The changes in biomarkers for necrotising enterocolitis in premature calves with respiratory distress syndrome

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Abstract: The aim of this study was to determine the changes of the biomarkers used for the diagnosis of necrotising enterocolitis of human neonates in premature calves with respiratory distress syndrome (RDS). Novel biomarkers including the intestinal fatty acid binding protein (IFABP), the liver-type FABP (LFABP), trefoil factor-3 (TFF3), actin gamma 2 smooth muscle (ACTG2), and Claudin-3 were investigated using bovine specific ELISA kits. Thirty premature calves with respiratory distress syndrome (the RDS group), seven premature calves without RDS (the non-RDS group), and seven healthy calves (control) were included in the study. Blood samples from all the groups were taken at 0 and 48 h for the blood gas and biomarker measurement. It was determined that IFABP ($P < 0.05$), LFABP ($P < 0.05$), TFF3 ($P < 0.05$), ACTG2 ($P < 0.05$), and Claudin-3 ($P < 0.05$) in the control group were significantly higher than those in the RDS and non-RDS groups at 0 hour. The LFABP and Claudin-3 concentrations in the control group were statistically higher ($P < 0.05$) than those in the RDS and non-RDS groups at 48 h, whereas the ACTG2 and TFF3 contents were significantly higher ($P < 0.05$) than the non-RDS group. A significant increase in the contents of IFABP ($P \leq 0.01$), LFABP ($P < 0.05$), TFF3 ($P < 0.05$), ACTG2 ($P < 0.05$) at 48 h was detected in the RDS group only. In conclusion, the changes in the biomarkers support the suspicion of intestinal damage such as necrotising enterocolitis (NEC) after enteral feeding in premature calves with RDS. Intestinal damage biomarkers such as IFABP, LFABP, TFF3, and ACTG2 may be useful in the diagnosis of intestinal damage in premature calves. These results also indicate that the plasma concentrations of the intestinal biomarkers change in new born calves with their gestational age.

Keywords: actin; claudin; FABP; hypoxia; trefoil factor

In prematurity, because of a surfactant deficiency, the sufficient gas exchange in the lungs is disrupted and it leads one to develop a respiratory distress syndrome (RDS). RDS and the associated complications are the major causes of death in premature calves. One of these complications is necrotising enterocolitis (NEC), which is related to immature intestinal interaction with aggressive enteral feeding, and intestinal epithelial damage due to hypoxia or altered intestinal blood flow. Some possible consequences of intestinal immaturity are an immature physiochemical luminal environment, decreased gut motility, ab-

normal microbiota, immature barrier function and, imbalance in the inflammatory responses to various agents (Neu 2005; Patole 2007; Neu et al. 2008; Gephart et al. 2012). Novel biomarkers such as the intestinal fatty acid binding protein (IFABP), the liver-type FABP (LFABP), trefoil factor-3 (TFF3), actin gamma 2, smooth muscle (ACTG2), and Claudin-3 are now being used to determine intestinal damage in infants with NEC (Kanda et al. 2011; Benkoe et al. 2014; Srivastava et al. 2015; Hong et al. 2017).

The fatty acid binding protein (FABP) family contains a number of cytoplasmic small size proteins with high organ specificity. The IFABP expression is reported to be the highest in the jejunum, whereas the LFABP expression is the highest in the colon of healthy cattle (Hayashi et al. 2013). The levels of LFABP and IFABP have been reported to be significantly higher in necrotising enterocolitis in infants (Ng et al. 2013). TFF3, secreted by the goblet cells of the small and large intestine, protects the intestinal mucosa against damage. Srivastava et al. (2015) have shown that the elevated levels of TFF3 are associated with a gastrointestinal tract injury, such as those in an inflammatory bowel syndrome. Smooth muscle actin-α (SMA-α) (ACTG2) arises from smooth muscle of the intestine (Fatigati and Murphy 1984; Hong et al. 2017) and its contents in the blood may increase when the smooth muscle layer of the gut is damaged, such as in severe NEC (Evennett et al. 2014). The loss of paracellular barriers and tight junctions is a sign of compromised intestinal integrity. These tight junctions consist of a large complex of intra- and extracellular proteins, including Claudin-3, an important sealing protein (Rahner et al. 2001; Turksen and Troy 2004; Thuijls et al. 2010), which disappears rapidly from the tight junctions following a haemorrhagic shock and inflammatory bowel disease (Zeissig et al. 2007). In previous human infant studies, IFABP, LFABP, TFF3, ACTG2, and Claudin-3 have been reported as intestine-specific markers (Thuijls et al. 2010; Ng et al. 2013; Evennett et al. 2014).

