CHARLES UNIVERSITY IN PRAGUE
FACULTY OF MEDICINE IN HRADEC KRÁLOVÉ
DEPARTMENT OF MEDICAL CHEMISTRY AND BIOCHEMISTRY

MICRODIALYSIS IN THE RAT GUT

- A BIOCHEMICAL STUDY OF NUTRITIONAL BLOOD FLOW AND MUCOSAL BARRIER

MUDR. NORBERT CIBIČEK

DOCTORAL THESIS

HRADEC KRÁLOVÉ 2008

Microdialysis in the Rat Gut

- a Biochemical Study of Nutritional Blood Flow and Mucosal Barrier

a doctoral thesis by

MUDr. Norbert Cibiček

Supervisors
Doc. MUDr. Pavel Živný, CSc.
Prof. MUDr. Zdeněk Zadák, CSc.

Opponents
Prof. MUDr. RNDr. Vilím Šimánek, DrSc.
Prof. MUDr. Antonín Jabor, CSc.

Hradec Králové 2008

O LORD, how manifold are thy works! In wisdom hast thou made them all: the earth is full of thy riches.

(Bible, Psalm 104:24)

To Ľubica, Albert and Elena

CONTENTS

1. **ABSTRACT** 5
2. **SOUHRN V ČEŠTINĚ** 6
3. **ORIGINAL STUDIES** 7
4. **ABBREVIATIONS** 8
5. **INTRODUCTION** 9
   5.1. Microcirculation, nitric oxide and gut barrier performance 9
   5.2. Metabolic, haemodynamic and mucosal permeability monitoring 11
6. **AIMS** 15
7. **MATERIALS AND METHODS** 16
   7.1. The in vivo microdialysis technique 16
   7.2. Substances used 18
   7.3. Animal models 18
   7.4. Experimental protocols 20
   7.5. Probe performance stability and calibration procedures 20
   7.6. Nutritive blood flow measurements 21
   7.7. Laboratory analyses 21
   7.8. Barier integrity determination 22
   7.9. Light microscopy 23
   7.10. Data analysis 23
8. **RESULTS** 24
   8.1. Study I 24
   8.2. Study II 24
   8.3. Study III 25
9. **GENERAL DISCUSSION** 26
   9.1. Methodological aspects 26
   9.2. Ischemic preconditioning 28
   9.3. Effects of caffeine 29
10. **CONCLUSIONS** 32
11. **FUTURE PERSPECTIVES** 33
12. **ACKNOWLEDGEMENTS** 34
13. **REFERENCES** 35
14. **APPENDIX** 39
   14.1. Fulltexts of original studies I-III 39
   14.2. The author 62

1. ABSTRACT

BACKGROUND

Microdialysis has been used to measure blood perfusion in almost all tissues but data from rat gut submucosa are missing. Lithium, previously suggested as a suitable flow marker has not been validated yet. Coffee impairs gastric mucosal barrier, but the effect of caffeine on gastric blood flow requires elucidation. All established *in vivo* methods of mucosal permeability assessment necessitate the functional involvement of bloodstream – the application of microdialysis as an alternative has not yet been tested.

AIMS

The aims were: firstly, to investigate the applicability of lithium microdialysis for monitoring blood flow changes due to ischemia/reperfusion in rat stomach and colon submucosa and to assess the systemic effects on selected enzymes and nitric oxide; secondly, to evaluate local impact of caffeine on gastric submucosal microcirculation, nitric oxide release and its systemic effect on oxidative stress-related marker malondialdehyde; and finally, to develop a microdialysis method of continuous mucosal permeability measurement in rat descending colon.

MATERIALS AND METHODS

Gastric and colon submucosal microdialysis technique plus colon single-pass luminal perfusion were used in pentobarbital-anaesthetized rats. As microdialysis perfusate, lithium, ethanol, Ringer or saline solution-containg media were applied. Luminal perfusate contained ^{51}Cr-EDTA-enriched Ringer solution with/out ethanol. Caffeine was applied i.p. in doses 1, 10 and 50 mg kg^{-1} b. wt. Ischemia and reperfusion were accomplished by temporary celiac artery occlusion.

RESULTS

Lithium microdialysis indicated a decrease in blood perfusion during celiac artery occlusion in stomach. During reperfusion, the ischemic stomachs showed a restoration of blood perfusion in contrast to the preconditioned ones. Colon microcirculation remained unaltered as did studied serum analytes (study I). Caffeine administration did not affect gastric submucosal microcirculation, nitric oxide production or serum malondialdehyde (study II). Colon mucosa exposed to ethanol presented with profound macro- and microscopical changes associated with increased tracer permeability (study III).

CONCLUSIONS

The aforementioned microdialysis and mucosal permeability techniques were successfully tested and found applicable in given experimental settings. Caffeine was found not to interfere with submucosal blood perfusion, malondialdehyde and Ca^{2+}-independent nitric oxide synthesis. Further studies are needed to account for the lack of gastric protective blood flow enhancement due to ischemic preconditioning and to explore possible mechanisms behind the effects of caffeine on gastric physiology in relation to irritant effects of coffee.

