SPOLEČNOST PRO VĚDU O LABORATORNÍCH ZVÍŘATECH

# Proceedings of the 20th Conference on Laboratory Animals Science

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The 20th Conference on Laboratory Animals Science, organized by the Czech Laboratory Animal Science Association (Společnost pro vědu o laboratorních zvířatech), was held on May 17-19, 2017, in EA Business Hotel, Jihlava, Czech Republic. More than 70 scientists, veterinary experts, representatives of biomedical research organizations, universities and animal welfare authorities participated in the meeting. The presented lectures were focused on laboratory animal welfare and protection, new experimental methods and procedures and the current status of alternative *in vitro* methods as replacement of animal experimentation according to the 3Rs principle. Representatives of animal welfare authorities shared information on the implementation and amendments of regulations concerning the protection of animals used for scientific purposes.

## THE MODEL OF EOSINOPHILIC ESOPHAGITIS IN MODULATION OF VISCERAL AFFERENT PATHWAYS

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Eosinophilic esophagitis (EoE) is a chronic immune inflammatory disorder of the esophagus characterized by symptoms (chest and abdominal pain, dysphagia, heartburn, vomiting, and food impaction) related to esophageal neural dysfunction. Here we aimed to establish a model of acute eosinophilic inflammation in the esophagus for the study of the inflammation-induced changes in the esophageal nerves. We used ovalbumin as an allergen. We evaluated various routes of administration and doses of allergen. The antigen ovalbumin (OVA) was inhaled daily for several days (5, 10, 15 or 20 days, respectively) in the one model or injected into the surgically exposed cervical esophagus in the OVA-sensitized guinea pigs in the second animal model. Middle portion of the esophagus was harvested at various time points (2-5-14 days) and the eosinophils were evaluated in transversal esophageal sections (12 µm) by using Giemsa staining. The expression of neurotrophins (NGF, BDNF)) was evaluated by quantitative RT-PCR. Repeated inhalations of ovalbumin in ovalbumin-sensitized animals failed to evoke consistent eosinophilic infiltration of esophageal mucosa. OVA inhalation revealed only a rare occurrence of eosinophils (1±0 per hpf) in mucosa but OVA injection into the esophagus in sensitized animals was highly effective to induce eosinophilic infiltration of the mucosa resulted in robust eosinophil infiltration of esophageal mucosa (97±24 eosinophils per hpf, n=10, $p<0.05$) on day 2 following the injection. The number of eosinophils was reduced on day 5 (27±16, n=4) and further reduced on day 14 (10±6, n=3), therefore the day 2 was selected for qRT-PCR analysis. On day 2 the expression of BDNF was 2-fold increased in the inflamed compared to control esophagus. We conclude that the injection of allergen into the esophagus of a sensitized guinea pig is an effective approach to induce massive eosinophilic infiltration of esophageal mucosa. This model should prove useful for those studying eosinophilic inflammation-induced changes in the esophagus.

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## THE EFFECT OF PROBIOTIC STRAINS ON *TRICHINELLA SPIRALIS* FECUNDITY *EX VIVO* AND *IN VITRO*

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Based on anti-parasitic effect of probiotic strains used in our study, where strains reduced the parasite burden in experimental trichinellosis, we focused to explain the interactions between the probiotic bacteria and intestinal parasites. Parasite infectivity is a result of the interplay of number of females that develop into adults, their fecundity, the length of their survival in the gut, and the period during which the muscle larvae remain viable. The aim of the study was to investigate the impact of selected probiotic (bacteriocinogenic) strains on female fecundity of *Trichinella spiralis ex vivo* and *in vitro*.
Bacterial strains of different origin (*Enterococcus faecium* EF55, *E. faecium* AL41-CCM8558, *E. faecium* 2019-CCM7420, *E. durans* 26E/7, *Lactobacillus fermentum* AD1-CCM7421, *L. plantarum* 17L/1) were administered daily in dose of $10^9$ cfu/ml in 100 µl and mice were infected with 400 larvae of *T. spiralis* on 7th day of treatment. Female adults of *T. spiralis* were isolated on 5th day post infection and used in fertility test *ex vivo.* The greatest inhibition in female reproductive capacity was caused by strains *E. faecium* AL41-CCM8558 and *E. durans* 26E/7 with 94 % reduction of newborn larvae. The high reduction of female fertility was recorded also after treatment with *L. fermentum* AD1-CCM7421 and *L. plantarum* 17L/1 (about 80 %). A direct impact of bacterial strains on fertility was examined *in vitro* at females isolated from untreated infected mice on 5th day post infection. The highest decrease in number of newborn larve was recorded after incubation of females with *L. fermentum* AD1-CCM7421 (93 %) followed by *E. faecium* AL41-CCM8558, *L. plantarum* 17L/1, *E. faecium* EF55 (about 80 %); and *E. faecium* 2019-CCM7420 and *E. durans* 26E/7 (about 60 %). Strain *E. durans* 26E/7 showed the highest difference between *ex vivo* and *in vitro* results. It could be caused by other factors, e.g. physiology of the intestine or immunomodulatory action of the bacterial strain.