We hypothesised that necrotising enterocolitis, which is commonly observed in human preterm infants, may develop in premature calves with RDS and can be detected by intestinal-specific biomarkers used in cases of NEC.

The aim of this study was to determine the presence of an intestinal injury in premature calves using serum biomarkers that define the loss of integrity in the intestinal wall and inflammation. Furthermore, the usefulness of the serum IFABP, LFABP, TFF3, ACTG2, and Claudin-3 in detecting enterocolitis in premature calves with RDS and screening the presence of NEC gastrointestinal symptoms like in human preterm infants are evaluated.

MATERIAL AND METHODS

Animals. The research procedure was carried out by the approval of the Institutional Ethics Committee of the Faculty of Veterinary Medicine, Selcuk University (No. 2015/16). Thirty spontaneous premature calves with RDS (group RDS) and 7 premature calves without RDS (non-RDS group) that were admitted to the Large Animal Hospital of the Faculty of Veterinary Medicine of Selcuk University for treatment were included in the study. Seven healthy calves (control) that had completed the gestational age were supplied from Selcuk University Veterinary Faculty Research and Application Farm. The gestational age range for the premature and healthy calves (according to the artificial insemination records) was 240–250 days and > 280 days, respectively. All the groups included Holsteins breed calves.

Clinical examination. The criteria for prematurity, such as short silky hair coat, low body weight, soft hooves, the incomplete eruption of incisors, weakness, lateral recumbency, and weak or no suckling reflex were recorded (Ok et al. 2000; Guzelbektes et al. 2012; Yildiz and Ok 2017). The respiratory rate, body temperature, and peripheral capillary oxygen saturation (SpO$_2$) of the calves were instantly monitored using a Compact 7 system (Medical Econet, Oberhausen, Germany) and the parameters were recorded at 0 and 48 hour.

Hypoxemia (arterial partial oxygen (PaO$_2$) less than 60 mmHg), hypercapnia (arterial partial carbon dioxide (PaCO$_2$) greater than 45 mmHg), tachypnoea (respiratory rate equal or greater than 45 breaths/min), and expiration accentuated by an abdominal lift and expiratory grunt were used as the criteria for grouping into the RDS group (Bleul 2009; Yildiz and Ok 2017). The premature calves which did not meet the RDS criteria were included in the non-RSD group. The healthy termed calves were evaluated during the first hours after birth and before the first feeding.

Standard treatment protocol for premature calves. The premature calves in all the groups

were administered standard support and an oxygen therapy protocol (Yildiz and Ok 2017). The premature calves received fresh or frozen colostrum (10% of the body weight, divided into four feedings) during the first 48 h from the birth. The calves with a weak suckling reflex received colostrum via a stomach tube instead of a nipple bottle (Yildiz and Ok 2017).

Sample collection and blood analyses. The blood samples were taken from all the groups at 0 and 48 hour. The arterial blood samples were taken anaerobically using a plastic insulin syringe containing sodium heparin from the *arteria auricularis* (Yildiz and Ok 2017). The arterial blood gases including the pH, the partial pressure of carbon dioxide ($PaCO_2$), the partial pressure of oxygen (PaO_2), and the oxygen saturation (SaO_2) was measured using an automated blood gas analyser (GEM Premier 3000, Inst. Laboratory, Lexington, USA) within 5 min after collection. Ten millilitres of blood were collected by jugular venepuncture and divided into two fractions. The first fraction was placed in tubes containing potassium ethylene diamine tetra-acetic acid (EDTA) for a complete blood count (CBC) using an automated cell counter (MS4e, Melet Schloesing Laboratories, Osny, France) and was measured within 5 min; the second was placed in tubes without an anticoagulant for the measurement of the intestinal biomarkers, allowed to clot and centrifuged at 2500 *g* for 15 min at 4 °C. The serum samples were stored at –80 °C until further analysis and defrosted immediately before the batch analysis. The sample collection phase of this study was completed within 12 months.

