ACTA MEDICA MARTINIANA

Jessenii Facultas Medica Martinensis
Universitatis Comenianae

Journal for Biomedical Sciences, Clinical Medicine and Nursing

2010
10/3

ISSN 1335-8421

Moderné vzdelávanie pre vedomostnú spoločnosť/Projekt je spolufinancovaný zo zdrojov EÚ
Modern education for modern society/Project is co-financed from EU sources

---

Vydanie ACTA MEDICA MARTINIANA 10/03
bolo podporené projektom

**Podpora rozvoja ľudských zdrojov
s využitím najmodernejších postupov
a foriem vzdelávania na JLF UK v Martine**

spolufinancovaným zo zdrojov EÚ
a Európskeho sociálneho fondu.

Publishing of Acta Medica Martiniana 10/03
was supported by project

**„Support of human resources development
using the most modern methods
and forms of education at JLF UK in Martin“**

co-financed from EU sources and European Social Fund.

ISSN 1335-8421 Acta Med Mart 2010, 10(3)

ACTA MEDICA MARTINIANA

Journal for Biomedical Sciences, Clinical Medicine and Nursing

Contents

Published by the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovakia

Editorial Office:

Acta Medica Martiniana
Jessenius faculty of Medicine, Comenius University
(Dept. of Physiology)
Mala Hora 4
036 01 Martin
Slovakia

Instructions for authors: http://www.jfmed.uniba.sk (Acta Medica Martiniana)

USE OF METHYLXANTHINES IN THE TREATMENT OF MECONIUM ASPIRATION SYNDROME

Mokra D.[1], Mokry J.[2], Drgova A.[3], Calkovska A.[1]

[1]Department of Physiology, [2]Department of Pharmacology, [3]Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

Abstract

In the pathophysiology of neonatal meconium aspiration syndrome (MAS), inflammation, pulmonary vaso- and bronchocontriction are of high importance. From this reason, various anti-inflammatory agents including methylxanthines have been tested in the treatment of MAS. This article provides a short review of mechanisms participating in the pathogenesis of MAS as a rationale for the use of anti-inflammatory drugs. Main part of the article is focused on pharmacological action of methylxanthines and their effects on the lung functions, inflammatory and hemodynamic parameters in experimental models of MAS, substantially based on the results of the author's own studies.

Key words: meconium aspiration, methylxanthines, aminophylline, inflammation

MECONIUM ASPIRATION SYNDROME (MAS)

MAS is a serious, life-threatening disorder of the term and post-term newborns, developing after perinatal aspiration of meconium into the lungs (1). Incidence of MAS in the well-developed countries stands around 2 per 1000 live births, in the developing countries this number may be considerably higher. The clinical picture varies from mild tachypnoea to respiratory failure, where about one third of the newborns with MAS require intubation and ventilatory support (2). Persistent pulmonary hypertension is a major complication of MAS, developing in more than one third of newborns with MAS. Vice versa, MAS is the most frequent cause of pulmonary hypertension in newborns, requiring the use of extracorporeal membrane oxygenation (ECMO) (3). In addition to acute effects, MAS may have also serious long-term consequences on the respiratory system, such as abnormal bronchial reactivity and wheezing (4).

PATHOGENESIS OF MECONIUM ASPIRATION SYNDROME – RATIONALE FOR THE TREATMENT

In an acute phase of the disease, mechanical obstruction of the airways by meconium may lead to alveolar atelectasis behind the plug. In case of ball-valve obstruction, air-trapping may occure with air leak into the interstitium. With initiation of ventilation, aspirated meconium may reach the alveoli, where inactivates the surfactant (5). Meconium disturbs surfactant synthesis (6), changes the viscosity and ultrastructure of surfactant (7), decreases the levels of specific surfactant proteins (8) and accelerate its conversion from large, surface active aggregates into small, less active forms (9). Since meconium components trigger also the inflammation, inactivation of surfactant is further potentiated by proteolytic enzymes and reactive species released from activated cells during the inflammation, as well as by plasma proteins leaking through the injured alveolocapillary membrane (10).

Address for correspondence:
Daniela Mokrá, M.D., Ph.D., Assoc. Prof., Department of Physiology, Jessenius Faculty of Medicine, Comenius University, Mala Hora 4, SK-03601 Martin, Slovakia
phone/fax: +421 43 4131426, e-mail: mokra@jfmed.uniba.sk

Meconium acts as a potent chemoattractant for neutrophils (11). Number of neutrophils in the lungs increases within several hours after the meconium aspiration and is linked with their decrease in the blood (12). In addition, meconium is a source of proinflammatory mediators (IL-1, IL-6, IL-8, TNFα etc.) (13) that may induce inflammation directly or indirectly through the stimulation of oxidative burst in neutrophils (14) and alveolar macrophages (15). Activated cells produce a wide spectrum of substances including leukotrienes, prostaglandins, platelet activating factor (PAF), proteolytic enzymes, and reactive oxygen and nitrogen species injuring the lung parenchyma and surfactant (16). In addition, meconium shows high activity of phospholipase A2 (PLA2), which may directly or through the arachidonic acid increase the production of lipid mediators and participate in the apoptosis of epithelial cells (17) and surfactant dysfunction (18). Meconium enhances also the expression of inducible cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the macrophages, epithelial and endothelial cells (19, 20).

Meconium aspiration is usually associated with pulmonary vasoconstriction from hypoxia and/or from vasoconstrictive effect of meconium and substances released during inflammation, what leads to pulmonary hypertension. Presence of meconium in the amniotic fluid is responsible for ischemic changes of the lungs, umbilical cord, and placenta (21) as well as for increased cellular apoptosis (22). Postnatal meconium instillation elevates the pulmonary artery pressure and vascular resistance in a concentration-dependent manner, linked with higher levels of thromboxane A2 (TXA2), leukotrienes, prostaglandins, and endothelin-1 (ET-1) (23, 24). Furthermore, release of bronchoactive substances like leukotrienes and PAF in meconium-induced inflammation is probably responsible for increased airway reactivity to methacholine and histamine (12, 25).

TREATMENT OF MAS

Conventional treatment of MAS covers the ventilatory support including high-frequency ventilation, oxygenotherapy, antibiotics, and support of the vital functions (24). If conventional therapy fails, novel therapeutic approaches are required. Various therapeutic interventions and drugs have already been applied or tested in animal models and/or in newborns with MAS (10, 26), among all, exogenous surfactant administered in the form of bronchoalveolar lavage (2, 27), inhalation of nitric oxide (28), liquid ventilation with perfluorocarbons (29) and administration of several anti-inflammatory agents, such as glucocorticoids (30-33), methylxanthines (34-36), selective phosphodiesterase (PDE) inhibitors (37, 38) and others (39, 40).

METHYLXANTHINE DERIVATIVES (MX)

Pharmacological action of MX

MX appear in several plants as natural alkaloids. The best known of them are caffeine with predominant central effects (stimulation of breathing and psychostimulation) and theophylline and theobromine with stronger peripheral (bronchodilation and cardiostimulation) effects. Because of these properties, MX, particularly theophylline, are widely used in the treatment of asthma and chronic obstructive pulmonary disease (COPD) (41, 42).

Mechanisms of MX action are still not fully elucidated (Fig.1). MX as non-selective PDE inhibitors increase concentrations of cAMP and cGMP in the cells leading to bronchodilation and vasodilation. In addition, MX decrease concentrations of calcium, acetylcholine, and monoamines in the cells and modulate release and action of various mediators of inflammation and bronchoconstriction including prostaglandins (41, 43). Due to similar chemical structure, MX compete with other purinergic substances for a binding site on the receptors and work as antagonists of adenosine receptors. Adeno-

sine is an endogenous purine nucleoside that modulates many physiological processes. Since adenosine makes bronchoconstriction and chronic inflammation in the airways (through released histamine and leukotrienes) and modulates action of neutrophils, eosinophils, lymphocytes, and macrophages (44), competitive inhibitors of adenosine receptors including MX work as bronchodilators. In addition, MX facilitate activity of mast cells and basophils, enhance production of surfactant and mucociliary clearance and ameliorate scavenging of ROS (43). However, properties of individual MX derivatives may differ according to their ability to influence the special families of phosphodiesterase and/or to an extent of interaction with adenosine receptors.

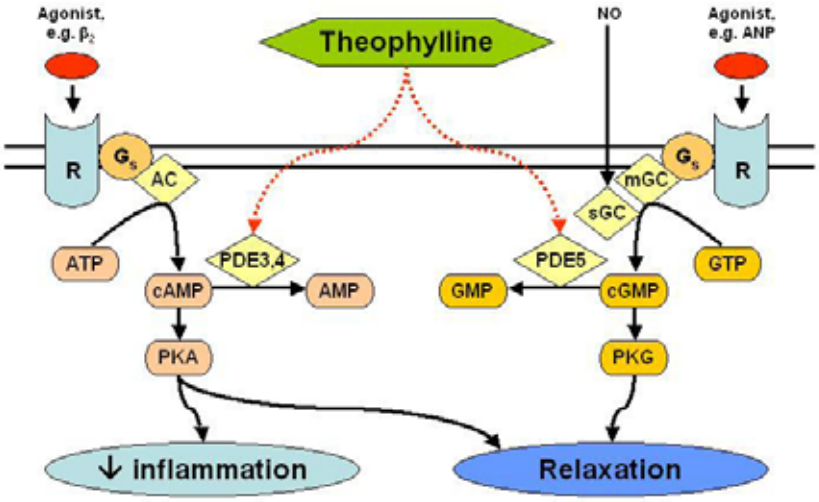


Fig. 1. Schematic action of theophylline on phosphodiesterases. AC: adenylatecyclase, AMP: adenosine monophosphate, ANP: atrial natriuretic peptide, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, GC: guanylatecyclase, GMP: guanosine monophosphate, Gs: stimulating G protein, GTP: guanosine triphosphate, mGC: membrane guanylatecyclase, NO: nitric oxide, PK: phosphokinase, PDE: phosphodiesterase, R: receptor, sGC: soluble guanylatecyclase.

Effects of MX in MAS

In the treatment of MAS, two MX derivatives have been used – pentoxiphylline and aminophylline. Both of them show potent anti-inflammatory action. Pentoxiphylline has suppressed production of oxidants by neutrophils after in vitro exposure to TNFα (45) and inhibited degranulation of polymorphonuclears and TNFα production also in in vitro incubation with meconium (34). In piglets with MAS, pentoxiphylline improved local ventilation:perfusion ratio and prevented increase of TNFα, proteins nad alveolar macrophages in BAL, with no effect on neutrophil accumulation in the lungs (35).

In comparison to pentoxiphylline, besides anti-inflammatory effects aminophylline (a mixture of theophylline and ethylenediamine, enhancing theophylline water-solubility) is able to influence bronchial and vascular tone very fastly, as well. In patients with asthma or COPD the therapeutic levels of theophylline may be obtained within several minutes, with maximum plasma concentration at 20 min following intravenous administration (46). These observations are consistent with our experimental findings where aminophylline treatment significantly improved gas exchange and reduced right-to-left pulmonary shunts in meconium-instilled rabbits within 30 min after the administration (Table 1) (36).

Table 1. Mean airway pressure (MAP, in kPa), oxygenation index (OI), right-to-left pulmonary shunts (RLS, in %) before and 0.5 h after meconium (M) instillation, and during the following 5-hour observation in low-dose (Mec+LD) and high-dose (Mec+HD) aminophylline-treated, and non-treated (Mec) groups of animals.

	Before M	After M	1 h Th.	5 h Th.
MAP				
Mec	0.30±0.01	0.91±0.03	1.00±0.03	1.09±0.02
Mec+LD	0.31±0.01	0.88±0.03	0.93±0.03[a]	0.91±0.03[b]
Mec+HD	0.28±0.02	0.82±0.04	0.82±0.05[e]	0.80±0.05[d]
OI				
Mec	0.77±0.06	14.9±1.02	14.7±0.94	18.1±1.33
Mec+LD	0.85±0.05	13.2±0.66	12.2±0.60[a]	11.0±1.08[c]
Mec+HD	0.76±0.07	12.4±0.82	9.56±1.06[fg]	8.14±1.16[f]
RLS				
Mec	16.9±3.12	54.8±6.02	57.7±4.34	63.1±3.42
Mec+LD	17.7±2.01	47.9±2.73	51.7±2.75	50.2±2.13[b]
Mec+HD	14.5±2.42	52.2±3.38	41.6±4.30[eg]	33.7±3.19[fh]

For between-group differences: for Mec+LD vs. Mec: [a]P<0.05, [b]P<0.01, [c]P<0.001; for Mec+HD vs. Mec: [d]P<0.05, [e]P<0.01, [f]P<0.001; for Mec+LD vs. Mec+HD: [g]P<0.05, [h]P<0.01. Data are expressed as means±SEM.

However, the action of theophylline is strongly dose-dependent with narrow therapeutic window (optimum plasma concentration of 8-15 μg/mL). Therapeutically used doses of theophylline with plasma concentrations between 10-20 μg/mL make bronchodilation and vasodilation, decrease permeability of the vascular wall and has several anti-inflammatory effects, such as increased release of IL-10, inhibition of NF-κB, and scavenging the ROS. These effects are mediated largely by PDE inhibition and adenosine antagonism (41). Higher theophylline plasma concentrations (>20 μg/mL) may be toxic and associated with nausea, headache and adverse effects on the gastrointestinal system (mediated via PDE inhibition) and cardiovascular system (mediated predominantly via adenosine A1 receptor antagonism) (43). On the other hand, low plasma concentrations (5-10 μg/ml) may exert anti-inflammatory and immunomodulatory action, which is not mediated by either PDE inhibition or adenosine receptor antagonism, but by direct activation of histone deacetylase activity leading to reduced transcription of inflammatory genes (41). Recently, we have compared effects of two different aminophylline doses in meconium-instilled rabbits (36). Repetitive administration of aminophylline at high-dose (2 mg/kg) has reduced shunting and requirements for artificial ventilation, enhanced gas exchange, decreased lung edema formation, number of neutrophils in BAL, tracheal reactivity to histamine, and peroxidation of lung lipids vs. non-treated group (Table 1, Table 2) (36). In agreement with our results, aminophylline given at a dose of 5 mg/kg and maintained at 0.5 mg/kg per hour ameliorated neutrophil sequestration in the lungs, decreased lipid oxidation, reduced IL-8 and TNFα, and improved oxygenation in patients undergoing valve replacement (47). In models of ALI, pretreatment with aminophylline (46, 48) as well as high-dose aminophylline (30 mg/kg) administered 80-90 min after phosgene exposure (49) diminished lung edema, lipid peroxidation, and levels of leukotrienes and reduced pulmonary artery pressure.

Table 2. Wet-to-dry lung weight ratio (W/D ratio), total number of leukocytes in the blood (WBC, x 109/l), relative number of neutrophils in the BAL fluid and blood (Ne, %), and concentration of marker of lipid peroxidation (TBARS, in nmol/mg protein) and fluorescence activity of markers of protein oxidation – dityrosine and lysine-lipid peroxidation (LPO) products (both expressed in arbitrary units A.U.) – in the lung homogenate in low-dose (Mec+LD) and high-dose (Mec+HD) aminophylline-treated, and non-treated (Mec) groups of animals at the end of experiments.

	Mec	Mec+LD	Mec+HD
W/D ratio	8.0±0.2	7.6±0.3	6.7±0.3[f]
WBC (blood)	1.0±0.1	1.7±0.1	4.9±1.2[eh]
Ne (BAL)	92.5±1.2	91.9±1.5	69.3±8.9[eg]
Ne (blood)	13.4±2.4	19.2±3.0	63.5±5.3[fi]
TBARS	1.8±0.1	1.3±0.1[c]	1.1±0.0[fg]
Dityrosine	31.2±1.0	22.6±1.5[c]	26.4±0.7[e]
Lysine-LPO	9.6±0.5	6.7±0.5[c]	6.5±0.2[f]

For between-group differences: for Mec+LD vs. Mec: [a]P<0.05, [b]P<0.01, [c]P<0.001; for Mec+HD vs. Mec: [d]P<0.05, [e]P<0.01, [f]P<0.001; for Mec+LD vs. Mec+HD: [g]P<0.05, [h]P<0.01, [i]P<0.001. Data are expressed as means±SEM.