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## VALIDATION OF ALTERNATIVE METHODS FOR THE TOPICAL TOXICITY ASSESSMENT IN THE EU

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Assessment of the topical toxicity belongs to the basic battery of toxicology tests. Due to the enormous efforts from test methods developers, validation centers and regulators, several alternative test methods have been developed, validated and regulatory adopted as potential of full replacements of the rabbit skin and eye irritation/corrosion tests. Skin is the main target of chemicals, pharmaceuticals and consumer products (including cosmetics). The decision on possible unwanted dermal effects, should be based on tiered strategy and a weight of evidence analysis, where all available information are taken into account to provide complete picture about the skin tolerance of an ingredient or product. This includes: information from databases (human and animal data), QSARs, pH considerations and results from *in vitro* tests. Use of animals for the assessment of cosmetics is no longer an option in the EU. To date, there are several formally validated and regulatory accepted alternative methods for assessment of skin corrosion and irritation potential of chemicals. Some of them are based on the use of reconstructed human epidermal models (OECD TG 431, OECD TG 439) others are using *ex vivo* skin of rat (OECD TG 430) or synthetic membrane (OECD TG 435). These assays underwent formal validation and regulatory acceptance at the OECD level and are used mostly for hazard assessment i.e. for classification and labeling purposes. The eye irritation testing *in vitro* is conducted as battery of tests followed by deep analysis of all available data. Following test methods are mostly forming such a testing battery: BCOP (Bovine Corneal Opacity and Permeability – OECD TG 437), ICE (Isolated Chicken Eye – OECD TG 438) and/or STE (Short Term Exposure – OECD TG 491) to distinguish Category 1 chemicals (severely irritating materials). EpiOcular EIT (EpiOcular Eye Irritation Test – OECD TG 492) can be used to distinguish between materials that do not require classification "Not classified" versus "classified" (Category 1/Category 2). To distinguish between Category 1 and Category 2, the following methods are relevant, but not fully regulatory accepted: histopathology in association with the BCOP and ICE, EpiOcular ET-50 (EpiOcular time-to-toxicity test), SMI (Slug Mucosal Irritation). The presentation will provide an overview of test methods applicable for the assessment of skin and ocular toxicity *in vitro* and will discuss testing strategies that could be used to correctly predict the skin and eye irritation/corrosion potential.

## HAZARD IDENTIFICATION IN NEWLY DEVELOPED ANTIMICROBIALS

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Protection of consumer products such as food contact plastics, coatings, cosmetics and textiles against undesirable microbial attack requires innovative agents with a wide spectrum of efficiency, long term stability

and safety of use. These requirements are often difficult to meet when using current biocides and antimicrobials. The aim of the currently performed reseach project ALTERBIO is to identify and select innovative and efficient antimicrobial agents, based on silver nanoparticles and photoactive phthalocyanine derivatives, able of covalent or ionic bond within a polymeric system and without undesirable effects on human health and the environment. Within the project, the promising agents with proved efficient and stable antimicrobial effects were subjected to a battery of toxicological tests to avoid local and systemic toxicity hazard. In compliance with the current European legislation restricting the use of experimental animals the toxicological methods employed in the project comprise exclusively *in vitro* procedures based on cellular and tissue models either of human origin or mimicking human tissues. The tests performed so far showed that AgNPs bound to montmorillonite are not irritant to skin or eye. FC as an ingredient is a skin irritant, phototoxic in the 3T3 phototoxicity test, but not phototoxic in the EpiDerm phototoxicity assay suggesting no penetration through stratum corneum. FC showed mutagenic potential in the reverse mutation test using bacteria in one of four used strains. None of the tested chemicals showed endocrine disruption potential in the XenoScreen YES/YAS Assay for the detection of estrogenic and androgenic endocrine disruptors. Further tests on acute toxicity, sensitization and skin penetration will follow in order to establish toxicological profiles of the novel antimicrobials.