The serum concentrations of the biomarkers (IFABP, LFABP, TFF3, Claudin-3, and ACTG2) were determined using commercially available bovine specific enzyme-linked immunosorbent assay kits (ELISA kits, MyBioSource, San Diego, USA) following the manufacturer's instructions and the absorbance was measured with a Universal Microplate Reader (ELX800, BIOTEK®, USA). The manufacturer reported the intra-assay and inter-assay coefficients of the variations (CV) and the minimum detectable concentrations (MDC) for the biomarkers are presented in Table 1.

Statistical analysis. The mean values and standard error of the mean (mean ± SEM) was calculated for each parameter. The Kolmogorov-Smirnov test was used to evaluate the data for normality. All the

Table 1. The manufacturer reported intra-assay and inter-assay coefficients of variations and minimum detectable concentrations for the biomarkers

Biomarkers	Intra-assay CV	Inter-assay CV	MDC
IFABP	≤ 8%	≤ 12%	0.05 ng/ml
LFABP	≤ 8%	≤ 12%	0.06 ng/ml
TFF3	5.9%	≤ 7.1%	< 0.26 ng/ml
ACTG2	≤ 15%	≤ 15%	0.1 ng/ml
Claudin-3	≤ 15%	≤ 15%	0.1 ng/ml

ACTG2 = Actin Gamma 2, smooth muscle; CV = coefficients of variations; IFABP = intestinal fatty acid binding protein; LFABP = liver-type fatty acid binding protein; MDC = minimum detectable concentrations; TFF3 = trefoil factor-3

parameters were normally distributed. A one-way analysis of variance (ANOVA) (with post-hoc Duncan's test) was used for comparing the difference between the groups. A paired *t*-test was used for comparing the difference within the groups. The *P*-value of < 0.05 was accepted as the level of statistical significance. All statistical analyses were performed using SPSS software for Windows, version 21.0.

RESULTS

Clinical findings

In the RDS group, twenty of 30 premature calves and all the non-RDS calves survived. In the non-surviving calves, hypothermia was recorded throughout the treatment process and these calves developed an abdominal distention after the oral feeding, they could not stand in the sternal position without support and had a prominent depressive appearance. All the recorded clinical indicators are presented in Table 2.

Blood gas and complete blood count

The measurements of the white blood cell (WBC) counts and arterial blood gas variables of the groups are presented in Table 3. The arterial blood gas analysis showed that, at the time of admission, the $PaCO_2$ of the RDS group was significantly higher

Table 2. Changes in the clinical indicators in the three groups (Mean ± SEM)

Indicator	Groups	0 h	48 h
Temperature (°C)	RDS	35.7 ± 0.36^B	37.7 ± 0.19^B
	non-RDS	37.2 ± 0.54^{AB}	38.1 ± 0.14^{AB}
	control	38.4 ± 0.08^A	38.5 ± 0.07^A
Respiratory rate breaths/min	RDS	60.9 ± 3.16^A	48.5 ± 4.04
	non-RDS	46.6 ± 7.10^{AB}	41.6 ± 5.01
	control	37.0 ± 1.19^B	36.7 ± 1.10
SpO$_2$ (%)	RDS	77.2 ± 1.73^B	85.2 ± 1.65^B
	non-RDS	91.7 ± 1.55^A	94.8 ± 0.82^A
	control	95.5 ± 1.13^A	97.2 ± 0.68^A

SEM = standard error of the mean; SpO$_2$ = peripheral capillary oxygen saturation

The different letters (^{A,B}) in the column are statistically significant ($P < 0.05$)

Table 3. Changes in the arterial blood gas indicators and the WBC value in the three groups (Mean ± SEM)

Indicators	Groups	0 h	48 h
PaCO$_2$ (mmHg)	RDS**	61.7 ± 3.30^A	47.9 ± 1.93^A
	non-RDS	43.8 ± 2.31^B	42.1 ± 2.14^{AB}
	control	41.5 ± 1.86^B	36.1 ± 2.24^B
PaO$_2$ (mmHg)	RDS***	25.9 ± 1.44^B	38.1 ± 2.19^B
	non-RDS	54.5 ± 2.57^A	61.7 ± 5.31^A
	control	53.7 ± 4.53^A	65.0 ± 7.72^A
Lactate (mmol/l)	RDS**	8.79 ± 0.84^A	3.75 ± 0.76
	non-RDS	4.68 ± 1.04^B	3.10 ± 1.31
	control**	2.78 ± 0.27^B	1.17 ± 0.21
SatO$_2$ (%)	RDS***	33.5 ± 4.35^B	70.8 ± 4.12^B
	non-RDS	84.4 ± 1.49^A	90.5 ± 1.61^A
	control	83.4 ± 5.55^A	86.8 ± 6.58^A
WBC (10^9/l)	RDS	16.7 ± 1.68	13.2 ± 1.59
	non-RDS	17.3 ± 1.26	10.4 ± 3.80
	control	12.5 ± 1.17	8.62 ± 0.87

