R&D-L/721/1

********** BIOCHEMISTRY REPORT **********

SERUM AMINO ACID QUANTITATION

TEST LAB NO.	REF. RANGE	UNITS	* May13/96 10:49H B61873
TAURINE		umol/L	38
ASPARTIC ACID		umol/L	35
THREONINE		umol/L	62
SERINE		umol/L	159
GLUTAMIC ACID		umol/L	79
GLUTAMINE		umol/L	352
GLYCINE		umol/L	662
ALANINE		umol/L	610
CITRULLINE		umol/L	39
AMN-N-BUT ACID		umol/L	13
VALINE		umol/L	168
CYSTINE		umol/L	11
METHIONINE/(HOMOCITRULLINE)		umol/L	34
ISOLEUCINE		umol/L	72
LEUCINE		umol/L	99
TYROSINE		umol/L	52
PHENYLALANINE		umol/L	56
ETHANOLAMINE		umol/L	20
TRYPTOPHAN		umol/L	3
ORNITHINE		umol/L	1446
LYSINE		umol/L	50
HISTIDINE		umol/L	42
ARGININE		umol/L	10482
PROLINE		umol/L	309
AMINO ACID BLOOD			BLOOD
Reviewed By:			AM

AM A.P.Mohammed

********** BIOCHEMISTRY REPORT **********

SERUM AMINO ACID QUANTITATION

TEST	REF. RANGE	UNITS	* May13/96 10:49H
LAB NO.			B61873
TAURINE		umol/L	38
ASPARTIC ACID		umol/L	35
THREONINE		umol/L	62
SERINE		umol/L	159
GLUTAMIC ACID		umol/L	79
GLUTAMINE		umol/L	352
GLYCINE		umol/L	662
ALANINE		umol/L	610
CITRULLINE		umol/L	39
AMN-N-BUT ACID		umol/L	13
VALINE		umol/L	168
CYSTINE		umol/L	11
METHIONINE/(HOMOCITRULLINE)		umol/L	34
ISOLEUCINE		umol/L	72
LEUCINE		umol/L	99
TYROSINE		umol/L	52
PHENYLALANINE		umol/L	56
ETHANOLAMINE		umol/L	20
TRYPTOPHAN		umol/L	3
ORNITHINE		umol/L	1446
LYSINE		umol/L	50
HISTIDINE		umol/L	42
ARGININE		umol/L	10482
PROLINE		umol/L	309
AMINO ACID BLOOD			BLOOD
Reviewed By:			AM

AM A.P.Mohammed

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2	TAURINE	2.71	2.219	0.389	37.97
3	UREA	4.97	16.333	29.532	21223.03
4	GLUCOSAMINIC A	6.37	11.228	0.444	219.28
5	ASPARTIC ACID	14.10	2.159	0.368	34.98
6	THREONINE	15.31	3.919	0.358	61.67
7	SERINE	16.44	10.309	0.351	159.23
8	GLUTAMIC ACID	19.65	5.403	0.331	78.81
9	GLUTAMINE	20.99	22.604	0.354	352.20
10	GLYCINE	31.07	44.648	0.337	661.81
11	ALANINE	32.86	33.843	0.409	609.61
12	CITRULLINE	34.55	1.815	0.492	39.30
13	AMN-N-BUT ACID	36.52	0.587	0.491	12.69
14	VALINE	38.01	9.740	0.392	168.19
15	CYSTINE	39.02	0.723	0.336	10.69
16	METHIONINE	40.36	2.319	0.337	34.39
17	ISOLEUCINE	43.95	4.318	0.377	71.58
18	LEUCINE	45.30	6.465	0.348	99.06
19	TYROSINE	48.03	3.070	0.385	52.04
20	PHENYLALANINE	52.80	3.067	0.413	55.73
21	ETHANOLAMINE	83.14	0.835	0.547	20.10
22	TRYPTOPHAN	85.04	0.122	0.491	2.63
23	AMMONIA	87.68	4.810	0.547	115.84
24	AEC	94.18	14.329	0.363	228.67
25	ORNITHINE	97.46	103.381	0.318	1445.84
26	LYSINE	101.24	3.659	0.308	49.65
27	HISTIDINE	105.91	2.957	0.322	41.94
28	ARGININE	126.14	634.597	0.375	10481.58
TOTALS			950.002		36376.21

