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The Effect of $ACTH_{4-10}$ synthetic on separated junction formation and CSF leukocyte number in LPS-induced meningitis

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Abstract : *Mortality and morbidity in bacterial meningitis remain high in children and adults. Previous studies suggested that inflammatory response to bacterial products apparently continues even after the bactericidal effect of antibiotics, resulting in destruction of the host tissue. Previous studies showed that $ACTH_{4-10}$ synthetic uses melanocortin receptors and it was considered that α-MSH shares the first 13 amino acids sequence with $ACTH_{4-10}$, $ACTH_{4-10}$ can be identified as α-MSH exogenous. αMSH has been known to have anti-inflammatory activity. Research method used experimental studies on Wistar rats to compare expression of intracerebral inflammatory through the number of leukocytes in CSS and separated junctions formation between endothelial cells after intracisternal LPS challenge and after intranasal administration of $ACTH_{4-10}$ .. The control group (n = 18) was given with LPS + Placebo and the experimental group (n = 18) was given with LPS + $ACTH_{4-10}$. Inflammatory characteristics were measured and analyzed statistically. The results show the number of CSS leucocytes were significantly different ($p < 0.05$) and there was a marked change in separated junction between the two groups ($p < 0.05$). It can be concluded that in this study, $ACTH_{4-10}$ synthetic administration significantly improved intracerebral inflammatory condition characterized by a decrease number of leukocytes and separated junction formation.*

Keywords– *ACTH4-10 synthetic, LPS, Leukocyte, Meningitis, Separated Junction*

I. Introduction

Mortality and disability caused by bacterial meningitis remain to be health problem in the world and still has not been improved despite the development of many specific antimicrobial[1,2]. In general, 34% mortality is caused by bacterial meningitis and more than 50% patients who survive have severe neurological sequelae[3]. Previous studies have shown that the inflammatory response to bacterial products apparently continues even after the bactericidal effect of antibiotics, resulting in the destruction of the host tissue[4].

The structure of Blood-brain barrier CNS is the most important thing for the defense against bacterial infections and it is established by intercellular Tight Junctions between cerebral microvascular endothelial cells, astrocytes cells, pericytes and basal membrane[5]. Destruction of this junction led to increased permeability of BBB and potentially disrupted intracranial homeostasis and ultimately destroyed brain cells. Research on intracerebral endotoxin challenge suggests that inflammatory responses cause damage on the junctional complex and increase paracellular transport between endothelial cells[6].

Based on that facts, the current therapeutic targets aimed not only to eradicate the invading bacteria but also to address the severe inflammatory reaction triggered by bacterial products (cell wall, capsule, DNA) from lysis of bacteria due to exposure to antibiotics[7]. Until now, many researches try to find new therapeutics to control excessive inflammatory response caused by bacterial products[3,8]. Analog $ACTH_{4-10}$ ($ACTH_{4-7}$ Pro-Gly-Pro) synthetic is the agent nootropik, a group of neuropeptides and acts as an endogenous neuromodulator function of the CNS[9] (Bashkatova et al., 2001). From previous studies it was found that Analog $ACTH_{4-10}$ synthetic had angioprotective, neurothrophic, antihypoxic effects on neuroinflammatory diseases[10].

The purpose of this study was to evaluate the effect of $ACTH_{4-10}$ synthetic after LPS exposure through the number of leukocytes in the CSS and change separated junction between cerebral microvascular endothelial cells.

II. Research Methods

Endotoxin.

Escherichia coli serotype 026:B6 lipopolysaccaride (LPS) was supplied by Sigma-Aldrich inc (Sigma, St Louis, Mo) and suspended in normal saline. Based on the results of previous study which demonstrated the maximal WBC concentrations in the CSF and percentage of BBBP, the applied dose was 20ng.

$ACTH_{4-10}$ synthetic.

$ACTH_{4-10}$ synthetic is a product of PT SEMAX axomedica developed by the Institute of Molecular Genetics, Russian Academy of Science Moscow, Russia and Lomonosov University in Moscow, with the concentration 0,1%. Based on the previous study, the used doses 1 drop for each nostril.