PaCO$_2$ = partial pressure of the arterial carbon dioxide; PaO$_2$ = partial pressure of the arterial oxygen; SatO$_2$ = oxygen saturation; SEM = standard error of the mean; RDS = respiratory distress syndrome; WBC = white blood cell

The different letters (^{A,B}) in the columns are statistically significant ($P < 0.05$)

The within-group differences between 0 and 48 h are expressed as *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

($P < 0.05$) than those in the non-RDS and control groups and remained significantly higher ($P < 0.05$) than in the control group at 48 hour. Also, it was apparent that the PaCO$_2$ levels in the RDS group at 48 h in comparison to the time of admission were significantly higher ($P < 0.05$) than those in the control and non-RDS groups, but this difference was not statistically significant. When the within-group PaCO$_2$ levels were evaluated at 0 and 48 h, the values of PaCO$_2$ decreased *($P \leq 0.01$)* in the RDS group at 48 h only (Table 3). The PaO$_2$ concentration in the RDS group was lower ($P < 0.05$) than those in the non-RDS and control groups at 0 and 48 hour. A significant increase in PaO$_2$ at 48 h ($P \leq 0.001$) was determined in the RDS group. When the blood lactate levels of all the groups were compared, the blood lactate levels in the RDS group were higher at 0 hour. However, compared to the 0 h sampling time, a statistically significant decrease in the blood lactate ($P \leq 0.01$) was observed in the RDS and control groups at 48 h (Table 3). The blood SatO$_2$ contents in the RDS group were found to be significantly lower ($P < 0.05$) than those in the non-RDS and control groups at 0 and 48 hour. A significant within-group increase in SatO$_2$ at 48 h ($P \leq 0.01$) was determined for the RDS group.

Intestinal damage biomarkers

The findings of the intestinal damage biomarkers are presented in Table 4. It was determined that, at the time of admission, (0 hour) IFABP, LFABP, TFF3, ACTG2, and Claudin-3 levels in the control group were significantly higher ($P < 0.05$) than in the RDS and non-RDS groups. There was not any significant differentiation between the RDS and non-RDS groups at the time of admission. The LFABP and Claudin-3 levels of the control group were significantly higher ($P < 0.05$) than the RDS and non-RDS groups, whereas the ACTG2 and TFF3 levels of the control group were significantly higher ($P < 0.05$) than the non-RDS group at 48 hour. The results of the intestinal damage biomarkers within the groups shown that a significant increase in the levels of IFABP ($P \leq 0.01$), LFABP ($P < 0.05$), TFF3 ($P < 0.05$), and ACTG2 ($P < 0.05$) from time of admission to 48 h were detected in the RDS group only. The RDS group also showed higher Claudin-3 levels at 48 h, but without any statistical significance.

Table 4. Changes in the intestinal damage biomarkers in the three groups (Mean ± SEM)

Indicators	Groups	0 h	48 h
IFABP (ng/ml)	RDS**	0.42 ± 0.02[B]	0.61 ± 0.06
	non-RDS	0.38 ± 0.01[B]	0.43 ± 0.03
	control	0.71 ± 0.10[A]	0.64 ± 0.08
LFABP (ng/ml)	RDS*	1.22 ± 0.03[B]	1.47 ± 0.10[B]
	non-RDS	1.17 ± 0.05[B]	1.23 ± 0.05[B]
	control	1.59 ± 0.06[A]	1.85 ± 0.09[A]
TFF3 (ng/ml)	RDS*	0.90 ± 0.10[B]	1.96 ± 0.40[AB]
	non-RDS	0.63 ± 0.11[B]	0.77 ± 0.13[B]
	control	2.21 ± 0.38[A]	2.4 ± 0.43[A]
ACTG2 (ng/ml)	RDS*	1.34 ± 0.05[B]	1.90 ± 0.20[AB]
	non-RDS	1.33 ± 0.07[B]	1.35 ± 0.06[B]
	control	2.57 ± 0.62[A]	2.62 ± 0.49[A]
Claudin-3 (ng/ml)	RDS	6.14 ± 0.15[B]	6.64 ± 0.51[B]
	non-RDS	5.82 ± 0.19[B]	5.43 ± 0.18[B]
	control	9.40 ± 2.99[A]	10.13 ± 1.77[A]