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2	PROLINE	28.93	7.043	0.998	309.27
TOTALS			8.222		527.17

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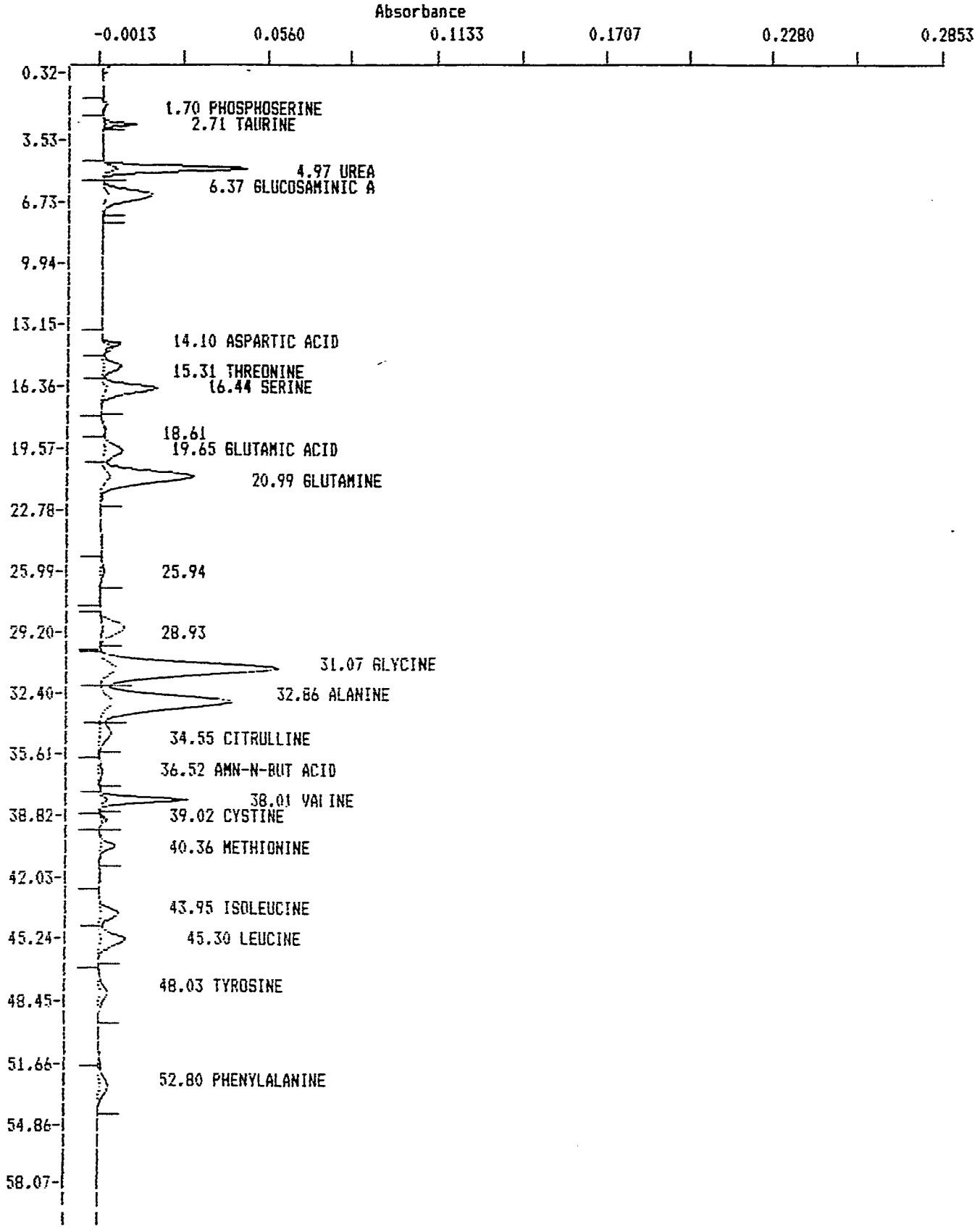

