Effect of age on nitric oxide level in Murrah buffalo (Bubalus bubalis) neonates


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Abstract Immune system of calf is more susceptible to oxidative stress during neonatal period due to immature defense system against superoxide radicals. Nitric oxide (NO), a component of bactericidal mechanisms of phagocytic leukocytes, plays a pivotal role in cell-mediated immunity. Present research has addressed many NO-related aspects of neonatal adaptation in the postpartum period. The experiment was conducted on twenty Murrah buffaloes selected from NDRI herd. Approximately 15 ml blood was drawn in sterile heparinised vacutainer tubes from each calf born from these buffaloes, by jugular veni-puncture on day 0 (Before colostrum feeding), followed by day 1, 3, 7, 14, 21, 28, 42, 56, 70, 84, 98, 112 and 126 post birth at 6.00 AM in the morning. Plasma was aliquoted and analysed for total NO by modified Griess Reaction as described by Shoker et al. (1997). Plasma nitrate and nitrite levels were significantly elevated (P<0.01) at birth in buffalo calves and decreased rapidly within first week of life with mean values decreasing more than 80%. The mean values declined from 797.08±43.62 μM/L (Precolostral levels) to 78.97±68.97 μM/L on day 126 post-birth. The levels afterwards though less than day 7 but were not comparable to mature buffaloes (1.09±0.41 μM/L to 7.63±2.75 μM/L) even after 126 days post birth exhibiting fluctuations in between. Therefore, we can say that buffalo neonates have an immature immune system as compared to the adult. Leukocytes from neonates produce unusually high concentrations of NO when compared with those produced by adult buffalo, thus, buffalo neonates have an immature immune system. Therefore, further research is needed in this aspect to explore the possibility of nitric oxide involvement in the extra-uterine adjustment of buffalo neonate.

Keywords: Buffalo, Immunity, Neonates, Nitric oxide

Introduction

The neonate has an immature immune system compared with that of an adult. Calf immune system is more susceptible to oxidative stress during neonatal period due to weak defense system against superoxide radicals (Inemani et al., 1999). Nitric oxide (NO) production is a component of the innate immune system that has not been well studied in buffalo neonates. Nitric oxide is a gaseous free radical, lipid soluble that reacts with a variety of molecules and mediates a large spectrum of biological effects (Nathan and Hibbs, 1991). Nitric oxide has pleiotropic functions in immune physiology. It has been reported that a wide array of dietary factors (amino acids, glucose, fructose, cholesterol, fatty acids, vitamins, minerals, phyto-estrogens, ethanol, and polyphenols), are either beneficial to health or contribute to the pathogenesis of chronic diseases partially through modulation of NO production (Bochsler et al., 1996). Nitric oxide besides being produced by various cells (monocytes, macrophages, endothelial cells and neurons), recently has been reported to be produced from lymphocytes in bovines. Nitric oxide synthases (NOS) produce NO by the oxidation of the 1-guanido nitrogen of L-arginine. Arginine is the substrate for endogenous NO formation because it can be metabolized to citrulline, whereby NO is formed. At least one inducible and two constitutional synthases (NOS) regulate the transformation of arginine to citrulline. Constitutive NOS is normally expressed in cells and generates small amounts of NO for short periods in response to increases in intracellular calcium. Absent in resting cells, expression of
inducible nitric oxide synthase (iNOS) can be induced in a variety of cell types, including monocytes, macrophages, keratinocytes, hepatocytes, and kidney cells by stimuli such as bacteria, cytokines, hormones, and lipoproteins (Dugas, 1995). Once expressed iNOS can generate large amounts of NO for extended periods. Nitric oxide acts as an intracellular signaling molecule or as a neurotransmitter when produced in low quantities. When produced in higher quantities for extended periods, NO is involved in the killing of microorganisms and tumor cells (Nathan, 1995) and in hematopoiesis (Ouaaz, 1995). Chronic production of NO in association with superoxide anion generates toxic radicals, which can damage cell membranes, cause inflammation, and induce apoptosis (Dugas, 1995). Yang and Shultz (1986) reported that lymphocytes from young piglets showed greater sensitivity to prednisolone than lymphocytes from 6-month old pigs. Other functional differences includes the capacity of peripheral blood mononuclear cell (PBMC) from young calves to produce iNOS, a component of bactericidal mechanisms of phagocytic leukocytes (Nonnecke et al., 2003), and reduced neutrophil function have been reported after birth (Dore et al., 1991; Higuchi et al., 1997). In bovine macrophages, iNOS is induced by heat-killed Gram-positive or Gram-negative bacteria and by combinations of endotoxin and cytokines (Adler et al., 1994; Zhao et al., 1996). Elevated plasma concentrations of total nitrate plus nitrite, the footprint of enhanced NOS-mediated NO production from arginine as well as several other recent lines of evidence, suggest that the NO axis plays a critical role in the neonate's adjustments to life. Nitrite (NO$_2^-$) and nitrate (NO$_3^-$) are stable products of nitric oxide metabolism in plasma. Therefore, the purpose of this study was to investigate the age-related changes in plasma nitric oxide in the buffalo neonates.

