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MINIREVIEWS

Preterm nutrition and neurodevelopmental outcomes

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Abstract

Survival of preterm infants has been steadily improving in recent years because of many recent advances in perinatal and neonatal medicine. Despite these advances, the growth of survivors does not reach the ideal target level of the normal fetus of the same gestational age. Postnatal weight gain is often not achieved because extrauterine growth has higher energy requirements than intrauterine growth, due to the intensive care environment, illness and inadequate nutrition. Although many other factors influence infant brain development, including family socioeconomic and educational background, the role of nutrition is considerable and fortunately, amenable to intervention. In the preterm neonate, the brain is the most metabolically demanding organ, consuming the largest proportions of energy and nutrient intake for its function and programmed growth and maturation. Weight gain, linear and head circumference growth are all markers of nutritional status and are independently associated with long-term neurodevelopment. Brain development is not only the result of nutrients intake, but in addition, of the interaction with growth factors which depend on adequate nutrient supply and overall health status. This explains why conditions such as sepsis, necrotizing enterocolitis and chronic lung disease alter the distribution and accretion of nutrients thereby suppressing growth factor synthesis. In this review, we will focus on the direct role of nutrition on neurodevelopment, emphasizing why it should be started without delay. The nutritional requirements of the preterm infant will be discussed, followed by the effects of general nutritional interventions and specific nutrients, as well as the role of nutritional supplements on neurodevelopment. The primordial role of human breast milk, breast milk fortifiers and human milk oligosaccharides will be discussed in detail. We will also examine the role of nutrition in preventing neonatal complications which can affect neurodevelopment in their own right.

Key Words: Brain; Nutrition; Preterm infants; Neurodevelopmental outcomes; Newborn;

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Breast milk

Core Tip: Survival of preterm infants has been steadily improving because of the many recent advances in perinatal and neonatal medicine. However, neither the growth of survivors reaches the ideal target level of the normal foetus of the same gestational age, nor is the optimum postnatal weight gain often achieved. In the preterm neonate, the brain is the most metabolically demanding organ. Growth is a marker of nutritional status and is also independently associated with long-term neurodevelopment. In this review, we will discuss the direct role of nutrition and the effect of general and specific nutritional interventions on neurodevelopment.

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INTRODUCTION

Survival of preterm infants has been steadily improving in recent years because of the many advances in perinatal and neonatal medicine, such as antenatal corticosteroids administration, surfactant therapy and novel mechanical ventilation modalities. Despite these advances, the growth of preterm infants, as indicated by anthropometric indices and body composition, does not reach the target level of the normal foetus of the same gestational age. Often optimal postnatal weight gain is not achieved because extrauterine growth has higher energy requirements than intrauterine growth, due to the intensive care environment, illnesses and inadequate provision of nutrition. The persisting suboptimal nutrition in survivors remains an issue and is associated with adverse outcomes, such as chronic lung disease (CLD), necrotising enterocolitis (NEC), sepsis and, more worrying, neurodevelopmental impairment, especially in the cognitive domains, which is increasing in prevalence[1]. These adverse developmental outcomes can be secondary to abnormal brain development, postnatal brain injury and suboptimal nutrition occurring during hospital admission. Therefore, preterm birth can be considered a nutritional emergency.

Improved neurodevelopmental outcomes in extremely low birth weight (ELBW) infants are associated with a reduction in the incidence of CLD, apnea, intraventricular hemorrhage (IVH) and any associated nutritional deficiency[2]. Although many other factors influence infant brain development, not least family socioeconomic and educational background, the role of nutrition is considerable and fortunately, amenable to intervention[3].

In the preterm neonate, the brain is the most metabolically demanding organ and consumes the largest amount of energy and nutrients for its function and programmed growth and maturation[4]. Starting in the prenatal period and continuing until the second year of life, the development of the cerebral white and gray matter structures of the central nervous system (CNS) involves many processes, especially between 22 and 42 wk post-conception. Cell replication, neurogenesis, neuronal differentiation, cell migration, myelination, synaptogenesis, progression and regression of specific structures in different periods of development and neuroplasticity, are some of these processes[4]. The number of cell replication cycles in the CNS is decreased in malnutrition, thereby reducing total brain DNA and leading to a restriction in dendritic arborization and a reduction in the connections between neurons[5]. These cellular mechanisms affect the pre-oligodendrocyte, the widely disseminated activated microglia and the reactive astrocytes. As they occur over a relatively long time period, neurorestorative interventions, such as better nutrition can improve brain development[6]. Also, alterations in dietary precursors may affect neurotransmitter levels, for example, serotonin, norepinephrine, dopamine and acetylcholine in specific brain regions. In addition, essential and nonessential lipid components play an important role in the structural composition of the brain and of myelin sheaths. The

resulting CNS impairment, caused by these nutrient deficiencies, involves motor and cognitive development and social abilities. They are also associated with alterations in the sleep-wake cycle organization, neurovegetative activities during sleep, waking electroencephalographic (EEG) activity and visual and auditory evoked responses.

The neurodevelopment of premature babies is very sensitive to nutrition in the first few weeks of life, and these effects may be long lasting until the age of eight years[7]. Cerebellar development is most affected by nutritional deprivation around the time of birth, and also, synaptic connectivity occurring before the third year of life. Nutrition is positively associated with weight gain, increased brain volumes and white matter maturation on magnetic resonance imaging at term equivalent age and neurodevelopment in infancy[7-9].

Adequate early nutrition may also attenuate the adverse effects of neonatal illness. Studies of EEG maturational age (EMA) during periods of quiet sleep which analysed spectral power, continuity and interhemispheric synchrony, have shown that a lower intake of calories and carbohydrate is associated with a greater reduction of spectral amplitude in the delta band. A lower protein intake was associated with higher discontinuity. In contrast, both higher proteins and lipid intake were associated with better developmental increase in interhemispheric synchrony and with brain activity maturation [10]. Increased caloric and protein intakes in preterm infants are associated with a higher fat free mass (a key marker for organ growth) and better neurodevelopment outcomes[11]. Protein and energy deficits in preterm nutrition are commonly associated with other nutritional deficiencies and also with psychosocial deprivation. Such interactions impact on the brain's structural development, disrupting neurodevelopment and adversely affecting cognitive performance[12].

Weight gain, linear and head circumference growth are all markers of nutritional status and are independently associated with long-term neurodevelopment. Brain development is not only a result of nutrients intake, but also of the interaction with growth factors, which depend on adequate nutrient status and overall health status. Sepsis, inflammation (occurring in NEC, CLD) and corticosteroids administration, alter the distribution and accretion of nutrients and suppress growth factor synthesis. Therefore, in addition to provision of adequate nutrients, strategies to reduce the incidence of these conditions are also required to optimise brain growth and development[4].

In this review, we will focus on the direct role of nutrition on neurodevelopment. First, we will discuss the nutritional requirements of the preterm infant, followed by the effects of general nutritional interventions, specific nutrients and nutritional supplements on neurodevelopment[9]. We will then examine the role of nutrition in preventing neonatal complications, such as sepsis, NEC and CLD, which can also affect neurodevelopment in their own right (Figure 1)[4,13].

NUTRITIONAL REQUIREMENTS

Macronutrients refer to carbohydrates, fats and proteins while micronutrients include trace elements, electrolytes, and vitamins. Nutritional requirements, based on the estimated average requirement of a specific population group, are defined as the type and amount of nutrients needed to support normal health, growth and development. However, as preterm infants do not constitute a homogeneous population, their requirements must be individualised based on their clinical condition and developmental stage[14].