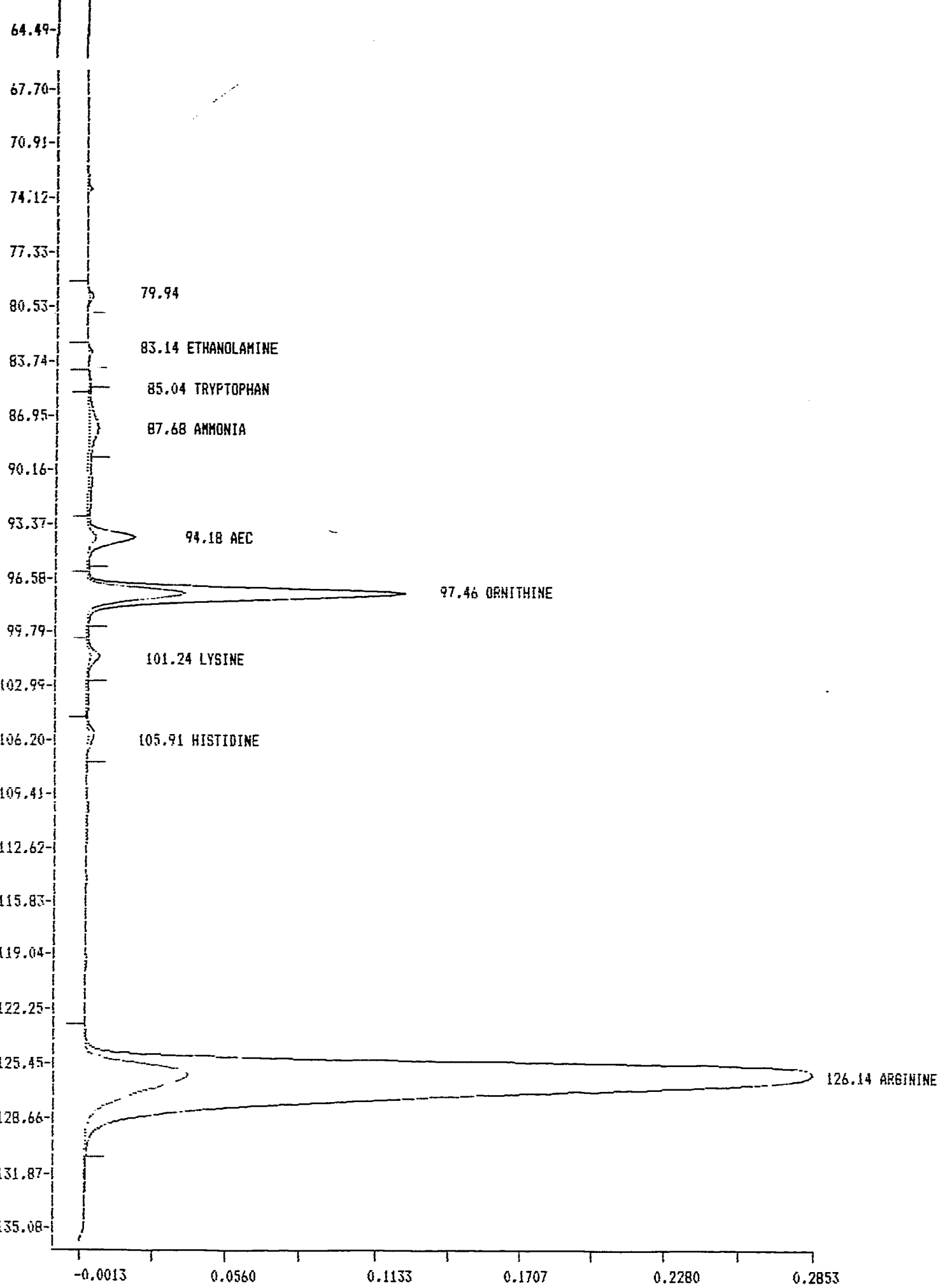
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105.91 HISTIDINE
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