On the other hand, treatment with low-dose aminophylline (1 mg/kg) diminished protein oxidation in the lungs and lung tissue reactivity to histamine, but showed milder effect on lung functions than high-dose (2 mg/kg) aminophylline and no significant effect on lung edema and number of lung neutrophils in rabbits with MAS (Table 1, Table 2) (36). In rats, low-dose aminophylline prevented bronchial hyperresponsiveness and inflammatory response in sensitized and ovalbumine-provoked rats (50), but failed to prevent endotoxemia-induced respiratory and hemodynamic manifestations of sepsis (51). In contrast to these experimental findings, low-dose theophylline reduced influx of neutrophils and eosinophils (52, 53) into the lungs of patients with asthma and reduced concentration of IL-8 and neutrophil chemotactic responses in patients with COPD (54).

Side effects of MX

Systemic administration of MX derivatives may be accompanied by undesirable cardiovascular side effects (51, 55). In meconium-instilled rabbits, intravenous administration of aminophylline at both low (1 mg/kg) and high (2 mg/kg) doses was associated with an acute increase of blood pressure, heart rate, and heart rate variability and more frequent cardiac arrhytmia (Mokra et al., unpublished observation). Side effects of MX derivatives may be eliminated by their local administration, decreasing the dose and/or by their suitable combination with other drugs (e.g. glucocorticoids). Rationale for combined use of MX and GCs in respiratory diseases is based on their properties and mechanisms of action. Synergistic effects of MX and GCs administered at low doses may finally lead to results comparable with high-dose monotherapy, but with lower side effects (42). For example, addition of theophylline to inhaled GCs was more effective in the control of asthma than a doubling the dose of GCs (56). In our recent experiments, combination of low-dose aminophyline and low-dose budesonide improved gas exchange and reduced shunting, lung edema and number of lung neutrophils in rabbits with MAS more effectively than single aminophylline, without significant cardiovascular effects (57).

CONCLUDING REMARKS

Methylxanthine derivatives are the substances with multiple action related to their chemical structure and their ability to influence individual phosphodiesterases and interact with adenosine receptors. Thanks to their bronchodilation, vasodilation, hemorheologic and anti-inflammatory effects as well as stimulation of breathing and production of surfactant MX derivatives possess great perspectives in the treatment of neonatal diseases.

Recent data has shown that MX derivatives – pentoxiphylline (58), theophylline (59), but particularly caffeine (59-61) may be beneficial in the treatment of apnoea of prematurity, may reduce the incidence of bronchopulmonary dysplasia and improve long-term neurological outcome.

Our results as well as results of other authors indicate that methylxanthine derivatives aminophylline (36) and pentoxiphylline (34, 35) may be useful also in the treatment of MAS. Nevertheless, because there is little data on the use of methylxanthine derivatives in MAS, further research is needed to evaluate effects of methylxanthines on the respiratory as well as on the cardiovascular parameters – firstly in meconium-instilled laboratory animals before their use in clinical study.

REFERENCES

1. Co E, Vidyasagar D. Meconium aspiration syndrome. Compr Ther 1990; 16: 34-9.
2. Dargaville PA, Mills JF. Surfactant therapy for meconium aspiration syndrome: Current status. Drugs 2005; 65: 2569-91.
3. Davis PJ, Shekerdemian LS. Meconium aspiration syndrome and extracorporeal membrane oxygenation. Arch Dis Child Fetal Neonatal Ed 2001; 84: F1-3.
4. Vázquez Nava F, Salas Ramírez E, Sánchez Núncio HR, Saldivar González AH, Beltrán Saldaña J, Cadena Mata D, Pérez Rodríguez P, Pérez Martin J, Almeida VM, Guidos Fogelbach G. Meconium aspiration syndrome, parental atopy and asthma symptoms in children under two years old. Rev Alerg Mex 2006; 53: 130-5.
5. Moses D, Holm BA, Spitale P, Liu M, Enhorning G. Inhibition of pulmonary surfactant function by meconium. Am J Obstet Gynecol 1991; 164: 477-81.
6. Janssen DJ, Carnielli VP, Cogo P, Bohlin K, Hamvas A, Luijendijk IH, Bunt JE, Tibboel D, Zimmermann LJ. Surfactant phosphatidylcholine metabolism in neonates with meconium aspiration syndrome. J Pediatr 2006; 149: 634-9.
7. Bae CW, Takahashi A, Chida S, Sasaki M. Morphology and function of pulmonary surfactant inhibited by meconium. Pediatr Res 1998; 44: 187-91.
8. Cleary GM, Antunes MJ, Cieselka DA, Higgins ST, Spitzer AR, Chander A. Exudative lung injury is associated with decreased levels of surfactant proteins in a rat model of meconium aspiration. Pediatrics 1997; 100: 998-1003.
9. Kakinuma R, Shimizu H, Ogawa Y. Effect of meconium on the rate of in vitro subtype conversion of swine pulmonary surfactant. Eur J Pediatr 2002; 161: 31-6.
10. Mokra D, Mokry J. Meconium aspiration syndrome. From pathomechanisms to treatment. New York: Nova Science Publishers; 2010, 130 p.
11. Yamada T, Minakami H, Matsubara S, Yatsuda T, Kohmura Y, Sato I. Meconium-stained amniotic fluid exhibits chemotactic activity for polymorphonuclear leukocytes in vitro. J Reprod Immunol 2000; 46: 21-30.
12. Mokry J, Mokra D, Antosova M, Bulikova J, Calkovska A, Nosalova G. Dexamethasone alleviates meconium-induced airway hyperresponsiveness and lung inflammation in rabbits. Pediatr Pulmonol 2006; 41: 55-60.
13. de Beaufort AJ, Bakker AC, van Tol MJD, Poorthius BJ, Schrama AJ, Berger HM. Meconium is a source of pro-inflammatory substances and can induce cytokine production in cultured A549 epithelial cells. Pediatr Res 2003; 54: 491-5.
14. Soukka HR, Ahotupa M, Ruutu M, Kääpä PO. Meconium stimulates neutrophil oxidative burst. Am J Perinatol 2002; 19: 279-84.
15. Craig S, Lopez A, Hoskin D, Markham F. Meconium inhibits phagocytosis and stimulates respiratory burst in alveolar macrophages. Pediatr Res 2005; 57: 813-8.
16. Speer CP. Inflammatory mechanisms in neonatal chronic lung disease. Eur J Pediatr 1999; 158: S18-22.

17. Holopainen R, Aho H, Laine J, Peuravuori H, Soukka H, Kääpä P. Human meconium has high phospholipase A2 activity and induces cellular injury and apoptosis in piglet lungs. Pediatr Res 1999; 46: 626-32.
18. Schrama AJ, de Beaufort AJ, Sukul YR, Jansen SM, Poorthuis BJ, Berger HM. Phospholipase A2 is present in meconium and inhibits the activity of pulmonary surfactant: an in vitro study. Acta Paediatr 2001; 90: 412-6.
19. Kytola J, Kääpä P, Uotila P. Meconium aspiration stimulates cyclooxygenase-2 and nitric oxide synthase-2 expression in rat lungs. Pediatr Res 2003; 53: 731-6.
20. Li YH, Yan ZQ, Brauner A, Tullus K. Meconium induces expression of inducible NO synthase and activation of NF-dB in rat alveolar macrophages. Pediatr Res 2001; 49: 820-5.
21. Burgess AM, Hutchins GM. Inflammation of the lungs, umbilical cord and placenta associated with meconium passage in utero. Review of 123 autopsied cases. Pathol Res Pract 1996; 192: 1121-8.
22. Korkmaz A, Tekinalp G, Oran O, Yurdakök M, Yiğit S, Cağlar M, Akçören Z, Onderoğlu L, Uçkan D. Placental apoptosis in pregnancies with intrauterine meconium passage. Am J Perinatol 2005; 22:133-8.
23. Soukka H, Viinika L, Kääpä P. Involvement of thromboxane A2 and prostacyclin in the early pulmonary hypertension after porcine meconium aspiration. Pediatr Res 1998; 44: 838-42.
24. Kuo CY, Chen JY. Effects of meconium aspiration on plasma endothelin-1 level and pulmonary hemodynamics in a piglet model. Biol Neonate 1999; 76: 228-34.
25. Khan AM, Elidemir O, Epstein CE, Lally KP, Xue H, Blackburn M, Larsen GL, Colasurdo GN. Meconium aspiration produces airway hyperresponsiveness and eosinophilic inflammation in murine model. Am J Physiol Lung Cell Mol Physiol 2002; 283: L785-90.
26. Zibolen M, Zbojan J, Dluholucky S Eds. Practical Neonatology (Slovak). Martin: Vydavatelstvo Neografie; 2001, 543 p.
27. Wiswell TE, Knight GR, Finer NN, Donn SM, Desai H, Walsh WF, Sekar KC, Bernstein G, Keszler M, Visser VE, Merritt TA, Mannino FL, Mastrioianni L, Marcy B, Revak SD, Tsai H, Cochrane CG. A multicenter, randomized, controlled trial comparing Surfaxin (Lucinactant) lavage with standard care for treatment of meconium aspiration syndrome. Pediatrics 2002; 109: 1081-7.
28. Friedlich P, Noori S, Stein J, Shin C, Burns C, Ramanathan R, Seri I. Predictability model of the need for extracorporeal membrane oxygenation in neonates with meconium aspiration syndrome treated with inhaled nitric oxide. J Pediatr Surg 2005; 40: 1090-3.
29. Jeng MJ, Soong WJ, Lee YS, Chang HL, Shen CM, Wang CH, Yang SS, Hwang B. Effects of therapeutic bronchoalveolar lavage and partial liquid ventilation on meconium-aspirated newborn piglets. Crit Care Med 2006; 34: 1099-105.
30. Holopainen R, Laine J, Halkola L, Aho H, Kääpä P. Dexamethasone treatment attenuates pulmonary injury in piglet meconium aspiration. Pediatr Res 2001; 49: 162-8.
31. da Costa DE, Nair AK, Pai MG, Al Khusaiby SM. Steroids in full term infants with respiratory failure and pulmonary hypertension due to meconium aspiration syndrome. Eur J Pediatr 2001; 160: 150-3.
32. Mokra D, Mokry J, Drgova A, Bulikova J, Petraskova M, Calkovska A. Single-dose vs. two-dose dexamethasone effects on lung inflammation and airway reactivity in meconium-instilled rabbits. J Physiol Pharmacol 2007; 58 (Suppl 5): 379-87.
33. Mokra D, Mokry J, Drgova A, Petraskova M, Bulikova J, Calkovska A. Intratracheally administered corticosteroids improve lung function in meconium-instilled rabbits. J Physiol Pharmacol 2007; 58 (Suppl 5): 389-98.
34. Tegtmeyer FK, Heilemann A, Reiss I, Fischer T. Inhibition of meconium induced activation of granulocytes from neonates and adults by pentoxyphylline. Klin Pädiatr 2002; 214: 347-52.
35. Korhonen K, Kiuru A, Svedstrom E, Kaapa P. Pentoxyfylline reduces regional inflammatory and ventilatory disturbances in meconium-exposed piglet lungs. Pediatr Res 2004; 56: 901-6.
36. Mokra D, Drgova A, Mokry J, Pullmann R, Redfors B, Petraskova M, Calkovska A. Comparison of low-dose vs high-dose aminophylline on lung function in experimental meconium aspiration syndrome. J Physiol Pharmacol 2008, 59 (Suppl 6): 449-59.
37. Shekerdemian LS, Ravn HB, Penny DJ. Intravenous sildenafil lowers pulmonary vascular resistance in a model of neonatal pulmonary hypertension. Am J Respir Crit Care Med 2002; 165: 1098-102.
38. Bassler D, Choong K, McNamara P, Kirpalani H. Neonatal persistent pulmonary hypertension treated with milrinone: four case report. Biol Neonate 2006; 89: 1-5.
39. Lukkarinen H, Laine J, Lehtonen J, Zagariya A, Vidyasagar D, Aho H, Kääpä P. Angiotensin II receptor blockade inhibits pneumocyte apoptosis in experimental meconium aspiration. Pediatr Res 2004; 55: 326-33.

40. Lu MP, Du LZ, Gu WZ, Yu ZZ, Chen XX, Yu ZS. Anti-inflammation and anti-oxidation effects of recombinant human superoxide dismutase on acute lung injury induced by meconium aspiration in infant rats. Zhejiang Da Xue Xue Bao Yi Xue Ban 2005; 34: 55-9.
41. Barnes PJ. Theophylline in chronic obstructive pulmonary disease. New horizons. Proc Am Thorac Soc 2005; 2: 334-9.
42. Barnes PJ. Drugs for asthma. Br J Pharmacol 2006; 147: S297-303.
43. Barnes PJ. Theophylline. New perspectives for an old drug. Am J Respir Crit Care Med 2003; 167: 813-8.
44. Spicuzza L, Di Maria G, Polosa R. Adenosine in the airways: Implications and applications. Eur J Pharmacol 2006; 533: 77-88.
45. Zheng H, Crowley JJ, Chan JC, Hoffmann H, Hatherill JR, Ishizaka A, Raffin TA. Attenuation of tumor necrosis factor-induced endothelial cell cytotoxicity and neutrophil chemiluminescence. Am Rev Respir Dis 1990; 142: 1073-8.
46. Sciuto AM, Strickland PT, Kennedy TP, Gurtner GH. Postexposure treatment with aminophylline protects against phosgene-induced acute lung injury. Exp Lung Res 1997; 23: 317-32.
47. Luo WJ, Ling X, Huang RM. Effects of aminophylline on cytokines and pulmonary functions undergoing valve replacement. Eur J Cardiothorac Surg 2004; 25: 766-71.
48. Hsu K, Wang D, Chang ML, Wu CP, Chen HI. Pulmonary edema induced by phorbol myristate acetate is attenuated by compounds that increase intracellular cAMP. Res Exp Med (Berl) 1996; 196: 17-28.
49. Sciuto AM, Hurt HH. Therapeutic treatments of phosgene-induced lung injury. Inhal Toxicol 2004; 16: 565-80.
50. Lin CC, Lin CY, Liaw SF, Chen A. Pulmonary function changes and immunomodulation of Th2 cytokine expression induced by aminophylline after sensitization and allergen challenge in brown Norway rats. Ann Allergy Asthma Immunol 2002; 88: 215-22.
51. Fakioglu H, Gelvez J, Torbati D, Glover ML, Olarte JL, Camacho MT, Wolfsdorf J. Aminophylline therapy during endotoxemia in anesthetized spontaneously breathing rats. Pharm Res 2004; 49: 45-50.
52. Kraft M, Torvik JA, Trudeau JB, Wenzel SE, Martin RJ. Theophylline: potential antiinflammatory effects in noctural asthma. J Allergy Clin Immunol 1996; 97: 1242-6.
53. Lim S, Tomita K, Caramori G, Jatakanon A, Oliver B, Keller A, Adcock I, Chung KF, Barnes PJ. Low-dose theophylline reduces eosinophilic inflammation but not exhaled nitric oxide in mild asthma. Am J Respir Crit Care Med 2001; 164: 273-6.
54. Culpitt SV, de Matos C, Russell RE, Donnelly LE, Rogers DF, Barnes PJ. Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in COPD. Am J Respir Crit Care Med 2002; 165: 1371-6.
55. Rudusky BM. Aminophylline: exploring cardiovascular benefits versus medical malcontent. Angiology 2005; 56: 295-304.
56. Evans DJ, Taylor DA, Zetterstrom O, Chung KF, O'Connor BJ, Barnes PJ. A comparison of low-dose inhaled budesonide plus theophylline and high-dose inhaled budesonide for moderate asthma. N Engl J Med 1997; 337: 1412-8.
57. Mokra D, Drgova A, Mokry J, Bulikova J, Pullmann R st., Durdik P, Petraskova M, Calkovska A. Combination of budesonide and aminophylline diminished acute lung injury in animal model of meconium aspiration syndrome. J Physiol Pharmacol 2008; 59 (Suppl 6): 461-471.
58. Harris E, Schulzke SM, Patole SK. Pentoxiphylline in preterm neonates: a systemic review. Paediatr Drugs 2010; 12: 301-11.
59. Henderson-Smart DJ, Steer PA. Caffeine versus theophylline for apnea in preterm infants. Cochrane Database Sys Rev 2010; (1): CD000273.
60. Schmidt B, Roberts R, Millar D, Kirpalani H. Evidence-based neonatal drug therapy for prevention of bronchopulmonary dysplasia in very-low-birth-weight infants. Neonatology 2008; 93: 284-7.
61. Schmidt B, Roberts R, Davis P, Doyle LW, Barrington KJ, Ohlsson A, Solimano A, Tin W. Long-term effects of caffeine therapy for apnea of prematurity. N Engl J Med 2007; 357: 1893-902.