Meningitis model

Adult wistar rats (approximately 3 weeks and 200g). were used in this experiment and divided into 2 groups. The first group functioned as the control group and the second as experiment groups. The wistar rat was anesthetized with intravenous injection of ketamine (7mg/kg) and xylazine (100mg). LPS was inoculated via cysternal puncture after the withdrawal of 50-75µl CSF. CSF was sampled at either 4 hours post inoculation with LPS, and WBC counts were determined. WBC concentration in CSF was determined by standard hemocytometer methods.

In this experiments $ACTH_{4-10}$ synthetic was administered intra nasally for second group and the placebo from $ACTH_{4-10}$ solvent was administered for the first group. CSF sample was taken at either 2 hours after administering $ACTH_{4-10}$ synthetic and placebo and WBC counts were determined again. Finally, the rats were decapitated and brain tissue sample was taken and preserved in 40% formaldehyde and then the separated junction formation between cerebral micro vascular endothelial cells was determined[11].

***Assessment of the WBC counts* in the CSS**.

4µl CSS was smeared on the object glass, dried for 10 minutes, fixated with 70% alcohol and dried again. And after the washing with the steril aqua, the CSF smear was stained with Giemsa solution for 30 minutes and after that it was washed again with steril aqua, dried and covered by glass. CSF smear was observed by using Olympus DX53 microscope with 200x magnification at 5 different angles and calculated with colonic counter[12].

Assessment of separated junction of micro vascular endothelial cell in Blood-brain barrier

Brain slice was stained with hematoxylin and Eosin (HE). Separated junctions were defined as areas where plasma membranes of adjacent endothelial cells, lining a capillary lumen, were completely separated. and in this experiment, DP25 camera olympus with full-resolution (2560x1920 pixels) and Olympus software Micro suite TM Net Cam was used to determined the separated junction. the separated junction was calculated in nm.

Statistical analysis.

The number of WBC and separated junction in the experimental and control groups were compared by using descriptive and inferential analysis, and it was considered as significance if $p<0.05$.

III. Results

Effects of ACTH4-10 synthetic at micro vascular cerebral separated junction

Control and experimental groups were distributed normally (Kolmogorov-Smirnov test, p> 0.05), so the analysis was performed with two-sample t test (independent t test) between the two groups, while the comparison with the group of normal rats used one-sample t test (one sample t test). The results of the following analysis, mean of the separated junction, can be seen in the table 1.

Group	n	The Separation of micro vascular endothelial cells				Independent t test
		$\bar{x}$	SD	Min	Max	
LPS	18	10152,4139	2338,18019	5538,19	14673,44	p=0,000*
Analog ACTH$_{4-10}$	18	4158,2172	835,23624	3137,32	6196,41	

Table 1. The ACTH4-10 effect on the separated junction of micro vascular cerebral endothelial cells
The results of one-sample t test showed a significant difference (p <0.05) on the control group compared with the experimental group, suggesting that the ACTH$_{4-10}$ synthetic could improve the separated junction of micro vascular cerebral endothelial cells (Fig 1 and Fig 2)

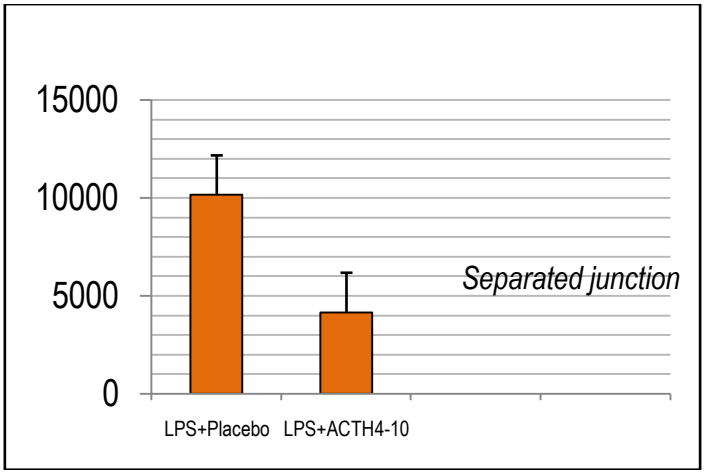

Figure 1. The mean differentiation of separated junction between the control and experimental groups