KEY WORDS

Microdialysis ● Blood Perfusion ● Lithium ● Gut ● Nitric Oxide ● Ischemic Preconditioning ● Caffeine ● Barrier ● Permeability

2. SOUHRN V ČEŠTINĚ

ÚVOD

Přestože mikrodialýzy bylo užito pro měření krevního průtoku v mnohých tkáních, data ze žaludeční submukózy potkana doposud chybí. V předchozí studii bylo jako nový marker průtoku navrženo lithium, které však zatím nebylo dostatečně validováno. Konzumace kávy poškozuje sliżniční bariéru žaludku, která závisí na přiměřeném krevním zásobení. Není však jasné, do jaké míry může být krevní průtok v žaludku ovlivněn kofeinem. Dosavadní metody měření střevní propustnosti vyžadovaly využití, resp. ovlivnění systémové cirkulace – mikrodialýza jako možná alternativa zatím nebyla v této aplikaci odzkoušena.

CÍLE

Cílem bylo zaprvé: zjistit využitelnost lithia při monitoraci změn krevního průtoku v submukóze žaludku a střeva daných ischemií/reperfúzí pomocí mikrodialýzy a zhodnotit systémové projevy pomocí aktivit vybraných enzymů a tvorby oxidu dusnatého; zadruhé: studovat lokální vliv kofeinu na mikrocirkulaci a tvorbu oxidu dusnatého v žaludeční submukóze a systémový vliv na oxidativní stres vyšetřením malondialdehydu; a konečně: zavést novou metodu kontinuálního měření slizniční propustnosti v sestupném tračníku potkana s využitím mikrodialýzy.

MATERIÁL A METODIKA

Bylo užito techniky žaludeční a střevní submukózní mikrodialýzy a single-pass luminální perfúze sestupného tračníku potkanů v celkové pentobarbitalové anestezii. Jako mikrodialyzační perfuzát byly použity roztoky obsahující lithium, ethanol, Ringerův a fyziologický roztok. Perfuzát střevního lumen obsahoval Ringerův roztok obohacený ^{51}Cr-EDTA s nebo bez přidání ethanolu. Kofein byl aplikovaný i.p. v dávkách 1, 10 and 50 mg kg^{-1} těl. hm. Ischemie a reperfúze bylo dosaženo dočasným uzávěrem *truncus coeliacus*.

VÝSLEDKY

Mikrodialýza s využitím lithia jakožto flow-markeru naznačila snížení krevní perfúze žaludeční submukózy během uzávěru *tr. coeliacus*. V reperfúzní fázi bylo v žaludcích bez ischemické přípravy dosaženo navrácení krevní perfúze k původním hodnotám na rozdíl od těch, u kterých tato příprava proběhla. Microcirkulace v sestupném tračníku zůstala beze změn podobně jako vyšetřené sérové analyty (studie I). Podání kofeinu nevedlo k významným změnám žaludeční submukózní mikrocirkulace, produkce oxidu dusnatého nebo sérového malondialdehydu (studie II). Sliznice sestupného tračníku vystavena působení ethanolu podlehla značným makro- i mikroskopickým změnám, které byly spojeny se zvýšením propustnosti pro ^{51}Cr-EDTA (studie III).

ZÁVĚR

Výše zmíněné experimentální techniky žaludeční a střevní mikrodialýzy včetně propustnosti střevní sliznice byly úspěšně zavedeny. Nebylo potvrzeno ochranné zvýšení krevního průtoku v žaludku v důsledku ischemické přípravy. Kofein neovlivňuje krevní průtok v submukóze žaludku, tvorbu malondialdehydu ani na Ca^{2+} nezávislou syntézu oxidu dusnatého. K objasnění role kofeinu v kontextu dráždivých účinků kávy na sliznici žaludku budou potřebné další studie.

KLÍČOVÁ SLOVA

Mikrodialýza ● Krevní průtok ● Lithium ● Žaludek a střevo ● Oxid dusnatý ● Ischemická příprava ● Kofein ● Slizniční bariéra ● Propustnost

3. ORIGINAL STUDIES

This thesis is based upon the following studies, which will be referred to in the text by their Roman numerals:

I. CIBIČEK N, MIČUDA S, CHLÁDEK J, ŽIVNÝ P, ZADÁK Z, ČERMÁKOVÁ E ET AL.
Lithium microdialysis and its use for monitoring of stomach and colon submucosal blood perfusion – a pilot study using ischemic preconditioning in rats.
Acta Medica (Hradec Králové) **2006**;49(4):227-231.

II. CIBIČEK N, ŽIVNÁ H, CIBIČEK J, ČERMÁKOVÁ E, VOŘÍŠEK V, MALÁKOVÁ J ET AL.
Caffeine does not modulate nutritive blood flow to rat gastric submucosa – a microdialysis study.
Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **2008**;152(1):83-90.