Acknowledgement
Supported by Project "Center of Experimental and Clinical Respirology" No. 26220120004, co-financed from the sources of EU.

Received: June, 28, 2010
Accepted: November, 11, 2010

CHANGES IN SURFACE ACTIVITY OF SURFACTANT INDUCED BY *IN VITRO* EXPOSURE TO MECONIUM ASSESSED BY CAPILLARY SURFACTOMETER

Mokra D., Tribulova E., Saffova I., Erneyova J., Calkovska A.

Department of Physiology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovakia

Abstract

Dysfunction of pulmonary surfactant may be assessed by surfactometers working on different principles. Capillary surfactometer detects surfactant dysfunction in conditions simulating peripheral airways. Since meconium acts as a potent surfactant inhibitor, these in vitro measurements were performed to evaluate changes of surfactant surface activity due to exposure to meconium in a model of terminal airways. Exogenous surfactant (Curosurf, Chiesi Farmaceutici, Italy) at concentrations of 0.5, 1 and 2 mg of phospholipids/ml was exposed to human meconium at concentrations of 1.0, 5.0 and 10.0 mg/ml and activity of samples was assessed by capillary surfactometer. Surface properties of surfactant were expressed by values of initial pressure necessary to transport the sample through the capillary and by percentage of the total time, for which the capillary was open (capillary patency). Addition of meconium to surfactant reduced the surface activity of surfactant, characterized by increased initial pressure and shortened capillary patency. Values of initial pressure increased with increasing meconium concentration, however, no linear relationship between initial pressure and meconium concentration was observed. On the other side, time of capillary patency gradually decreased with increasing meconium concentration. In conclusion, capillary surfactometer as a model of terminal bronchioles was first shown to be an effective tool in evaluation of surfactant inactivation by meconium. With this method, capillary patency is a more sensitive parameter of surfactant dysfunction than initial pressure.

Key words: surfactant, surface activity, capillary surfactometer, meconium

INTRODUCTION

Pulmonary surfactant is a mixture of phospholipids (PLs), neutral lipids, proteins, and saccharides coating the alveoli and conducting airways down to the terminal airspaces. Natural surfactant consists of more than 50 different PLs, with dipalmitoylphosphatidylcholine (DPPC) as a main component. Surfactant PLs reduce the surface tension at air:liquid interphase in the alveoli and terminal bronchioles and keep it at low values at the end of expiration preventing their collapse. Other components of surfactant – specific proteins (SPs) – regulate the metabolism and secretion of surfactant compounds, enhance the properties of PLs and participate in the immune response. Forming a barrier between the environment and the body, surfactant plays an important role also in the fluid balance and gas exchange (1, 2).

Dysfunction of pulmonary surfactant has been described in many respiratory disorders (3-5). Primary surfactant deficiency is an underlying cause of the idiopathic respiratory distress syndrome (RDS). In premature newborns the surfactant production by alveolar type II cells is insufficient due to immaturity of the lungs. Secondary surfactant deficiency is in various forms of acute lung injury (ALI) caused by inactivation of the surfactant system in patients with mature lungs. In neonatal meconium aspiration syndrome (MAS), surfactant function is inhibited by components of meconium, as well as by plasma proteins leaking through an injured alveolocapillary membrane and by mediators, enzymes and reactive oxygen species released during meconium-induced

Address for correspondence:
Daniela Mokra, MD., PhD., Assoc. Prof., Department of Physiology, Jessenius Faculty of Medicine, Comenius University, Mala Hora 4, SK-03601 Martin, Slovakia
phone/fax: +421 43 4131426, E-mail: mokra@jfmed.uniba.sk

inflammation (6). However, little is known about deteriorated activity of surfactant in the airways, although airway dysfunction is an important pathomechanism of MAS (7, 8).

Changes in surfactant surface activity may be assessed by different methods. Langmuir-Wilhelmy balance measures spreading rate of the surfactant film on the sensor plate (9). Pulsating and captive bubble surfactometers can evaluate biophysical activity in a dynamic system mimicking in vitro a lung alveolus, with (10) or without (11) communication of the generated bubble to ambient air. On the other hand, capillary surfactometer enables to detect surfactant dysfunction under the conditions simulating those in terminal conducting airways (12).

The aim of this study was to evaluate whether the capillary surfactometer is a suitable method for evaluation of surfactant function in in vitro exposure to meconium.

MATERIAL AND METHODS

Measurements of in vitro samples

Meconium was collected from 20 term neonates, pooled, lyophilized and stored at -20 °C. Before use, meconium was suspended in 0.9 % NaCl at concentrations of 1.0, 5.0 and 10.0 mg/ml. Exogenous porcine surfactant Curosurf (Chiesi Farmaceutici SpA, Italy) was diluted in 0.9 % NaCl at concentrations of 0.5, 1 and 2 mg PLs/ml. Mixing both, 12 combinations originated: Curosurf only (Cur0.5, Cur1, and Cur2) and Curosurf mixed with meconium (Cur0.5+Mec1, Cur0.5+Mec5, Cur0.5+Mec10; Cur1+Mec1, Cur1+Mec5, Cur1+Mec10; Cur2+Mec1, Cur2+Mec5, and Cur2+Mec10). Samples were freshly prepared. If mixed with meconium (Cur+Mec combinations), samples of Curosurf were incubated with meconium for 20 min at a room temperature. Then, measurements of surface activity were done, n=5 in each combination.

Measurement of surface activity by capillary surfactometer

Surface activity of the samples was evaluated by capillary surfactometer CS 2005 (Calmia Medical, Canada; Fig.1).

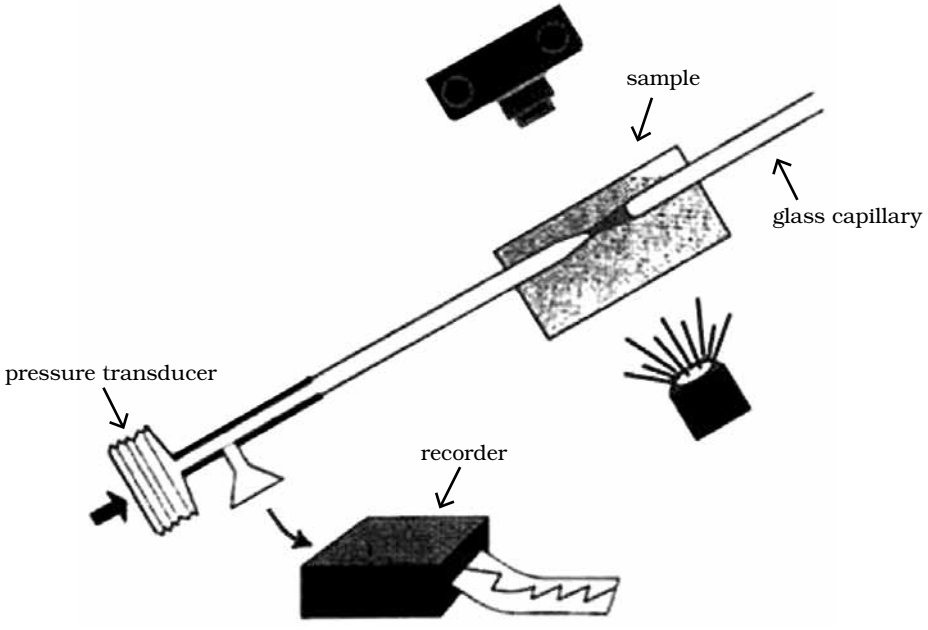


Fig. 1. Scheme of the measurement by capillary surfactometer (with permission of Calmia Medical, Canada). Sample of fluid containing surfactant is placed into the narrow part of the capillary using a micropipette. The

other end of the capillary is connected to a bellows and a pressure transducer. Initial pressure is automatically generated to extrude the sample from the narrow part of the capillary and the actual values of the pressure, as well as the time during which the capillary was opened are recorded. For more detailed explanation, see the text.

For the measurement, concentration of surfactant PLs should range between 0.5-2.0 mg PLs/ml. Ability to maintain the patency of the capillary that simulates the terminal conducting airways was assessed by values of initial pressure (expressed in cm H_2O) necessary to transport the sample through the narrow part of the capillary and by percentage of the total time (120 s) for which the capillary was open (capillary patency, expressed in %). Before measurement, samples were pre-heated to 37 °C. Small volume of the liquid (0.5 µl) was sucked by micropipette and placed into the upper part of the capillary with narrow section (ID 0.25 mm) simulating terminal airways (10, 12). The other end of the capillary was connected to a bellows and a pressure transducer. Gradually increasing values of initial pressure are automatically generated to extrude the sample from the narrow part of the capillary (Fig. 1). If the sample contains well-functioning surfactant, the liquid is squeezed out and will not return to the narrow part of the capillary, pressure is abruptly lowered to zero and capillary will be open till the end of the measurement (Fig. 2A). In sample with dysfunctional surfactant, the liquid will return repeatedly into the narrow part of the capillary, initial pressure will repeatedly increase and time of capillary patency will be reduced (Fig. 2B).

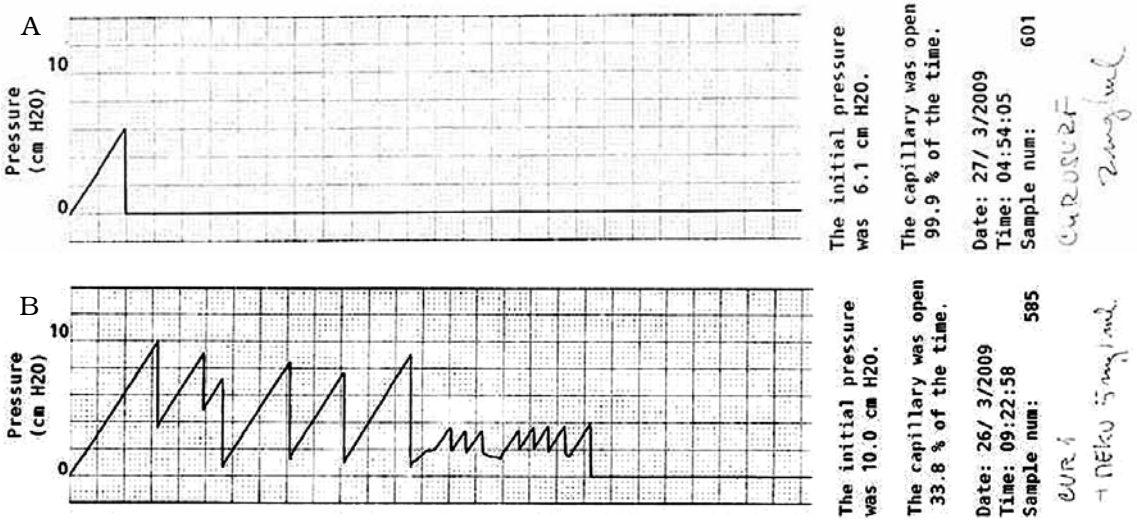


Fig. 2. Original recordings of the surface activity measured by the capillary surfactometer CS 2005. A. Sample of Curosurf at a concentration of 2.0 mg PLs/ml. Value of initial pressure was 6.1 cm H_2O, what was sufficient to extrude the sample through the narrow part of the capillary for the first time. Till the end of the measurement, the capillary was opened, i.e. the capillary patency was 99.9 %. B. Curosurf at a concentration of 1.0 mg PLs/ml mixed with meconium at a concentration of 5 mg/ml. Despite the automatically generated initial pressure was higher (10.0 cm H_2O), sample returned repetitively to the narrow part of the capillary and time of opened capillary was only 33.8 %.

Statistics

Statistical analysis was made by statistical package Systat for Windows. Between-group differences were evaluated by Analysis of Variance (ANOVA) with post-hoc Fisher's LSD test. A $P<0.05$ was considered statistically significant. Data are expressed as means±SEM.

RESULTS

There were no significant differences in initial pressure and capillary patency in Curosurf-only samples at different concentrations of phospholipids (all P>0.05; Fig. 3 and Fig. 4).

Addition of meconium to surfactant did not change initial pressure at concentration of 0.5 and 1 mg PLs/ml. Statistically significant increase in initial pressure was only seen in samples with highest Curosurf and meconium concentrations (2 mg surfactant PLs/ml vs. 5 and 10 mg of meconium/ml) (Fig. 3).

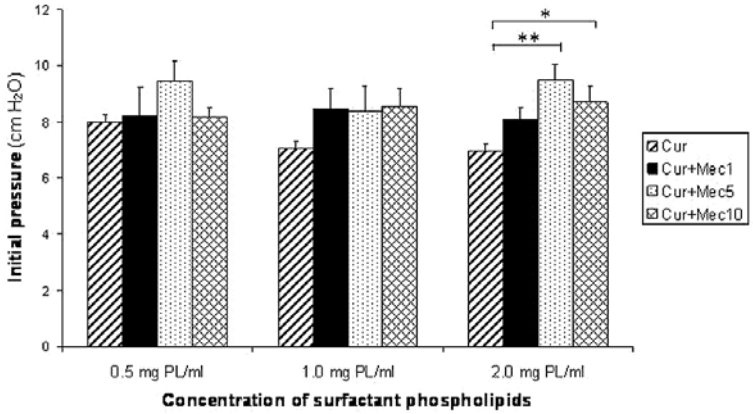


Fig. 3. Initial pressure. Cur: Curosurf at concentrations of 0.5, 1.0 and 2.0 mg PLs/ml, Mec: meconium at concentrations of 1, 5 and 10 mg/ml. For between-group comparisons: *P<0.05, **P<0.01.

At each surfactant concentration, capillary patency was significantly reduced at lowest surfactant:inhibitor ratio (e.g. at meconium of 10 mg/ml) (all P<0.05 and 0.01, respectively, Fig. 4). The typical finding of concentration-dependent surfactant inhibition was demonstrated at highest content of surfactant phospholipids (2 mg/ml). A gradual shortening of capillary patency was observed with increasing meconium concentration (Fig. 4). Capillary patency was shorter in Cur+Mec10 vs. Curosurf (P<0.01), as well as vs. samples with lower meconium concentrations (vs. Cur+Mec1, P<0.01 and vs. Cur+Mec5, P<0.05; Fig. 3 and Fig. 4).

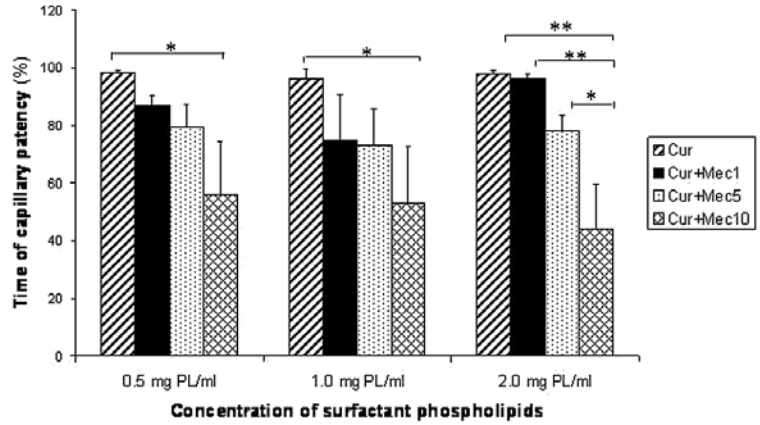


Fig. 4. Time of capillary patency. Cur: Curosurf at concentrations of 0.5, 1.0 and 2.0 mg PLs/ml, Mec: meconium at concentrations of 1, 5 and 10 mg/ml. For between-group comparisons: *P<0.05, **P<0.01.

DISCUSSION

Capillary surfactometer is only tool available to study the pulmonary surfactant function in the conditions mimicking the terminal airways (10, 12). By this method, surface properties of the BAL fluid have been evaluated in newborns with respiratory failure (13), in patients with asthma (14) or chronic airway inflammation (15), as well as in various animal models of acute lung injury (16, 17).

According to recommendations of the producer (Calmia Medical, Canada), in the measurement by capillary surfactometer CS 2005 samples containing surfactant should be at concentration of 0.5 – 2.0 mg of PLs/ml. In our study, surface properties of surfactant samples were evaluated at three surfactant concentrations – 0.5, 1.0 and 2.0 mg PLs/ml. We found no significant differences in initial pressure and capillary patency in Curosurf-only samples at different concentrations of surfactant PLs, demonstrating that all the concentrations used in our study are suitable for the measurement by capillary surfactometer.

