THE ROLE OF INTESTINAL TIGHT JUNCTIONS IN PATHOGENESIS OF NECROTIZING ENTEROCOLITIS

ROLA POŁĄCZEŃ ŚCISŁYCH W PATOGENEZIE MARTWICZEGO ZAPALENIA JELIT

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Summary: Tight junctions are multiprotein junctional complexes fastening intestinal epithelial cells together. Their function is to regulate paracellular permeability and to maintain integrity of intestinal epithelial barrier. Disruption of this barrier is believed to be involved in necrotizing enterocolitis development in preterm infants. In this review article we would like to present recent studies involving the role of tight junctions in the pathogenesis of necrotizing enterocolitis and also analyse impact of breastmilk components and probiotics on this process.

Keywords: tight junctions, necrotizing enterocolitis, breast milk, probiotics, preterms

Streszczenie: Połączenia ścisłe to składające się z wielu białek połączenia międzykomórkowe łączące komórki nabłonka jelitowego. Uczestniczą one w regulacji przepływu międzykomórkowego i utrzymaniu ciągłości bariery nabłonkowej jelit. Uszkodzenie tej bariery u wcześniaków może prowadzić do wystąpienia martwiczego zapalenia jelit. W tej pracy przeglądowej chcielibyśmy przedstawić rolę połączeń zamykających w patogenezie martwiczego zapalenia jelit i przeanalizować wpływ na ten proces zarówno składników mleka kobiecego, jak i probiotyków

Słowa kluczowe: połączenia ścisłe, martwicze zapalenie jelit, mleko kobiece, probiotyki, wcześniaki

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INTRODUCTION

Necrotising enterocolitis (NEC) is one of the most common and serious diseases in the neonatal intensive care unit (NICU) [58], affecting mostly very low birth weight infants with the prevalence of 7% [27] and mortality of 20-40% [27, 79]. The characteristic feature of the disease is necrosis of the small intestine caused by inflammation and ischemia [58]

While pathophysiology of NEC is still unclear [46], there are some specific risk factors including prematurity [77], hypoxia [19], formula feeding [14, 23, 31], abnormal bacterial colonization, and many others, that predispose to this disease [63]. Some studies mention also that impairment and immaturity of tight junctions might play role as risk factor of NEC [60]. On the other hand, breastfeeding [31] and specific probiotics [69] may significantly decrease the risk of this disease.

Thus, in this shortly review we would like to discuss briefly the role of tight junctions (TJ) in the pathogenesis of NEC and to analyse major factors that may have impact on this process.

STRUCTURE AND DEVELOPMENT OF THE INTESTINAL EPITHELIAL BARRIER

Intestinal epithelial barrier is a structure that separates intestinal lumen from the underlying lamina propria and its main role is to prevent the transition of bacteria and other harmful intestinal content, while ensuring appropriate absorption of nutrients. Dysfunction of this structure can lead to bacterial translocation, NEC and even sepsis [4, 47]. R.C. Anderson [7] distinguished four components in the intestinal epithelial barrier: physical, chemical, immunological and microbial. The physical barrier is built by a single layer of epithelial cells, which are connected by four types of junctional complexes: tight junctions, adherents junctions, desmosomes, and gap junctions [28]. Because of the complexity and multifunctionality of the intestinal epithelial barrier, its development starts in the first trimester and ends around the 4th year of life [59]. During the 8th week of gestation a single layer of enterocytes arises, later in the 10th week of gestation TJs start to appear and in twenty-sixth week of gestation the intestine obtain partial absorption capacity [34]. Despite immaturity of the intestine in preterm infants, intestinal permeability is higher (compared to term newborns) only during the first two days of life, and it is not correlated with gestational age nor birth weight [25].

Therefore, intestinal epithelial barrier is a multi-dimensional and comprehensive structure where each factor affects one another and may have a direct or intermediate influence on the tight junctions.

TIGHT JUNCTIONS

To begin with structure description, tight junctions are composed of cytoplasmic TJ proteins consisting of cingulin and zonula occludens (ZO-1, ZO-2 and ZO-3); and trans-membrane TJ proteins including occludin, claudins and junctional adhesin proteins [5, 49]. Myosin light chain kinase (MLCK) is the enzyme that regulates TJ's functions [49]. TJs maintain connection between adjacent cells and as a sealing element provide primary barrier for intracellular space [21, 80]. Therefore, the main role of this structure is to regulate paracellular permeability [3, 52].