ACTG2 = Actin Gamma 2, smooth muscle; IFABP = intestinal fatty acid binding protein; LFABP = liver-type fatty acid binding protein; RDS = respiratory distress syndrome; SEM = standard error of the mean; TFF3 = trefoil factor-3
The different letters ([A,B]) in the columns are statistically significant ($P < 0.05$)
The within-group differences between 0 and 48 h are expressed as *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

DISCUSSION

To our knowledge, this is the first explanatory study about the changes in the diagnostic biomarkers for necrotising enterocolitis in premature calves with RDS. We believe that these findings may provide basic knowledge for further studies about NEC in the veterinary field.

It has been reported that the ideal indicators to evaluate the hypoxia caused by lung function insufficiency are the arterial PaO_2, $SatO_2$, SpO_2, lactate, $PaCO_2$ and the respiratory rate (Yildiz and Ok 2017) in premature calves with RDS. In the RDS group, the arterial PaO_2 (< mean 38 mmHg) was lower than the critical value (60 mmHg) (Pickel et al. 1989; Bleul et al. 2008; Yildiz and Ok 2017), while the arterial $SatO_2$ (mean 70.8%) and SpO_2 (mean 85%) were below the desired level (88–95%)

(Yildiz and Ok 2017); the arterial $PaCO_2$ value was above the critical value (> 45 mmHg) as reported in the previous studies (Bleul et al. 2008; Aydogdu et al. 2016; Yildiz and Ok 2017; Yildiz et al. 2017). Furthermore, the lactate level (mean 3.75 mmol/l) was close to critical value (4 mmol/l) (Yildiz et al. 2017) and the respiratory rate was above the critical value (> 45 breaths/min) after the treatment (48 h) in the RDS group. These results suggested that persistent hypoxia in the RDS group might cause a decrease in the intestinal oxygenation and result in regional ischemia. Yildiz and Ok (2017) reported that premature calves with hypoxia fail to pass the meconium combined with a progressive abdominal distention after feeding. In addition, the autopsy findings of non-surviving premature calves revealed bleeding and oedema in both the abomasum and intestines (Yildiz and Ok 2017). The previous studies (Bleul et al. 2008; Guzelbektes et al. 2012; Canikli Engin et al. 2017; Yildiz and Ok 2017) have also reported that an abnormal pulmonary function is not the only critical factor in the premature calves. Therefore, the inflammation of the mucosa of the forestomach and abomasum should also be taken into consideration when treating these animals. Enteral feeding plays a key role in the pathogenesis of NEC. There are many physical barriers against bacteria in the mature intestine, such as gastric acid, proteolytic enzymes, peristalsis and intestinal mucus; tight junctions and cell surface glycoconjugates provide more advantages for the adherence and translocation for the pathogenic organisms (Berseth 1994; Claud 2009). Earlier studies have identified that the introduction of enteral feeding causes a disruption to the mucosal integrity, blood flow, and motility, and it has a pivotal role in the development of NEC (Pietz et al. 2007; Gregory et al. 2011). Yildiz and Ok (2017) observed that premature calves with hypoxia develop an abdominal distension after the oral feeding. Guzelbektes et al. (2012) reported relaxation in the lower oesophageal sphincter, suggesting a gastroesophageal reflux. The reason for the abomasal fluid escaping into the oesophagus may be related to an abdominal distension and a decrease in the intestinal motility. Canikli Engin et al. (2017) reported that premature calves have abomasal hypomotility and the administration of erythromycin before the oral feeding may be useful for emptying the abomasal contents. Normal motility is crucial for the prevention of the stasis and

subsequent diseases in the gastrointestinal tract and for the digestion and absorption of nutrients for the development of healthy organs and systems (Gregory et al. 2011). An abnormal peristaltic activity in preterm infants may increase the bacterial adhesion and allow bacterial overgrowth that increases the endotoxin exposure and risk of NEC (Berseth 1994; Claud 2009).