After surfactant exposure to meconium, increased initial pressure necessary to transport the sample through the narrow part of the capillary and shortened time of capillary patency, especially at the highest concentration of meconium, were observed compared to Curosurf only. In *in vitro* exposure to meconium, surfactant dysfunction is caused solely by substances present in meconium – by hydrophilic fraction containing mucopolysaccharides and gastrointestinal enzymes including pancreatic phospholipase A_2, as well as by hydrophobic fraction consisting of cholesterol, free fatty acids, triglycerides and others. Effect of both fractions of meconium is additive, since the hydrophobic fraction has about 10-times stronger inhibitory potential than the hydrophilic one (18). An extent of surfactant dysfunction depends on concentration of both – inhibitors and surfactant. Thus, at low concentration of surfactant even low concentrations of inhibitors may cause surfactant inactivation. On the other side, surface properties of surfactant in high concentration may remain intact despite addition of higher concentrations of inhibitors (18, 19). Moses et al. (18) found that even highly diluted meconium (20 μg/ml) is able to inhibit function of surfactant with concentration of 1.5 mg PLs/ml. In the study by Bae et al. (20) meconium concentration of at least 1 mg/ml was needed to inhibit the function of surfactant with concentration of 1.5 mg PL/ml. In our measurements, three different concentrations of surfactant (0.5, 1 and 2 mg PLs/ml) were exposed to three concentrations of meconium (1.0, 5.0 and 10.0 mg/ml). At each of the surfactant concentrations, increasing meconium concentrations gradually reduced the time of capillary patency. However, no clear relationship between increasing surfactant concentration and duration of capillary patency was found. Similarly, addition of meconium to surfactant increased values of initial pressure, but no concentration-dependent relationship was observed. Interesting is also the finding of the most pronounced negative effect of meconium at the highest surfactant concentration. It is possible that discrepancy between our results and the above-mentioned studies (18-20) may be caused by different type of exogenous surfactant used for analysis and/or by assessment method. Moses et al. (18) and Sun et al. (19) used pulsating bubble surfactometer, while we used capillary surfactometer for analysis. Furthermore, we may speculate that absence of additional improvement in surfactant function with its increasing concentration may be related to biophysical limitations of the transport of highly-concentrated sample through the narrow part of the capillary, i.e. to increased viscosity of the sample. Similar results (i.e. shorter capillary patency in mixture of surfactant, meconium and dextran in high concentrations) were found also in the previous study made in our laboratory (21). Moreover, there have been big inter-individual differences in the measured samples (expressed as standard error of the mean, SEM) found, that indicate the necessity to have a number of measurements higher than 5 in each combination.

In conclusion, meconium acts as a potent inhibitor of surfactant function also in a model of the terminal airways. Capillary surfactometer was shown as fast and effective tool for evaluation of changes in the surfactant surface activity in the conducting airways. In addition, with this method, capillary patency was shown to be more sensitive and valuable parameter than initial pressure.

REFERENCES

1. Daniels CB, Orgeig S. Pulmonary surfactant: the key to the evolution of air breathing. News Physiol Sci 2003; 18: 151-7.
2. Dargaville PA, Mills JF. Surfactant therapy for meconium aspiration syndrome. Drugs 2005; 65: 2569-91.
3. Greenough A. Neonatal chronic lung disease and exogenous surfactant therapy. Eur J Pediatr 1998; 157 (Suppl 1); S16-8.
4. Hallman M, Glumoff V, Rämet M. Surfactant in respiratory distress syndrome and lung injury. Comp Biochem Physiol A Mol Integr Physiol 2001; 129: 287-94.
5. Finer NN. Surfactant use for neonatal lung injury: beyond respiratory distress syndrome. Paediatr Respir Rev 2004; 5 (Suppl A): S289-97.
6. Mokra D, Mokry J. Meconium aspiration syndrome: From pathomechanisms to treatment. New York: Nova Science Publishers; 2010.
7. Khan AM, Elidemir O, Epstein CE, Lally KP, Xue H, Blackburn M, Larsen GL, Colasurdo GN. Meconium aspiration produces airway hyperresponsiveness and eosinophilic inflammation in murine model. Am J Physiol Lung Cell Mol Physiol 2002; 283: L785-90.
8. Mokry J, Mokra D, Antosova M, Bulikova J, Calkovska A, Nosalova G. Dexamethasone alleviates meconium-induced airway hyperresponsiveness and lung inflammation in rabbits. Pediatr Pulmonol 2006; 41: 55-60.
9. Robertson B, Schürch S. Assessment of surfactant function. In: Methods in pulmonary research (Eds. S. Uhlich and A. E. Taylor), Basel: Birkäuser Verlag; 1998, p. 349-83.
10. Enhorning G. Pulmonary surfactant function studied with the pulsating bubble surfactometer (PBS) and the capillary surfactometer (CS). Comp Biochem Physiol A Mol Integr Physiol 2001; 129: 221-6.
11. Schoel WM, Schürch S, Goerke J. The captive bubble method for the evaluation of pulmonary surfactant: surface tension, area, and volume calculations. Biochim Biophys Acta 1994; 1200: 281-90.
12. Enhorning G. Surfactant in airway disease. Chest 2008; 133: 975-80.
13. Landmann E, Gortner L, Reiss I, Weller E, Tegtmeyer FK. Protein content and biophysical properties of tracheal aspirates from neonates with respiratory failure. Klin Padiatr 2002; 214: 1-7.
14. Hohlfeld JM, Schmiedl A, Erpenbeck VJ, Venge P, Krug N. Eosinophil cationic protein alters pulmonary surfactant structure and function in asthma. J Allergy Clin Immunol 2004; 113: 496-502.
15. Braun A, Steinecker M, Schumacher S, Griese M. Surfactant function in children with chronic airway inflammation. J Appl Physiol 2004; 97: 2160-5.
16. Dani C, Martelli E, Tronchin M, Buonocore G, Longini M, Di Filippo A, Giossi M, Rubaltelli FF. Bilirubin influence on oxidative lung damage and surfactant surface tension properties. Pediatr Pulmonol 2004; 38: 179-85.
17. Dani C, Pavoni V, Corsini I, Longini M, Gori G, Giannesello L, Perna A, Gritti G, Paternostro F, Forestieri A, Buonocore G, Rubaltelli FF. Inhaled nitric oxide combined with prostacyclin and adrenomedullin in acute respiratory failure with pulmonary hypertension in piglets. Pediatr Pulmonol 2007; 42: 1048-56.
18. Moses D, Holm BA, Spitale P, Liu MY, Enhorning G. Inhibition of pulmonary surfactant function by meconium. Am J Obstet Gynecol 1991; 164: 477-81.
19. Sun B, Curstedt T, Robertson B. Surfactant inhibition in experimental meconium aspiration. Acta Paediatr 1993; 82: 182-9.
20. Bae CW, Takahashi A, Chida S, Sasaki M. Morphology and function of pulmonary surfactant inhibited by meconium. Pediatr Res 1998; 44: 187-91.
21. Calkovska A, Mokra D, Stransky L. The use of capillary surfactometer for evaluation of surfactant preparations – a methodological study. Neonatology 2008; 93: 336.

Acknowledgement
This work was supported by the project "Center of Excellency for Perinatology Research" No. 26220120016, co-financed from EU sources.

Received: October, 28, 2010
Accepted: November, 24, 2010

IS FLOW CYTOMETRY (ALONE) SUFFICIENT FOR DIAGNOSIS OF BERNARD – SOULIER SYNDROME: A CASE REPORT

Arabi A., Brahimi M., Bekadja M.A.

Department of Hematology and Bone Marrow Transplantation
University Hospital 1st November 1954, Oran, Algeria

Abstract

This case-report relates the medical story of a 45 years-old woman (B.Mk) with familial haemorrhagic feature, thrombocytopenia and presence of large size platelets. These findings evocate a Bernard-Soulier Syndrome. CD42b, measured by flow cytometry, was absent. This laboratory method seems to confirm the diagnosis and permitted to examine the other family members: two brothers (B.B and B.M) and two sisters (B.S and B.F). So, is this method enough for the diagnosis of platelet dysfunction related to glycoprotein defects ?

Key words: familial haemorrhage, thrombocytopenia, platelets large size, flow cytometry, Bernard Soulier syndrome

INTRODUCTION

Bernard-Soulier Syndrome (BSS), also known as Hemorrhagiparous Thrombocytic Dystrophy, is a hereditary bleeding disorder affecting the megacaryocyte/platelet lineage and characterized by bleeding tendency, giant blood platelets and low platelet counts (1).

This syndrome is extremely rare: only about 100 cases have been reported in the literature. The first case has been reported by Jean Bernard and Jean Pierre Soulier, two French haematologists, in 1948 (2).

The syndrome is transmitted as an autosomal recessive disorder. The underlying defect is a deficiency or dysfunction of the glycoprotein GPIb-V-IX complex a platelet-restricted multisubunit receptor required for normal primary haemostasis (3). Diagnosis method are usually (4): skin bleeding time moderately to severely prolonged, presence of small number of very large size platelets, platelet count from 20 to 100 x109/l, isolated defect in Ristocetin-induced aggregation.The diagnosis is confirmed by flow cytometry analysis using specific monoclonal antibodies (CD42a-d). We report case of the BSS family determined by flow cytometry alone .

MATERIAL AND METHODS

1.) Clinical findings

A 45 years – old woman (B.Mk) presented since many years severe bleeding symptoms, essentially menorrhagia, with secondary anaemia. She received corticoid, hormonal drugs and iron therapy. Two brothers of this woman died in childhood of haemorrhage. Two others (B.B and B.M) are alive, and one of them (B.B) presents a mild haemorrhagic syndrome (epistaxis). Two sisters (B.S and B.F) are alive and present mild menorrhagia.

2.) Laboratory tests

Microcytic and hypochromic anaemia:

– Haemoglobin: 11g/dl; MCV:78fl; MCHC: 0,30

Thrombocytopenia:

– Platelet count: 20 x109/l

– Presence of very large size platelet (Fig. 1):

Address for correspondence:
Arabi A., Médecin Hématologiste, service d'hématologie et de Thérapie cellulaire, Etablissement Hospitalier Universitaire (EHU) 1er Novembre 1954, Oran, Algérie
Tel: +213 41 42 95 00 ; + 213 74 11 66 06; e-mail:abdessameddz@yahoo.fr

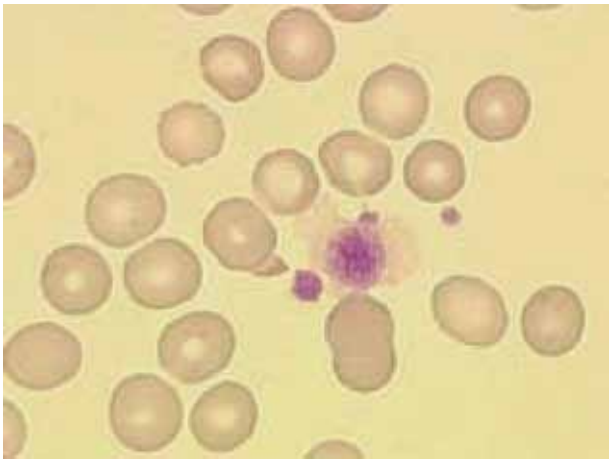
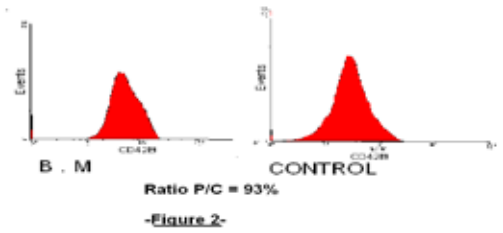


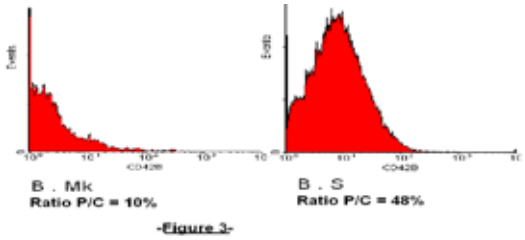
Fig. 1. A very large size platelet in blood film

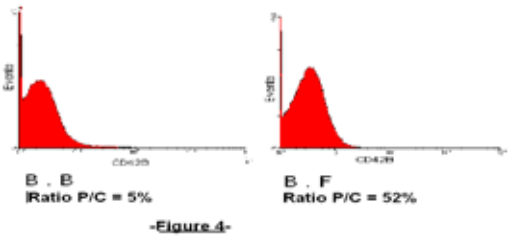
– Prothrombin time (PT): 12 s (control plasma: 12 s)
– Activated Partial Thromboplastin Time (aPTT): 32s (control plasma: 30s)
– von Willebrand factor activity: 120%
– von Willebrand factor antigen: 130%

3.) Flow cytometry results

GPIb glycoprotein test (CD42b), in the family members, is absent or present with a lower expression (Figures 2, 3, 4).







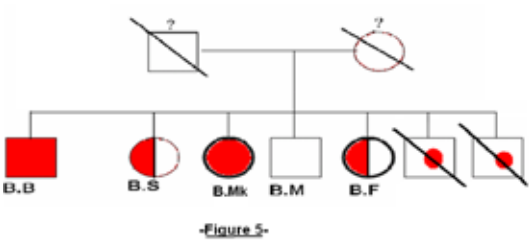


Fig. 5 summarizes the family investigation .

DISCUSSION

Since platelets were first identified, there has been continuous and recently accelerating progress in our basic understanding of platelet function. Many clinical tests of platelet function exist: platelet aggregometry, plasma assays for platelet factor 4, b-thromboglobulin, and soluble P-selectin, plasma and urine assays for thromboxane A2 metabolites, etc. Recently, there have been an increasing number of published studies that have attempted to use whole blood flow cytometry as a platelet function method in clinical settings (5): platelets in suspension are labelled with a fluorescent conjugated monoclonal antibody (like CD42b) which is linked to glycoprotein complex Ib-V-IX).

In the flow cytometer, the suspended cells pass through a flow chamber and, at a rate of 1,000 to 10,000 cells per minute, through the focus of laser; the emitted fluorescence of each cell can be measured; it is proportional to the amount of GPIb-V-IX (6).

In this family, the patient B.Mk presents severe haemorrhagic findings, and flow cytometry demonstrates that she is homozygote for the disease (10% of CD42b emitted fluorescence). Her brother B.B is also homozygote (5% of CD42b emitted fluorescence), but he presents only mild haemorrhagic findings: It seems that there is no correlation between the degree of deficiency of GPIb glycoprotein and severity of haemorrhage.

In the literature, the same observance is reported by F.Lanza (7): “as for other deficiencies, it is difficult to correlate genetic defects with propensity to bleed and severity of bleeding”.

B.M is normal (93% of CD42b emitted fluorescence), B.S and B.F present a heterozygote form of the disease (48% and 52% of CD42b emitted fluorescence). So, this method has the advantage to lead rapidly to sure diagnosis and to clasify the patient as homozygote or heterozygote.

The analysis of platelet by flow cytometry is becoming more common in both research and clinical laboratories.The pathogenesis and molecular defects of many primary thrombopathies are well known and related to defects in structural or functional glycoproteins (8).

REFERENCES

1. Nurden AT. Qualitative disorders of platelets and megakaryocytes. J Thromb Haemost 2005, 3:1773-1782
2. Bernard J, Soulier JP. Sur une nouvelle variété de dystrophie thrombocytaire hemorragipare congenitale. Sem Hop Paris 1948, 24: 3217-3222
3. De la Salle C, Lanza F, Cazenave JP. Biochemical and molecular basis of Bernard-Soulier Syndrome: A review. Nouv Rev Fr Hematol 1995, 37: 215-222
4. Bernard-Soulier Syndrome (http: //www.bernardsoulier.org). Site last updated February 2007.
5. Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelet in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987, 70: 307-315.
6. Michelson AD. Flow Cytometry: A Clinical test of platelet function. Blood 1996, 87: 4925-4936
7. Lanza F. Bernard-Soulier Syndrome. Orphanet Journal of Rare Diseases 2006, 46:1-6
8. Brown M, Wittner C. Flow Cytometry: Principles and clinical applications in Haematology. Clinical Chemistry 2000, 4:1221-1230.