The high growth rate of premature infants requires a proportionally very high intake of all nutrients. With the general assumption that adequate postnatal growth should approximate the in-utero growth of a normal foetus, nutrient requirements were initially derived from postmortem measurement of the size and body composition of fetuses at varying gestational ages. The resulting nutritional intakes were estimated as the amounts required to replicate intrauterine rates of growth and nutrient accretion. The traditional method for estimating protein requirement is the factorial method, based on the body composition of the foetus with an estimate of the inevitable urinary nitrogen losses (occurring even in the absence of nitrogen intake) and of the amount deposited in-utero for accretion of tissue, corrected for efficiency of absorption and deposition[15]. Improvements to this model now take into account, not only the estimation of intrauterine accretion rate, organ development and factorial estimates of requirements, but also nutrient interactions, supplemental feeding and long-term developmental outcomes[16].

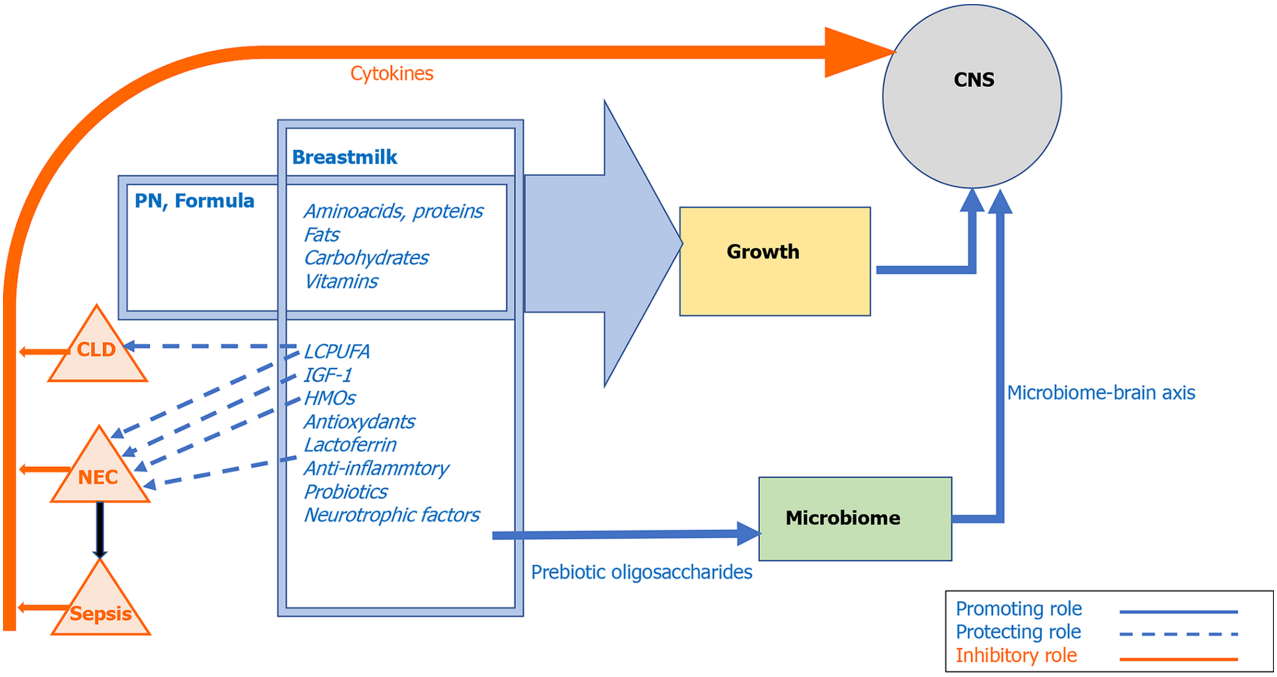

Figure 1 Direct effect of nutrients on brain development of the preterm infant and indirect impacts in preventing some neonatal complications, which can affect neurodevelopment in their own right. PN: Parenteral nutrition; LCPUFA: Long chain polyunsaturated fatty acids; IGF-1: Insulin-like growth factor 1; HMOs: Human milk oligosaccharides; CLD: Chronic lung disease; NEC: Necrotising enterocolitis.

Energy, stored in the body in the form of adenosine triphosphate (ATP), is required for several cellular and organ functions. Preterm infants have low energy reserves because they are born before adequate accumulation of fat and glycogen has occurred. In healthy premature infants, a recommended daily energy intake of 110-130 kcal/kg/day allows a growth rate similar to that of the intrauterine growth rate. For the neonate, carbohydrates are the main energy source (4 kcal/gram), in the form of glucose. Glucose synthetic rates in preterm infants are much higher at 6-8 mg/kg/min compared to 3-5 mg/kg/min in term infants. Because of their high energy-density (9 kcal/gram), fats or lipids are a very good source of energy. In addition, they also provide essential fatty acids indispensable for brain and visual development. Proteins are essential for normal growth and development (see below). After the initial free water diuresis, 2-3 mmol/kg/day of sodium and potassium (as a minimum) will be required to maintain serum levels in the normal range. Preterm infants often develop low sodium levels in the second week of life due to renal immaturity resulting in sodium loss. A normal serum sodium level is a requirement for adequate growth. Calcium, magnesium and phosphorus are essential for tissue structure and function. Vitamins are important for growth and development facilitating many reactions in intermediary metabolism. Preterm infants have low body stores of fat-soluble vitamins at birth due to limited transfer of lipid-soluble substrate across the placenta and, therefore, provision of the latter should be started as soon as lipid containing parenteral nutrition is initiated. A summary of the high nutrient requirements of the preterm infant is shown in Table 1. Human milk alone cannot provide the nutritional needs (especially protein) of preterm infants and requires fortification.

Providing preterm infants with adequate nutritional support presents unique challenges. Failure to provide adequate nutrient intakes at all stages of development places them at risk of impaired neurodevelopment. Therefore, every effort must thus be made to meet their complete nutritional needs from birth[15]. The main and most important reasons for growth failure in premature infants are the physiological limitations preventing the provision of nutrients by enteral feeding. This, in turn is compounded by the very high nutritional needs. Provision of nutrients has to take into account the immaturity of the intestinal tract, a major risk factor for the development of NEC. To overcome this, parenteral nutrition is used during the early days and often weeks of life. Although there are problems associated with parenteral nutrition, especially that of infection, failure to provide nutrients parenterally would place these infants at high risk of impaired neurodevelopment and host defenses. Providing early nutrient intakes by the parenteral route or with breast milk when possible, are therefore crucial to optimise long-term health outcomes[3].

Table 1 Nutritional requirements[1]

Body weight	500-1000 g	1000-1500 g	1500-2200 g	2200-3000g
Foetal weight gain (g/kg/d)	19.0	17.4	16.4	13.4
Protein(g/kg/d)	4.0	3.9	3.7	3.4
Energy (kcal/kg/d)	106	115	123	130
Protein/energy (g/100 kcal)	3.8	3.4	3.0	2.6
Glucose	6-8 mg/kg/min			
Lipids	2.5-3.5 g/kg/d			
Calcium	60-90 mg/kg/d			
Phosphorus	40-70 mg/kg/d			

[1]Estimated by factorial and empirical methods, derived from several references in this article[14-16].

INDIVIDUAL NUTRIENTS DIRECTLY AFFECTING NEURODEVELOPMENT IN PRETERM INFANTS

Caloric intake

Caloric intake in a large population of extremely premature infants (born < 28 wk' gestation) was associated with the development of retinopathy of prematurity (ROP) [17]. In preterm infants, caloric deprivation is associated with a significantly low composite score on Bayley-III scales performance at 18-24 mo corrected age[13]. In the same study, late onset sepsis, NEC, and CLD were also found to be risk factors for impaired neurodevelopment. Improving nutritional support, specifically total caloric intake is therefore necessary to prevent neurodevelopment impairment.