TIGHT JUNCTIONS IN THE ANIMAL MODELS OF NEC

To get a better understanding of NEC pathophysiology several studies in the animal models were conducted, demonstrating the influence of NEC on TJs, however there are some discrepancies in their results. In the study by Clark et al. [20] occludin and claudin-3 gene expression in the rat pups with NEC was measured. They found that both the protein levels and mRNA expression of these components were significantly higher in NEC group and correlated with progression of the ileal injury. Moreover, the level of occludin and claudin-3 proteins was markedly increased in the NEC group ileum rats. Another experiment performed on rats [67] reports significantly increased level of claudin-1 and claudin-3 in the NEC group. However, in contrast to the previous paper, there was no significant difference in the protein level of ZO-1 and occludin, nor in the gene expression of any of those proteins (claudin-1, claudin-3, occludin, ZO-1) between the NEC group and the control group. Shiou et al. [76] reported significant decrease of claudin-3 and ZO-1 but with no statistical difference for claudin-1 in the NEC group compared to control. Bergmann et al. [10] measured gene expression of the claudin-2, -4, -7 in the small intestine of the mouse pups. All of them were markedly down regulated except for claudin-2 gene, which was significantly increased in the NEC group. In Western Blot analysis only claudin-2 was markedly increased in the NEC affected mice. Those changes were not observed in case of

occludin, claudin-4 and -7. This study also showed that in the immunohistological stained enterocytes occludin and claudin-2, -4, -7 were internalized in the NEC affected mice. Hogberg et al. [38] reported significantly increased gene expression of the claudin-8 and down regulation of the claudin-1, -14, -15.

TIGHT JUNCTIONS IN NEC-HUMAN STUDIES

There are also studies which show how NEC affects tight junctions in the humans. Bergmann at al. [10] compared samples of the small intestine and colon of newborns affected with NEC with the samples taken from healthy infants. In his study, claudin-2 expression was significantly increased in NEC small intestine and colon whereas there was no significant difference in the expression of claudin 4, occludin, and ZO-1 between control and NEC group. Sevastiadou et al. [72] discovered that NEC is more likely to appear in preterm newborns with higher intestinal permeability. Moore et al. [55] made the first study which directly examined the intestinal barrier function in human NEC. He measured the transepithelial resistance (TER) of intestinal mucosal tissues and found that in NEC group, both in the margin and affected area, TER was significantly lower than in the control group. It is known that lower TER correlates with loss of TJ function.

In the same study they found that the expression of the occludin's mRNA, measured in the RT PCR, was markedly decreased in the NEC affected tissue compared to the control one. The last feature that was measured in the study was the myosin light chain kinase (MLCK). Expression of the mRNA of MLCK in the RT PCR was significantly higher in the NEC group than in the control group.

A recent study performed by Bein et al. [8] focused on expressions of TJ proteins and changes in NEC. They found that expression of TJ's genes for ZO-1, occludin, cingulin and claudin was significantly downregulated in the NEC group. Moreover, immunohistological staining of the intestinal samples also showed that ZO-1 and occludin proteins were markedly down regulated in jejunum and ileum of NEC patients compared to the control group. What is interesting in this study is the fact that changes in the expression of TJ's genes correlated with the severity of the injury in NEC affected intestine. Additionally, the authors carried out the in-vivo experiment with two samples of the healthy intestinal tissue under hypoxic conditions. They observed that the fractures of adjusted epithelial cells in the samples exposed to the hypoxic conditions were similar to those found in the NEC affected tissue. Conversely, these changes were not found in the control group. Immunohistochemical staining performed with anti-occludin antibodies during this experiment may also suggest that hypoxia disrupts junctions between cells in epithelium.

BREAST MILK AND OTHER PROTECTING FACTORS

Intestinal mucosal integrity develops in response to the nutrients' exposure. The maturation of this tissue promotes also the growth of proper intestinal microbiota. Peptides that support this process are mostly derived from the proteins delivered to gut with breast milk. All these elements are responsible for promoting the gastrointestinal homeostasis [41]. Whenever this homeostasis becomes unregulated the risk for NEC increases.

Human breast milk not only prevents from NEC but also can influence the severity of this disease. The beneficial effects of human breast milk in the prevention of NEC were analysed by Boyd et al. [14]. Human milk's immunomodulating properties and its physiological activity are still deeply investigated.

As proved in some studies [15] breast milk, or more specifically its components, create the best environment for the intestinal cells, stimulating the integrity of gastrointestinal tract and mucosa [50]. Exclusive human-milk diet decresases the incidence of NEC [23, 53, 78]. It also ensures the protection against LPS-mediated disruption of intestinal tightness by modulating the expression of TJs-, integrin- and actin-mediated signalling [64].

It is worth mentioning that fortification of the human breast milk, that is recently used for almost all VLBW and LBW newborns, possibly does not increase the risk for NEC, although further studies are required [17, 74].

It has been proved that donor breast milk used together with mother's owns breast milk is also associated with lower risk for NEC, when compared to the formula [66] .Further studies are needed to investigate if exclusively used donor breast milk lowers the prevalence of NEC itself. So far it has been shown that at least 50% of total feeds needs to be of mother's own milk to significantly reduce the risk of NEC [70]. In the animal based trial, bovine colostrum was shown to be a protective factor against NEC by stabilizing intestinal cells architecture and junctions [75]. Majority of human milk components were studied carefully in order to find their properties. Many of them seem to be associated with lowering the risk of NEC by different mechanisms.