125.45
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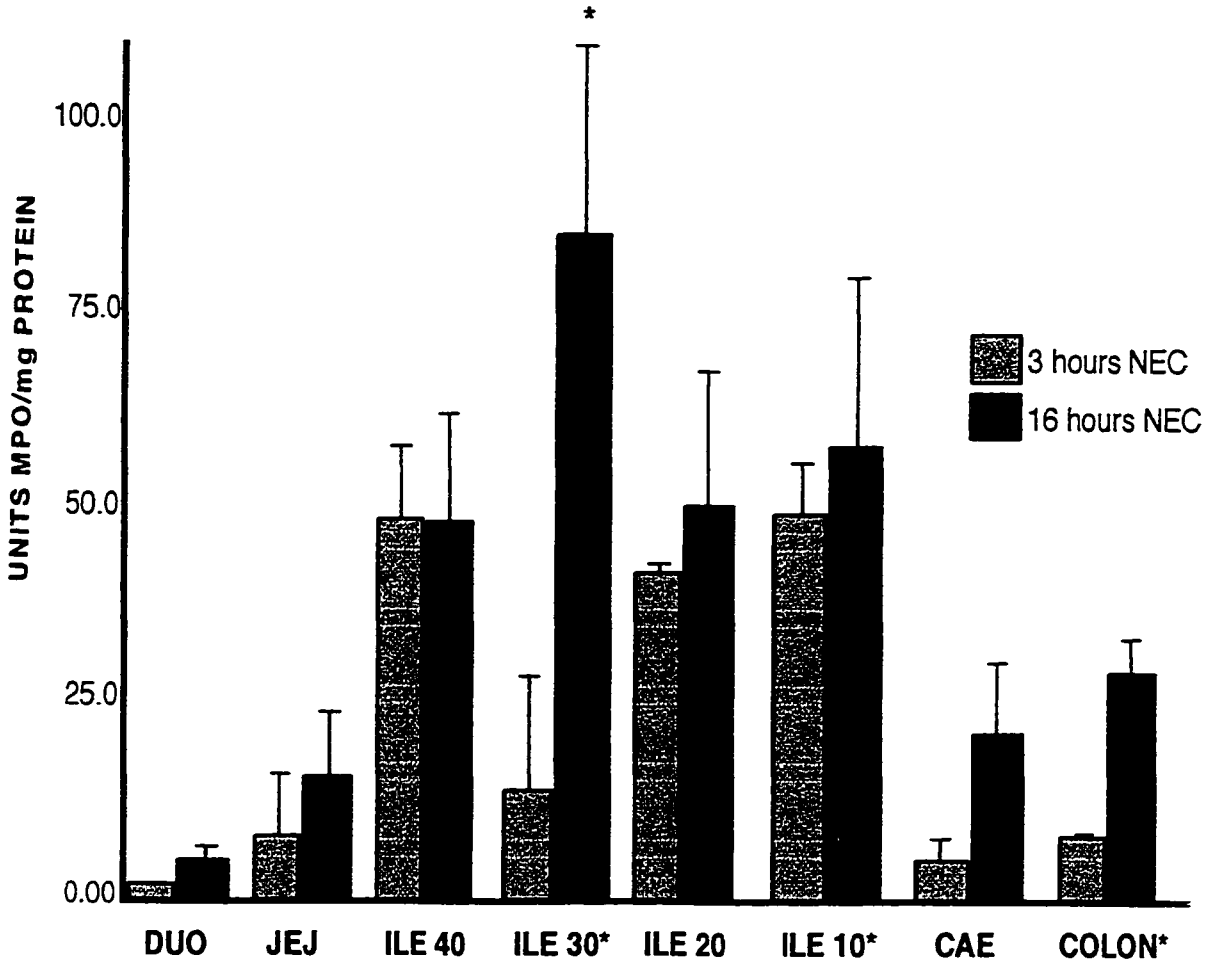
APPENDIX VI

