Necrotizing Enterocolitis in Rat Offspring Exposed to Placental Insufficiency: Role of Aldosterone, Oxidative Stress and Leptin

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Abstract

**Background:** Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in the neonatal intensive care unit among premature and low birth weight infants. Formula feeding, cold exposure, and altered intestinal bacterial colonization are frequently associated with this condition. However, there are few studies linking factors associated with placental function and necrotizing enterocolitis.

**Aim:** To investigated the role of aldosterone, oxidative stress and leptin in the development of NEC. The hypothesis proposes that Aldosterone, Oxidative Stress and Leptin are associated with development of NEC in rat offspring exposed to placental insufficiency.

**Method:** Premature low birth weight rat’s offspring were exposed to induced-NEC immediately after birth during 24hs. Leptin, aldosterone, and oxidative stress markers (superoxide dismutase activity, total antioxidant capacity and lipid peroxidation) were assessed at birth. Intestinal injury was evaluated after 24 hr of induced-NEC. Male and female offspring were divided in 4 groups with “n”=8, per group.

**Results:** Plasma levels of Aldosterone and Oxidative Stress markers were significantly greater, and leptin levels were significantly lower in premature low birth weight offspring compared to normal offspring (P<0.05). Intestinal injury was greater, and survival rate was lower in premature low birth weight offspring compared to normal offspring (P<0.05).

**Conclusion:** These results suggest that plasma levels of aldosterone, oxidative stress and leptin at birth are associated with the susceptibility to develop necrotizing enterocolitis in premature low birth weight offspring in a rat model of placental insufficiency.

Keywords: Placental insufficiency; Necrotizing enterocolitis; Aldosterone; Oxidative stress; Leptin

Introduction

NEC is an acute inflammatory disease of the intestine of neonates and can result in intestinal necrosis, systemic sepsis and multi-system organ failure [1,2]. The incidence of NEC is inversely related to birth weight and gestational age [3,4], and when intestinal necrosis is already established the process is rarely reversible and mortality rates can reach up to 50-80% in severe cases [1,2]. Despite, the effort of physicians and researchers the morbidity and mortality of NEC have increased in the last decade, with more severe complications and poor response to treatment [5,6]. This is partially explained by the improvement in health care practices to maintain alive premature infants, but it does not explain individual differences in outcomes within the same institutional setting [7]. One of the factors involved in infant’s susceptibility to develop NEC is perinatal morbidity, which is strongly associated with fetal-placental unit function [8]. Currently, there are no identified factors linking placental function and the risk to develop NEC; therefore, identification of these factors could help to develop perinatal preventive strategies to decrease the morbidity and mortality associated with NEC.

Experimental and epidemiological studies suggest a multifactorial etiology for NEC including predisposing factors such as enteral feeding, hypoxia and or hypothermia, but with unclear pathogenesis [9,10]. We induced NEC in premature low birth weight (LBW) rat’s offspring from a rat model of placental insufficiency [11]. Premature birth is the major determinant of NEC [4,12]. Increased oxidative stress is among the factors associated with NEC in premature infants [13,14]. Oxidative stress has been observed in several maternal conditions associated with placental insufficiency [15]. Experimental studies report a direct correlation between increased oxidative stress and Aldosterone plasma levels in newborns [16]. Moreover, epidemiological studies reported increases in plasma aldosterone levels associated with LBW and preterm delivery [17-19]. Aldosterone can regulate oxidative stress [20], hence it can be suggested that increased levels of aldosterone and oxidative stress may be associated with NEC in premature and low birth weight infants exposed to placental insufficiency. Aldosterone is involved in the developmental changes of Na+ electrogenic transport in immature intestines [21], resulting in alteration in the homeostasis of gastrointestinal mucus barrier [21,22], and abnormal microbial colonization of the gastro intestinal tract with exacerbated inflammatory response and necrosis [23]. The digestive and absorptive capacity of the gastrointestinal tract is also compromised in premature infants [23,24]. Experimental studies report that postnatal leptin treatment enhances digestive function in intrauterine growth restricted offspring [25,26], by increasing cell mitosis and promoting growth of intestinal mucosa [27]. Leptin levels were reduced in animal models of placental insufficiency induced by reduced uterine perfusion. [28,29]. Therefore, leptin levels at birth could be associated with growth capacity and maturation of gastrointestinal tract in newborns.