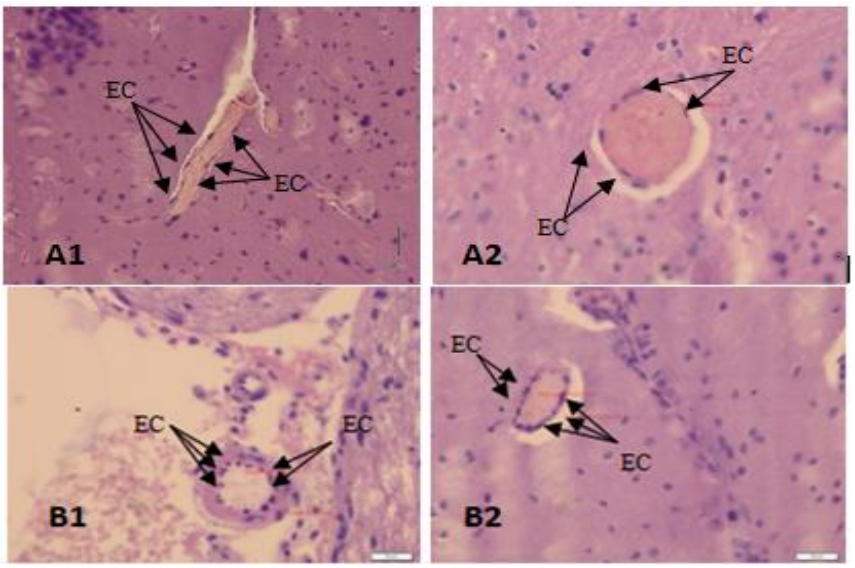

Fig 2. The increased separated junction was seen in the sagital view of microvaskular cerebral area (A1) and transversal view (A2), and after administration of ACTH4-10 synthetic, the separated junction improved (B1 and B2)

The number of leukocyte in CSF

Cerebrospinal fluid sample collected by intracisternal puncture was performed to determine the number of leukocyte in normal rats of control and the experimental groups. This study showed the significant increase of leucocytes number in the control group when compared with the normal rats and after administration of ACTH4-10 synthetic, the number of leukocyte was nearly normal (Table 2).

The data for the number of leukocytes in the CSF for experimental group was normally distributed (Kolmogorov-Smirnov test, $p > 0.05$), but the variance of the data between the groups was not homogeneous (Levene's test, $p < 0.05$), similar to the result of the analysis conducted by Brown-Forsythe test. The results of the analysis is presented as follows.

Table 2. The Effect of ACTH4-10 on the number of leukocytes in the cerebrospinal fluid

Group	n	The Separation of micro vascular endothelial cells				Independent
		$\bar{x}$	SD	Min	Max	t test
LPS	18	10152,4139	2338,18019	5538,19	14673,44	p=0,000*
Analog ACTH$_{4-10}$	18	4158,2172	835,23624	3137,32	6196,41	

Brown-Forsythe test results showed a significant difference ($p < 0.05$) from the comparison between control group and the experimental group. The Administration of ACTH4-10 appears to decrease the number of leukocytes (Fig 3 and 4).

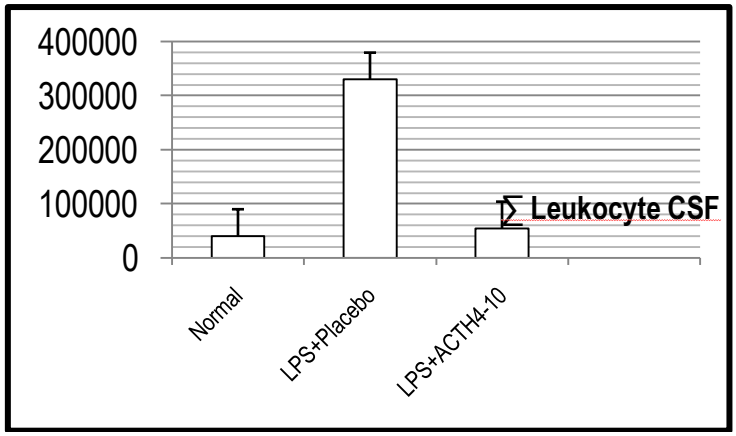

Figure 3. The comparison of the leukocyte number between normal , control and experimental rats.
Figure 4. The differentiation of the leukocyte number in (A) normal rat; (B) control groups, (the number of leukocyte increased) and (C) experimental groups (the leukocyte was almost similar to the normal rats)