**Materials and Methods**

**Ethical permission**

The experiment was approved by the Institutional Animal Ethics Committee (IAEC) constituted as per the article number 13 of the CPCSEA-rules, laid down by Government of India. Norms regarding the ethical treatment of animals during the whole operation were strictly followed.

**Selection of animals**

The experiment was conducted on twenty Murrah buffaloes selected from National Dairy Research Institute (NDRI) herd, apparently healthy and in advance state of pregnancy. Calves born from these buffaloes were removed from their dams before colostrums ingestion and housed in calf’s pen separately after weighing and ear tagging. The colostrums of the same dam were fed to the newborn within 2 hours of birth, which was designated as 0 h and followed subsequent feedings at an interval of 12 h for five days. Calves were fed via nipple bottle and amount of colostrum was supplied as per the body weight of the calf. After five days, all the calves were fed whole milk @ 1/10th of their body weight up to one month (in two equal feedings). For the second month, whole milk was reduced to 1/15th and skim milk was provided @ 1/25th of their body weight. Concentrate mixture and some green fodder was offered after three months. The composition of the concentrate mixture was crushed maize 30 parts, whole oats 16 parts, groundnut cake 34 parts, wheat bran 17 parts, mineral mixture 2 parts and common salt 1 part.

**Collection of blood and deproteinization of plasma**

Approximately, 15 ml of blood was drawn in sterile, heparinized vacutainer tubes (BD Franklin, USA) from each calf by jugular venipuncture on day 0 (before colostrums feeding), followed by days 1, 3, 7, 14, 21, 28, 42, 56, 70, 84, 98, 112 and 126 post birth at 6.00 AM in the morning. Blood was centrifuged at 3000 rpm for 40 minute at 4°C for plasma separation. Acetonitrile (1:1, v/v) was used for deproteinizing of plasma proteins as described by Ghasemi et al. (2007). The process was slightly modified to suit our requirements as detailed below.

About 500μl blood plasma sample and same volume of acetonitrile (1:1, v/v) were taken in 1.5 ml ependorf tube. The contents were vortexed and incubated at room temperature for a minimum of 2 h for complete deproteinization of proteins present in plasma. The proteins were removed by centrifuging the ependorf tubes at 7000 rpm for 7 minute. The supernatant was pipetted into a new ependorf tube (1.5 ml). The contents were evaporated to dryness at 37°C. The dried contents were redissolved in 500μl Milli Q water and kept for 1 h at room temperature for complete solubilisation.

**Determination of total nitrite in deproteinized plasma**

The nitric oxide levels were quantified using modified Griess Reaction as described by Shoker et al. (1997). About 100μl of deproteinised plasma aliquot from each sample was pipetted into a 96-well microplate (F-bottom, microplate) to which 100μl of VCL (III) dissolved in 1N HCL was added. This was rapidly followed by addition of 100μl Griess reagent prepared by mixing Griess-I and Griess-II reagents in 1:1 ratio. The plates were incubated at 37°C for 30 minute for colour development yielding pinkish blue colour. The standard (sodium nitrite - NaNO$_2$) was serially diluted from 0 to 100μmol/L with milli Q water; 100μl of each was pipetted in duplicate in different wells and processed as unknowns. Blank wells consisted of milli Q water in place of sample or standard. After incubation, the absorbance was read at 540 nm wavelength in ELISA plate reader (Microscan MS-5608A). The concentration was calculated from the standard curve using linear regression equation.
Statistical Analysis

All analysis was done using Systat 12 software package. Data from different experiments are presented as mean±SE. Analysis of variance of the data was done using RBD factorial design. Significance was considered at P<0.05 or is mentioned otherwise. The following model was used for analysis:

\[ Y_{ij} = \mu + D_i + e_{ij} \]

Where, \( Y_{ij} \) is the response for plasma nitrite, \( \mu \) is the overall mean, \( D_i \) is the day's effect and \( e_{ij} \) is the residual with the usual assumptions for errors. All independent variables were tested against the residual mean squares.