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## SAFE ANESTHESIA OF RATS FOR EXACTING SURGERIES

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The combination of ketamine/xylazine is considered a gold standard to anesthetize most animals, including rats. Nevertheless, general anesthesia (GA) induced with this combination has short duration (surgical anesthesia 20-40 min) and can be associated with severe complications, including respiratory depression and difficult reversion which makes it inappropriate for some demanding and time-consuming surgeries. The aim of this study was to evaluate a combination of propofol/medetomidine/nalbuphine (PMN) in rats undergoing a vascular surgery. Total 12 Long-Evans rats (both sexes, age 7-12 months) were anesthetized by intraperitoneal bolus of propofol (100 mg/kg), medetomidine (0.1 mg/kg) and nalbuphine (0.1 mg/kg). The animals were continually supplied with oxygen *via* face mask and subjected to the one-side, end-to-end anastomosis of *arteria carotis*. During the surgery animals were monitored using pulse oximetry; depth of anesthesia was verified by pedal withdrawal and corneal reflexes. After 40-60 min, reduced dose (20 % of the initial dose) of anesthetic mixture was administered again. The additional reduced doses were given as necessary, usually each 20-30 min. After finishing the surgery, atipamezole (0.5 mg/kg IM) was administered to induce reversion. Intraperitoneal administration of PMN induced anesthesia within 5-10 min achieving surgical anesthesia within next 5-10 min. The surgical anesthesia lasted for 35-45 min without loss of corneal reflex and it was able to extend it up to 150 min by administration of reduced doses of PMN without any harmful effects on vital functions. The surgical stage of GA was characterized only by reversible decrease in pulse rate below 200 bpm which showed reliable marker of the depth of GA. Administration of atipamezole (0.5 mg/kg IM) led to rapid reversion (5-10 min) of animals. Intraperitoneal PMN showed to be reliable and safe reversible anesthesia in rats undergoing time consuming and exacting vascular surgeries.

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## GENDER DIMORPHISM IN COUGH RESPONSE – A NEED OF NEW MODEL FOR BASIC COUGH RESEARCH

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Epidemiological studies indicate that overwhelming majority of patients treated on specialized cough clinics for chronic cough are postmenopausal women. Moreover, homogeneity amongst these patients worldwide suggests a distinct clinical entity, etiopathogenesis of which remains unclear. In basic cough research, only models utilizing male animals are used and upon further investigation why, the literature databases fail to provide an answer. Therefore, we decided to characterize cough response in female guinea pigs, which could provide a model suitable for study of hormonal influences on cough physiology. First experiment utilized Dunkin-Hartley guinea pigs (8 females and 9 males – control group) which were repeatedly exposed to aerosols of 0.4 M citric acid, 50 μM capsaicin and distilled water for 10 min in whole-body plethysmograph. Airflow traces and sounds were simultaneously recorded. Aim of the second experiment was the construction of dose-response curves for citric acid and capsaicin utilizing another 5 female and 5 male guinea pigs. Average number of coughs to citric acid in females (12.5±3.5 – 24.5±6.5 – 18.5±6) did not differ from males (13±5.5 – 18±2.5 – 19±4). Number of coughs to capsaicin did not differ between females (15.5±3 – 16±2.5 – 15±6) and males (8±2 – 10±2.5 – 14±6), neither did number of coughs in response to distilled water (females: 5±2 – 7.5±3.5 – 5.5±3.75; males: 5±2 – 5±3 – 6±2). Cough latency showed similar tendencies. Dose-response curves did not differ significantly between genders. Based on our results we conclude that cough response obtained in naïve female guinea pigs over time is relatively stable and comparable to that of male guinea pigs, which is documented by similar cough response and its variability. However, these experiments have to be conducted in sensitized animals, because hormonal influences can be more evident in pathologic conditions.