•**Lactoferrin** has antibacterial activity. In animal studies it was shown to decrease the risk for NEC by preventing activation of proinflammatory response [36, 37]. The proposed mechanism of action is competition with LPS in binding to the Toll-like receptor 4, that further inhibits the genes responsible for the cell response. It is worth underlining that Toll-like receptor 4 is widely present in the

foetal intestines although it is not so common among adults. This may also suggest its role in the pathogenesis of NEC [1, 8]. The Cochrane review has shown that lactoferrin treatment can decrease the NEC stages II and III up to 30% [61].

•**Human milk oligosaccharides (HMOs)** are known to play a key role in promoting and stabilizing the gut microbiome [81]. Ex vivo HMOs were shown to promote the development of the intestinal mucosal immune system and by that they can possibly prevent NEC and other inflammatory responses in guts [35].

Mothers are known to differ in the level of fucosylation of glycans in their milk [81]. This might influence the probability of NEC development in low and non-secretory infants. Morrow has proved that in preterm infants with fucosyltransferase-2 low secretor phenotype, the prevalence of NEC is ten times higher than in other newborns. [57].

•**Transforming growth factor-β2 (TGF-β2)**, present in the biggest amount in colostrum (comparing to matured human breast milk), was observed to be lowered in infants with NEC. TGF-β2 stimulates the villi growth on the intestinal cells, but also promotes the expression of TJs and cell proliferation. That increases the tightness of intestinal tract and prevents from NEC [16, 30, 51].

•**Immunoglobulines**, although widely known as beneficial and present in the human milk, were analyzed by Cochrane review which summarizes that they have no influence on NEC and it's prevention [29]. The single IgA was not included in the Cochrane review and up to now it is the only one that was suggested to support and regulate the paracellular barrier function in the intestines and through that prevent from NEC [4].

•**Glutamine, arginine and citrulline** also have the influence on TJs located in the intestinal cells. Arginine and citrulline were proved to protect guts from the consequences of ischemic injury and by in the same way they also play a role in protection from NEC. The suggested mechanism includes NO-inducing path initialised by metabolism of arginine and citrulline that keeps TJs stable, although further studies have shown that it is rather the inhibition of iNO that protects the intestinal cohesion [18]. Arginine was also observed to be lower in plasma of newborns undergoing NEC, but not enough studies were conducted to conclude, whether supplementation might be beneficial [73]. Glutamine supports the villi structure in adults' intestines and restores the structures of Tjs by increasing the expression of Claudine-1. Glutamine has also shown an outstanding feature of improving the TJs protein expression after cell injury performed with antimetabolic agent [11, 12, 39]. By this mechanism it was suggested to be beneficial also

in NEC prevention and modulation but Cochrane review showed no benefits from glutamine supplementation in newborns so far [54]. According to that, human milk components should not be administered independently as the NEC prevention. Intestinal homeostasis is also regulated by the inhibition of the nitric oxide (NO) production [82], cyclooxygenase activity [48] and epithelial growth factor (EGF) [20].The regulation of the NO production is correlated with erythropoietin, which is present in the human breast milk [44].

•**Erythropoietin (Epo)** is the natural component of human milk [42]. The large cohort study concerning neonates receiving recombinant human erythropoeietin (rhEpo) for prevention or treatment of anemia of prematurity proved that in the group of very low birth weight preterm newborns (500 to 1,250 g) receiving rhEpo, administered via injection, the incidence of NEC was significantly lower, than in the untreated control group [45]. The presence of Epo receptors on enterocytes described in the 90's has remained a mystery for over twenty years [43] until the study of Sheng-Ru Shiou et al. that confirmed the efficacy of enteral administration of rhEpo [76]. Nowadays it is known that Epo not only regulates erythropoiesis but also strengthens epithelial cell barrier by inducing expression of the tight junction protein zonula occludens (ZO-1) that interacts and binds other tight junction proteins. It is proved that decrease of ZO-1 protein level precedes NEC development and it can be detected before the appearance of any histological changes. The authors of the previously mentioned study suggest that supplementation of rhEpo in doses detected in human milk is essential for adequate ZO-1 expression, gut barrier preservation and consequently reduction of NEC incidence [71, 76].

•**EGF** increases cell density and normalises the expression of TJ proteins (occludin, claudine) which promotes the integrity, migration and mucosal barrier and thus was shown to lower the risk of NEC in the animal model [22]. Currently due to possible side effects of its supplementation in the preterm infants the routine use is not recommended except for the clinical trials. The study conducted recently on rats proved that soybean-expressed EGF decreases the risk of NEC by weakening JAM-A and ZO-1 proteins from the tight junctions. It was also shown to reduce the NO production and COX-2 mRNA transcription [20, 40].