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Received: Septemebr 16, 2015; Accepted: October 17, 2015; Published: October 24, 2015


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The identification of factors associated with the risk to develop NEC in infants exposed to placental insufficiency will help to develop preventive strategies and modify current paradigms in postnatal care to prevent development of NEC.

Materials and Method

Animals

All experimental procedures were conducted in accordance with National Institutes of Health guidelines for the Care and Use of Laboratory Animals with approval by the Animal Care and Use Committee at the University of Mississippi Medical Center. Timed pregnant Sprague Dawley rats were purchased from Charles River Laboratories International, Inc. (Raleigh, NC) and housed in a temperature-controlled room (23°C) with a 12:12-hour light/dark cycle with food and water available ad libitum. Day 14 of gestation, rats underwent reduced uterine perfusion surgical procedure as described below or sham procedure. Dams were allowed to either deliver at term for a normal gestational period for the Sprague Dawley rat (day 21-23 of gestation) [30], or Dams underwent cesarean section to surgically deliver the offspring at day 20 of gestation. C-section at day 20 of gestation ensures that offspring will not have access to dams’ milk or been nurtured by dams. Offspring from dams that underwent C-section were placed in an incubator with controlled temperature and humidity under the care of investigators and exposed to Formula feeding, Asphyxia and Cold during the first 24 hrs of post-natal life.

Dam Control-DN: Normal Birth Weight-Dams Nurtured (NBW-DN); Dam RUP-DN: Reduced Uterine Perfusion-Dams Nurtured (RUP-DN); Offspring NBW+DN: Normal Birth Weight-Offspring Nurtured (NBW+DN); Offspring NBW+FAC: Normal Birth Weight-Formula Asphyxia and Cold (NBW-FAC).

Low Birth Weight-Dams Nurtured (LBW-DN); Low Birth Weight-Formula Asphyxia and Cold (LBW-FAC). All animals undergoing surgical procedures were anesthetized using 2-5% Isoflurane gas by inhalation. The experiment was stopped at 24 hours after birth due to the high mortality rate in LBW offspring exposed to FAC (Table 1).

Reduction in uterine perfusion

Dams were exposed to reduced uterine perfusion by placing silver clips around abdominal aorta and both branches of ovarian arteries at 14 days of gestation as previously described [32]. This procedure leads to fetal intrauterine growth restriction and results in low birth weight offspring as previously reported [11]. Low birth weight was defined as the birth weight equal or below the tenth percentile of birth weights of offspring delivered from control litters from dams exposed to sham surgical procedure.

Formula feeding, asphyxia and cold (FAC)

The protocol previously described by Caplan et al. [33] with modification was utilized to induce NEC. Briefly, the offspring selected for FAC were delivered via C-section at 20 days of gestation. After delivery normal birth weight and low birth weight rat offspring selected for FAC were placed in an incubator with controlled temperature and humidity and without contact with the dam. Formula feeding was initiated between 5 to 10 minutes after delivery using commercially available puppy milk (ESBILAC Pet-Ag) prepared according to manufacturers at a dose of 100 every 3 hours by gavage. Asphyxia exposure was accomplished by placing the offspring in a refrigerated chamber at 4°Celsius for 5 minutes every 12 hours initiated at 1 hour post-delivery. Cold exposure was done by placing offspring in a refrigerated chamber at 4°Celsius for 5 minutes every 12 hours initiated at 1 hour post-delivery.

Circulating aldosterone and leptin levels

Plasma levels of aldosterone, and leptin were measured using commercially available kits (Siemens and R&D Systems,) from umbilical cord blood obtained at delivery. To increase the volume of our samples we pooled samples from 2 offspring of the same sex from the same litter per each animal group.