Myeloperoxidase activity in NEC model.



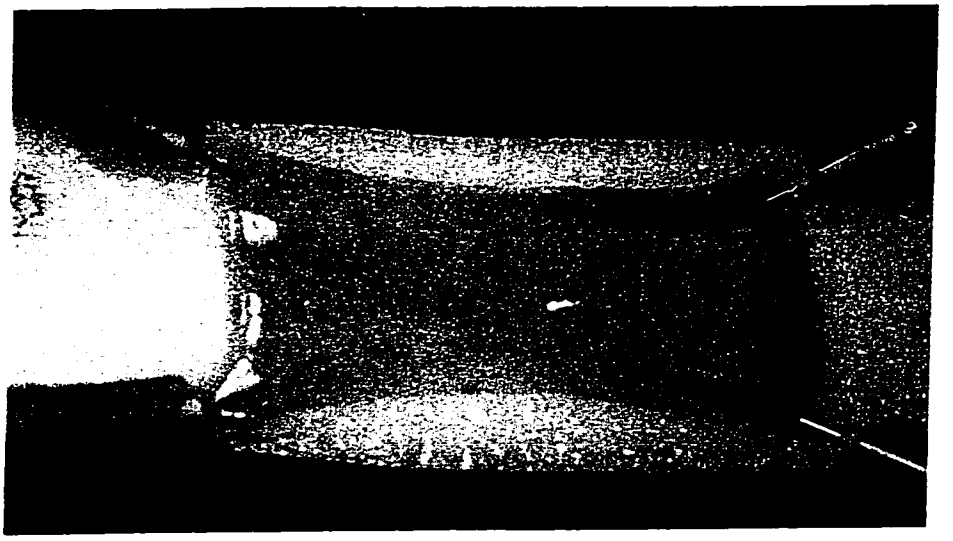
HISTOGRAM OF MYELOPEROXIDASE ACTIVITY IN <3DAY OLD PIGLETS AT 3 AND 16 HOURS AFTER NEC INDUCTION IN DIFFERENT GUT REGIONS

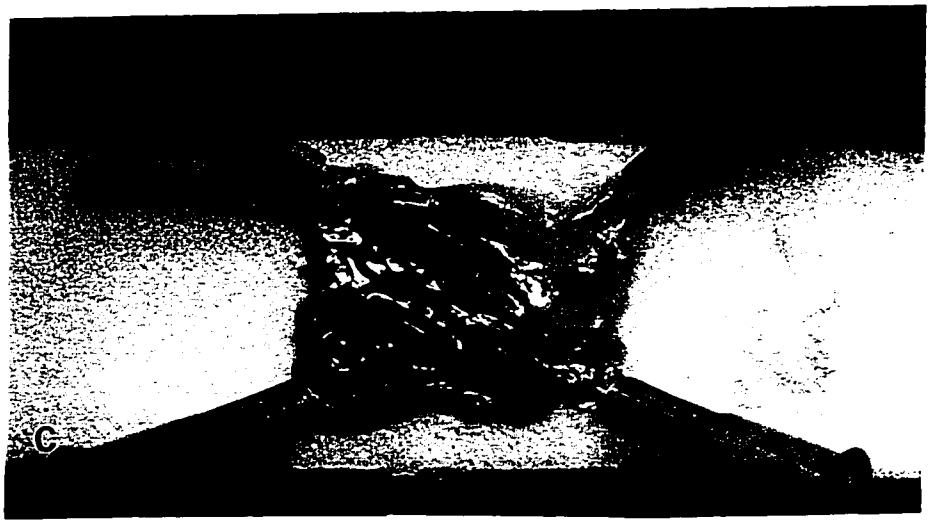
The only activity which was significantly higher compared to duodenum and jejunum at both 3 and 16 hours of NEC was found in the 30 cm ileal loop at 16 hours after NEC induction.

*$p<0.05$
ILE 30*, ILE 10*, COLON*: test loops

APPENDIX VII

Macroscopic appearance of luminal aspect of acidified casein-induced colon damage in term new-born piglet at 3 hours (A), after concomitant i.v. administration of L-arginine (B) or D-arginine (C) at 600 mg/kg/hr. Note the marked visible attenuation of damage with L-arginine and the lack of effect of D-arginine treatment.

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