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## TRANSCRIPTIONS OF NLRP3 INFLAMMASOME, NOD1 AND NOD2, AND INDUCTIONS OF INFLAMMATORY CYTOKINES IL-1 BETA, IL-10, AND IL-12/23 P40 IN THE ILEUM OF GNOTOBIOTIC PIGLETS

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The gastrointestinal tract harbors a majority of body microbiota. Gnotobiotic animals are derived germ-free. Possible protective effects of *E. coli* Nissle (EcN) and *Lactobacillus amylovorus* (LA) against infection with *Salmonella* Typhimurium (ST) in gnotobiotic piglets were compared. Germ-free piglets (GF) were colonized with EcN or LA four hours after hysterectomy. A part of one-week-old piglets was infected with ST (ST, LA+ST and EcN+ST) for 24 h. Bacterial colonization of the intestine (CFU counting), secretion of IL-1 beta, IL-12/23 p40, and IL-10 (xMAP technology) and transcriptions of NLRP3 inflammasome, NOD1, and NOD2 (RT-qPCR) were monitored. The piglets infected with ST suffered from bacteremia, somnolence, anorexia, and diarrhea and showed increased levels of IL-1 beta, IL-12/23 p40, and IL-10. The cytokine levels were not decreased in the group of piglets preliminary associated with LA (LA+ST) but the piglets associated with EcN (EcN+ST) had them suppressed. Transcriptions of NLRP3 inflammasome, NOD1 and NOD2 were increased in ST and LA+ST groups but in EcN+ST group were comparable with GF or monoassociated LA and EcN groups. The preliminary association of GF piglets with EcN suppressed symptoms of *Salmonella*-induced gastroenteritis and levels of the inflammatory mediators but LA suppressed it partially only. Mechanisms of the suppression will be studied in the future.

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## DECREASE OF *SALMONELLA TYPHIMURIUM RFAL* MUTANT VIRULENCE FOR GNOTOBIOTIC PIGLETS

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A lipopolysaccharide (LPS) is a part of a cell wall of Gram-negative bacteria and their virulence factor. *Salmonella typhimurium* causes self-limiting gastroenteritis in the human and the pig. HMGB1 was found to be a marker of a severity of enteric infection and its neutralization can ameliorate consequences of infections. One-week-old hysterectomy-derived germ-free (GF) piglets were infected with *S. typhimurium* (ST) or its isogenic *rfaL* mutant for 24 h. *Salmonella* CFU counting, HMGB1, IL-8 and TNF-alpha (ELISA) and transcriptions of HMGB1 receptors RAGE, TLR2, TLR4, and TLR9 (RT-qPCR) in the ileum were evaluated. Both ST and its *rfaL* mutant successfully colonized the ileum of the gnotobiotic piglets. Intestinal levels of HMGB1, IL-8, and TNF-alpha levels were increased in *Salmonella*-infected piglet but lower values were found in the *rfaL* group. While transcription of RAGE and TLR9 were decreased in the presence of both *Salmonella*-infected groups TLR2 and TLR4 were increased. A rough LPS of *rfaL* mutant induced lower levels of HMGB1, IL-8, and TNF-alpha in the ileum of the gnotobiotic piglets. The transcriptions of HMGB1 receptors showed different trends. Detections of soluble decoy receptors RAGE, TLR2, TLR4, and TLR9 can contribute to clarifying of the importance of the HMGB1 regulatory role in enteric infections. Other experiments will be necessary to evaluate *rfaL S. typhimurium* mutant as a live inducer of an innate immune mechanism for the protection against infection with virulent wild strain.