•**The tissue inhibitor of metalloproteinase – 1 (TIMP-1)** inhibits intestinal matrix metalloproteinases and in this way it plays a role in keeping TJ and the guts integrity. TIMP-1 was found in higher amounts in breast milk of mothers of preterm newborns and can be an explanation for protective role of human breast milk against NEC [9].

Heat shock protein-70 (Hsp70) is an important, intestinal stress protein. Its expression induced physiologically by the mother's milk, was proved to prevent form stress-induced permeability, promoting the epithelial integrity in conjunction with greater expression of ZO-1 [68].

Interleukin-10 is also considered a protecting factor for NEC [26] as it has been proved to decrease the stage of NEC in the animal model. IL10 reduces the expression of NO and in this mechanism it stabilises the integrity of intestinal barrier [26].

The study on experimental NEC model was focused on looking for upregulation of TJ protein (occludin) by the **Rho kinase** blockade. Both in vitro as in vitro model was protective against NEC after inhibiting the Rho kinase. [32].

PROBIOTICS

When discussing NEC etiology we certainly need to take into account probiotics, which are in these days one of the hot topics among neonatal researchers. They are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [56]. It is know that they play a huge role in the gastrointestinal tract and probably also protect from the NEC development. They have metabolic functions such as fermentation of fibre that leads to short-chain fatty acids production, synthesis of vitamin K or ion absorption. Secondary short-chain fatty acids regulate the process of epithelial cells proliferation and differentiation. What is more bacteria are part of gastrointestinal immune system. They create a barrier effect that prevents colonisation of the pathogenic bacteria species [33].

Over the years many parallel hypotheses emerged, among them one focused on TJs. A study from New Zealand confirmed that Lactobacillus plantarum strengthens the expression of genes associated with tight junction signalling. Tight junction-related genes encoding proteins such as occludin and its associated plaque proteins, ZO-1, ZO-2 and cingulin were subject of microarray analysis, qRT-PCR. Moreover the fluorescent microscopy confirmed higher protein concentration in cells treated with L. plantarum than in the untreated control group [6].

Another similar study has proved the efficacy of Lactobacillus sp. application in Caco-2 cells which is human intestinal epithelial cell line. Cells treated with Lactobacillus sp. were more resistant to lipopolysaccharide (LPS) isolated from *Escherichia coli,* that caused cell membrane disruption than untreated control group [13, 65]. It is still a matter of debate whether NEC itself causes increased

permeability of intestine epithelial cells or just contrary increased permeability is responsible for NEC. In a study considering probiotic influence of Bifidibacterium sp., augmented intestinal permeability was observed 12 hours before any histological changes occurred. It suggested cellular malfunction, confirmed in the immunofluorescence and Westernblot method, concerning internalization of claudins 2, 4, and 7 and occludin from enterocyte to cytosol, which resulted in TJs increased permeability. Permeation of pathological antibodies through the TJs causes inflammatory response in mucosa, an important step in NEC pathogenesis [10].

Additionally it was proved in different studies that L. plantarum efficacy is strain dependent and dose dependent considering gene expression enhancement as well as ability to supress the disruptive influence of enteroinvasive *E. coli* on tight junction [62, 65].

Nevertheless, it is worth mentioning that in a few papers there is an evidence of the connection between probiotic supplementation and bacteraemia or even sepsis. It is pointed out that the most vulnerable group of patients that can develop septic complications are underweight (VLBW- very low birth weight <1500 g) preterm babies, below 30 weeks of gestational age. As it was previously said it is the same risk group as for NEC development [24, 84]. Hyperpermeability caused by TJ structure changes including increase in claudin-2 and JAM-A expression and decrease in claudin-5 and occludin expression leads to bacteria and bacterial metabolites translocation [83].

To conclude there is strong evidence based on review of many randomised trials, that probiotics decrease the risk of critical NEC as well as other fatal diseases among preterms. It confirms the efficacy of supplementation for Lactobacillus alone or in combination with Bifidobacterium species. Even though there is still a need for evaluation of dosage and formulation of probiotics in therapeutic use [2].

SUMMARY

Despite numerous studies, demanding difficult research efforts, the pathogenesis of necrotizing enterocolitis is still unknown. Tight junctions, that are responsible for the intestinal permeability and alteration in protein composition, seem to play an important role in the onset, course and treatment of NEC. With regard to the citied researches, the positive effect of probiotics and breast milk components on reducing occurrence of NEC, may be explained by stabilizing TJs and sealing the intestinal epithelial barrier. The attempts of understanding the role of TJs is not only a matter of microscopic examinations but mainly a chance to find out preventive measures for NEC.

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