Results and Discussion

Nitric oxide concentrations were highest at birth and then decreased (P<0.05) up to day 7, tended to increase transiently (P<0.5) at 14 days in both male and female calves. The nitric oxide mean values declined from 797.08±43.62 μM/L (precolostral levels) to 78.97±68.97 μM/L on day 126 post-birth. In male calves mean values declined from 888.09±52.82 μM/L (pre colostral levels) to 153.88±87.37μM/L, while in female calves decline was 706.06±63.07 μM/L to 112.25±89.61 μM/L on day 126 (Figure 1). Plasma nitrate and nitrite levels were significantly elevated (P<0.01) at birth in calves of both sex and decreased rapidly within first week of life with mean values decreasing more than 80% on day seven. In both the sex, we recorded a significant decline (P<0.01) in NO concentration at 24h and afterwards. The ANOVA indicated that plasma nitrate and nitrite levels were unaffected by the sex of calf but significantly declined (P<0.01) with age in both male and female calves.

The primary finding of this study is the surprisingly very high plasma NO concentrations in both male and female buffalo calves. Concentrations in male calves were numerically higher than in female calves, suggesting that there may be more stress in male calves. Nitric oxide (NO), a component of bactericidal mechanisms of phagocytic leukocytes, plays a pivotal role in cell-mediated immunity. Various authors have addressed many NO-related aspects of neonatal adaptation in the time-period immediately following birth in humans and bovines (Nathan, 1995; Ouaz, 1995; Rajaraman et al., 1998; Biban, et al., 2001; Gow et al., 2002; Gow et al., 2004; Colnaghi et al., 2003; Huang et al., 2003; Levy et al., 2005; Christen et al., 2007). We observed very high levels of NO in plasma after birth which exhibited fluctuations even up to 126 days. Nitric oxide concentration was not in general agreement with Huoza et al. (2012) who reported NO level in range of 1.09±0.41 μM/L to 7.63±2.75 μM/L in mature buffaloes. The present data is in agreement with other previous reports on cattle (Christen et al., 2007). Blum et al. (2001) reported very high NO concentration in blood plasma, saliva and urine in newborn calves before the first meal. Christen et al. (2007) suggested that activation of the NO axis was required during the transition period that eventually caused an increase in endogenous production of NO at birth for neonates of cattle and many other species. Elevated plasma concentrations of total nitrate plus nitrite, the footprint of enhanced NO mediated NO production from arginine (Gow et al., 2002, 2004). Nitric oxide involve in various physiological processes viz killing of microorganisms and tumor cells (Nathan, 1995).

![Figure 1: Plasma Nitric Oxide (μM/L) concentration in growing buffalo calves](image-url)
hematopoiesis (Ouaaz, 1995), cardiovascular adjustments (Manak, 1986). The biological importance of the high NO in Murrah buffalo neonate is presently not clear. The elevated NO status may cause enhanced methemoglobin formation (Hapke, 1988). An elevated NO status, combined with an already high methemoglobin status, may explain why neonatal and young calves are particularly susceptible to methemoglobin intoxication after NO ingestion (Hapke, 1988).

Nevertheless, the enhanced NO production by endothelial cells would be expected to influence blood pressure (Lancaster 1992; Anggard, 1994). In addition, enhanced NO production by the autonomous nervous system would be expected to influence the motility of the gastrointestinal tract. It also involves in controlling of local blood flow, which ensures adequate tissue perfusion (Gow et al., 2002, 2004; Huang et al., 2003) suggesting that the NO axis plays a critical role in the neonate's adjustments to the extra-uterine life.

Conclusions

In conclusion, present study shows that both male and female buffalo neonates are characterized by a high NO status. Peripheral blood mononuclear cells from neonates produce unusually high concentrations of nitric oxide when compared with those produced by adult buffalo, thus, buffalo neonates have an immature immune system. Therefore, further research is needed in this aspect to explore the possibility of nitric oxide involvement in the extra-uterine adjustment of buffalo neonate.

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References