IV. Discussion

In bacterial meningitis, one of morphological changes of blood-brain barrier (BBB) is the total separated junction between vascular endothelial cells. These changes have an impact through increased permeability via paracellular pathway. Normally, endothelial cells are connected with the other cell through junctional complex that consists of Tight Junction (TJ), Adherence Junction (AJ) and cytoskeleton [5,13,14]. This structure relates to BBB functions as a regulator of intracranial

homeostasis that should be able to restrict the influx of the cells, hormones, enzymes, bacteria and the others from blood into the brain [15].

Separated junction causes leukocyte influx into CSS. The mechanisms of separated junction formation relates to balance between contractile force of cytoskeleton and adhesive force in the junctional complex [5,16]. Under normal conditions, cerebral microvascular endothelial cells interacts closely one another[17]. In this study, we did not perform histological examination of normal brain tissue by ethical considerations, thus data distance between endothelial cells is not obtained.

The separated junction formation consists of 2 processes that occur simultaneously : (1) changes in the adhesive property of TJ and AJ and (2) reorganization of actin cytoskeleton Separated junction formation process consists of two processes occurring simultaneously: (1) changes protein phosphorylation in the adhesive properties of TJ and AJ and (2) reorganization of the actin cytoskeleton that consists of polymerization actin filament, assosciation actin myosin and the formation of intracellular contractile force causing dislocation of transmembrane and AJ protein[18,19]. In general, alterations of TJ protein (Ocludin, ZO-1, ZO-2 and claudin-5) could change the protein transmembrane localization, induce redistribution this proteins and alter the junctional complex between endothelial cells[20,21].

Increasing of BBB permeability could be caused by many factors, (1) proinflammatory mediators released by cells within the brain and leucocytes into the brain, (2) iNOS within the neutrophils and macrophages[22,23] and (3) bacterial products such as LPS and peptidoglycan which can stimulate the release of proinflammatory cytokines (Abbott et al., 2006). In inflammatory conditions, LPS could damage BBB through direct manner with actin cytoskeleton that induces morphological changes in the inner surface of the intercellular junction[24,25] (Lai et al., 2005; Eswarappa et al., 2008) and indirectly through cytokine-induced release of pro-inflammatory cytokines (IL-1, IL -6, IL-8 and TNFα) from endothelial cells and glial cells. TNFα and IL-1β are known as a potent activator of NFκB which is a transcriptional activator of genes involved in the production of proinflammatory cytokines, chemokines and adhesion molecules. NFκB activation triggers the overexpression of proinflammatory mediators and increases both adhesion molecule expression in endothelial cells (ICAM-1) and leukocyte resulting in withdrawal, leukocyte adhesion to the abluminal side of cerebral microvascular endothelial cells, diapedesis and massive leukocyte influx into space subarachnoid[26,27]. This is very different from the normal condition that the expression of ICAM-1 and VCAM-1 in microvascular endothelial cells is very limited[28] (Gan et al, 1999). Consistent with the results of this study, LPS alters the junctional complex with separated junction formation (Fig 2), and following this formation, the number of significant leucocytes influx to CSS can be seen when compared with the normal rats ($p<0,05$) (Table 2,