Protein

Adequate protein intake is crucial for satisfactory growth and neurodevelopment in preterm infants. It has been recommended that a high protein intake starts on day 1 of life to limit extrauterine growth restriction, which in itself negatively affects neurodevelopment. Brain magnetic resonance imaging (MRI) studies in the first month of life in extremely preterm infants, who had received early nutrition, demonstrated larger brain volumes in those who had received a higher protein and caloric intake than controls[18]. In very low birth weight (VLBW) newborns, serial cranial ultrasound measurements of the size of several brain structures in the first month of life showed a positive correlation between enteral protein intake and brain volume[19]. Supplemental enteral proteins were also shown to reduce postnatal growth restriction and improve neurological outcomes in preterm infants < 1000 g and in small for gestational age (SGA) infants (birth weight < 10th percentile)[20]. Similarly, parenteral amino acid intake within the first two weeks of life was correlated with neurodevelopmental outcomes at 2 years, with higher language and motor scores on the 2-year Bayley Scales of Infant and Toddler Development (Bayley III)[21].

However, not all studies have reported a consistently positive relationship between early protein intake and neurocognitive outcomes. Protein supplementation in very low birth weight infants (VLBW) (birth weight < 1500 g) showed a significant positive correlation with neurological development in communication, auditory, verbal language, cognitive function, social connection and gross motor development, but not with fine motor, problem solving or personal-social relationships in the third year of life[22]. However, this study involved small population numbers and protein supplementation was stopped when the infants weighed 1500 g. In preterm infants born before 32 wk' gestation or with birth weight < 1500 g, addition of fortifier to expressed human milk to increase protein intake, was associated with better head growth and weight gain at 40 wk' postmenstrual age. However, this intervention had no benefits on long-term growth and neurodevelopment at 12 to 18 mo corrected age[23]. Likewise, early aggressive parenteral nutrition with high-dose amino acids and mixed fat emulsions did not improve neurodevelopmental scores of VLBW infants, nor decrease the incidence of major disabilities[24]. Again this study included a small study population divided into 5 groups with different parenteral feeding regimes.

Fats and long-chain polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LCPUFA), including docosahexaenoic acid (DHA) and arachidonic acid (ARA), are essential for neurodevelopment and normal vision. In-utero DHA accretion occurs primarily in the last trimester of pregnancy supporting rapid growth and brain development. Premature infants, born before the completion of this process are therefore relatively deficient in these essential fatty acids. This deficiency persists for a long period after birth because of ineffective conversion from precursor fatty acids, lower fat stores, and limited nutritional provision of DHA and ARA. Besides long-term visual and neurodevelopmental risks, very preterm infants have significant morbidity and mortality from diseases specific to premature birth, including CLD, NEC and ROP. There is increasing evidence that DHA has protective benefits against these complications[25]. The ratio of n-3 to n-6 LCPUFAs, which generates eicosanoids, is important in moderating the effects of hypoxia, inflammation, infection, thrombosis and oxidative damage of key organs such as the lungs, brain and retina[26]. These actions improve the prognosis for ELBW and VLBW infants. By providing LCPUFAs, including DHA, breastfeeding improves cognitive development, especially in infants with a proven lower activity of DHA synthesis[27].

The outcomes of supplementation with PUFAs are still contested. Although early PUFAs supplementation in preterm infants had a positive impact on early childhood psychomotor development and visual acuity, they had no significant effects on global intelligence quotient (IQ) in childhood[28]. Similarly, a metanalysis of LCPUFAs supplementation in preterm infants showed a significant improvement in their neurodevelopment (assessed by the Mental Development Index of the Bayley Scales at one to three years of age). Supplementation during lactation accelerated neurodevelopment but their final developmental outcome remained unchanged[29]. Other studies have shown no beneficial effect of early high-dose mixed fat emulsions on neurodevelopment of VLBW infants[24,30]. Studies of formula milk supplemented with LCPUFAs showed mixed results, some reporting better psychomotor outcomes [31] while others showed no long-term benefits on visual or psychomotor development[32]. Infants enrolled in the supplemented formula study were mature in general and the quality of evidence was low for both visual and neurodevelopmental outcomes. Current recommendations for follow-on formula suggest that they should contain omega-3 DHA equal to the mean content of human milk, and also AHA[33].

Iodine supplementation

Iodide supplementation in babies born before 31 wk' gestational age provided no benefit to neurodevelopment measured at 2 years of age[34]. Similarly, a large high-quality study showed no beneficial effects of iodine supplementation for preterm infants[35].

Iron supplementation

In infants born ≥ 34 wk gestational age, latent in-utero iron deficiency (serum ferritin < 75 ng/mL at birth) was associated with abnormal auditory neural maturation[36]. The age at which to begin iron supplementation remains controversial. It is often given from day 28 of life onwards, assuming until then, the infant has sufficient iron stores from the breakdown of fetal hemoglobin. As breast milk does not provide sufficient iron to sustain a premature infant's growth and normal haemopoietic function, breast-fed infants require iron supplementation. Formula-fed babies do not need iron supplements as milk formulas are iron fortified.

BREAST MILK

Fresh maternal milk contains factors involved in antioxidant and anti-inflammatory defense[35], gut microbiome establishment and the maturation of immune defenses [37,38], feed tolerability and metabolism[39] and also protects infants against NEC [16]. For these and other reasons, human milk is the preferred choice for feeding premature infants. It reduces the risk of morbidities and promotes growth through elevated serum levels of insulin-like growth factor 1 (IGF-1), an essential intrauterine hormonal mediator of growth[40].

Human milk contains critical nutrients and other neurotrophic factors that benefit the brain of preterm infants[41]. A systematic review of neurodevelopment outcomes of ex-preterm children found better test scores in those who had been fed breast milk compared with formula feeds, when evaluated in the first three years of life and also,

between the age of five to eleven years[42]. In a prospective study of 316 VLBW infants, correcting for gestational age (GA), being born SGA, and for complications at birth and during hospital admission, better neurodevelopment at 24 mo corrected age was associated with breast milk feeds[43]. VLBW infants fed fortified human milk had increased head circumference velocity and decreased regional white matter diffusivity when assessed by magnetic resonance diffusion tensor imaging suggesting improved maturation of the cerebral connective tracts[44]. They also had improved visual perception as judged by their response to global motion[45]. Brain MRI studies at term equivalent age, using network-based statistics, tract-based spatial statistics and volumetric analysis of white matter water diffusion parameters, reported that feeding with breast milk in the weeks after preterm birth was associated with improved structural connectivity of developing networks and greater fractional anisotropy (FA)-weighted connectome in major white matter fasciculi[46]. There were also better outcomes observed in infants fed breast milk for more than 90% of their hospital admission duration compared with those who received breast milk for 75% of the same period.

When a mother expressed breast milk is unavailable, donor breast milk is used widely to feed premature babies. The methods used to process donor milk reduce the levels of protective and growth promoting factors found in fresh breast milk. However, donor milk still offers protection against NEC but remains inferior to fresh human milk in promoting the growth and development of very preterm infants[47].

Breast milk fortification

Human milk, although the preferred feeding for premature infants because of its protective effects, does not provide adequate amounts of nutrients to support the rapid growth and development of the premature baby and therefore, must be fortified. Failure to fortify mother's milk places the baby at risk of neurodevelopmental impairment. As mentioned earlier, an enhanced nutrient supply with breast milk fortification to VLBW infants was associated with increased head circumference growth and decreased regional white matter mean diffusivity seen on magnetic resonance diffusion tensor imaging, suggesting improved maturation of cerebral connective tracts[44]. A recent study of long-term outcomes of preterm infants fed an exclusive human milk-based diet using a donor human milk-based fortifier reported an absence of severe cognitive developmental delay using a cutoff score of 70 (Bayley Scales of Infant and Toddler Development III) measured at 18-22 mo corrected gestational age (GA)[48].