Received: July, 19, 2010
Accepted: October, 5, 2010

MULTIPLE OCCURRENCE OF BASAL CELL CARCINOMA OF THE SKIN: A COMPARATIVE STUDY

Bartos V.[1], Pokorny D.[1], Zacharova O.[1], Haluska P.[1], Doboszova J.[1], Kullova M.[2], Adamicova K.[3], Pec M.[4]

[1]Department of Pathological Anatomy, Faculty Hospital in Zilina, [2]Department of Dermatovenerology, Faculty Hospital in Zilina, [3]Department of Pathological Anatomy, Jessenius Faculty of Medicine in Martin and [4]Department of Medical Biology, Jessenius Faculty of Medicine in Martin, Comenius University, Slovakia

Abstract

Patients with basal cell carcinoma (BCC) of the skin demonstrate considerable clinico-morphological diversity. One of the most interesting characteristics of this cancer is a marked variation in the tumors number and locality between the individuals. The purpose of the this study was to evaluate a prevalence and pathomorphological features of patients with multiple cutaneous BCCs, and to compare them with the patients with diagnosed one BCC only. Pathological specimens and clinical data from consecutive 514 patients with BCC were analysed retrospectively and cathegorised into two groups. Group A consisted of patients with only one bioptically verified BCC, group B included those with diagnosed 2 and more BCCs. In group A (413 members, 80.3% of all cases), female gender predominated (230 women – 55,7%, 183 men – 44.3%). The vast majority of cancers were located on the head and neck regions (n = 298, 72.2%), followed by trunk (n = 73, 17.6%), and extremities (n = 42, 10.2%). The most commonly affected area was a face. In group B (101 members, 19.7 % of all cases), we observed inverse gender ratio (56 men – 55.5%, 45 women – 44.5%). Topographical sites of tumors were as follows: head and neck regions (n = 185, 64.2%), trunk (n = 73, 25.4%), and extremities (n = 30, 10.4%). In contrast to group A, we generally noted a lower prevalence of cephalic, and higher prevalence of extracephalic (especially truncal) localities. Furthermore, there was inverse involvement of the facial and extrafacial areas on the head and neck regions, and more frequent affection of the upper than lower extremities. The number of tumors per patients ranged from 2 to 13. Individuals with two diagnosed tumors represented the most common subset (n = 70) followed by those with three (n = 12), four (n = 9), five (n = 4), six (n = 1), seven (n = 2), eleven (n = 1), twelve (n = 1), and thirteen (n = 1) BCCs. Twenty-one individuals manifested themselves by clusters presentation. Histologically, the indolent-growth tumor variants generally occurred more commonly than in previous group. In conclusion, many patients with BCC have a tendency of developing multiple lesions. Recent advances in molecular biology and pathology will help to explain a relationship between BCC carcinogenesis and crucial intrinsic and extrinsic etiological factors. A better understanding of why some persons have many lesions are needed for identification and selection of such cases and starting adequate preventative strategies.

Key words: basal cell carcinoma, single, multiple, tumor clusters

INTRODUCTION

Basal cell carcinoma (BCC) of the skin is recently the most common malignancy in human population. It has very heterogeneous clinical manifestation, histomorphology, and biological behaviour. Therefore, BCC patients generally demonstrate considerable phenotypic diversity (1, 2, 3, 4). One of the most interesting characteristics of this cancer is a marked variation in the tumors number, sites and accrual (number of tumors per year from first presentation) between the individuals (5, 6, 7). Some patients have only one diagnosed BCC with no impact on their health status. Conversely, the others may suffer many BCCs during their life that significantly increases overall morbidity (8, 9). A tendency to develop multiple BCCs at an early age is a characteristic feature of some rare hereditary disorders, of which Gorlin-Goltz syndrome (10), Bazex syndrome (11) and xeroderma pigmentosum (12) are the most important. Multiple BCCs have been also described in successive family generations without relationship to any syndrome and with

Address for correspondence:
MUDr. Bartos Vladimir, Str. P. Mudrona 30/16, Martin, 036 01, Slovak Republic
E-mail: bartos@jfmed.uniba.sk, vladimirbartos@post.sk

no additional cutaneous or extracutaneous anomalies (13). Regardless whether single or multiple, sporadic or hereditary, all patients with once diagnosed BCC are at high risk of suffering further ones (8, 9). Mostly a presentation with tumor clusters is associated with an predisposition to develop many additional lesions (1). A cluster is defined as a presence of two and more new, primary BCCs at the same locality. Based on this we distinct individuals with a single presentation phenotype (SPP) and multiple cluster presentation phenotype (MPP). The former includes those patients, that may have totally more than one cancer, but with only single lesion during one clinical manifestation. The latter demonstrates the individuals, that are manifested by large number of tumors at the first or further presentation (1, 7). Naturally, depending on different number and topographical locations of tumors it can be also expected different histological subtypes preponderance in individual persons and thus, distinct biological behaviour and clinical outcome.

The purpose of the present study was to evaluate the prevalence and pathomorphological features of patients with multiple (2 and more) BCCs of the skin and to compare them with the patients with diagnosed one BCC only.

MATERIAL AND METHODS

Pathological specimens and clinical data from consecutive 514 patients (275 women, 239 men) with cutaneous BCC diagnozed from January 2007 to October 2010 at the Department of Pathology in Faculty Hospital in Zilina were analyzed retrospectively. Based on the tumor number the individuals were categorized into two basic groups. Group A (single BCC) consisted of 413 patients with only one bioptically verified cutaneous BCC present in our complete database program. Group B (multiple BCC) included remaining 101 patients who had diagnosed 2 and more BCCs registered in our database system. The latter group was further subcategorized according to the total number of BCC in individual patients. A distinction between recurrence (BCC in contact or within the scar after the removal of previous tumor) and a new primary BCC was made on the basis of clinical reports. Cases of primary and corresponding recurrent (including re-recurrent) carcinomas diagnozed during observed period were considered as one tumor. Skin lesions were excised at the several clinical departments (department of surgery, dermatology, ophthalmology, and otorhinolaryngology). Cancers were obtained by total surgical excision, partial (probatory) excision, punch biopsy, and curretage. A biopsy material was fixed in buffered formalin, embedded in paraffin blocks, stained with hematoxylin and eosin and slides were reviewed by pathologists in the light microscope. Histological BCC types were classified according to WHO classification of skin tumors (14) and subcategorized into the aggressive-growth and indolent-growth variants. Aggressive-growth variants included the infiltrative (morpheic) type, micronodular BCC, and metatypical carcinoma, whereas indolent-growth variants comprised superficial, nodular and BCC with adnexal differentiation (2, 15). Information about patients were received from the hospital clinical records, or by consultations with the clinicians. No patient suffered from Gorlin-Goltz (multiple basal cells nevus) syndrome.

RESULTS

Group A – single BCC individuals

Out of all studied cohort members, patients with only one histologically proven BCC of the skin (n = 413) represented 80.3 % of cases. They included 230 women (55,7 %) and 183 men (44.3 %), thus, female gender predominated (male: female ratio 1: 1.25). The age of patients varied from 25 to 97 years (mean age 68.3 years). We confirmed 19 cases (4.6 %) of tumor reccurrence. Topographically, the vast majority of carcinomas were located on head and neck regions (n = 298, 72.2 %) (fig 1), followed by trunk (n = 73, 17.6 %), and extremities (n = 42, 10.2 %). On the head and neck localities, the most commonly affected area was a face reflecting the ratio between facial and extrafacial

regions 1.7: 1. On the trunk, the ratio between posterior (truncal) and anterior part was 1.9: 1, and finally, the ratio between upper and lower extremities was 1.2: 1. One tumor arose on the hand. Histomorphological types of the individual BCCs were as follows: 40 superficial (9.6 %), 188 nodular (45.5 %), 20 mixed superficial-nodular (4.8 %), 68 mixed nodular-infiltrative (16.4 %), 37 infiltrative including morpheic (8.9 %), 26 BCCs with adnexal differentiation (6.3 %), 9 metatypical (2.1 %), 9 micronodular (2.1 %), and other rare types and combination of various subtypes represented remaining 16 cases.

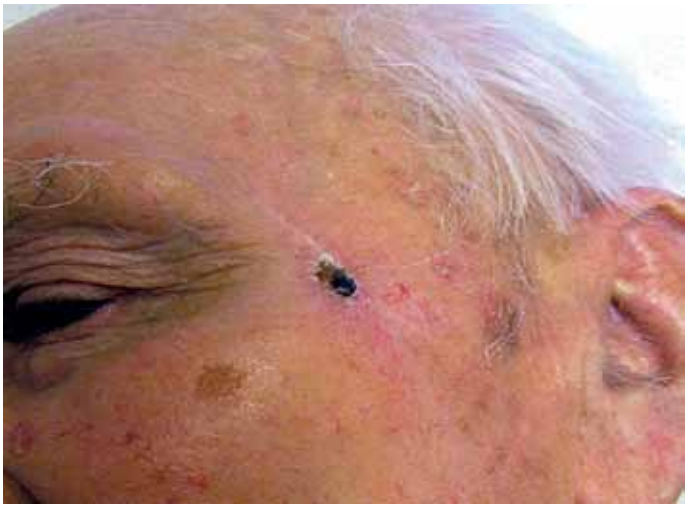


Fig. 1 Single BCC localized on the left temporal region

Group B – multiple BCCs individuals

Patients with multiple cutaneous BCCs constituted 19.7 % of all studied members and represented a total of 288 tumors. Mean age of individuals at the time of the last diagnosed BCC was 71.6 years. There was observed inverse gender ratio in comparison with group A, because this set consisted of 56 men (55.5 %) and 45 women (44,5 %) (male: female ratio 1.24: 1). We diagnosed 18 cases (6.25 %) of tumor reccurrence. Topographical sites of cancers were as follows: 185 on the head and neck regions (64.2 %), 73 on the trunk (25.4 %), and 30 on the extremities (10.4 %) (Table 1). Although the most common anatomical sites were also head and neck regions, in contrast to group A, we generally noted a lower prevalence of cephalic, and higher prevalence of extracephalic (especially truncal) localities. Furthermore, on the head and neck regions, there was inverse affection of the facial and extrafacial areas (the ratio between facial and extrafacial parts was 1: 1.6). Although percentual involvement of the extremities was nearly equal in both groups, we showed much more frequent affection of the upper than lower extremities (ratio between upper and lower extremities 4: 1). No BCC was found on the hands.

Table 1. Differences in the prevalence, gender and topographic localisation of BCCs in both groups. Group A – single BCC individuals, Group B – multiple BCCs individuals

	Group A	Group B
Prevalence		
– number of patients	413 (80.3 %)	101 (19.7 %)
– number of tumors	total of 413	total of 288
Gender		
– males	183 (44.3 %)	56 (55.5 %)
– females	230 (55.7 %)	45 (45.5 %)
Locality		
• **head and neck**	298 (72.2 %)	185 (64.2 %)
– facial regions	190	70
– extrafacial regions	108	115
• **trunk**	(17.6 %)	(25.4 %)
– anterior parts	25	25
– posterior parts	48	48
• **extremities**	(10.2 %)	(10.4 %)
– upper	23	24
– lower	19	6

The number of tumors per patients ranged from 2 to 13, thus, we could classify them into nine subsets – individuals with 2, 3, 4, 5, 6, 7, 11, 12, and 13 BCCs. Mean number of lesions per patients was 2.8. As expected, individuals with two diagnosed tumors represented the most common subset (70 cases) followed by those with three (12 cases), four (9 cases), five (4 cases), six (1 case), seven (2 cases), eleven (1 case), twelve (1 case), and thirteen (1 case) BCCs. Among them, twenty-one individuals (12 men, 9 women) manifested themselves by clusters presentation characterised by two (18 cases), three (2 cases) (Fig. 2), and four (one case) cancers growing simultaneously at the same locality during one clinical manifestation (Table 2). They were associated with extracephalic sites (66.6 %) and tumors in clusters mostly exhibited identical histological type (71.1 %), of which superficial type was the most common. In the remaining patients (SPP individuals), all tumors grew successively after a certain time interval.

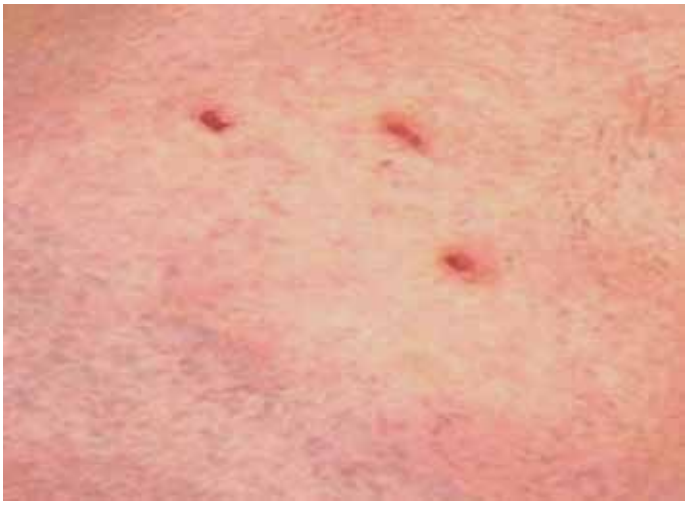


Fig 2. Cluster of three superficial BCCs localised on the upper part of the trunk.

Tab 2. Number of tumors and prevalence of the individual subsets of patients with multiple BCCs of the skin.

Number of tumors	Number of patients	Male	Female
2 BCCs	70 (69.30 %)	39	31
3 BCCs	12 (11.88 %)	8	4
4 BCCs	9 (8.91 %)	2	7
5 BCCs	4 (3.96 %)	2	2
6 BCCs	1 (0.99 %)	1	0
7 BCCs	2 (1.98 %)	2	0
11 BCCs	1 (0.98 %)	1	0
12 BCCs	1 (0.99 %)	0	1
13 BCCs	1 (0.99 %)	1	0
total	101	56	45

As for microscopical picture, we revealed distinct ratio in some BCC types in contrast to group A. There was marked higher prevalence of superficial (n = 70, 24.3 %) (Fig. 3), and partially even mixed superfical-nodular (n = 22, 7.6 %) type. On the contrary, we diagnosed only two micronodular (0.7 %), no metatypical carcinoma, and less frequency was also noted in mixed nodular-infiltrative (n = 31, 10.8 %) BCC. The other histological types generally exhibited similar prevalence. When we summarized percentage rate, the indolent-growth variants generally occurred more commonly than in previous group. Histomorphological varietes of tumors in both groups are summarized in Table 3.

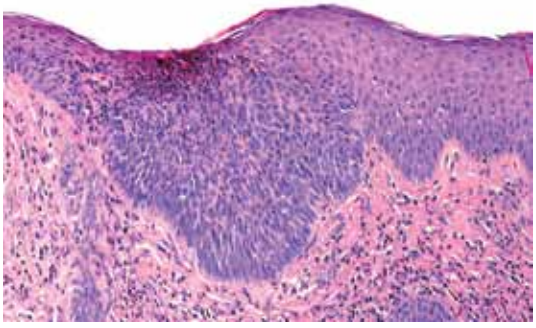


Fig 3. Superficial BCC is characterised by proliferation of well circumscribed small nests of atypical basaloid cells that remain in contact with epidermis (H&E, 200x)

Tab 3. Comparision of the histomorphological types and percentage of BCCs in both groups.