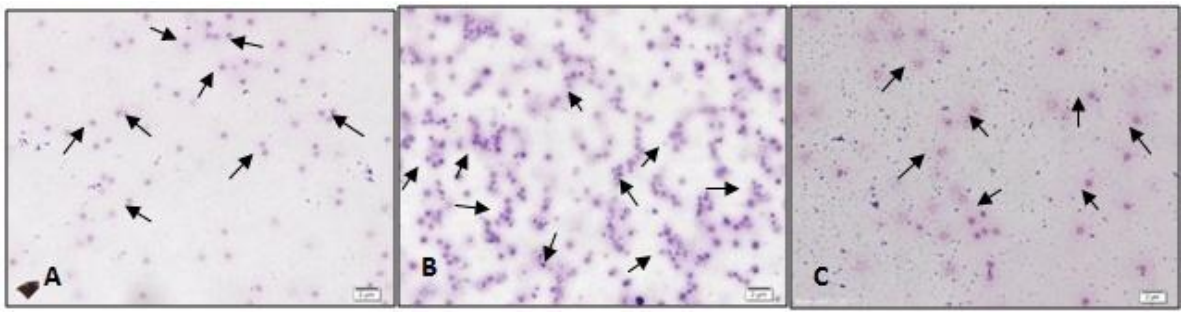

Fig 3,4).

Analog ACTH$_{4-10}$ synthetic consists of a synthetic amino acid sequence Met-Glu-His-Phe-Pro-Gly-Pro, which is identical to the amino acid fragment of α-MSH by eliminating the effect of hormones melanocytes[10,29]. Alpha-MSH has potent anti-inflammatory effects when it is administered systemically or locally. Anti-inflammatory effects are mediated either directly on cells of the immune system and indirectly through its effect on the function of the cells of non-immune. Thus, α-MSH can influence multiple pathways involved in the regulation of immune responses such as NF-κB activation, expression of adhesion molecules and chemokine receptors, the production of pro-inflammatory cytokines and other mediators of non-cytokine[30].

According with the junctional complex, $ACTH_{4-10}$ synthetic can significantly improve ($p<0,05$) the separated junction (Table 1, Fig 1,2). The underlying mechanism relates to $ACTH_{4-10}$ synthetic structures and melanocortin receptors are used[10] (Gusev and Skvortsova, 2003). $ACTH_{4-10}$ synthetic improves the paracellular pathway via inhibition mechanism activation NFkB through ERK1/2 and p38 signalling pathways[31]. After that, the decreased expression of TNFα and IL-1β will cause the decreased induction redistribution of ZO-1, Ocludin and lower TER>80% approximately[32,33]. Another mechanism relates to the ability of $ACTH_{4-10}$ to reduce the increased NO, oxidants that have the ability to impair BBB functions[34] through the destruction of tubulin and actin. It proves that administering $ACTH_{4-10}$ synthetic can improve the BBB function as a regulator of cerebral homeostasis[10,35] (Table 1, Fig 1,2).

According with number of leukocyte in the CSF after LPS induction that is very different from the result after administration of $ACTH_{4-10}$ synthetic, the exact mechanism is still unclear, the possible explanation here is that $ACTH_{4-10}$ synthetic could improve BBB permeability so the leukocyte influx could be stopped and at the same time, leukocyte apoptosis in the CSF accelerates. The result of this procesess is the decreased number of leukocyte. Neutrophil phagocytosis that has undergone apoptosis by macrophages has anti-inflammatory effects. At the time of phagocytosis, macrophages release TGFβ and IL-10[36]. TGFβ plays role to maintain the balance between activation of leukocytes to combat bacterial and leukocyte deactivation to prevent potential damage that causes tissue injury with the reduction of leukocytes withdrawal, the suppression of the inflammatory response that precedes the activation of leukocytes and endothelial cells, the expression of adhesion molecules, the production of pro inflammatory cytokines, the suppression of the release of H_2O_2, and the expression of iNOS[37,38]. The administration of $ACTH_{4-10}$ synthetic results in an increase of TGFβ compared to placebo[10]. This finding supports the results of this study in which the administration $ACTH_{4-10}$ significantly reduced the number of leukocytes in the CSS ($p <0.05$).