III. CIBIČEK N, ŽIVNÁ H, ZADÁK Z, KULÍŘ J, ČERMÁKOVÁ E, PALIČKA V.
Colon submucosal microdialysis: a novel in vivo approach in barrier function assessment - a pilot study in rats.
Physiol Res **2007**;56(5):611-617.

4. ABBREVIATIONS

ANOVA – analysis of variance
ALT – alaninaminotransferase
AMYL – amylase
AR – absolute recovery (of a microdialysis probe)
AST – aspartataminotransferase
CAO – celiac artery occlusion
cGMP – cyclic guanosine monophosphate
CHE – cholinesterase
CM – control medium (as used in study III)
cpm – counts per minute
CT – computer tomography
ECF – extracellular fluid
EDTA – ethylenediamine tetra-acetate (^{51}Cr-EDTA – ^{51}Cr-labelled EDTA)
ELISA – enzyme-linked immunosorbent assay
EM – ethanol medium (as used in study III)
GC-MS – gas chromatography (gas chromatograph) associated with MS detection
HE – hematoxylin-eosin
HPLC – high performance liquid chromatography
IP – (gastro-) intestinal permeability
IPC – ischemic preconditioning
IR – ischemia/reperfusion
LDF – laser doppler flowmetry
LDH – lactatdehydrogenase
LIP – lipase
LM – lithium microdialysis
L-NAME – N-ω L-arginine methyl ester
LPS – lipopolysaccharide
MDA – malondialdehyde
MS – mass spectrometry (mass spectrometer)
NMR – nuclear magnetic resonance
NO – nitric oxide
NOS – nitric oxide synthase (nNOS – neuronal, eNOS – endothelial, iNOS – inducible, cNOS – constitutive NOS isoform)
PET – positron emission tomography
rIPC – remote ischemic preconditioning
RR – relative recovery (of a microdialysis probe)
SEM – standard error of mean
TBA – thiobarbituric acid
TS – test solution (as used in IP tests)
ULS – doppler-ultrasound

5. INTRODUCTION

Over the past three decades, the significance of gut either for the maintenance of whole body homeostasis on one hand or as a motor of disease on the other has been largely distinguished. Gut mucosa with its enormous area for absorption of nutrients is in parallel a life-long battlefield with the external world where keeping away the toxins and fighting the pathogens are everyday routine of the epithelial and immunocompetent cells. Therefore, intact mucosal barrier and functional defence mechanisms such as adequate blood flow, motility, production of mucus and immunoglobulin A secretion are crucial not only for well-being of gut *per se*, but are of major importance for the entire organism. In these aspects, the study of mucosal barrier and its role in health and disease attracts much attention.

Owing to its vulnerability, the gut necessitates multifaceted protection (Tab. 1). Mucosal barrier function may be injured in numerous manners: by inadequate blood perfusion, lack of luminal and blood nutrients, trauma, chemical and immunological irritants, biological agents with associated toxins and stress of physical or mental nature. The impact on the afflicted individual may hence vary from mere gastrointestinal discomfort (dyspepsia), diarrhoea and bleeding to severe states resulting in multiple organ failure (Doig *et al.* 1998). Even though the question of prevention and treatment of diseased gut has previously been addressed with various experimental modalities, the employment of microdialysis – a novel and advantageous method of tissue chemistry and blood flow monitoring – to a large extent remains a challenge.

Gut barrier *sensu stricto*	Enterocytes *per se* and their connection by tight junctions
Immunologic components	Immunoglobulin A (its production and secretion into the bile and on the luminal surface of the enterocytes)
Non-immunologic components	The presence of HCl and pepsin in the stomach lumen
	Normal gut peristalsis
	The presence of pancreatic and intestinal proteases in the chyme
	Normal intestinal mucus production
	Unstirred water layer on the epithelial surface
	Adequate mucosal blood perfusion

Tab. 1. *The components of the gut barrier sensu lato (according to Kohout 2002)*

5.1. MICROCIRCULATION, NITRIC OXIDE AND GUT BARRIER PERFORMANCE

Ischemia-reperfusion injury and ischemic preconditioning

Ischemia/reperfusion (IR) injury of the gut is an important factor associated with high morbidity and mortality in both surgical and trauma patients (Koike *et al.* 1993). The underlying causes typically include surgical interventions (abdominal aortic aneurism surgery, cardiopulmonary bypass and intestinal transplantation), disease states (strangulated hernias, neonatal necrotizing enterocolitis) or shock (sepsis or hypovolemia). Interruption of blood supply results in ischemic injury which rapidly damages metabolically active or otherwise predisposed tissues. Intestinal mucosa, due to its vascular anatomy creating a counter-current exchange system within the villi, is very much prone to this type of damage. Paradoxically, restoration of blood flow to ischemic tissue initiates a cascade of events that may lead to additional injury known as reperfusion injury, which is reactive oxygen and nitrogen species-mediated and often exceeds the original ischemic insult. It is widely recognized that the microcirculation, particularly the endothelial cells are very susceptible to deleterious consequences of IR injury. Indeed, IR-induced microvascular dysfunction has been described in most organs as a potentially serious problem associated with molecular and biochemical changes characteristic for acute inflammatory response (Granger 1999, Grisham *et al.* 1998). The intensity of this immunological reaction can be of such a grade that may affect other distant organs leading to systemic inflammatory response syndrome and multiple organ failure. Therefore, effective prevention of gut microvascular dysfunction accompanying major operations would be of high value.