Table 1: Experimental Design

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gestation day 14</th>
<th>Gestation day 20</th>
<th>Gestation day 21-23</th>
<th>Post-natal hour 0</th>
<th>Post-natal hours 1-24</th>
<th>End Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam Control-DN</td>
<td>Sham Surgery</td>
<td>Delivery</td>
<td>Nurturing Offspring</td>
<td>Nurturing Offspring</td>
<td>Euthanized</td>
<td></td>
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<tr>
<td>Dam Control-FAC</td>
<td>Sham Surgery</td>
<td>C-Section Euthanized</td>
<td>Delivery</td>
<td>Nurturing Offspring</td>
<td>Euthanized</td>
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<td>Dam RUP-DN</td>
<td>RUP Surgery</td>
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<td>Nurturing Offspring</td>
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<td>Dam RUP-FAC</td>
<td>RUP Surgery</td>
<td>C-Section Euthanized</td>
<td>Delivery</td>
<td>Nurturing Offspring</td>
<td>Euthanized</td>
<td></td>
</tr>
<tr>
<td>Offspring NBW+DN</td>
<td>Normal Birth</td>
<td>Collection Umbilical Blood</td>
<td>Dam nurtured</td>
<td>Euthanized Tissue Collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Offspring NBW+FAC</td>
<td>Premature Birth</td>
<td>Collection Umbilical Blood</td>
<td>FAC exposed</td>
<td>Euthanized Tissue Collection</td>
<td></td>
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<tr>
<td>Offspring LBW+DN</td>
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</table>
Oxidative stress markers

We utilized commercially available ELISA kits (Cayman Chemicals) following manufacturer assays techniques to measure Extracellular Superoxide Dismutase (E-SOD), Total Antioxidant Capacity (TAC) and Lipid Peroxidation (T-BARS) in plasma from umbilical cord blood. To increase the volume of our samples we pooled samples from 2 offspring of same sex from the same litter per each animal group.

Tissue morphology

Intestines were harvested for histological assessments after euthanasia at the end of experiment at 24 hours post-delivery and after exposure to FAC in the group of induced NEC. A section of 2 cm of the distal ileum was selected for evaluation in each offspring. Each intestinal section was placed in 10% phosphate buffered formalin, embedded in paraffin, sectioned (4 um thickness) then stained with hematoxylin and eosin (Suripath Medical Industries, Leica, IL). Evaluation of the slides was performed in a blinded manner to samples identity using a modified semiquantitative evaluation previously published by Dvorak et al. [34]. For scoring, all slides were examined under light microscope at 400X magnification. The magnitude of necrosis, and structural damages were scored as follows: 0 (normal), no damage; 1 (mild), slight submucosa and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria, and/or edema in submucosa and muscular layers; 3 (severe), severe separation of submucosa and/or lamina propria, and/or severe edema in submucosa and muscular layers region, villous sloughing; 4 (necrosis), loss of villi and necrosis. Scores were reported as the average of 10 different fields per slide from each sample.

Most representative microphotographs were taken at 200X magnification to show details of the structure of mucosa villous.

Statistical analysis

GraphPad 5 and SPSS 22 statistical software were utilized to perform data analysis of the results. ANOVA with multivariate analysis was used to calculate differences between groups. Survival rate was calculated using Prism survival curves by performing both the log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. P values were calculated using both test, and Log-rank (Mantel-Cox) Chi square test were reported.

Results

Birth weight and weight gain

As previously reported offspring from dams exposed to reduced uterine perfusion were born with low birth weight (LBW) (Figure 1A). Weight gain within 12 hours postnatal was significantly lower (P<0.05) in LBW offspring exposed to experimental FAC compared to other groups; however, no difference in weight gain was observed between LBW and NBW in the groups nurtured by dams. (Figure1B).

Plasma levels of leptin at birth

Premature LBW offspring showed significantly lower plasma levels of leptin at birth compared to NBW offspring in both DN and FAC groups (P<0.05) (Figure 2).

Plasma levels of aldosterone at birth

Premature LBW offspring showed significantly higher plasma levels of aldosterone at birth compared to NBW offspring in both DN and FAC groups (P<0.05) (Figure 3).

Plasma levels of oxidative stress markers

Premature LBW offspring showed significant (P<0.05) lower levels of extracellular superoxide dismutase activity (Figure 4A), higher levels of lipid peroxidation (Figure 4B), and lower total antioxidant capacity (Figure 4C) at birth compared to NBW offspring.

Survival rate after FAC

LBW offspring had a significantly lower survival rate after exposure to FAC in comparison to NBW offspring (χ²=20.53, df=3, P=0.0001). The survival percentage for LBW exposed to FAC was 75% at 12 hours, 38% at 15 hours and 0% at 18 hours (Figure 5).