Kanda et al. (2011) determined a cut-off value (3.1 ng/ml) of IFABP in the diagnosis of an intestinal ischaemia, which is a reliable and non-invasive method for evaluating the risk of ischemia in the small intestine of the patients with acute abdominal complaints. It has been stated that the evaluation of the concentrations of IFABP in the abdominal fluids and plasma may be useful for predicting survival in horses and the need for abdominal surgery in horses with colic (Nieto et al. 2005). Niewold et al. (2004) reported that the plasma IFABP levels were significantly increased in the intestinal damage of pigs and it is a reliable parameter that could be used to detect intestinal damage and monitor the health of the intestines. In human infants with necrotising enterocolitis, the LFABP concentration was found to be high in the initial phase of the disease, whereas the IFABP level was high in the advanced phase (Guthmann et al. 2002). Ng et al. (2013) reported 45% mortality in necrotising enterocolitis in infants and that the LFABP, IFABP and TFF3 levels of the infants were significantly higher than the surviving infants. It has been determined that the level of TFF3 increases significantly during ulcerative colitis (Srivastava et al. 2015). IFABP and Claudin-3 significantly increased in new-borns suspected of NEC. They promise to be new non-invasive markers for the early detection of NEC (Thuijls et al. 2010). Evennett et al. (2014) has reported that SMA is a clinically useful marker of transmural intestinal necrosis. Plasma SMA has been reported to be able to provide information about the need for surgical intervention in neonates with elevated plasma IFABP concentrations, but further research is needed to examine its use in conditions that may cause intestinal necrosis (Evennett et al. 2014).

In this study, we assessed certain intestinal damage biomarkers in healthy mature calves and premature calves with and without RDS. The contents of IFABP, LFABP, TFF3, ACTG2, and Claudin-3 in the healthy calf group were significantly ($P < 0.05$) higher than both the premature calves with and

without RDS (Table 4). In a study, investigating IFABP and brain-caspase-3 responses in preterm and term mice in which an experimental asphyxiated model was established, it was reported that the expression of these markers in the term mice was higher than the preterm ones. It was indicated that the expression of the markers by the gestational age may be related to the different levels of the expression (Figueira et al. 2016). It has been reported that a significant portion of TFF3 in rats is expressed late in term pregnancy (Lin et al. 1999). It was also reported that the expression of TFF3 was relatively deficient in immature rats and an insufficient TFF3 expression may play a role in the development of NEC in premature infants (Lin et al. 1999). The results from the present study indicate that the plasma level of intestinal markers changes in the new-born calves with the gestational age. However, the intestinal tissue expressions of these markers still need to be investigated in calves.

In the present study, a significant elevation was found in the contents of IFABP, LFABP, TFF3, and ACTG2, while there was a non-significant increase in the level of Claudin-3 in premature calves with RDS only at 48 hour. The absence of an increase in the intestinal biomarkers in non-the hypoxic premature and control calves at 48 h suggests that this may be a condition like NEC in human infants triggered by intestinal hypoxia after oral feeding in premature calves with RDS. As previous studies report (Berseth 1994; Kleigman 2003; Caplan et al. 2005; Claud 2009; Henry and Moss 2009; Gregory et al. 2011), an immature intestinal system, the lack of a protective tight junction protein expression, hypomotility and hypoxia-induced microcirculation degradation in premature calves with RDS trigger pathogenic bacterial growth and abdominal distention after enteral feeding and cause further intestinal damage.

In preterm calves, it was seen that intestinal damage may occur after enteral feeding in cases when RDS persists. Intestinal damage biomarkers such as IFABP, LFABP, TFF3, and ACTG2 may be useful in the diagnosis of intestinal damage in premature calves. The results indicate that the serum contents of the intestinal biomarkers change in the new born calves with their gestational age. When the clinical signs and intestinal damage markers were evaluated in premature calves with RDS, a disease similar to necrotising enterocolitis in human infants can be identified. However, there are several

limitations of this study. A histopathology was not performed in order to correlate the amount of intestinal damage with the contents of IFABP, LFABP, TFF3, ACTG2, and Claudin-3 in order to gauge how well they indicate *in vivo* necrotising enterocolitis. No samples could be taken from the non-surviving calves because their death times could not be foreseen. Therefore, the significance of the specified biomarkers as mortality indicators could not be demonstrated. In the premature calves with RDS, a low survival rate is predicted to be associated with complications, such as in the gastrointestinal tract. It has been observed that oral nutrition management should be considered in premature calves. There is need for further research to improve the treatments for the increase in the intestinal microcirculation, motility and reduction of the intestinal tissue damage due to pathogenic bacterial colonisation.

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