Stimulated by the original study of Murry *et al.* (Murry *et al.* 1986), many authors have congruently confirmed that functional reserves or viability of the splanchnic organs exposed to ischemic insult can be positively affected by ischemic preconditioning (IPC) taking place prior to sustained devastating ischemia (McCallion *et al.* 2000, Mallick *et al.* 2005, Koti *et al.* 2002, Dembinski *et al.* 2003). IPC refers to a process by which a (series of) brief ischemic episode(s) confers a state of protection against injury evoked by subsequent prolonged IR. The time window of IPC is characterized by a biphasic pattern. The acute or early phase, being protein-independent, acts immediately following non-lethal ischemia and lasts for 2-3 h, whilst delayed IPC starts at 24 h until 72 h after brief arterial closure and requires *de novo* protein synthesis preceded by genomic activation (Post and Heusch 2002, Carden and Granger 2000). Depending on the relationship between the preconditioned tissue and the tissue subjected to severe ischemia, the efficacy of this phenomenon can be examined with respect to one specific organ, or considering other organs or organ systems at a distance. The former differentiates between local i.e. classic or conventional (Murry *et al.* 1986) and regional IPC (Przyklenk *et al.* 1993), whereas the latter defines an inter-organ or remote IPC, rIPC (Liem *et al.* 2002). At present, besides a number of extraabdominal organs, published papers document examples of IPC in the liver (Cavalieri *et al.* 2002), pancreas (Dembinski *et al.* 2003), small intestine (Hotter *et al.* 1996) and stomach (Pajdo *et al.* 2001). Remote IPC in colon has not earned much interest, so far. Even though the exact mechanism of IPC has not been fully elucidated until recently, an array of neurohumoral mediator pathways have been proposed, where nitric oxide (NO) plays a central role (Peralta *et al.* 2003).

Mucosal barrier integrity and nitric oxide paradox

NO is a free-radical molecule with dichotomous character participating in both maintenance and derangement of gut mucosal homeostasis. Physiological levels of NO were found to be essential in mucosal integrity maintenance (Alican and Kubes 1996). Blockage of endogenous NO synthesis may aggravate gut barrier impairment resulting from IR (Kubes 1993), platelet activating factor (MacKendrick *et al.* 1993) or endotoxin administration (Hutcheson *et al.* 1990), whereas NO-donors ameliorate gut mucosal damage in similar models (Lopez-Belmonte *et al.* 1993, Payne and Kubes 1993). Besides vasodilation, the proposed mechanisms of beneficial action of NO include the prevention of leukocyte adhesion and secretion (Niu *et al.* 1994), decreased mast cell degranulation (Kubes *et al.* 1993), reduced platelet adherence and secretion, stimulation of mucus secretion by gastric epithelial cells and increased gastric mucus gel thickness (Brown *et al.* 1992). Furthermore, antioxidant role of NO in the intestinal epithelium was confirmed *in vitro* (Chamulitrat 1998) and suggested also *in vivo* (Szlachcic *et al.* 2001).

On the other hand, excessive amounts of NO produced by local infusion of exogenous NO donors produce macroscopic and morphologic mucosal injury (Lopez-Belmonte *et al.* 1993) and exacerbate gastric damage from luminal irritants like bile or ethanol (Helmer *et al.* 2002). On the cellular level, NO donors promote actin-based cytoskeletal derangement and dilate tight junctions whereby permeability of Caco-2 epithelial monolayers is elevated (Salzman *et al.* 1995, Han *et al.* 2003) and viability of rat gastrointestinal mucosal cells dwindles (Tepperman *et al.* 1994, Tripp and Tepperman 1996). Even though the mechanisms behind these cytopathic effects of NO have not been fully explained yet, it is probable that NO *per se* is not the toxic moiety. The likely candidates are rather the products of its reactions with superoxide ($O_2^{-\cdot}$), namely peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH) (Huie and Padmaja 1993, Menconi *et al.* 1998). These substances may be responsible for the initial steps in the collapse of mucosal barrier function – inhibition of mitochondrial respiration, diminished ATP synthesis (Fink 1997, Gross and Wolin 1995) and ATP-dependent Na^+/K^+ channel failure (Sugi *et al.* 2001).