Some controversies still exist. An earlier study showed no significant differences at 18 mo corrected age in mean Bayley-III cognitive composite score, language composite score, or motor composite score in VLBW infants fed fortified donor milk when compared with formula fed babies[49]. Similarly, a study of the balance of risks and benefits of feeding formula *vs* donor breast milk for preterm or LBW infants found no evidence of an effect on long-term neurodevelopment, although the level of evidence was moderate[50]. This may be explained by the effects of the pasteurisation process on donor milk causing reduced levels of the non-nutritive content. Even fewer data are available for growth and developmental outcomes assessed beyond infancy and many do not show consistent effects. Feeding with a nutrient enriched formula had no effect on Bayley Mental Development Index scores at 18 mo post term or on cerebral palsy, and there were no effects on long-term development[51].

Human milk oligosaccharides

Human breast milk has two types of carbohydrates, lactose and oligosaccharides. HMOs are indigestible unconjugated complex carbohydrates forming the third most abundant component of breast milk, after lactose and lipids. They are a complex mixture of over 200 non-digestible and non-nutritional carbohydrates. There are three main HMO categories based on their building blocks. The first category comprises non-fucosylated neutral (core) HMOs, which are the foundations upon which other HMOs are built, lacto-N-tetraose isomer (LNT) being the most abundant representative in this category. The fucosylated neutral HMOs form the second category, with 2'FL the most abundant fucosylated HMO and DFL (LDFT) among the 10 most abundant representatives in this group. The third category is composed of the sialyated acidic HMOs, 6'SL is the leading representative of this group followed by 3'SL.

In animal studies, using a genome-wide profiling of the intestinal epithelial transcriptome in response to HMOs, the latter were found to protect against NEC in part by altering the differentiation of the crypt-villus axis host transcriptome in 225 target genes involved in cell proliferation and differentiation, including upregulation

of the stem cell differentiation marker HMGCS2. HMOs also directly induced a series of biological processes to protect the intestine[52]. In another experiment, milk supplementation with 2'-FL and 6'-SL, but not lactose, prevented NEC in mice and piglet models and attenuated NEC inflammation in the ileum, in part through TLR4 inhibition with reduced apoptosis and inflammation[53].

In neonates, HMOs have been shown to decrease the incidence of late-onset sepsis and NEC, both conditions being associated with neurodevelopment impairment. Reducing the risk of these complications leads to less disruption of feeds which is also beneficial for normal neurodevelopment. In the premature baby's gut, HMOs and the microbiome of breast milk act as immunomodulatory agents that provide intestinal homeostasis through regulation of the microbiome and protection of the intestinal barrier, thereby protecting the premature baby at risk of NEC[54]. The concentrations of different HMOs found in neonates vary according to the lactation period and maternal secretory phenotype status[55]. Fucosyl--N-hexose (FDSLNH) was associated with a reduced risk of late-onset sepsis in VLBW infants[55].

An animal study examined the effect of HMOs on cognition, novel object recognition task, brain development, and hippocampal gene expression using magnetic resonance imaging procedures to assess structural brain development and hippocampal tissue analysis for mRNA expression. The results showed improved recognition memory and better absolute and relative volumes of the cortical and subcortical brain regions. Hippocampal mRNA expression of GABRB2, SLC1A7, CHRM3, and GLRA4 were also affected by HMOs[56].

Relative abundances of several individual HMOs were associated with normal growth and neurodevelopment in infants, mainly motor development at 12 mo, including ability to stand or walk alone. They were also associated with language skills, socioemotional development, executive function and working memory at 18 mo, mostly among secretors[57].

Microbiota and probiotics

While the newborn term microbiome is normally dominated by Bifidobacterium species, the microbial composition and functions in preterm babies has a higher initial percentage of Lactobacillaceae[58]. It has been established that intestinal dysbiosis, (abnormal microbial colonisation), can occur as a result of exogenous factors such as mode of delivery, formula feeding, and exposure to antibiotics. These factors predispose preterm infants to sepsis and NEC in association with an already existing impaired intestinal barrier and immature immunity. The prolonged use of antibiotics and parenteral nutrition were reported to have significant adverse effects on the Lactobacillus and Bifidobacterium levels in the gut of preterm infants[59].

Inflammation and perinatal infection play a crucial role in the pathogenesis of white matter injury in preterm infants. Therefore, nutritional components with immunomodulatory and/or anti-inflammatory effects may serve as neuroprotective agents. There is growing evidence of the existence of a microbiome-gut-brain axis in which the microbiome interacts with the brain through immunological, endocrine, and neural pathways. Consequently, nutritional components that influence gut microbiota may also exert beneficial effects on the developing brain. Based on these properties, probiotics, prebiotic oligosaccharides, and certain amino acids may offer neuroprotection. Certain nutritional components may also have a neuroprotective role against white matter injury through modulation of inflammation and infection and may influence the microbiome-gut-brain axis[60].

As stated above, the normal term newborn microbiome is dominated by Bifidobacterium species but the establishment of abnormal microbial colonisation (intestinal dysbiosis) can be influenced by several factors, thereby increasing the risk of developing sepsis and NEC. Probiotics have been shown to reduce rates of necrotising enterocolitis (NEC), sepsis, and mortality[61]. However, research in this area has been complicated by studying different bacterial strains, administration of different doses of probiotics and for different time periods of administration. Provided all safety issues are met, it is currently recommended that either Lactobacillus rhamnosus GG ATCC53103 or the combination of Bifidobacterium infantis Bb-02, Bifidobacterium lactis Bb-12, and Streptococcus thermophilus TH-4 would help reduce the rates of NEC[62]. It is essential that probiotic strains should be devoid of transferable antibiotic resistance genes.

MEDIUM AND LONG-TERM EFFECTS OF CONDITIONS INFLUENCED BY NUTRITION WHICH HAVE AN IMPACT ON NEURODEVELOPMENT

Growth

The long-term effect of inadequate early postnatal growth (EPG) is a growing concern. Poor head growth during hospital admission and post discharge is associated with delayed motor and cognitive development between 16 and 36 mo of age[63]. A systematic review of the relationship between EPG before 3 years of age and neurodevelopmental outcome, showed that EPG was positively associated with good neurodevelopmental outcome in a non-linear association, with a plateau attained with higher weight gain, suggesting a possible ceiling effect[64]. Similarly, post-discharge failure to thrive was found to be significantly correlated with poor neurodevelopmental outcomes. There was an association between lower body weight (< 3rd percentile) at corrected ages of 6, 12, and 24 mo and poor neurodevelopmental outcomes among 30% of VLBW premature infants[65]. Extra uterine growth retardation (EUGR) of VLBW infants is significantly associated with low mental development index (MDI) at a corrected age of 24 mo[66].

Studies examining the relationship between body composition and neurodevelopment in VLBW infants reported higher growth, fat mass and fat-free mass were associated with larger cerebellar volumes at term[67]. Also, greater fat-free mass (measured by air displacement plethysmography) gained by VLBW premature infants as inpatients, was associated with improved cognitive and motor scores at 12 months corrected age (CA)[68]. Increased provision of protein and calories during the first week of life is positively associated with fat-free mass gains, but not fat mass gains. Frondas-Chauty *et al*[69] reported a deficit of fat-free mass in VLBW infants at discharge was associated with neurological impairment at 24 mo (corrected age), independent of sex, GA and BW.