V. Conclusion.

Administration of ACTH4-10 synthetic has proved to improve the altered BBB function, especially improving the separated junction formation that reduces the number of leukocytes in the CSS

References

[1]. Hudeckova H, Jesenak M, Maria A, Svihrova V, Banovcin P. *National Analysis of Bacterial Meningitis in Slovakia, 1997–2007, Public Health Rep, 125(1), 2010, 129–36*

[2]. Gessner BD, Mueller JE, Yaro S. *African meningitis belt pneumococcal disease epidemiology indicates a need for an effective serotype 1 containing vaccine, including for older children and adults, BMC Infect Dis, 10, 2010, 22*

[3]. Van de Beek, D., de Gans, J., Tunkel, A.R., Wijdicks, E.F. *Community-aquired bacterial meningitis in adult, N Engl J Med, 354, 2006, 44-53*

[4]. Wang WW, Dentle L, Borchardt RT. *VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly, Am J Physiol Heart Circ Physiol, 280, 2001, 434-40*

[5]. Stamatovic, S.M., Keep, R.F., Andjelkovic, A.V. *Brain Endothelial Cell-Cell Junctions: How to "Open" the Blood Brain Barrier, Current Neuropharmacology, 6, 2008, 179-92*

[6]. Lush, C.W., Cepinskas, G., Kvietys, P.R. *LPS tolerance in human endothelial cells: reduced PMN adhesion, E-selectin expression, and NF-κB mobilization, Am J Physiol Heart Circ Physiol, 278, 2000, 853–61*

[7]. Bucki, R., Levental, I., Janmey, P.A. *Antibacterial peptides—a bright future or a false hope, Anti-Infective Agents, Med Chem, 6, 2007, 175–84*

[8]. Garber, K. *Protein C may be sepsis solution, Nat Biotechnol, 18, 2000, 917-8*

[9]. Bashkatova, V.G., Koshelev, V.B., Fadyukova, O.E., Alexeev, A.A., Vanin, A.F., Rayevsky, K.S., et al. *Novel synthetic analogue of ACTH 4–10 (Semax) but not glycine prevents the enhanced nitric oxide generation in cerebral cortex of rats with incomplete global ischemia, Brain Res, 894, 2001, 145–9*

[10]. Gusev, E., Skvortsova, V.I. *Brain ischaemia, kluwer academic/plenum pub, New york, 299, 2003*

[11]. Coumans ABC, Middelanis J, Garnier Y, Vaihinger HM, Leib SL, Von Duering MU, Hasaart THM, Jensen A, Berger R, 2003. Intracisternal Application of Endotoxin Enhances the Susceptibility to Subsequent Hypoxic-Ischemic Brain Damage in Neonatal Rats, *Ped Res*. **53**(5): 770-5

[12]. Shandaus LM, Ciarlini P, Kidric D, Dillman C, O'Riordan MA, 2010. Automated Cerebrospinal fluid cell counts using the Sysmex XE-5000: is is time for new reference ranges?, Am J Clin Pathol,134(5):734-8

[13]. Abbott NJ, Ronnback L, Hansson E. *Astrocyte-endothelian interactions at blood-brain barrier, Nat Rev, 7, 2006, 41-53*

[14]. Hawkins BT, Davis TP. *The blood-brain barrier neurovascular unit in health and disease. Pharmacol. Rev, 57, 2005, 173–85.*

[15]. Ohtsuki S, Terasaki T. *Contribution of carrier-mediated transport systems to the blood–brain barrier as a supporting and protecting interface for the brain; importance for CNS drug discovery and development. Pharm. Res, 24, 2007, 1745–58.*

[16. Van Hinsbergh VW, van Nieuw Amerongen, G.P. *Intracellular signalling involved in modulating human endothelial barrier function. J. Anat, 200(6), 2002, 549-60*

[17]. Zhang Y, Yuyang CSWL, Johnson K, Poe J, Johnson S, Bobrowski W, Garrido R, Madhu C. *Porcine Brain Microvessel Endothelial Cells as an in Vitro Model to Predict in Vivo Blood-Brain Barrier Permeability, Drug Metab Dispotision, 3(11), 2006, 1935-1943*

[18]. Bazzoni, G., and Dejana, E. *Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol. Rev, 84, 2004, 869–901*

[19]. Smythe, E., Ayscough, K.R. *Actin regulation in endocytosis. J. Cell Sci, 119(22), 2006, 4589-98*

[20]. Haorah, J., Ramirez, S.H., Schall, K., Smith, D., Pandya, R., Persidsky, Y. *Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. J. Neurochem, 101(2), 2007, 566-76*

[21]. Kago, T., Takagi, N., Date, I., Takenaga, Y., Takagi, K., Takeo, S. *Cerebral ischemia enhances tyrosine phosphorylation of occludin in brain capillaries. Biochem. Biophys. Res. Commun, 339(4), 2006, 1197-1203*