NO is produced by nitric oxide synthase (NOS) in a wide variety of cell types from the terminal guanidine nitrogen atom of L-arginine giving L-citrulline as a second product. Three isoforms of NOS have been recognized – neuronal (nNOS, type I), endothelial (eNOS, type III) and inducible (iNOS, type II). The first two, Ca^{2+}-dependent, incessantly produce moderate amounts of NO and are constitutively expressed wherefore they are classified as constituive (cNOS). In contrast, the last type lacks Ca^{2+}-dependency, necessitates *de novo* protein synthesis to release vast amounts of NO in response to cytokines or lipopolysaccharide (LPS) and is thus referred to as inducible (Stuehr and Griffith 1992). Regulation of physiologic functions and protective roles have been attributed to NO originating from constitutive isoforms (Whittle *et al.* 1990). In the gut, eNOS is bound predominantly to plasma membranes of the endothelium of submucosal blood vessels with responsibility for mucosal

blood flow maintenance. Neuronal NOS is principally a cytosolic enzyme localized mainly in the superficial epithelial cells, where it represents the most important generator of NO (Price *et al.* 1996, Price and Hanson 1998). On the contrary, iNOS, found in the cytosol of several cell types, has been considered pathologic, since it leads to a reduction of rat intestinal epithelial cell viability (Tepperman *et al.* 1993) and enhancement of lesion formation in many experimental models of mucosal irritation and stress (Nishida *et al.* 1997, Tanaka *et al.* 1999, Mercer *et al.* 1998, Ferraz *et al.* 1997). However, the debate on iNOS is still open as its increased expression was found to have also gastroprotective consequences (Barrachina *et al.* 1995, Franco and Doria 1998, Konturek *et al.* 1998, Tepperman and Soper 1994, Mercer *et al.* 1998).

Caffeine and gastric physiology

The maintenance of gastric mucosal barrier integrity is dependent on the balance between aggressive and protective factors represented by hydrochloric acid on one side and adequate mucosal blood flow with sufficient mucus production on the other. Caffeine, the most consumed stimulant drug worldwide, has long been known for its powerful acid secretagogue ability (Alonso and Harris 1965) and suspected from causing mucosal hypoperfusion due to (micro)vascular impairment (Roth and Ivy 1945) that was later supported by electron microscopy (Pfeiffer and Roth 1970). More recent observations document also its suppressive effect on acetylcholine-induced mucus production (Hamada *et al.* 1997) and gastric mucosal transmembrane potential difference (Dziaduś-Sokołowska *et al.* 1989). This barrier-braking and irritant conception of caffeine was completed by epidemiological associations of gastro-esophageal reflux, ulceration and cancer with the consumption of coffee (Marotta and Floch 1991, Terry *et al.* 2000). However, the aforementioned notion was challenged by experimental observations showing enhancement of mucosal blood flow by caffeine (Ozturkcan *et al.* 1974) and protective influence of this drug on mucosal barrier integrity (Wittmers *et al.* 1998) suggesting actually a preventive role of caffeine in gastric mucosal injury (Koyama *et al.* 1999).

Caffeine is a methylxanthine with pluripotent, concentration-dependent (Fredholm *et al.* 1999) and hence possibly opposing pharmacological actions. It is a nonselective adenosine receptor antagonist, phosphodiesterase inhibitor, ryanodine-sensitive Ca^{2+} channel activator and soluble guanylate cyclase inhibitor. As a consequence, these actions may, besides others, intervene with NO production and/or its second messenger cyclic guanosine monophosphate (cGMP) pathway leading to modulation of a wide spectrum of mucosal barrier-related (patho)physiological effects ascribed to NO including vascular tone regulation or modulation of oxidative stress. Indeed, the latest findings indicate that caffeine may decrease NOS expression *in vivo* (Corsetti *et al.* 2007) and attenuate glutamate-induced NO synthesis *in vitro* (Godfrey *et al.* 2007). Caffeine ingestion was found to decrease exhaled NO (Bruce *et al.* 2002) and negate the protective effect of IPC, i.e. reactive hyperemia due to the hypoperfusion-induced accumulation of adenosine and enhanced NO production (Riksen *et al.* 2006). In contrast, aortal endothelium responds to caffeine by promotion of NO synthesis (Hatano *et al.* 1995). Despite generally recognized vasoconstricting role of caffeine in the brain (Couturier *et al.* 1997), heart (Bottcher *et al.* 1995), limb (Casiglia *et al.* 1991) or gut (Hoecker *et al.* 2002) vasculature, literature is inconsistent as far as gastric (sub)mucosal perfusion is concerned (see above). Moreover, conflicting data are available also on the effect of caffeine on endothelial function (Papamichael *et al.* 2005, Umemura *et al.* 2006). Quite understandably, these discrepancies are reflected in contradictory results regarding induced gastric mucosal injury (Yano *et al.* 1982, Parmar *et al.* 1985, Koyama *et al.* 1999). Hence, the limited data describing caffeine´s effect on gastric (sub)mucosal microcirculation is inconsistent and parallel monitoring of local NO release following caffeine administration is, thus far, lacking. Similarly, the putative effect of caffeine on oxidative stress awaits clarification.