Histomorphological type	**Group A** (number of cases)	**Group B** (number of cases)
superficial type	40 (9.6 %)	70 (24.3 %)
nodular type	188 (45.5 %)	105 (36.5 %)
superficial-nodular type	20 (4.8 %)	22 (7.6 %)
infiltrative type	37 (8.9 %)	29 (10.0 %)
nodular-infiltrative type	68 (16.4 %)	31 (10.8 %)
metatypical type	9 (2.1 %)	0 (0 %)
micronodular type	9 (2.1 %)	2 (0.7 %)
BCC with adnexal differentation	26 (6.3 %)	21 (7.6 %)
other (mixed) types	16 (3.8 %)	7 (2.5 %)

DISCUSSION

Biological mechanisms that determine development and number of BCCs of the skin in individual persons are relatively poorly understood (8). Popular belief is that this neoplasia is caused by cumulative life exposure to ultraviolet (UV) radiation (9, 16). However, a relationship between dose exposure and risk of BCCs development is complex, and the exact etiologic factors responsible for the topographical distribution and number of tumors are obscure (5, 6, 8, 16, 17, 18). Although the sun-exposed areas are more prone to the occurrence of BCC in contrast to sun-protected sites, up to one third of BCC occur in normally protected areas. On the other hand, BCCs develop extremely rarely on the dorsum of the hands despite significant lifetime solar radiation in the vast majority of people (17), as it was demonstrated also in our study. It has been suggested (19, 20), that lesions occuring on these different areas can have a fundamentally distinct biological characteristics. This phenotypic heterogeneity is probably not the result of differences in UV exposure alone, but reflects the presence of subgroups of patients with individual host characteristics, of which some are associated with susceptibility to multiple BCCs (5, 6, 16). Individual response to UV-induced intracellular oxidative stress and effectiveness of DNA reparative mechanisms modify a process of BCC carcinogenesis (5, 16). Some authors suggest (6, 7) that a rate of tumors appearance is mediated by efficiency of local immune surveillance in recognising preexisting micro-tumours, and interaction between various host (intrinsic) and environmental (extrinsic) factors determines whether they become clinically evident. One of the crucial factor seems to be an imunological status, because immunocompromised patients have a higher propensity to develop multiple BCCs in contrast to immunocompetent individuals (21). Moreover, some authors showed (4), tissue microenvironment of BCC displayed a strong predominance of Th2-lymfocyte related cytokines. Thus, a local immunosuppression may have a significant effect on the growth of BCC. Even role of HPV infection of the skin in development of multiple BCCs have been discussed (22). There is also well-known an influence of some carcinogen-metabolizing enzymes polymorphisms on susceptibility to this cancer. For example, it has been demonstrated a relationship between glutathione S-transferase (GSTM1) null genotype (1, 5, 23) or cytochrome P450 polymorphism and number and accrual of BCCs in individual patients (1, 5). Furthermore, an association between HLA DR4 (24) or HLA-DR1 (25) and multiple BCCs presentation was confirmed two decades ago.

In our study we found that nearly 20 % of all BCC patients had suffered from two or more tumors during their life. This incidence is somewhat lower in comparison with the results of Kiiski et al. (26) and Mantese et al. (27) who described that 31.1 % and 26.0 % of BCC individuals had multiple lesions, respectively. In spite the fact, the overwhelming majority of BCC are usually located on the face, we could show that BCCs, among patients who developed multiple lesions, appeared more frequently on the trunk and upper extremities than on the face, compared to the individuals with a single tumor only. We also noted association between male gender and multiple occurrence of this neoplasia. Although patients with diagnosed BCC are generally at high risk of further BCCs development, the rate of the occurrence of new cancers after the first manifestation is highly variable (8). It has been estimated that among individuals with at least one previous BCC, the 1-, 3-, and 5-year risk of a new BCC is 17 %, 33 %, and 41 %, respectively (28). More detailed observations showed (8), that 5-year risk of further BCC development in relation to number of prior BCC is rising as follows: 27 % in 1 BCC, 49 % in 2 BCCs, 68 % in 3 BCCs, 73 % in 4-5 BCCs, 78 % in 6-9 BCCs, and 90 % risk in 10 or more BCCs. A probability of the appearence of new tumors during lifetime also depends on other factors, of which localization, age, and tumor clusters presentation are the most important. It has been shown that the individuals who were relatively young at the time of their first BCC diagnosis (26, 29), and with tumors on the trunk (6, 8, 16) or on the upper extremities (26) had a higher relative risk of developing multiple lesions. According to Ramachandran et

al. (1, 7) especially a finding of tumor clusters is a critical event that is followed by markedly increased accrual of further tumors and it probably suggests a reduced immune surveillance. MPP is not the consequence of excessive UV exposure alone, but reflects the presence of genetically distinct tumor subgroups (1, 7). Indeed, results of recent genetic studies demonstrated (30), that BCCs in patients with multiple lesions are probably polyclonal and multiple tumors at topographically different body sites may not arise from the same progenitor cell. In our observations over 20 % of patients manifested themselves by clusters presentation with simultaneous growth of two, three, or four carcinomas located mostly on the extracephalic sites. In the vast majority, tumors within one cluster exhibited the same histomorphology, preponderantly indolent-growth features. Even a higher incidence of superficial type found in our multiple BCC patients corresponded with a more common appearence of tumors in the extracephalic localities, because this type tends to occur more frequently on the trunk (4, 27, 31). From a practical point of view it should be noted, that simultaneous or consecutive developing of new BCCs leads to the more adverse prognosis and has a negative impact on clinical outcome. Wilson et al. (32) found that removal of multiple BCCs during one surgical procedure had been significantly associated with incomplete resection of lesions. Especially accumulation of multiple tumors in close proximity to one another results in difficulty of their total surgical excision. What is also important, consistent evidences indicate that patients with personal history of BCCs have an elevated risk of developing other primary cutaneous and non-cutaneous malignancies (33, 34). This can suggest a generalized role of some carcinogenic factors not only in the BCC pathogenic pathways, but even in another tumors (29). Therefore, an association between BCCs and increased probability for subsequent appearence of other cancers determine the necessity of adequate monitoring these individuals.

In conclusion, many patients with diagnosed BCC of the skin have a tendency of developing further new lesions. The exact reason of this prognostically unfavorable feature is unclear, but recent advances in molecular biology and pathology will help to explain a relationship between BCC carcinogenesis and crucial intrinsic and extrinsic etiological factors. It is evident that besides UV radiation even local immunosuppression, different skin microenvironment and genetic factors play important role in this process. A better understanding of why some persons have many lesions are needed for identification and selection of such cases at the first presentation and starting adequate preventative strategies.

REFERENCE

1. Ramachandran S, Fryer AA, Strange RC. Genetic factors determining cutaneous basal cell carcinoma phenotype. Med Pediatr Oncol 2001; 36 (5): 559-63.
2. Crowson AN. Basal cell carcinoma: biology, morphology and clinical implications. Modern Pathology 2006; 19: S127-S147.
3. Tilli CM, Van Steensen MA, Krekels GA et al. Molecular etiology and pathogenesis of basal cell carcinoma. Br J Dermatol 2005; 152 (6): 1108-1124.
4. Elamin I, Zečević RD, Vojvodić D et al. Cytokine concentrations in basal cell carcinomas of different histological types and localization. Acta Dermatoven APA 2008; 17 (2): 55-59.
5. Lear JT, Heagerty AHM, Smith A et al. Multiple cutaneous basal cell carcinomas: glutathione S-transferase (GSTM1, GSTT1) and cytochrome P450 (CYP2D6, CYP1A1) polymorphisms influence tumour numbers and accrual. Carcinogenesis 1996; 17 (9): 1891-1896.
6. Ramachandran S, Lear JT, Ramsay H et al. Presentation with multiple cutaneous basal cell carcinomas: association of glutathione S-transferase and cytochrome P450 genotypes with clinical phenotype. Cancer Epidemiol Biomarkers Prev 1999; 8 (1): 61-67.
7. Ramachandran S, Fryer AA, Smith AG et al. Basal cell carcinoma: Tumor clustering is associated with increased accrual in high risk subgroups. Cancer 2000; 89 (5): 1012-18.
8. Karagas MR, Greenberg ER. Unresolved issues in the epidemiology of basal cell and squamous cell skin cancer. In: Mukhtar H (ed). Skin Cancer: Mechanisms and Human Relevance. Boca Raton: CRC Press; 1995. pp. 79-86.
9. Wallberg P, Kaaman T, Lindberg M. Multiple basal cell carcinoma. A clinical evaluation of risk factors. Acta Derm Venereol (Stockh) 1998; 78: 127-129.

10. Ljubenovic M, Ljubenovic D, Binic I. et al. Gorlin-Goltz syndrome. Acta Dermatoven APA 2007; 16 (4): 166-69.
11. Valdivielso M, Longo I, Sauárez R et al. Acrokeratosis paraneoplastica: Bazex syndrome. J Eur Acad Dermatol Venereol 2005; 19 (3): 340-44.
12. Giannotii B, Vanzi L, Difonzo EM, Pimpinelli N. The treatment of basal cell carcinomas in a patient with xeroderma pigmentosum with a combination of imiquimod 5% cream and oral acitretin. Clin Exp Dermatol 2003; 28: (Suppl 1): 33-35.
13. Guarneri B, Borgia F, Cannavò SP et al. Multiple familial basal cell carcinomas including a case of segmental manifestation. Dermatology 2000; 200 (4): 299-302.
14. Kossard S, Epstein EH jr, Cerio R et al. Basal cell carcinoma. In: LeBoit P (Eds). World Health Organization Classification of Tumours, Pathology and Genetics of Skin tumours, IARCPress, Lyon, 2006; pp. 13-19 ISBN 92-832-2414-0
15. Bartos V, Adamicova K, Pec M. Aggressive-growth types of basal cell carcinoma of the skin. Acta Med Mart 2009; 9 (3): 24-31.
16. Lear JT, Tan BB, Smith AG et al. Risk factors for basal cell carcinoma in the UK: case-control study in 806 patients. J R Soc Med 1997; 90 (7): 371-374.
17. Vandeweyer E, Herszkowicz A. Basal cell carcinoma of the dorsum of the hand. Acta Chir Belg 2003; 103: 300-303.
18. Bartos V, Kullova M, Pokorny D et al. Histomorphological study of periocular basal cell carcinomas and overview of some clinically relevant aspects of the disease. Acta Med Mart 2010; 10 (2): 16-22.
19. Walling HW, Fosko SW, Geraminejad PA. Aggressive basal cell carcinoma: Presentation, pathogenesis, and management. Cancer Metastasis Rev 2004; 23: 389–402.
20. Crowson AN, Magro CM, Kadin M et al. Differential expression of bcl-2 oncogene in human basal cell carcinoma. Hum Pathol 1996; 27: 355-359.
21. Harwood, C.A., Proby, C.M., McGregor, J.M., et al. Clinicopathologic features of skin cancer in organ transplant recipients: A retrospective case-control series. J Am Acad Dermatol 2006; 54 (2): 290-300.
22. Sass U, Theunis A, Noël JC et al. Multiple HPV-positive basal cell carcinomas on the abdomen in a young pregnant woman. Dermatology 2002; 204: 362-364.
23. Heagerty A, Smith A, English J et al. Susceptibility to multiple cutaneous basal cell carcinomas: significant interactions between glutathione S-transferase GSTM1 genotypes, skin type and male gender. Br J Cancer 1996; 73 (1): 44-48.
24. Czarnecki D, Nicholson I, Tait B, Nash C. HLA DR4 is associated with the development of multiple basal cell carcinomas and malignant melanoma. Dermatology 1993; 187 (1): 16-8.
25. Czarnecki D, Lewis A, Nicholson I, Tait B. Multiple basal cell carcinomas and HLA frequencies in southern Australia. JAAD 1991; 24 (4): 559-561.
26. Kiiski V, de Vries E, Flohil SC et al. Risk factors for single and multiple basal cell carcinomas. Arch Dermatol 2010; 146 (8): 848-855.
27. Mantese OAS, Gomides ADM, Berbert VCLA, Rocha A. Basal cell carcinoma – analysis of 300 cases observed in Uberlândia – MG, Brazil. An Bras Dermatol 2006; 81 (2): 136-42.
28. Karagas MR, Stukel AT, Greenberg ER et al. Risk of subsequent basal cell carcinoma and squamous cell carcinoma of the skin among patients with prior skin cancer. JAMA 1992; 267: 3305-3310.
29. Milán T, Pukkala E, Verkasalo PK et al. Subsequent primary cancers after basal-cell carcinoma: A nationwide study in Finland from 1953 to 1995. Int J Cancer 2000; 87 (2): 283-288.
30. Heitzer E, Quehenberger F, Wolf P. Polyclonality of multiple sporadic basal cell carcinomas. J Invest Dermatol 2009; 129: 1586–1589.
31. Betti R, Radaelli G, Mussino F et al. Anatomic location and histopathologic subtype of basal cell carcinomas in adults younger than 40 or 90 and older: any difference ? Dermatol Surg 2009; 35 (2): 201-206.
32. Wilson AW, Howsam G, Santhanam V et al. Surgical management of incompletely excised basal cell carcinomas of the head and neck. Br J Oral Maxillofac Surg 2004; 42: 311–14.
33. Troyanova P, Danon S, Ivanova T. Nonmelanoma skin cancers and risk of subsequent malignancies: a cancer registry-based study in Bulgaria. Neoplasma 2002; 49 (2): 81-85.
34. Whelees L, Black J, Alberg AJ. Nonmelanoma skin cancer and the risk of second primary cancers: a systematic review. Cancer Epidemiol Biomarkers Prev 2010; 19 (7): 1686-95.

Acknowledgments:
The authors would like to thank Kadlecova Maria, Kagerova Zofia, Kafunova Dana, Davidikova Jana, Melova Daniela, and Bologova Jolana for their outstanding technical assistance.
Received: October, 26, 2010
Accepted: December, 6, 2010

PUBLIC HEALTH PROBLEM OF MYCOTOXIN OCCURRENCE IN NUTRITION OF POPULATION WITH EMPHASIS ON OCHRATOXIN A AND BREAST MILK

[1]Cajdova J., [2]Hudeckova H., [2]Dostal A.

[1]The Healthcare Surveillance Authority, Department of Forensic Medicine, [2]Department of Public Health, Jessenius Faculty of Medicine in Martin, Comenius University, Slovak Republic

Abstract

Mycotoxins are known as causal agents of many serious diseases. This group of substances as products of ubiquitous genera of microscopic fungi can occur normally, though often in sublimit but detectable concentrations on almost all levels of the food chain, including the first food of human.

Ochratoxin A as one of the most frequent mycotoxins produced by representatives of the genus Aspergillus and Penicillium can be produced under a wide range of temperature and humidity conditions and is able to penetrate from nursing mother's organism to breast milk. We review recent information on the xenobiotics with emphasis on ochratoxin A and focus on circumstances when the national food control authorities cannot control these contaminants in food.

Key words: health risks – mycotoxins – ochratoxin A – nutrition – breast milk

INTRODUCTION

The current population is continuously exposed to many risk factors which include a variety of substances contaminating the environment and entering the food chain, whether as a result of human activity or as a result of biological processes in the body. And just such agents are also secondary metabolites of microscopic filamentous fungi – mycotoxins. Increasingly wider information about the conditions, under which microscopic fungi with ability to produce mycotoxins can grow and reproduce, gets in public awareness through Public Health activities. However, due to underestimation of the problem or unrecognation of the risk of possible contamination of food by microscopic fungi at not quite appropriate handling, then such food becomes a suitable substrate for mould settlement and thus for the production of toxins. In this case not only a direct consumer is in a risk, but through breast milk also one of the most vulnerable population, infants.

1. ENVIRONMENTAL RISK FACTORS OF POPULATION

Public health is the result of complex factors – genetics, economic and social situation, availability of adequate medical care, lifestyle and the environment and nutrition. The quality of the environment, as well as eating habits are crucial factors affecting health.

For several decades the more apparent adverse environmental changes are known. The role of Public Health is to define all relevant risk factors. In addition to physical risk factors, an increasing number of hazardous chemicals contaminating water and food, indoor and outdoor air is a major reason of environment-related diseases.

Because of the fact that many contaminants harmful to human health has been clearly demonstrated, it is necessary to effectively monitor the incidence and dynamics of accumulation of these substances in the environment and to assess the health state of exposed populations through health monitoring, systematic data collection, analysis

Address for correspondence:
RNDr. Jela Cajdova, The Healthcare Surveillance Authority, Department of Forensic Medicine in Martin
Kuzmányho 27, Martin, 036 01, Slovak Republic
Phone: 00421 43 4260015; e-mail: jela.cajdova@udzs.sk

and dissemination of information on health, diagnosis of health problems and risks. Information obtained in this step is used for awareness and education of the population, mobilization of public interest in the development of health policies at local, regional and national level [1].

Since 1992 information on the status and trends of various environmental components of the Slovak Republic has been the objective of the Information system of environmental monitoring. The subjects to the monitoring have become the air, water, soil, fauna and flora, forests, geological factors, wastes, contaminants in food and feed, meteorology, climatology and radioactivity [2].

Processes of improving of environmental health in the countries of Europe started after the International Forum on Environment and Health in Helsinki in 1994, where an important document the *Action Plan for Environment and Health for Europe* was adopted. The Slovak Republic as one of the first countries developed the *National Action Plan* (NEHAP), which was adopted in 1997. Most of the tasks was to develop and adopt codes and standards compatible with the legislation of the European Union (EU). In 2000 the Government approved the update NEHAP II, where the main goal was to minimize the impact of environmental risks and to keep it at such a state that does not damage and does not endanger human health.