Discussion

The main findings in this study are that offspring exposed to placental insufficiency have 1) Low birth weight 2) Increased plasma levels of aldosterone at birth, 3) Increased levels of oxidative stress markers at birth, 4) Decreased plasma levels of leptin at birth, 5) Decreased survival rate after exposure to formula feeding, asphyxia and

Tissue morphology

The intestinal sections from offspring nurtured by dams showed normal structural parameters in both NBW and LBW offspring. Offspring exposed to FAC showed intestinal structural damage, which was significantly greater in LBW offspring compared to NBW offspring. LBW offspring exposed to FAC exhibited tissue damage score with an average of 3.6 ± 0.2 (P<0.05) vs. all other groups (Figure 6A). Most representative microphotographs show intestine section of LBW offspring exposed to FAC with intestinal villi disintegration, loss of cell nuclei, edema in the muscular wall and lamina propria and necrosis with perforation (Figure 6B).

Figure 3: Plasma Levels of Aldosterone at Birth. Umbilical cord blood samples were collected with capillary tubes at delivery from all groups for determination of aldosterone plasma levels. NBW and LBW offspring were separated in two group, DN and FAC, with "n"=8 for each group. *P<0.05 vs. NBW counterpart. Values represent mean ± SD.

Figure 4: Plasma Levels of Markers of Oxidative Stress at Birth. Umbilical cord blood samples were collected with capillary tubes at delivery from all groups for determination of plasma levels of Superoxide Dismutase Activity (4A), Lipid Peroxidation (4B) and Total Antioxidant Capacity (4C). NBW and LBW offspring were separated in two group, DN and FAC, with "n"=8 for each group. *P<0.05 vs. NBW counterpart. Values represent percentage of survival.

Figure 5: Survival rate. Survival rate was assessed each 3 hours postnatal, coincidently with feeding time. NBW and LBW offspring were separated in two group, DN and FAC, with "n"=8 for each group. *P<0.05 vs. all other groups. Values represent percentage of survival.

Figure 6: Histology Assessment. Histological parameters were assessed using an established method of semi quantitative evaluation of 10 fields randomly selected per each examined sample. The magnitude of damages were scored as: 0 (normal), no damage; 1(mild), slight submucosa and/or lamina propria separation; 2 (moderate), moderate separation of submucosa and/or lamina propria, and/or edema in submucosa and muscular layers; 3 (severe), severe separation of submucosa and/or lamina propria, and/or severe edema in submucosa and muscular layers, region villous sloughing; 4 (necrosis), loss of villi and necrosis (6A). All data are expressed as mean ± SD. * P<0.05 vs. NBW counterpart. Most representative pictures show histological structures from NBW and LBW offspring in DN and FAC groups. HE. Magnification 200X. Scale Bar=200 um (6B).
cold temperature. This is the first time that an experimental model of NEC induced by formula feeding, asphyxia and cold exposure is utilized in a rat model of premature LBW induced by placental insufficiency. This model is mimicking the human condition of NEC in premature and LBW infants.

NEC is one of the leading causes of morbidity and mortality in the neonatal intensive care unit (NICU) [35]; it is almost an exclusive disease of pre-term and LBW infants, with unclear mechanistic pathways involved in its etiology. Moreover, preventive strategies are inadequate and the prognosis of NEC in the NICU is often associated with long-term consequence [36]. Therefore, diagnostic tools to identify infants “at risk” to develop NEC may help prevent this condition and improve survival outcomes. Other investigators have examined other factors implicated in the etiology of NEC utilizing animal models [2]. However, few reports have examined potential biomarkers for early identification of infants at risk to develop NEC [37,38]. We designed our study to investigate potential early biomarkers that may predict an increased risk for NEC in premature LBW offspring.

In this study, LBW offspring were more susceptible to develop NEC in comparison to NBW offspring from dams exposed to sham operation. The experimental model to induce NEC utilized in this study is a modification of the protocol developed by Caplan et al. [39]. The protocol used by Caplan et al. induces NEC in newborn rat offspring via exposure to formula feeding, asphyxia and cold for a period of 48 to 96 hours until NEC is developed. However, in the current study the survival rate was lower in LBW offspring compared to NBW offspring. Premature LBW offspring showed clear evidences of development of NEC with abdominal distention and lethargy as early as 12 hours after initiation of formula feeding, asphyxia and cold exposure. The group of LBW exposed to FAC resulted in no survivors after 18 hours of exposure. This finding suggests that premature low birth weight offspring are more susceptible to the development of NEC with a lower survival rate in comparison with NBW offspring.