5.2. METABOLIC, HAEMODYNAMIC AND MUCOSAL PERMEABILITY MONITORING

Tissue chemistry monitoring

It has long been acknowledged that for understanding dynamic processes taking place in particularly vulnerable organs (such as the brain) systemic blood or local tissue withdrawal may either be inaccurate, inadequate or even become significantly organ-damaging. An approach was pursuited that would provide more specific information describing the physiology and chemistry of organ in question in a minimally invasive way. After years of experience with push-pull cannulas (Fox and Hilton 1958), semi-permeable dialysis sacs (Bito *et al.* 1966) and their combination in the form of dialytrodes

(Delgado *et al.* 1972), the progression of *in vivo* tissue chemistry measurement has settled on continuous perfusion of hollow dialysis fibres (Ungerstedt and Pycock 1974) later displaced by microdialysis needle probes (Tossman and Ungerstedt 1986) that have been in use until recently. The major advantages of microdialysis over the former techniques were in the prevention of tissue pressure build-up (push-pull systems) while providing relatively continuous monitoring of analytes (instead of mean concentrations of solutes over long periods of time as was the case with dialysis sacs). At present, the unique characteristics of microdialysis make it a considerable challenge for traditional golden diagnostic standards, i.e. repeated withdrawal of fluids from living organisms either in form of systemic blood, urine and other materials or somewhat more tissue-specific liquids such as bile or cerebrospinal fluid. In this aspect, microdialysis is starting to be established in clinical routine, especially neurointensive care as elegant and inexpensive means of peri- and postoperative metabolic monitoring (Tisdall and Smith 2006). Even though some experimental and clinical data are available also on its applications in metabolic monitoring of splanchnic areas like the gut or peritoneal cavity (Kitano *et al.* 2000, Solligård *et al.* 2004, Jansson K *et al.* 2004), the experience with gut microdialysis is still insufficient.

Blood flow measurements

Due to intraabdominal location, existence of peristalsis and high variation in the microcirculation, there is at present no golden standard technique of gastrointestinal blood flow measurement that would be widely accepted for clinical use. In addition, gut viability-relevant information requires knowledge of microcirculatory alterations, since local perfusion does not necessarily correlate with total blood flow to the organ (Thoren *et al.* 2000) i.e. flow via macrovessels. In human and experimental medicine, serum D-lactate, doppler-ultrasound (ULS), laser doppler flowmetry (LDF), tonometry, multislice spiral computer tomography (CT), nuclear magnetic resonance (NMR) angiography, positron emission tomography (PET), fluorescein method, oximetry, dye (aminopyrine, aniline, neutral red) dilution technique (Jacobson *et al.* 1966, Curwain and Holton 1973, Szelenyi 1981), intravital microscopy and inert (hydrogen) gas or microsphere clearance methods (Murakami *et al.* 1980, Dregelid *et al.* 1986) have been employed. However, regarding microcirculation-based gut viability these methods are either insufficiently specific and/or sensitive (D-lactate, CT, ULS), liable to subjective interpretation (fluorescein method), lacking clinical validation (oximetry) or applicability (dye dilution, gas or microsphere clearance methods), burdened with gut-specific methodological drawbacks (LDF, intravital microscopy) or too costly for routine use (NMR, PET) (Sommer 2004).

The original rationale behind the application of microdialysis technique for nutritive blood flow measurement was the development of mininvasive method of local blood flow monitoring in skeletal muscle *in vivo*. The proposed method was based on negative correlation between capillary blood perfusion and efflux of added indicator from the probe (Hickner *et al.* 1992). The main advantage of the technique is its low invasiveness, direct contact with the extracellular space in the vicinity of microvessels and surrounding cells allowing for parallel metabolic monitoring and pharmacological studies. In order to be suitable for experiments and particularly for clinical use, blood flow indicators or markers must fulfill numerous criteria – they must be soluble in water, safe to use (with well-described toxicity), apyrogenic in character, easy to prepare under sterile conditions, have very good tissue distribution (small molecule), low interaction with the components of the microdialysis system, established sensitive analytical technique available and last but not least – must be cheap. The original method advocated by Hickner *et al.* made use of ethanol. The ethanol efflux technique consistently responded to variations in blood flow to skeletal muscle both during rest and during hyperemia. No influence of ethanol (0.005 – 1 mol l^{-1}) on local blood flow or metabolism was documented. In addition, the technique was validated against ^{133}Xenon clearance and showed a high correlation therewith (Hickner *et al.* 1994). Although in theory it is possible to calculate the interstitial blood flow quantitatively (Wallgren *et al.* 1995), in most circumstances it should be considered a rather qualitative method. The drawbacks of the method are volatile and possibly also radioactive (Stallknecht *et al.* 1999) character of ethanol (problematic pre-analytical phase) and less available analytical instruments such as gas chromatography associated with mass spectrometric detection, GC-MS (analytical phase). Thus, in our previous work, lithium has been used to describe blood perfusion variations in liver, kidney and muscle interstitium after partial hepatectomy or nefrectomy (Hrubá *et al.* 2004). Unfortunately though, the actual relationship of this marker to blood flow is hypothetical and requires validation (e.g. by a controlled hemorrhagia or IR).