One of a priority area has become the safety of food, soil, free air, provision of drinking water, housing, health services and education in environmental health. Since 2005 this area has been enlarged to eliminate the effect of risk factors on child health [3]. The risk factors of growing interest are often substances capable of accumulation in plant and animal tissues, which are characterized by their resistance to degradation and by their acute and chronic toxicity. The groups of substances listed below do not have similar health effect but we can generally include them in the group of xenobiotics. In connection with the assessment of health risks most countries have legislation limiting their maximum levels in parts of the food chain and in food commodities of risk.

Undoubtedly, medically significant xenobiotics that have received attention are particularly compounds of the type of persistent organic pollutants (POPs) such as dioxins, polychlorinated biphenyls (PCBs) and DDT insecticide. Also toxic minerals, which include radioactive materials, metals, metalloids and other inorganic compounds, have a similar meaning in terms of health impact. The substances get into components of the environment from various sources, particularly in relation to human activities.

In addition to those contaminants, another important group of xenobiotics causes serious health problem, many of which are characterized by carcinogenic and mutagenic activities and a high toxic potential. They may occur through a chemical conversion of natural ingredients of raw food, food itself or feed during processing and storage, but also through the activity of microorganisms at improper practices or mishandling. Toxins excreted by pathogenic bacteria and microscopic filamentous fungi have been the cause of many serious diseases.

2. NUTRITION AS A POTENTIAL RISK FACTOR

Health of the population is closely linked to the field of nutrition. Good nutrition is essential for healthy human development.

Human food is chemically very complicated material. According to a rough estimate, about five hundred thousand different chemical compounds have been found in fresh food. A much higher number of compounds has occurred by enzymatic and nonenzymatic reactions during storage and processing up to finished dishes. The most important components of food are nutrients – proteins, lipids, carbohydrates, vitamins and minerals that determine its energy and nutritional value. In addition to nutrients also other substances are normally found there, usually only with sensory significance. As a part of food there can also be some antinutritional substances, with their negative

impact on nutritional availability. However, many foodstuffs contain as their natural components various substances that are toxic to some or all subjects [4]. According to the Codex Alimentarius, it is allowed at food production to use preservatives, antioxidants, colorants, sweeteners and other additives which are utilized to improve food shelf life, conditions of production, processing, treatment and transport. [5]. Under certain conditions agents endangering human health can be formed in the food or can be penetrated there from outside. Council Regulation No 315/93 EEC considers these contaminants any substance added not intentionally to food but which arises due to improper processing, treatment, packing, transport and storage or as a result of environmental contamination [6].

There is a potential risk of serious health consequences for human from each substance that does not occur naturally in food. In Slovakia, the conditions of food production and handling, as well as management of the food supervision are provided in Act No 152/1995 Coll. on food, as amended. Other adopted legislation enables state authorities to control and regulate the contaminants. The legislation determines maximum contents of selected risk factors for defined commodities. National food control authorities have carried out systematic monitoring of additives and contaminants in food. One of the advantages of the present is the connection to the Rapid Alert System for Food and Feed (RASFF), where it is possible to receive and send information about incidence of unsafe food. The RASFF serves either to report on food and feed of risk in order to avoid putting them into circulation or as means to withdrawing such food from the European market. Reports of the European warning system have brought information about frequent hits of chemical as well as microbiological contamination of food and feed. The highest number of warnings, however, has been for natural metabolites of toxinogenic strains of molds – mycotoxins.

Mycotoxins are toxins of various risk categories, of various composition and toxicity, which are able to contaminate food and feed. It is difficult to eliminate them from contaminated food. Many of them are not affected by cooking procedures due to their resistance to high temperatures used in processing and preparing food. Especially food and feed of plant origin can be the most contaminated commodities. The raw materials may be attacked by microscopic filamentous fungi during vegetation, but particularly in their processing or storage. Cereal derivatives, maize, nuts, roasted coffee, wine, beer, vine fruits and fruit juice are considered to be the most risky final products.

Mycotoxins may occur also in food of animal origin – liver, kidney, muscle, blood, resulting from the ingestion of contaminated feedstuffs. These xenobiotics are able to penetrate into animal tissues and depot fat, to remain and accumulate there because they are mostly fat soluble and not readily excreted [7]. Animal products can also be significantly contaminated during the production process and storage.

In 2008 RASFF received from a total of 3099 reports up to 931 reports of limit-exceeding concentrations of mycotoxins, a majority of which related to aflatoxins. In other cases the most frequent identified risk was for ochratoxin A in the number of twenty reports, four reports of deoxynivalenol, three of patulin and both fumonisins as well as zearalenone in the number of two reports. This issue does not have a downward trend, in 2007 from a total of 2976 up to 754 reports related to mycotoxins, which included the leadership of aflatoxins and the second of the order was again ochratoxin A. [8], [9]

Because of the fact that these substances have a highly negative impact on the human body, many countries have adopted legislation to regulate them. The SR has in force the general European legislation establishing the maximum levels of xenobiotics in the individual commodities of risk. The maximum levels of mycotoxins in such commodities are set in the Commission Regulation (EC) No 1881/2006. The patulin content is limited in fruit products, particularly in juice, fruit compote, puree and baby food based on apple. It can occur as a result of bad manufacturing practices when mouldy raw material was used. Other mycotoxins – aflatoxins, ochratoxin A, zearalenone, fumonisins and

trichothecenes such as deoxynivalenol and T-2 toxin are limited especially in maize, cereals and cereal derivatives. In addition to these food the maximum levels of aflatoxins are also set for dried fruit, milk, spices, nuts and groundnuts. The Commission Regulation (EC) No 165/2010 amends the maximum levels of aflatoxins for other oilseeds, such as sunflower seeds [10], [11]. As to ochratoxin A, the Commission Regulation (EC) No 1881/2006 establishes the maximum value for both corn and maize, but also for a special group of dried fruits – raisins as well as other commodities at risk – grape juice, wine and coffee – roasted, and instant. The Commission Regulation (EC) No 105/2010 amends the maximum value in spices. According to the recommendations in this regulation, it is appropriate to keep on monitoring of the ochratoxin A levels in food for which the maximum of ochratoxin A has not been set yet, but in case of repeated findings of elevated levels the setting should be considered [10], [12]. To the food which are expected to have a higher incidence of the contaminant belong cocoa and cocoa products, grapes and grape products, beer, dried fruits, both raisins and figs, legumes, green tea, but also pork, pork blood and guts [13], [14]. Potatoes in terms of ochratoxin A are at low risk [15].

It has been found that the representatives of microscopic filamentous fungi especially from the ubiquitously occurring genera Aspergillus, Penicillium and Fusarium are mycotoxin-producing strains. For their enzymatic equipment they can adapt to almost any substrate. However, in general they are not phytopathogens, so it is not characteristic for them to colonize plants at the stage of vegetation, with the exception of some plant, and their presence in healthy fruits before harvest is not typical. Most of them are found in such food products which do not comply with proper processing procedures for collecting, processing, drying, storage or transport. Although significant progress has been made in many countries in making food safer, it is impossible to provide adequate protection to the consumer by mere inspection at the final stage and rejection of a hazardous final product. Better sense makes the introduction of preventive measures to minimize risks at all stages of food production and distribution chain, when unsuitable products can be identified earlier. Such an approach includes the system of hazard analysis and method critical control points (HACCP) which was developed for the National Air and Space Agency (NASA) in the USA in the sixties of the last century to ensure the protection of all food and nutritional products for astronauts against mechanical damage, toxic, chemical or physical contamination. Since 1992, HACCP has become a global standard and has been incorporated into EU legislation. The Council Directive 93/43/EEC on the hygiene of foodstuffs was the base for implementation of the system for our conditions as it is done in the Codex Alimentarius SR [16]. The criteria used for observation of mycotoxins include temperature, time, humidity, pH and sensory parameters such as appearance.

In terms of food safety, specific attention is paid to food products for infants and young children, as a particularly vulnerable group of population. Absorption, distribution, metabolism and excretion of xenobiotics in their organism are different from the adults. Key factors in their diverse operation are other sensitivity of receptors, maturation of tissues, gastrointestinal motility, immaturity of the biotransformation processes, the composition of intestinal microflora, and also the membrane permeability and renal processes. Furthermore, with regard to body weight, children receive more food than the adults [17]. Exposure to contaminants and thus to the mycotoxins can disrupt their normal development and cause them permanent damage. The aim of adoption of the Slovak and European legislation is to minimize the impact of these risk factors on children‘s health through strict requirements on the composition and levels of contaminants in products for infants and young children.

The Health State Authority of SR (UVZ SR) issued data of monitoring of mycotoxin content in food for infants and young children, collected from 2005 to 2007, where from a total of 1001 of samples 28 ones were with positive evidence of aflatoxin B1, from a total of 1018 of samples 81 ones were with positive evidence of patulin where even in 2 samples the content was limit-exceeding . As to ochratoxin A, from 133 samples of food

for infants and young children 43 were with detectable evidence of the mycotoxin [18]. [19]. [20]. Although these levels, with exception the limit-exceeding concentrations of patulin, were not high at all and the method of high performance liquid chromatography with a great sensitivity was employed for measures that enabled to find also very low concentrations of the xenobiotics, the presence of the mycotoxins itself confirmed the fact of possibility to receive repeated intake of low doses of the toxic substances.

The food control system does not concern with harmlessness of such foodstuffs of plant or animal origin which individuals produce on their own. It also does not concern with food after purchase from commercial network, which is stored at home in various manners. The lack of knowledge about microorganisms action and failure to keep the food at the right conditions can lead to the mycotoxin contamination. What is more, not everyone is aware that mycotoxins can migrate deep into the substrate so they can be found in high concentrations also in such parts of affected food which seem to be healthy. And due to the ability of mycotoxins to transfer into breast milk, in such a manner not only the health of a direct consumer himself is endangered but also a breastfed infant. This group of substances can occur normally, though often in sublimit but detectable concentrations on almost all levels of the food chain, including the first food of human – breast milk.

The risk of mycotoxins in breast milk

Breast milk is the first child nutrition providing an easily digestible form of optimally balanced composition of substances essential for child's healthy growth. It is species-specific and all substitute feeding preparations differ considerably from it, making breast milk uniquely superior for infant feeding [21]. Its medical importance is strongly evident, particularly for children under the age of the first six months of life. Numerous studies have reached the results that breastfeeding decreases the incidence and severity of a wide range of infectious diseases, allergies, asthma, arthritis, diabetes, cardiovascular diseases, obesity, the risk of various types of cancer, whether in childhood of breastfed infants or later in adulthood, compared with the non-breastfed. The studies have also shown a reduction of likelihood of Sudden Infant Death Syndrome (SIDS) during the first year of life. Breastfed infants have an increased immunity that is provided through substances contained in milk of nursing mothers [21], [22].

During lactation nursing mother's diet itself does not significantly affect the representation of essential nutrients in breast milk because they keep their optimal ratio even if the food of the mother is not ideal. Changes in the composition would occur only in the case of strong malnutrition of the nursing woman [23]. Significantly, however, the diet influences both the content of vitamins and minerals, but also the risk of occurrence of contaminants that have been many times proven and determinated in measurable concentrations in breast milk. Then the essential food can become a serious health risk factor for breastfed infants.

It is necessary for nursing mothers to consume only safe food. The results of our questionnaire survey where we addressed about 100 mothers of the newborns suggest that information on the issue of mycotoxins are still not sufficient for public. Despite an effort of many mothers to take good regimen, the risk of consumption of contaminated food and thus possibility of mycotoxin transition into breast milk still continues.

Although the ubiquitous presence of toxinogenic mould spores is a common phenomenon, there is only low probability of a direct danger from an income of their toxins by consumption of visibly moldy food. The risk of acute toxicity of such disgusting products is in fact minimal. However, a cause of worry is the risk of repeated low doses of toxic mycotoxins. The multiple intake of low doses of these secondary metabolites may occur especially in the first days of the growth of toxin-producing moulds when they are too small to be seen with the naked eye and it is unable to recognize their occurrence without an expertise. So a nursing mother can consume some contaminated food, although macroscopically intact, without any evidence of presence of microscopic fungi.

As to mycotoxin content in the organic food, that is recommended to pregnant women and nursing mothers as healthy one, the question is the subject of intense discussions because it has been shown that it also is not totally free of contaminants. Although compared with products of conventional agriculture the organically grown food is expected to have a lower content of substances derived from synthetic agrochemicals such as pesticide residues, heavy metals and nitrates, in certain circumstances it can contain higher levels of natural toxins. Under adverse weather conditions or when there is a mechanical damage or insect infestation of organic food, more intense microbial contamination is more probable in comparison with the chemically treated products, which can lead to an increased content of toxic secondary metabolites of filamentous fungi, capable of transfer into breast milk [24].

At the present the high current issue is the quality of food which could be in contact with floodwater, mud and sludge. The Ministry of Health SR on its website has issued the basic sanitary requirements for health protection after the floods explaining also the handling of food and agricultural products. Following the instructions it was necessary the flooded food considered unhealthy, with the exception of those that had remained sealed [25]. The problem is the consumption of food that do not show any signs of damage or changes in its external appearance, but the hermetic packaging is in dispute. In addition to bacterial contamination, such food may also be affected by toxin-producing microscopic fungi and endanger the health of the population consuming this food directly, as well as a nursing infant through breast milk contaminated in mother's body.

3. TOXICITY OF MYCOTOXINS WITH EMPHASIS ON OCHRATOXIN A

Mycotoxins mostly belong to compounds of natural origin, toxic to humans and animals. Their chemical structures vary considerably, but they all are characterized by relatively low molecular weight. They are non-protein products of biochemical reactions of microscopic filamentous fungi, which serve neither as an energy source, nor as stock agents and therefore are referred as secondary metabolites. Their total number is not completely known, so far there has been identified more than 300 mycotoxins. Some authors estimate the number of all the secondary metabolites of moulds in the order of thousands. Knowledge of other products of these microorganisms, effective as potential toxins, is constantly growing. But not all the identified mycotoxins were found to be of significant influence on the development of disease in humans or animals. Many of them do not occur under natural conditions because they were obtained during laboratory experiments with cultures of microscopic filamentous fungi. In terms of toxicity and frequency of the findings, there are the greatest risk from aflatoxins, ochratoxin A, patulin, trichothecenes, fumonisins, zearalenone and ergot alkaloids, but also cyclopiazonic acid, sterigmatocystine and several others [13], [26].

The principal impetus for the development of mycotoxin research was the incident in Great Britain in 1960, when there was a series of mass mortalities around 120 000 birds of poultry. Investigation showed the relationship with the administration of mouldy food and as the agents of intoxication were identified previously not known toxins of microscopic fungi [13], [27]. In the period following the outbreak of the disease, induced by the high acutely toxic aflatoxins, much information about the xenobiotics have been produced.

Microscopic filamentous fungi are multicellular or unicellular eucaryote organisms with heterotrophic nutrition. They obtained nutrients by absorbing from the environment, what enables them to grow almost anywhere where organic matter is. They are mostly saprophytic organisms that have the role as destruents in ecosystems and contribute significantly to the cycle of matter and energy in nature. Only a minor part is adapted to parasitism of other organisms, including humans [13]. Until now about 350 species of toxin-producing moulds are known, many of them can produce more than one toxin [27]. The same mycotoxin may also be a secondary metabolite of microscopic fungi of other genera or species. Not under all conditions all toxinogenic moulds produce my-

cotoxins, but all strains of microscopic filamentous fungi which once have been found to produce a mycotoxin are considered to be potentially toxinogenic. Mycotoxin formation is dependent on the synergy of enzymatic activities in cells. Besides the species of microscopic fungi the process depends also on the physical, chemical and biological conditions of the substrate and the surrounding area. The most important physical properties influencing the mycotoxin formation include humidity and temperature [4], [28].

We can say that all living species could be negatively affected by these toxic substances. However, the sensitivity and severity of harm depends on several factors such as age, sex, physical and nutritional status of the individual, species, concentration levels of mycotoxins and extent of exposure as well as possible synergistic effects of other present chemicals. Most mycotoxins entering the body mainly by ingestion of infected food or feed or less frequently by absorption through the skin. Since mycotoxins were found in airborne particles containing mould spores in certain environmnet where some susceptible commodities were handled, also inhalation of the bioaerosol was regarded as a further route of exposure. Exposure to mycotoxins can cause wide range of dose-dependent acute and chronic diseases in humans and animals, with common name mycotoxicoses.