[22]. Nau, R., Eiffert, H. *Modulation of Release of Proinflammatory Bacterial Compounds by Antibacterials: Potential Impact on Course of Inflammation and Outcome in Sepsis and Meningitis, Clin Microbiol Rev, 15, 2002, 95-110*

[23]. Singer II, Kawka DW, Scott S, *et* al. *Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease, Gastroenterol, 111, 1996, 871–85.*

[24]. Lai, C.H., Kuo, K.H., Leo, J.M. *Critical role of actin in modulating BBB permeability, Brain Res Rev, 50(1), 2005, 7-13*

[25]. Eswarappa SM, Pareek V, Chakravortty D. *Role of actin sitoskeleton in LPS-induced NF-kappaB activation and Nitric Oxide production in murine macrophages, Innate Immun, 14(5), 2008, 309-18*

[26]. Miller DW. *Immunobiology of the blood-brain barrier, J NeuroVirol, 5, 1999, 570- 8*

[27]. Scheld, W.M., Koedel, U., Nathan, B., Pfister, H.W. *Pathophysiology of bacterial meningitis: mechanism(s) of neuronal injury. J Infect Dis, 186(2), 2002, 225–33*

[28]. Gan X, Zhang L, Berger O, Stins MF, Way D, Taub DD, Chang SL, Kim KS, House SD, Weinand M, Witte M, Graves MC, Fiala M. *Cocaine enhances brain endothelial adhesion molecules and leukocyte migration. Clin Immunol, 91, 1999, 68-76*

[29]. Valentina, B.G., Vladimir, K.B., Olga, F.E., Alexandr, A.A., Anatolii, V.F., Kirill, R.S., et al. *Novel synthetic analogue of ACTH $_{4-10}$ (Semax) but not glycine prevents the enhanced nitric oxide generation in cerebral cortex of rats with incomplete global ischemia, Brain Res, 894, 2001, 145–9*

[30]. Catania A. *The melanocortin system in leukocyte biology, J Leuko Biol, 81, 2007, 383-92*

[31]. Catania A, Lonati C, Sordi A, Carlin A, Leonardi P, Gatti S. *The melanocortin system in control inflammation, Sci World J, 10, 2010, 1840–53*

[32]. Kimura K, Teranishi S, Nishida T. *Interleukin-1β–Induced Disruption of Barrier Function in Cultured Human Corneal Epithelial Cells, Invest Ophthalmol Vis Sci, 50, 2009, 597– 603*

[33]. Peng S, Gan G, Rao VS, Adelman RA, Rizzolo LJ. *Effects of proinflammatory cytokines on the claudin-19 rich tight junctions of human retinal pigment epithelium, Invest Ophthalmol Vis Sci, 53(8), 2012, 5016-28*

[34]. Banan A, Fields JZ, Zhang Y, *et al. iNOS upregulation mediates oxidant-induced disruption of F-Actin and the permeability barrier of intestinal monolayers. Am J Physiol, 280, 2001, 1234–46*

[35]. Barth BM, Stewart-Smeets S, Kuhn TB. *Proinflammatory Cytokines Provoke Oxidative Damage to Actin in Neuronal Cells Mediated by Rac1 and NADPH oxidase, Mol Cell Neurosci, 41(2), 2009, 274–85*

[36]. Klein, M., Paul, R., Angele, B., Popp, B., Pfister, H.W., et al. *Protein expression pattern in experimental pneumococcal meningitis, Micro Infect, 8, 2006, 974–83*

[37]. Weisfelt, M., van de Beek, D., Spanjaard, L., Reitsma, J.B., de Gans, J. *Clinical features, complications, and outcome in adults with pneumococcal meningitis: a prospective case series, Lancet Neurol, 5, 2006, 123-9*

[38]. Malipiero, U., Koedel, U., Pfister, H.W., Leve´en, P., Bu¨ rki, K., Reith, W., Fontana, A. *TGFb receptor II gene deletion in leucocytes prevents serebral vasculitis in bacterial meningitis, Brain, 129, 2006, 2404–15*