Necrotising enterocolitis, intestinal failure, and sepsis

NEC often occurs in premature infants at a time of rapid brain growth and development and is associated with poor neurodevelopmental outcomes. The pathogenesis of neurodevelopmental impairment following NEC is likely multi-factorial, involving both nutritional and non-nutritional factors[70]. A variety of circulating cytokines are known to be associated with white matter injury. There is also increased risk of secondary blood stream infections and subsequent nutritional compromise[71]. Breast milk, probiotics and lactoferrin have all been shown to reduce the incidence of NEC and late-onset sepsis[72].

NEC is also a major cause of intestinal failure (IF), which in turn is associated with developmental delay in multiple domains. While gross motor skills appear to be most significantly affected, cognitive deficits become more apparent at 26 months[73]. In children with IF assessed before 15 mo corrected age, 88% had abnormal general movements and over 50% had suspect or abnormal gross motor scores. Factors significantly associated with poorer outcomes at 12-15 mo included prematurity, low birth weight, CNS co-morbidity, longer neonatal intensive care admission, NEC, number of operations and conjugated hyperbilirubinemia. Thus, multiple risk factors contribute to early developmental delay in children with IF and close developmental follow-up is essential in these patients[73].

GENERAL DIETARY INTERVENTION

Early feeding

As mentioned earlier, the preterm brain is very vulnerable to undernutrition in the first few weeks of life, even over a period as short as 4 wk, and these effects persist up to the age of 8 years[74]. Suboptimal nutrient intakes permanently affect later cognitive attainment and therefore, early nutrient intakes and breast milk provision are essential[3]. A positive association between several components of early nutrition, growth, brain volumes, white matter maturation, and neurodevelopment in infancy has been shown[7,75]. Immediate breast milk feeding results in elevated insulin-like growth factor 1 (IGF-1), an essential intrauterine hormonal mediator of growth, measured at term, in the serum of preterm infants[40]. Early administration of optimal postnatal parenteral and enteral nutrients helps to prevent neurodevelopmental impairment caused by extrauterine growth restriction, NEC, sepsis, CLD dysplasia, and ROP[72].

Early introduction of enteral feedings to stimulate or prime the gut even in the acutely ill preterm infant, is well tolerated[76,77]. Several metanalyses have shown the safety of early trophic feeding with breast milk within 48 h after birth and progressing the volume of enteral feeding before four days of life at a rate of 24 mL/kg/d in clinically stable very preterm and VLBW infants. These measures did not lead to higher mortality or an increased incidence of NEC[77,78].

However, despite the introduction of more aggressive early feeding guidelines and improved energy and nutrient intakes, many preterm infants do not achieve their recommended nutrition intake in the first week of life, especially those with morbidities[79].

FEEDING IN PRACTICE

During the immediate postnatal period, the aim of nutritional support is twofold: i) to provide an early and uninterrupted flow of nutrients to ensure the anabolic state that existed in-utero continues with minimal or no interruption and ii) to stimulate the immature gastrointestinal tract to undergo maturation. However, as described above, early provision of the preterm infant with the recommended nutritional intakes is fraught with difficulties. The latter include comorbidities which preclude normal enteral feeding such as respiratory distress, haemodynamic instability and sepsis. Co-morbidities also indirectly affect the delivery of adequate nutrition because a significant proportion of the total daily fluids may be used to deliver several medications, thereby limiting the amount of actual nutrition given to the baby. This problem can be overcome to some extent by concentrating the constituents of TPN in a smaller volume. In addition, gut immaturity limits the amount of enteral feed the baby will tolerate and is also a major risk factor for NEC, which will further impair enteral feeding. Furthermore, the high nutritional requirements cannot be fully provided immediately after birth and thus need progressive and cautious escalation. All these factors contribute to the risk of undernutrition with its resulting outcomes.

Strategy for nutritional support

Nutritional support of preterm infants occurs in four distinct phases, each with its own risks and challenges. During the early phase, nutrients are almost exclusively provided *via* the parenteral route, while small enteral feedings (gastrointestinal priming or trophic feeds) are used to stimulate the intestinal tract towards maturation. During the subsequent transition phase, enteral feeding is slowly advanced as the intestinal tract shows evidence of maturation, and parenteral nutrition is gradually phased out. During the late phase, infants are exclusively enteral fed and are expected to grow normally. If provided with the necessary nutrients, preterm infants may also show catch-up growth, making up for lost time during the early phase. Preterm infants continue to have special nutritional needs after discharge from hospital.

The importance of providing early nutrition has led to the use of parenteral nutrition immediately after birth with the knowledge that it differs from enteral nutrition in nutrient composition and carries the risk of infection. Nevertheless, the use of total parenteral nutrition (TPN) has contributed to improving survival rates and at its most fundamental level, it helps the newborn premature infant maintain a neutral nitrogen balance.

While nutrients are provided parenterally immediately after birth, starting trophic feeds with breast milk from day 1 of life, with volumes as low as 2 mL every 4 to 6 h, is the safest and most effective method of stimulating the intestinal tract and accelerating its maturation.[18]

When breast milk is not available, pasteurised donor milk (free of viruses such as HIV and cytomegalovirus) can be used for gastrointestinal priming. Although pasteurisation diminishes some of the protective and trophic factors of human milk, donor milk still remains protective against NEC and sepsis while maintaining trophic effects. Using gastrointestinal priming has been shown to lead to earlier establishment of full feedings and to earlier hospital discharge without an increase in morbidities. Moreover, earlier achievement of full feedings has been shown to decrease the risk of sepsis.

Increasing enteral feed volumes

Once intestinal tract maturation occurs, enteral nutrients can be progressively increased while parenteral nutrition is proportionally reduced before being discontinued. Gastrointestinal motility is a marker of gut maturation and is monitored by the

assessment of gastric residuals. Improving gastric emptying (facilitated by breast milk) reflects the improving ability to digest and absorb nutrients. Feeding volumes can be gradually increased by 20-25 mL/kg each day as gastric residuals permit. The absence of significant gastric residuals should be ascertained before feeding volume is further increased. Although more rapid increases in feed volumes have been achieved safely, intestinal maturation requires time and therefore more rapid increases are not necessary. Parenteral nutrition is usually maintained until enteral feedings reach 120 mL/kg/day after which it may be discontinued, provided the baby is tolerating enteral feeds.

Breast milk fortification

When feeding volumes of 80-100 mL/kg/day are tolerated, fortification of breast milk is usually initiated, although in some neonatal units, fortification is started at an earlier stage. Half-strength fortification may be tried before proceeding to full strength.

Once full feed volumes (≥ 150 mL/kg/day) are established, the aim is to facilitate a growth rate similar to intrauterine growth. If the growth rate is not satisfactory, enteral intake may be increased by 10%-20%. The best nutrition is fortified human mother's milk or, when not available, fortified donor milk or special formulas with higher protein content (3.3-3.6 g/100 kcal). Babies should also be given multivitamins and, if being exclusively fed with expressed breast milk, iron supplements from day 28 onwards. It is important to measure serum sodium and phosphate levels regularly (minimum once weekly). Serum sodium levels should be kept in the normal range to facilitate the action of IGF1 at cellular level. Serum phosphate levels should be at least 2.0 mmol/L as the premature baby's requirement for phosphate is high, not only for bone development but also for intermediary metabolism.

After discharge from hospital, preterm infants continue to have high nutrient requirements in addition to potential accrued deficits in bone mineral content. There is therefore a need for continued fortification of human milk and mineral and vitamin supplementation or to feed with an enriched post-discharge formula.