Despite the fact that mycotoxicoses were observed already in ancient times, mycotoxins as the etiological agents were not known for a long time. The first identified mycotoxicosis was probably ergotism, widespread in the Middle Ages throughout Europe. The human disease was caused by eating cereals usually in the form of bread, infected with sclerotia of Claviceps purpurea containing ergot alkaloids. Two forms of ergotism have been recognized, convulsive and gangrenous. The convulsive ergotism affects the central nervous system and is characterized by neurological symptoms such as mental disorientation, convulsions, blindness and paralysis, while the gangrenous form due to vasoconstrictive activity of ergot alkaloids affects the blood supply to the extremities and is charecterized by necrosis and gangrene of the limbs. The disease is also called St. Anthony‘s fire because it causes burning symptoms. Over 370 hospitals were built in the name of St. Anthony for those suffering from this disease [29].

Another mycotoxicosis with serious consequences is Alimentary toxic aleukia (ATA) which has many forms including leukopenia, necrotic lesions on the oral cavity, sepsis, haemorrhagic diathesis and depletion of the bone marrow. The outbreak of the disease was recorded in Russia especially during the World War II where it was induced by consuming overwintered mouldy grain. The fungi responsible for the disease belong to the genera Fusarium and Cladosporium, producing toxic trichotecenes. [30]. The strain Penicillium citreoviride as a frequent contaminant of rice producing citreoviridine was the causal agent of another disease called Yellow rice poisoning. It is known mainly from Japan after World War II when rice had to be imported from various countries. The ingestion of ”yellow rice” caused vomiting, convulsions, ascending paralysis and was characterized by high mortality [13], [30], [31].

At the present the modern food processing and storage as well as induction of HACCP should minimize the risk of outbreaks of the serious acute mycotoxicoses but from time to time they occur sporadically among the population with poor level of nutrition. However, clinical and veterinary practices know many other pathological manifestations connected with repeated intakes of small doses of the xenobiotics through feed and food that lead to chronic poisoning often with severe or fatal consequences. Their occurrence depends on geographical area, season, climate and dietary habits. Mycotoxins or their metabolites have the ability to be accumulated in the body and in the case of presence of greater number of contaminants they are capable of synergistic effects. Numerous findings of the xenobiotics in human serum, urine and which is extremely serious even in breast milk show evidence of the human exposure [21].

In terms of biological effects and target organs of experimental and domestic animals affected by exposure to mycotoxins, some of the xenobiotics belong to hepatotoxins as it is in the case of aflatoxins or sterigmatocystin, but also to nephrotoxins, represented

mainly by ochratoxin A and citrinin. Fumonisins, penitrem A and fumitremorgens have neurotoxic effects and trichothecenes affect toxically gastrointestinal tract. The immune system can be attacked by aflatoxins, ochratoxin A, patulin and trichothecenes as well. Haematotoxic effect has been proven for aflatoxins, ochratoxin A, zearalenone and trichothecenes. When tested on guinea pig skin, several trichotecenes exhibited also dermatotoxic reaction [13], [26].

Biological effects of mycotoxins may occur also later, since some of them have mutagenic, embryotoxic and carcinogenic potential. Aflatoxin B1 with its sufficient evidence for the carcinogenity is classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans. Ochratoxin A, fumonisin B1 and sterigmatocystin are classified as probably carcinogenic to humans [13], [32], [33]. But conclusions of animal studies on rats showed that also the others, such as citrinin or patulin, are capable of inducing deoxyribonucleic acid damage [13], [34].

Overview of the toxic effects of aflatoxin B1 and ochratoxin A on animal species and humans are summarized in Table 1 and 2.

Table 1 Toxic effects of aflatoxin B1 [13], [14], [26], [32]

Effect	**Animal species**						**Human**
	Mouse	Rat	Rabbit	Pig	Poultry	Monkey	
Hepatotoxicity		+	+	+	+	+	+
Immunotoxicity		+		+	+		
Hepato carcinogenicity		+			+	+	+
Reproductive and developmental effects	+	+			+		
Genotoxicity	+	+					+ (human cells)

Table 2 Toxic effects of ochratoxin A [13], [14], [26], [37]

Effect	**Animal species**				**Human**
	Mouse	Rat	Rabbit	Pig	
Nephrotoxicity	+	+	+	+	Association with endemic nephropathy
Neurotoxicity	+	+	+		
Immunotoxicity		(+)		(+)	
Renal carcinogenicity	+	+			Inadequate evidence
Reproductive and developmental effects	+	+	+		
Genotoxicity	No clear evidence				

+ Evident, (+) Observed in limited studies

Aflatoxin B1, ochratoxin A, and trichothecenes are considered to be the etiological factor for some serious diseases from dietary exposure, but from inhalation of contaminated bioaerosol as well. It is possible that aflatoxin B1 in addition to hepatocarcinocenic potential is also able to cause tumors in the respiratory tract [13]. The acute renal failure of a patient working in the workplace where grain contaminated by Aspergillus ochraceus was stored is attributed to inhalation of ochratoxine A. But only little data of

a similar type are available so to answer the question about the possible effects of mycotoxins entering the respiratory system requires further study of this issue [35].

In order to protect public health many international institutions concern with the xenobiotics in their programs. Because of new toxicological knowledge about the harmful effects on living organism and also on the dietary exposure recently estimated, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) several times reviewed the potential risks associated with mycotoxin contaminated food intake, modified the values of the lowest toxic doses (LOAEL) and derived the values of the acceptable daily and weekly intake of several mycotoxins, including one of the most wide-spread and the most toxic natural toxins, ochratoxin A.

3.1 Mycotoxins from a group of ochratoxins

Ochratoxins are a group of closely related derivatives of isocoumarin linked to L-phenylalanine and classified according to biosynthetic origin as pentaketides within the group of polyketides [31]. They are produced either by biochemical reactions of some microscopic filamentous fungi or they occur as a result of the metabolism of thus created toxins after entering living organisms

In addition to ochratoxin A, which is excreted by several species of the genus Aspergillus in tropical regions and of the genus Penicillium in temperate regions, the group consists of other secondary metabolites of the moulds, namely ochratoxin B which is the chlorine-free analogue of ochratoxin A, also ochratoxin C, ethyl ester of ochratoxin A, and the main metabolite of ochratoxin A which is chlorinated isocoumarin carboxylic acid called ochratoxin α and its chlorine-free derivative ochratoxin β [36]. Also other products of the metabolism of ochratoxin A integrate the group – 10-hydroxyochratoxin A and two ochratoxins D, which are the epimers 4S-and 4R-hydroxyochratoxin A [13].

From a toxicological point of view, the most dominant among the group is ochratoxin A because hydroxyochratoxins and ochratoxinu a are of lower toxicity. Ochratoxin B is about 16 times less toxic, although according to the European Food Safety Authority (EFSA) there is only little data available for this claim. Ochratoxin C is as toxic as ochratoxin A, but its occurrence is rare. It has, however, genotoxic effect [37].

3.2 Risk assessment and toxic effects of ochratoxin A

Many authors attributed some of the toxic effects to metabolites of ochratoxin A. However, the results of subsequent studies have shown that ochratoxin A is the very toxic agent that is active at a higher rate than any of its metabolites.

The most serious toxic effect recorded in several animal species exposed to ochratoxin A is nephrotoxicity. Its hepatotoxicity, neurotoxicity, immunotoxicity, teratogenicity and probably also mutagenicity have been demonstrated too. The IARC, based on the significant correlations between the geographical distribution of Balkan endemic nephropathy inducted probably by ochratoxin A and a high incidence of tumors in the urogenital tract in patients from these areas, as well as on the basis of the carcinogenic effects on the laboratory animals, classified ochratoxin A in group 2B as a possible carcinogen to humans [33].

When studying the mechanism of action of ochratoxin A there have been demonstrated the inhibition of protein synthesis, increased lipid peroxidation, damage of carbohydrate and calcium metabolism and impaired mitochondrial function. Results of experiments show the evidence that phenylalanine as the structural component of ochratoxin A is probably responsible for many serious health activities of the compound. The dihydroisocoumarin moiety can be responsible for other unexplained yet toxicological processes [13]. Some authors stated the hypothesis of the toxic action of chlorine atom substituting in the aromatic ring [28].

Acute and chronic toxicity is derived from the ability of ochratoxin A to inhibit protein synthesis by competition with phenylalanine in the reactions catalyzed by phenylalanine-t-RNA synthetase. The phenylalanine part of the molecule is changed by the

transfer RNA for phenylalanine. It is, however, bound to the coumarin part of ochratoxin A that blockes its binding into a protein chain. Thus the protein synthesis is stopped. Ochratoxin A inhibits almost all reactions involving phenylalanine. It is also able to inhibit mitochondrial respiration and ATP production. It can be concluded that the acute toxicity is not too significant, but chronic and late effects may be serious and must be taken into account when assessing the risk exposure of the population [28].

CONCLUSION

From the number of xenobiotics present in the components of the environment and food chain, mycotoxins have been a serious public health problem for a long time. Attention, what many agencies and organizations pay to mycotoxins in their programs is appropriate, because they are a significant group of natural contaminants, many of which are characterized by carcinogenic and mutagenic activity and high toxicity. Among those deserving special attention is besides aflatoxins, fumonisins, zearalenone, patulin and trichothecenes also the secondary metabolite almost everywhere occuring microscopic filamentous fungi genera Aspergillus and Penicillium, ochratoxin A.

Serious health effects of ochratoxin A is related to its high toxic potential, as well as the features that enable long-term persistence in the human body. It is a mycotoxin, which occurs commonly in food and feed in both cold and tropical zones including in the diet which is generally considered to be healthy.

Work was intended to highlight the risks associated to mycotoxins with emphasis on mycotoxins ochratoxin A and also to provide an overview of current knowledge related to this issue. In Slovakia, the content of mycotoxins and thus also of ochratoxin A in selected food commodities and animal feed is routinely monitored within the health surveillance but it is impossible to control either the products manufactured under domestic conditions, or those purchased from commercial network that are already stored at home. Repeated intake of the low contaminant concentrations contributes to the burden of the population also through improper handling and storage of food at risk often due to insufficient knowledge or underestimation of the problem. The occurrence of the xenobiotics in human serum, urine and which is extremely serious even in breast milk show evidence of the human exposure. Levels of contaminants contained in breast milk can be determinated only in the targeted studies with voluntary donors and can not be monitored regularly. It is essential that the nursing mother has to consume exclusively healthy food. Information available to common people is still insufficient and it is therefore necessary to extend education mainly to pregnant women and nursing mothers.

REFERENCES

1. Hudečková H, Rovný I. Public-health approach to hygiene and epidemiology. In: Ághová Ľ, editor. Living conditions and health. Bratislava: Public Health Authority; 2005. p.16-19. (in Slovak)
2. Ministry of Environment SR/Slovak Environmental Agency. The Information system for environmental monitoring. 2007. [online] [cit. 2009-06-21] < http://enviroportal.sk/ism/>
3. Halzlová K. Action Plan for Environment and Health. Enviromagazín 2006; MČ 2: 8-9. (in Slovak)
4. Velíšek J. Food Chemistry. Food Chemistry 1. Tábor: OSSIS; 1999. (in Czech)
5. Ministry of Agriculture, Ministry of Health. Food additives. Title 12. In: Codex Alimentarius SR 2008. (in Slovak)
6. Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. Official Journal of the European Union 1993; L 037: 1-3.
7. Pitt J I. Toxigenic fungi and mycotoxins. British Medical Bulletin 2000, 56 (1): 184-192.
8. European Commission. Rapid Alert System on Food and Feed (RASFF) Annual Report. Luxembourg: Office for Official Publications of the European Communities; 2009.
9. European Commision. Rapid Alert System on Food and Feed (RASFF) Annual Report. Luxembourg: Office for Official Publications of the European Communities; 2008.
10. European Commission. Commission Regulation (EC) No 1881/2006 of 19 December 2006, setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union 2006; L 364: 5-24.

11. European Commission. Commission Regulation (EC) No 165/2010 of 26 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Official Journal of the European Union 2010; L 50: 8-12.
12. European Commission. Commission Regulation (EC) No 105/2010 of 5 February 2010, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. Official Journal of the European Union 2010; L 35: 7-8.
13. Malíř F, Ostrý V. Filamentous fungi (moulds), mycotoxins and human health. Brno: National Centre of Nursing and non-physician disciplines; 2003. (in Czech)
14. Food and Agriculture Organization/World Health Organization. Safety Evaluation of Certain Food Additives and Contaminants prepared by the sixty-eight meeting of JECFA. Geneva: WHO Press; 2008.
15. Bayman P, Baker J L. Ochratoxins: A global perspective. Mycopathologia 2006; 162: 215-223.
16. Ministry of Agriculture, Ministry of Health. Good manufacturing practice. Title 8. In: Codex Alimentarius SR 2000. (in Slovak)
17. Jakušová Ľ, Dostál A. Xenobiotics and their contribution to the pathogenesis of some diseases in childhood. In: Řehulka E, editor. School and Health 21, 3/2008. Brno: Pedagogical Faculty of Masaryk University; 2008.
18. Public Health Authority of the Slovak Republic. Annual Report 2005. Bratislava: UVZ SR; 2006. (in Slovak)
19. Public Health Authority of the Slovak Republic. Annual Report 2006. Bratislava: UVZ SR; 2007. (in Slovak)
20. Public Health Authority of the Slovak Republic. Annual Report 2007. Bratislava: UVZ SR; 2008. (in Slovak)
21. World Health Organization. The International Code of Marketing of Breast-Milk Substitutes: frequently asked questions. Geneva: WHO Press; 2008.
22. American Academy of Pediatrics. Policy statement, organizational principles to guide and define the child health care system and/or improve the health of all children. Breastfeeding and the use of human milk. Pediatrics 2005; 115 (2)
23. Mead M N. Contaminants in HumanMilk. Weighing the Risks against the Benefits of Breastfeeding. Environmental Health Perspectives 2008, 116 (10): 427-434.
24. Hajšlová J, Schulzová V. Comparison of organic and conventional farming. Professional Studies in Chemical Technology in Prague. Department of Agricultural and Food Information: Praha; 2006. (in Czech)
25. Ministry of Health of the Slovak Republic. [online] [cit. 2010-08-27] < http://www.health.gov.sk >
26. Council for Agricultural Sciene and Technology. Task Force Report No. 139, 2003. Mycotoxins: Risk in Plant, Animal and Human Systems. Ames: Iowa USA; 2003.
27. Velíšek J. Food Chemistry. Food Chemistry 3. Tábor: OSSIS; 1999. (in Czech)
28. Šimunek J. Mycotoxins. [online] [cit. 2009-06-21] <http://www.med.muni.cz/prelek/MYKOTW/mtidx.htm> (in Czech)
29. Miraglia M et al. Mycotoxins. In: Nollet LML, editor. Handbook of Food Analysis. Residues and Other Food Component Analysis. Volume 2. New York, Basel: Marcel Dekker, Inc; 2004.
30. Van Egmond HP editor. Mycotoxins In Daiary Products. London and New York: Elsevier applied science; 1989.
31. Peraica M et al. Toxic effects of mycotoxins in humans. Bulletin of the World Health Organization 1999; 77 (9): 754-766.
32. World Health Organization/International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risk to humans. Volume 82. Some traditional herbal medicines, some mycotoxins, naphtalene and styrene. Summary of data reported and evaluation. Lyon: IARC Press; 2002.
33. World Health Organization/International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 56. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. Summary of data reported and evaluation. Lyon: IARC Press; 1993.
34. World Health Organization/International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans. Volume 40. Some naturally occurring and synthetic food components, furocoumarins and ultraviolet radiation. Summary of data reported and evaluation. Lyon: IARC Press; 1999.
35. Ward MDW, Chung YJ, Selgrade MK. Toxicology associated with respiratory exposures to fungi (moulds). In: Salem H, Katz SA. Inhalation toxicology. USA: CRC Press; 2006.
36. FAO,WHO,UNEP. Mycotoxins of growing interest. Ochratoxins. Joint FAO/WHO/UNEP International Conference on mycotoxins, Tunis, Tunisia, 3-6 March 1999.
37. European Food Safety Authority. Opinion of the Scientific Panel on contaminants in the food chain on a request from the commision related to ochratoxin A in food. Question No EFSA-Q-2005-154. The EFSA Journal 2006; 365: 1-56.

Received: September, 7, 2010
Accepted: October, 27, 2010