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## NON-INVASIVE AND TELEMETRIC METHODS FOR MEASUREMENT OF BLOOD PRESSURE AND ECG IN RODENTS

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Arterial blood pressure and heart rate are basic parameters, which are assessed in order to evaluate function of the heart. The golden standard for non-invasive measurement of blood pressure in human medicine is auscultatory method. However, oscilometric method is more feasible in case of rodents. The golden standard for proper assessment of heart rate is ECG recording. Both blood pressure measurement and ECG recording are dependent on compliance of examined subject. For non-invasive measurement of blood pressure in laboratory rat, CODA 8 system (Kent Scientific, France) was used. The measurement is based on oscilometric method using tail cuff. During the measurement, animal was fixed in plastic chamber to restrict its movements and placed on a heated pad. Five measuring cycles were performed. The value of systolic and diastolic pressure was defined as a mean calculated from valid measuring cycles in each measurement. For non-invasive ECG recording, ecgTUNNEL (Emka Technologies, France) was used. During the recording, animal was fixed on measuring pad. At least 1 min lasting ECG record was recorded in 3 limb leads for each animal and then analyzed offline. Analysis was performed on ecgAUTO software (v3.3.3.10, Emca Technologies, France). Periods with motion artifacts were excluded from analysis. For telemetric monitoring of blood pressure and ECG, Stellar telemetry system (TSE Systems, USA) was used. Transponder was implanted into abdominal cavity. Catheter for direct measurement of blood pressure was inserted into the aorta. Two subcutaneous electrodes for ECG recording were placed on the chest of the animal. Record lasting 20 s was recorded each 15 min during 24 h. Records were automatically analysed in AcqKnowledge software (Biopac Systems, Inc., USA). In animal studies, measurement of blood pressure and ECG recording are usually provided under general anesthesia. However, most drugs used for premedication and anesthesia affect cardiac functions. Recently, devices for non-invasive measurement of blood pressure and ECG recording in conscious animals were introduced. Such devices enable measurement without effects of anesthetics. However, the stress from manipulation and restriction of the movements during measurement may affect the results. Careful handling in order to habituate animals to measuring procedure is crucial. For long-lasting studies, telemetric measurement might represent better alternative.

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## MICROBIAL MONITORING OF LABORATORY MICE ENVIRONMENTS

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Microbial monitoring of animal environments is a part of biosecurity introduced in our breeding and use facility. The goal of this lecture is to present our achieved results. We concentrated on the state of microbial contamination in cages of mice and their wire-bar lids before and after sanitation was performed. (Methods) Healthy adult laboratory mice (strain BALB/c, C57BL/6, DBA x BL/6, ICR) with defined microbial and genetic profiles were kept in thermo-plastic cages (size 20 x 10 x 15 cm). The sanitation procedure was as follows: conventional system – bedding, cages and wire-bar lids were changed once a week. Dirty cages and their accessories were hand washed in detergent solution, immersed in freshly-prepared disinfectant solution, rinsed in clean water and left to drain. Barrier system – dirty cages and their accessories were machine washed in freshly-prepared detergent solution, rinsed in clean water and then autoclaved. Animal rooms in both systems of breeding were cleaned daily and disinfected by the same procedures using freshly-prepared disinfectant solutions, which were regularly changed. Microbial monitoring of the cage sanitation procedures was performed on a random selection of cages and wire-bar lids. Sterile swabs were taken from inner surface cages and wire-bar lids (sampling area about 25 $cm^2$) before and after sanitation. Smears were transfered on different cultivation media (Trypticase Soy Agar, Sabouraud Agar, Baird-Paker agar, Endo Agar). After an incubation period, all colonies were counted and representative colonies were picked for Gram staining, biochemical studies conducted to indentify the genera of bacteria. The microbiological examination of cage samples after sanitation did not reveal presence of bacteria or fungi; however, mild bacterial and fungal contamination of wire-bar lids samples was found. Aforementioned contamination is explained by the fact that the sampling of wire-bar lids was not performed immediately after sanitation. According to general accepted cirteria (bacterial and fungi colonies count was <5 colonies per plate, no incidence of Gram negative rods and Gram positive cocci), it is possible to consider our sanitation procedures as excellent. Microbiological examination of cages and wire-bar lids samples before their sanitation revealed microbial contamination, without presence of bacteria of genera *Enterobacteriaceae* and *Staphylococcus aureus*. The isolated bacteria (*Bacillus sp., Enterococcus casseliflavus, Micrococcus sp., Staphylococcus sciuri, ssp. Lentus* and others) belong among bacteria present in intestinal microflora of adult, heathy mice and also among contaminants of the animal environment. Based on the achieved results it is possible to conclude that microbial monitoring of animal environments as a part of biosecurity measures contributes to preservation of required health quality of our laboratory mice.