DIRECTIONS FOR FUTURE RESEARCH

Despite the major improvements already made in preterm infant nutrition, several unknowns remain and should direct future research efforts. Many studies report historical data in small populations.

There is a lack of a standardised approach to report nutritional intake data and measures of outcomes. Also, there is substantial variation in methods used to estimate and calculate nutritional intakes, making comparison amongst studies difficult and meta-analysis unreliable. Future research should focus on the development of minimal reporting sets of standardised nutritional interventions, and an agreed checklist for standardised reporting of neonatal nutrition and core outcomes.

More research is needed to determine the optimal nutrition, growth rates and body composition in preterm infants that are associated with the best neurocognitive benefits while minimising the long-term risk of chronic diseases.

Adequately powered randomised controlled trials on the late commencement of parenteral nutrition in term and late preterm infants who have IF as well as trials of intensified initial parenteral nutrition are required. Also, large-scale adequately powered randomised controlled trials should determine the optimal intake of amino acids and the effects of caloric balance in parenteral nutrition on the brain and neurodevelopment. The comparison of different types and amounts of protein in multi-component fortifiers on long-term neurodevelopment merits further investigation.

Further studies investigating the interactions between maternal HMOs and the intestinal microbiome, and the identification of specific pathways by which individual HMO structures exert protective actions are needed. More information on the development of a simple, high-throughput method to allow full characterization of HMOs is required and also, to determine if a causal relation exists between HMOs and neurodevelopmental outcome.

That LCPUFAs, particularly DHA, provide beneficial effects on preterm infants needs further exploration. These potential effects include better neurological outcomes at 2 years and reduction in the incidence and severity of neonatal morbidities such as CLD, NEC, ROP and possibly sepsis. Knowing how LCPUFAs affect different steps of the immune and anti-inflammatory response would contribute to determining the optimal LCPUFA requirements for good neurodevelopmental outcomes.

CONCLUSION

Despite the many difficulties encountered in nutrition research, several studies to date have demonstrated a positive relationship between aspects of nutrition when optimised and growth and neurocognitive outcomes for premature babies. Survival rates of premature infants, including those born at the extremes of viability, have improved enormously but future research needs to address how better growth and neurodevelopmental outcomes may be achieved. As more knowledge becomes available, neonatologists and all those involved in neonatal care, can significantly improve nutritional management, particularly in terms of quality, thereby making a major contribution to the lives of individuals, families and society.

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ORIGINAL ARTICLE

Retrospective Study

Comparison of indirect immunofluorescence and western blot method in the diagnosis of hantavirus infections

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Abstract

BACKGROUND

Serologic cross-reactivity between hantaviruses often complicates the interpretation of the results.

AIM

To analyze the diagnostic value of indirect immunofluorescence assay (IFA) and western blot (WB) in the diagnosis of hantavirus infections.

METHODS

One hundred eighty-eight serum samples from Puumala (PUUV) and Dobrava (DOBV) orthohantavirus infected patients were analyzed. Serology was performed using commercial tests (Euroimmun, Lübeck, Germany).

RESULTS

Using IFA, 49.5% of acute-phase samples showed a monotypic response to PUUV, while 50.5% cross-reacted with other hantaviruses. The overall cross-reactivity was higher for immunoglobulin G (IgG) (50.0%) than for immunoglobulin M (IgM) (25.5%). PUUV IgM/IgG antibodies showed low/moderate reactivity with orthohantaviruses Hantaan (12.3%/31.5%), Seoul (7.5%/17.8%), DOBV (5.4%/

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28.1%), and Saaremaa (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with Hantaan (76.2%/95.2%), Saaremaa (80.9%/83.3%), and Seoul (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in most cross-reactive samples (89.5%).

CONCLUSION

The presented results indicate that WB is more specific than IFA in the diagnosis of hantavirus infections, confirming serotype in most IFA cross-reactive samples.

Key Words: Hantaviruses; Serology; Cross-reactivity; Indirect immunofluorescence; Western blot

Core Tip: Serologic cross-reactivity among hantaviruses often complicates the interpretation of the results. The overall cross-reactivity is generally higher for immunoglobulin G antibodies than for immunoglobulin M antibodies. Western blot seems to be a more specific serology method than indirect immunofluorescence assay in the diagnosis of hantavirus infections, confirming serotype in the majority of cross-reactive samples detected by indirect immunofluorescence assay.

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INTRODUCTION

Hantaviruses represent a group of serologically related rodent-borne RNA viruses that belong to the genus *Orthohantavirus* of the family *Hantaviridae*. Two different diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), are caused by hantaviruses in humans[1]. Orthohantaviruses Hantaan (HTNV), Dobrava (DOBV), Puumala (PUUV), Seoul (SEOV), and Saaremaa (SAAV) cause HFRS with varying degrees of severity. While HTNV and DOBV cause a severe form of HFRS in Asia and Europe, SEOV causes less severe disease worldwide[2,3]. SAAV is also found to be responsible for a relatively mild human disease in Europe[4]. PUUV is a causative agent of nephropathia epidemica, the mildest form of the disease, endemic in Western Europe and Scandinavia[2].

Diagnostic methods for hantavirus infections include serology, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and virus isolation [5].

Vero E6 cell culture has been used to isolate hantaviruses causing HFRS and HPS. Hantaviruses usually are not cytopathic in cultured cells; therefore, the detection of infection is confirmed using an immunofluorescence antibody test for viral antigen. Virus isolation is not performed as part of routine hantavirus diagnostics, since it is laborious and time-consuming and requires biosafety level 3 and 4 laboratories[6].

Serology is the main method for the diagnosis due to the hazardous nature of hantaviruses and a short-term viremia in infected humans[7,8]. Enzyme-linked immunosorbent assay and indirect immunofluorescence assay (IFA) are broadly used serologic tests used for detection of hantavirus immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies[9]. Immunoblot tests [western blot (WB) and line immunoassay] are also used in some laboratories[10].

Hantavirus nucleocapsid (N) protein is the major antigen in early humoral response in patients with hantavirus infection[11,12]. N protein is highly cross-reactive between different hantaviruses due to its conserved nature[11,13]. Overall, serologic cross-reactivity within the genus *Orthohantavirus* is the highest among viruses associated with (phylo)genetically closely related rodent species. DOBV is genetically and antigenetically related to other orthohantaviruses transmitted by *Murinae* rodents (Old

World mice and rats) such as HTNV, SEOV, and SAAV. PUUV is more distantly related to this group since its reservoirs belong to the *Arvicollinae* rodents (voles and lemmings)[14-16]. The interpretation of serology results is often complicated by the cross-reactivity, especially in areas where different hantaviruses co-circulate. Virus neutralization test is still the gold standard serologic test. Since this test has to be performed in biosafety level 3 laboratory, it is confined mainly to the reference laboratories[17].

Molecular diagnostic methods, including classic and real-time RT-PCR, are also widely used for the diagnosis of hantaviruses. Hantavirus RNA is detectable in blood early after the onset of symptoms; therefore, RT-PCR is a sensitive method for detecting hantavirus infections before the appearance of IgM antibodies. Primers specific for the hantavirus S and M segments have been used in different studies. The advantage of the molecular methods is that the RT-PCR product may be sequenced to identify the virus and perform phylogenetic analysis[5,18].

In Croatia, PUUV and DOBV have been demonstrated in humans[19-23], while SAAV and Tula orthohantavirus were also documented in rodents[24,25]. This study aimed to analyze the diagnostic value of IFA and WB methods in the diagnosis of hantavirus infections.