Gut barrier function assessment

As far as gut barrier *sensu stricto* (i.e. the integrity of its luminal surface that normally hinders or prevents the transepithelial passage of macromolecules) is considered, it is tested as the facility with which the intestinal mucosal surface can be penetrated by the unmediated diffusion of specified constituents – the (gastro-) intestinal permeability (IP). IP tests are based on passive unmediated diffusion of various substances (termed markers or probes) across the mucosal surface in both directions. With the exception of proteins, ideal markers should be biochemically inert, should cross the gut epithelium by non-mediated diffusion through defined pathways, should be qualitatively recoverable after oral or i.v. administration and conveniently and reliably measured in biological fluids. Typical probe molecules include sugars (sucrose, lactulose, mannitol), polyethylene glycols, ^{51}Cr-labelled ethylenediamine tetra-acetate (^{51}Cr-EDTA), horseradish peroxidase and various protein markers such as bovine serum albumin (Uil 1996). The markers are selected respecting their site of absorption or degradation as ingested fluid constituents (e.g. sucrose for stomach and duodenum, lactulose and mannitol for the small intestine and sucralose for the large intestine). In the case of lumen to blood pathway, the markers are usually measured after their oral ingestion and absorption into the bloodstream either in the systemic blood or collected urine. Oral IP tests have been employed with convenience in animals as well as in humans (Červinková *et al.* 2002, Cibiček *et al.* 2004). However, there are plenty of confounding factors (beginning with the quality and delivery of test solution and ending up with sample preservation) that may influence the urinary recovery of orally ingested probes (Tab. 2).

Delivery	Test solution (TS) content and osmolarity
	Test conditions
Premucosal	Completeness of TS ingestion
	TS dilution in the stomach and intestines
	Unstirred water layer on the enterocytes
	Gastric emptying, intestinal transit time
	Degradation of the TS in the gut (by bacteria and gut enzymes)
Mucosal	Gut permeability *sensu stricto* (permeation pathways)
	Mucosal area (for absorption)
Postmucosal	Splanchnic blood- and lymphatic flow
	Systemic (tissue) distribution
	TS metabolism
	Endogenous production of substances similar to TS components
	Renal functions (clearance)
	Completeness and timing of urine collection
Other preanalytical	Sample preservation (bacterial degradation of TS in urine)
Analytical	Analytical accuracy
Postanalytical	Result interpretation

Tab. 2. *Factors that may influence the outcomes of oral IP tests (adopted with modifications from Kohout 2002 and Uil 1996).*

These problems may to a large extent be reduced by using a calculated ratio of individual recoveries of two differently absorbed probes (e.g. lactulose and mannitol), or by studying IP in an (anaesthetized) animal model with luminal perfusion (Fihn *et al.* 2003). If the model considers lumen to blood clearance, repeated blood withdrawals are necessary. If the opposite (i.e. blood to lumen) route is the case, the probes are injected intravenously and determined in the luminal perfusate. These experimental methods are sometimes assisted by the detachment of kidneys by ligatures to avoid the undesirable loss of the marker. On the other hand, they allow for monitoring of short-term changes (in minutes or hours) in the IP in constrast to the former (oral) methods that require longer sampling (5 - 24 hours) and are hence suitable for detection of changes taking place over days or weeks. The most

obvious but perhaps not the most important drawback common to all these techniques is their absolute dependence on the bloodstream. Theoretically, if the blood perfusion of the gut falls (close) to zero, virtually no marker will be transported and the results will hence be misleading. This may hold true especially for short-term experiments on (anaesthetized) animals, where ischemia, particularly if induced for a longer time, may put the reliability of the results under question. In this aspect, submucosal microdialysis might prove an advantageous approach since it is blood perfusion-independent and may, in parallel, bring additional (biochemical, pharmacological or microcirculatory) information.

6. AIMS

The overall objective of the present thesis was to employ microdialysis to study haemodynamic and metabolic events in the splanchnic region of rats. In addition, the intention was exploration of a brand new field in using microdialysis – barrier function monitoring.

The specific aims were:

I. To investigate the applicability of lithium microdialysis (LM) in rat stomach and colon submucosa for monitoring of blood flow changes due to IR.

   To study the protective effect of local (in glandular stomach) or remote (in descending colon) IPC on nutritive blood flow.

   To assess the systemic effects of celiac artery occlusion (CAO) and IPC using selected enzyme activities and NO production.