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## CURRENT APPLICATION OF ALTERNATIVE TOXICOLOGICAL METHODS *IN VITRO* FOR HAZARD AND RISK ASSESSMENT

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Directive 2010/63/EU on the protection of animals used for scientific purposes emphasizes reduction of animals in tests, refinement limiting their suffering and replacement of animal testing by alternative *in vitro* methods utilizing cells and tissues of human origin. According to the Directive, the member states are obliged to ensure support of alternative methods and dissemination of information. Ministry of Agriculture of the Czech Republic has nominated as a contact point to provide advice on the regulatory relevance and suitability of alternative approaches and a specialized laboratory for validation studies the National Institute of Public Health (NIPH) in Prague, namely the National Reference Laboratory for Experimental Immunotoxicology. Following a selection procedure, the nominated laboratory at NIPH has been appointed into the network of national reference laboratories (NETVAL). Due to the joint efforts of validation centers worldwide (ECVAM, ICCVAM, JaCVAM), a number of *in vitro* methods have been validated and accepted into the legal system in recent years. As an example, the Draize test for skin and eye irritation testing has been fully replaced by methods using cell and tissue models. The number of animals used for acute toxicity testing may be significantly reduced when the 3T3 NRU cytotoxicity test to identify substances not requiring classification for acute oral toxicity (cut-off $LD_{50}$>2,000 mg/kg b.w.), is used. Regarding endocrine disruptors, *in vitro* transactivation assays based on human cells or yeast strains are applied for screening purposes. Currently, an *in vitro* test battery to predict skin sensitizers based on key events of sensitization (protein reactivity, keratinocyte responses, dendritic cells activation and T-cell proliferation) is being finalized. For classification, a strategy is being assessed based on a combination of methods detecting interaction of potential haptens with peptides (Direct Peptide Reactivity Assay), keratinocyte responses (KeratinoSens assay measuring activation of cytoprotective genes in keratinocytes), and dendritic cell activation (MUSST assay employing human myeloid U937 cells and h-CLAT using human monocytic leukemia cell line THP-1). The progressive methods are being continually implemented at NIPH.

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## VERIFICATION OF ANTIBIOTIC LEVELS IN LUNG TISSUE DURING SURGERY IN PRECLINICAL EXPERIMENT (ANIMAL MODEL - PIG)

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Minithoracotomy in cardiac and thoracic surgery is more and more freguent, safe and effective procedure being equivalent to the traditional medial sternotomy. These operations are performed using the technique called one-lung ventilation by two-lumen endotracheal intubation in order to fully expose to the operating field. The aim of this pilot study was to monitor the impact of selective ventilation and atelectasis of lung on tissue concentrations of prophylactic antibiotic (cefuroxim) in peripheral lung tissue during surgery by interstitial microdialysis in preclinical experiment. These antibiotic concentrations were compared with plasma concentrations of cefuroxim. After introduction of anestethesia a double-lumen endotracheal cannula with blocker was inserted and the left main bronchus was obturated. Than bilateral minithoracotomy with explosure of lung was performed and special microdialysis probes (CMA) were inserted into the lung tissue and continuously perfused. Cefuroxim in dose 40 $mg.kg^{-1}$ of weight was administered intravenously and microdialysis was started. Samples of dialysates and samples of blood were collected in the same intervals, each 30 min to the end of surgery. Concentrations of cefuroxim were determined by the fluid chromatography method and corrected by *in vivo* recoveries of the microdialysis probes. The evaluated results showed to us the different concentrations of the antibiotics in the monitored compartments at times up to 180 min from the start of the surgical intervention. From this time, antibiotic concentrations in blood plasma as well as blocked and ventilated lung were decreased under the MIC determined for $G^-$ bacteria. At 210 min from the start of the experiment, the antibiotic concentration is higher by 0.5 $mg.l^{-1}$ from the borderline MIC for $G^+$ bacteria. For the remainder time of the experiment, the antibiotic level does not meet the prophylactic concentration in blood plasma and lung tissue.
This method brought new information about administering drugs in lung tissue during the surgery with atelectasis of the lung and about blood plasma antibiotic concentration. The limitation of this pilot study is the small number of animals. To verify these results in clinical human practice it is necessary to perform further studies.

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