MATERIALS AND METHODS

A total of 188 serum samples from patients with serologically confirmed acute hantavirus infection (2015-2019) tested at the National Reference Laboratory for Arboviruses and Hantaviruses, Croatian Institute of Public Health were included in the study. Serologic tests were performed using a commercial IFA (Hantavirus mosaic; Euroimmun, Lübeck, Germany) to detect IgM/IgG antibodies of the most common hantaviruses: PUUV, DOBV, HTNV, SEOV, and SAAV. A fluorescence occurring as fine droplets in the cytoplasm of infected cells in a dilution 1:100 was considered a positive result.

Cross-reactive samples were further tested for hantavirus IgG antibodies using a WB (Euroline Hantavirus profile, Euroimmun). WB test strips were coated with nucleocapsid PUUV; DOBV and HTNV antigens. Band signal intensity at least as of IgG control was considered a positive result. According to the band intensity, results were interpreted as follows: strong positive-very strong band (+++); positive-medium to strong band (+/++); borderline-very weak band (+/-).

The study was approved by the Ethics Committee of the Croatian Institute of Public Health (Decision number: 030-02/17-10/1). Informed consent was obtained from all subjects included in the study.

RESULTS

PUUV was confirmed in 146 (77.6%) and DOBV in 42 (32.4%). Using IFA, 93 (49.5%) of 188 acute-phase serum samples reacted only with the homologous PUUV antigen, while in 95 (50.5%) samples, cross-reactive IgM and/or IgG antibodies were found. The overall cross-reactivity was higher for IgG antibodies (94/188; 50.0%) than for IgM antibodies (48/188; 25.5%). Among 95 cross-reactive samples, 55 (57.9%) were confirmed as PUUV and 30 (31.6%) samples as DOBV using a WB.

Cross-reactive patterns to different hantavirus antigens in PUUV- and DOBV-infected patients detected using IFA are presented in Figures 1 and 2. Among PUUV positive samples, a low/very low IgM reactivity was observed with HTNV (18/146; 12.3%), SEOV (11/146; 7.5%), DOBV (8/146; 5.4%), and SAAV (7/146; 4.8%). PUUV IgG antibodies showed a moderate reactivity with HTNV (46/146; 31.5%) and DOBV (41/146; 28.1%), while reactivity with SEOV and SAAV was low (26/146; 17.8% and 23/146; 15.7%, respectively).

In DOBV positive samples, both IgM and IgG antibodies showed a high degree of cross-reactivity. Among IgM positive samples, the highest cross-reactivity was observed with SAAV (34/42; 80.9%), 33/42 (78.6%) with SEOV, and 32/42 (76.2%) with HTNV. In 12 samples (28.5%), cross-reactive antibodies with PUUV were found. DOBV IgG antibodies showed the highest reactivity with HTNV (40/42; 95.2%). Almost equally high reactivity was found with SEOV and SAAV (36/42; 85.7% and 35/42, 83.3%, respectively), and moderate reactivity was found with PUUV (16/42; 38.1%). The majority of DOBV-positive samples (IgM 24/42, 57.1%; IgG 35/42; 83.3%) showed reactivity with all three hantavirus antigens (HTNV + SEOV + SAAV).

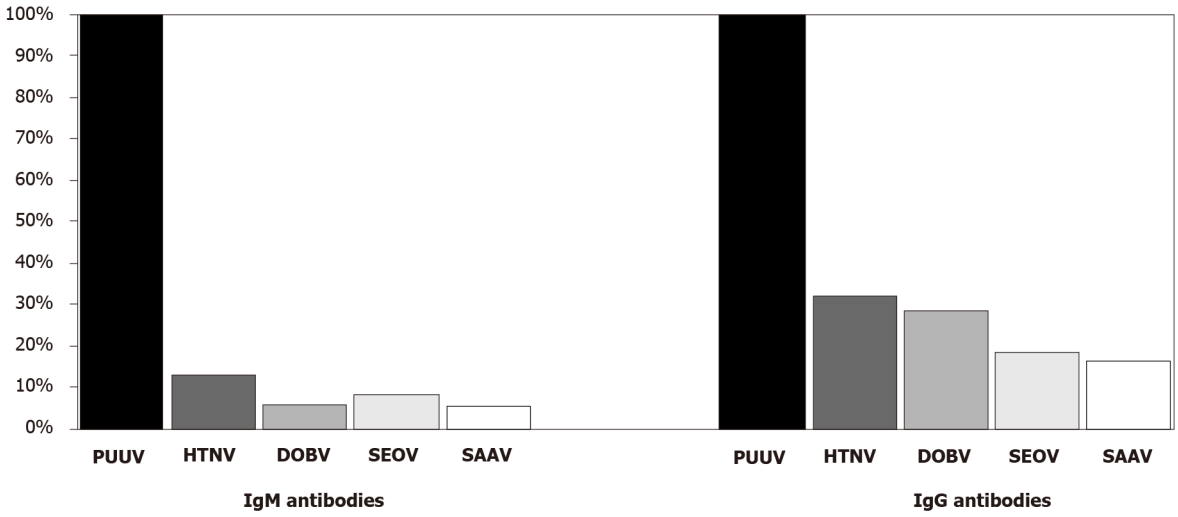

Figure 1 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Puumala-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

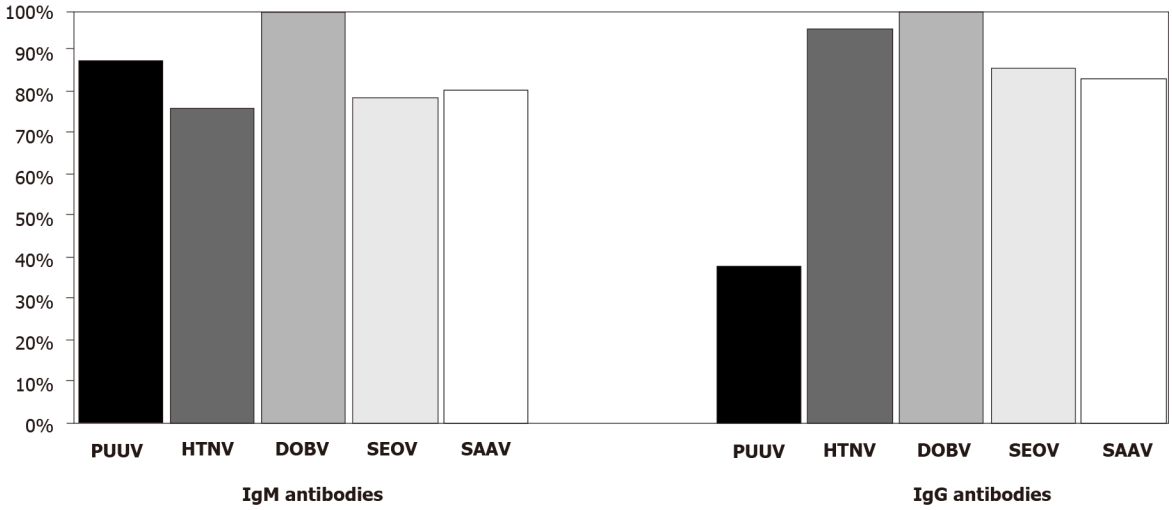

Figure 2 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Dobrava-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

Forty-six of 172 (24.5%) IgG-positive samples cross-reacted with other hantaviruses by WB. However, based on signal intensity, a very strong band to the homologous viral antigen was observed in most cross-reactive samples compared to a weak/medium band of the related hantavirus antigens (Figure 3). Among PUUV positive samples, 8 (5.5%) tested borderline to HTNV and 10 (6.8%) to DOBV. Among DOBV positive samples, 19 (45.2%) tested positive/borderline to HTNV and 5 (9.5%) to PUUV. Only 8 PUUV positive samples (5.5%) showed a very strong band to PUUV and DOBV antigens. Additionally, two DOBV positive samples (4.7%) showed a very strong band to both DOBV and HTNV antigens (Table 1). The detection of PUUV and DOBV IgM antibodies by IFA in these samples indicated acute PUUV and DOBV infection, respectively.