Oxidative stress is strongly involved in the etiology of morbidity in premature and LBW infants. A reduction in antioxidant capacity leading to the formation of free radicals may contribute to increased morbidity in preterm and LBW infants including NEC [14]. Increased oxidative stress induce a state of imbalance in the normal cell redox homeostasis resulting in shifting cell metabolism from anabolic to catabolic and increased apoptosis [20]. The fast cell turnaround observed in the intestinal mucosa is affected by oxidative stress resulting in disruptions of intercellular tight junctions and more susceptibility to shear stress [14]. There are strong evidences supporting that oxidative stress biomarkers in cord blood could help in the early identification of infants at risk to develop NEC [14].

There are several epidemiological studies reporting an increases in plasma aldosterone levels associated with LBW and preterm delivery [17-19]. Experimental studies report that aldosterone can mediate the regulation of oxidative stress [16] by decreasing the activity of glucose-6-phosphate dehydrogenase [20] the rate-limiting enzyme in the pentose phosphate pathway for production of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) [20]. NADPH limits the production of reactive oxygen species (ROS) and reduces oxidative stress [20].

Additionally, aldosterone is the most important factor responsible for electronegative Na+ absorption in the immature gut, and this action has a genomic effect [40]. Therefore, an elevation in aldosterone levels could result in deregulation of Na+ absorption and alterations in water and electrolyte homeostasis in the intestinal epithelium. A fully developed intestinal barrier provides selective permeability and controlled bidirectional fluid flow to flush away pathogens and toxins from the intestinal lumen [41]. Plasma aldosterone levels are elevated in infants delivered prematurely and in infants with perinatal asphyxia [17,19], and these two conditions are also associated with NEC [42]. Therefore, we investigated the association between aldosterone levels and NEC in our experimental model.

We found that LBW offspring have increased levels of aldosterone in plasma samples obtained from the umbilical cord at the time of delivery. The significant elevation of plasma aldosterone observed in LBW offspring could be a contributing factor for the development of NEC. Further studies will be necessary to investigate this paradigm and to test a causality effect.

Immaturity of the gastrointestinal tract is another factor involved in the etiology of NEC. The injury in NEC begins with a breach in the intestinal mucosal barrier leading to microbial colonization and exacerbated inflammatory response [23]. Intestinal digestive and absorptive capacity are also impaired in premature infants creating a favorable environment for bacterial colonization [23,24]. Leptin is a peptide with neuroendocrine actions and regulatory effects on several systems related to growth and maturation [43,44]. Leptin is known for promoting growth and maturation during fetal and early postnatal life [25,26], and it is important in proper infant development [45,46]. Experimental studies report that postnatal leptin treatment enhances digestive function in intrauterine growth restriction offspring [25,26]. Leptin is also associated with the cell mitosis to apoptosis ratio and enhanced growth of the mucosa in newborns [27]. Epidemiological studies report that prematurity and LBW are associated with low leptin levels at birth [47,48]. Consequently, we examined the correlation of leptin levels at birth and NEC in our model of low birth weight offspring.

In this study plasma leptin levels were lower in umbilical cord samples obtained from LBW rat offspring at the time of delivery. Leptin actions and regulatory effects are extended to several systems related to fetal growth [43,44], and it seems to have a protective effect against cell apoptosis and autophagy in the neonatal gut epithelium [27]. Moreover, other investigators reported that leptin treatment promotes small intestine growth and significantly increases the total surface area of Peyer’s patches in growth restricted piglets [26]. Low leptin levels found in LBW offspring in our study could be associated with immaturity of gut epithelium, impairment of digestive and absorbance functions, and increased apoptosis [1,36] often associated with NEC.

In conclusion the findings from this study demonstrate that the rat model of LBW induced by placental insufficiency is a suitable model to investigate NEC and the factors associated in the link between placental insufficiency and NEC. We found parallelisms between our data and prior humans reports that low birth weight fetuses have higher aldosterone and lower leptin levels at birth. In addition, findings from this study indicate that LBW induces a significant susceptibility to a secondary insult that contributes to the development of NEC. We speculate that alterations in aldosterone and leptin levels at birth may be critical factors in predisposing premature and low birth weight infants to an increased risk to develop NEC. It is important to point out that the measurement of aldosterone, oxidative stress and leptin were performed at birth in offspring not exposed yet to formula feeding, asphyxia and cold temperature. The limitation of this study is related to the investigation of causality effects between the biomarkers measured at birth and the development of NEC. Further studies are warranted to
examine causality effects and potential mechanistic pathways linking these biomarkers at birth with NEC.

References