DISCUSSION

Results of this study indicated broadly cross-reactive patterns of hantaviruses detected by IFA, which were found to be much higher for DOBV compared to PUUV. One published multicenter study on the simultaneous detection of hantaviruses showed a high cross-reactivity of serum samples from DOBV-infected patients with SAAV, HTNV, and SEOV (60%-100%), while cross-reactivity with PUUV was moderate (up to

Table 1 Cross-reactive patterns of hantavirus immunoglobulin G antibodies by western blot

Band intensity	PUUV	HTNV	DOBV
PUUV-infected patients (n = 146)			
Strong positive (+++)[1]	-	0 (0%)	8 (5.5%)
Positive (+, ++)[2]	-	0 (0%)	0 (0%)
Borderline (+/-)[3]	-	8 (5.5%)	10 (6.8%)
DOBV-infected patients (n = 42)			
Strong positive (+++)[1]	0 (0%)	2 (4.7%)	-
Positive (+, ++)[2]	1 (2.4%)	8 (19.0%)	-
Borderline (+/-)[3]	4 (9.5%)	11 (26.2%)	-

[1]Very strong band.
[2]Medium to strong band.
[3]Very weak band.
PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan.

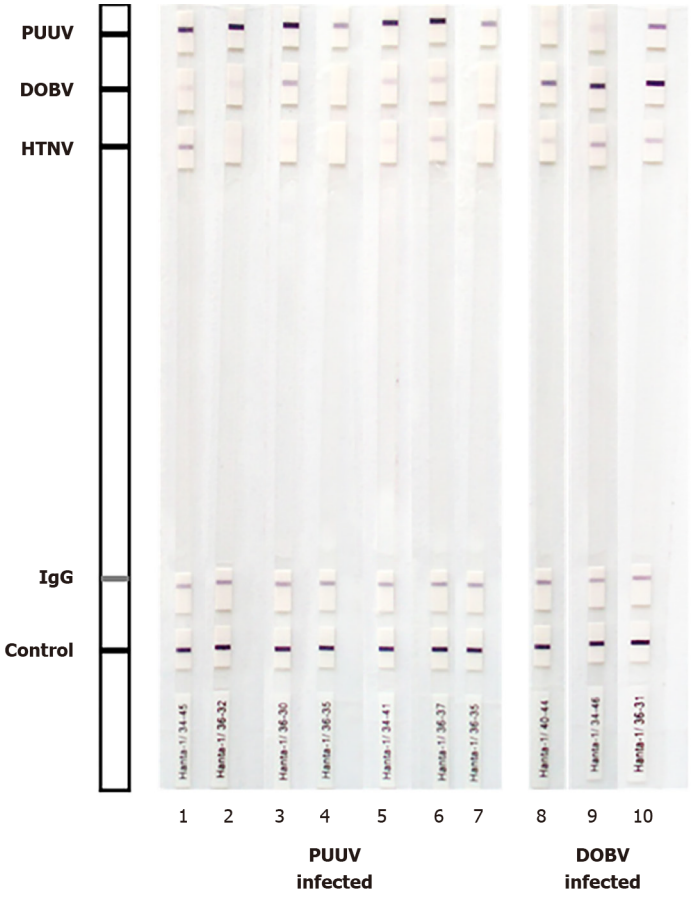

Figure 3 Western blot analysis of Puumala and Dobrava- infected patients. The test strips were coated with the affinity purified nucleocapsid Puumala (PUUV); Dobrava (DOBV) and Hantaan (HTNV) antigen. A correctly performed test for immunoglobulin (Ig)G antibodies against hantavirus antigens is indicated by a positive reaction of the control band and the IgG band. Some samples (strips 1, 3, 5, 6, 8-10) cross-reacted with other hantaviruses, however, based on signal intensity, a very strong band to the homologous virus antigen was detected compared to a very weak/weak band of the related hantavirus antigens.

43%) using IFA[26]. This study observed a remarkably high cross-reactivity for both DOBV IgM/IgG antibodies with SAAV, HTNV, and SEOV antigens (IgM 76.2%-80.9%, IgG 83.3%-95.2%). In addition, 57.1% IgM and 83.3% IgG positive samples cross-reacted with all three hantavirus antigens. These results are in accordance with the phylogenetic relatedness of hantaviruses. However, a substantial cross-reactivity was also found with PUUV (IgM 28.5%, IgG 38.1%), although PUUV is phylogenetically

distantly from DOBV.

IgM/IgG antibodies of PUUV-infected Croatian patients reacted moderately with HTNV (12.3%/31.5%). In a study by Lederer *et al*[26], even higher cross-reactivity between PUUV and HTNV IgM/IgG was found (49%/79%), while the reactivity to other tested hantaviruses was low, similar to our results.

In this study, a lower degree of cross-reactivity was also found by WB (24.5%). However, in all but 8 samples, differentiation of hantavirus serotype was possible based on powerful signal intensity to homologous antigen compared to weak/medium signal intensity to heterologous antigens. Some other studies which used WB for result confirmation showed similar results[27,28].

Since the clinical course and prognosis differ in PUUV and DOBV infection, the determination of hantavirus serotype is important for diagnosing acute HFRS cases. In addition, due to specific rodent hosts, identification of currently circulating hantavirus serotype is also useful for planning rodent control programs. Using IFA, serotype identification in seroepidemiological studies is often difficult because of extensive cross-reactivity among IgG antibodies. In DOBV infected individuals, considerable cross-reactivity was also observed between IgM antibodies. Using WB, differentiation of hantavirus serotype was possible in most cases by comparing the signal intensity in most IFA cross-reactive samples.

CONCLUSION

Although cross-reactivity among hantaviruses was detected in both IFA and WB, the results of this study showed that WB seems to be more specific than IFA, confirming hantavirus serotype in 89.5% of cross-reactive samples detected by IFA.

ARTICLE HIGHLIGHTS

Research background

The cross-reactivity among hantaviruses often complicates the interpretation of serology results, especially in areas where different hantaviruses co-circulate.

Research motivation

Data on the comparison of different serologic methods in the diagnosis of hantaviruses are scarce.

Research objectives

This study aimed to analyze the diagnostic value of indirect immunofluorescence (IFA) and western blot (WB) methods in diagnosing hantavirus infections.

Research methods

A commercial IFA was used to detect immunoglobulin M (IgM)/immunoglobulin G (IgG) antibodies to the most common orthohantaviruses: Puumala (PUUV), Dobrava (DOBV), Hantaan (HTNV), Seoul (SEOV), and Saaremaa (SAAV). Cross-reactive samples were additionally tested by a commercial WB using PUUV, DOBV, and HTNV antigens.

Research results

Using IFA, 49.5% of acute-phase serum samples reacted only with the homologous PUUV antigen, while in 50.5% samples, cross-reactive IgM and/or IgG antibodies were found. PUUV IgM/IgG antibodies cross-reacted with HTNV (12.3%/31.5%), SEOV (7.5%/17.8%), DOBV (5.4%/28.1%), and SAAV (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with HTNV (76.2%/95.2%), SAAV (80.9%/83.3%), and SEOV (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in 89.5% cross-reactive samples.

Research conclusions

WB seems to be more specific than IFA, confirming hantavirus serotype in the majority of cross-reactive samples detected by IFA.

Research perspectives

Further studies on a large sample caused by different hantavirus serotypes are needed.

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