II. To evaluate possible impacts of caffeine on gastric submucosal microcirculation and NO release.

   To measure plasma malondialdehyde (MDA) as a marker of systemic oxidative stress (lipid peroxidation) in response to increasing doses of caffeine.

III. To develop a microdialysis method of continuous mucosal permeability measurement in rat descending colon.

   To verify the hypothesis, that the method detects barrier function impairment due to intraluminal perfusion with concentrated ethanol.

7. MATERIALS AND METHODS

7.1. THE *IN VIVO* MICRODIALYSIS TECHNIQUE

The principle of microdialysis

The basic principle of microdialysis technique is to mimic blood capillaries. After introduction of a microdialysis probe (catheter) into the investigated tissue and its perfusion with liquid (termed the perfusate), equilibration with the surrounding tissue fluid takes place by passive diffusion of solutes (metabolites, xenobiotics etc.) across the probe´s semi-permeable membrane in both directions creating a dialysate (Fig. 1). Providing the perfusate has acceptable hydrostatic pressure and is chemically matched to the extracellular tissue fluid, there is no ultrafiltration or net water and ion exchange. The principal limitation for diffusion is the membrane´s pore size characterized by cut-off value (usually ranging between 5 - 30 kDa), which normally allows for transport of low-molecular substances and excludes macromolecules such as proteins and small molecules bound to them. Hence, this advantageous characteristics applies also to enzymes which would otherwise possibly cause degradation of analytes and/or elongation of the preanalytical phase with inevitable sample loss. Due to the physical properties of the dialysis membrane, highly lipophilic substances attach to the system and cannot be measured.

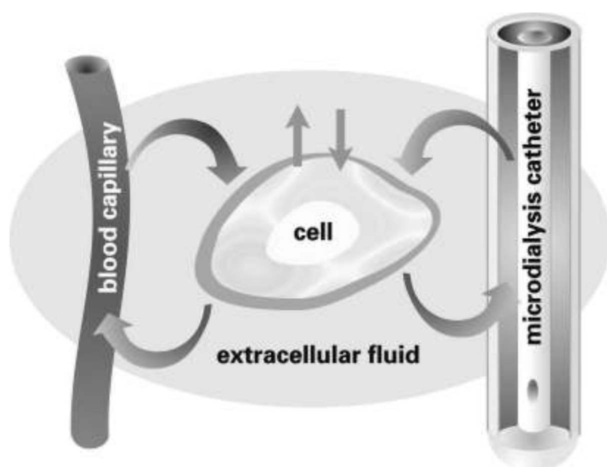

Fig. 1.
The principle of microdialysis is to mimic a capillary blood vessel. The catheter typically consists of two concentric tubes with semi-permeable membrane being the end part of the outer. The perfusate is pumped down the inner tube, changes direction as it enters the outer tube through a hole at its tip and is transported upwards to allow diffusion in both directions.

The microdialysis system

The microdialysis system consists of a probe with inlet tubing connected to a perfusion pump-driven syringe and outlet tubing draining the dialysate into microvials (or directly into the analysator/detector in so called „on-line“ systems). Since the dialysate is collected in preset time intervals, the samples contain mean concentrations of analytes harvested over time giving cumulative results unlike blood samples that provide point measurements. From a variety of catheter types only flexible concentric „needle“ probes were employed in the present experiments (Fig. 1). Due to generally low perfusion rates (0.1 - 5 $\mu l \ min^{-1}$) and short sampling intervals (minutes) resulting in small sample volumes containing diluted analytes, sophisticated and highly sensitive analytical and detection methods (such as high performance liquid chromatography, HPLC or capillary electrophoresis with electrochemical or MS detection) are generally utilized. If one wants to circumvent the problem with analysis, he may use higher perfusion rates and/or longer sampling time. However, both of these approaches have their drawbacks as the former limits the equilibration of perfusate with interstitial fluid (further dilutes the studied substance) and the latter decreases the technique´s time resolution, i.e. its ability to discern short-term alterations. Therefore, the right selection in these parameters in praxis is usually a compromise.

The basic probe characteristics

If microdialysis data are to be quantified in absolute terms and interpreted correctly, one needs to be aware of what is known as probe´s recovery. Recovery characterises probe´s function and may be defined as the (dynamic) determinant of the degree to what the composition of dialysate reflects the

composition of interstitial fluid surrounding the probe. It may be viewed from two aspects – as absolute (termed mass and expressed in mol of recovered substance) and relative (termed fractional extraction and expressed in %) recovery. Absolute recovery (AR) increases with the perfusion rate, whereas relative recovery (RR) is negatively correlated to the flow of perfusate. The recovery of substances from the extracellular fluid (ECF) depends, besides the perfusion flow rate, also on the diffusion area (given by the dimensions of the probe´s dialysis membrane), physical properties of the membrane and substance-specific tissue diffusion characteristics. The latter will in turn depend on the solute´s interstitial pool, which is determined by the rate of its production or uptake by the surrounding cells as well as its delivery or elimination by local microcirculation. The exact mathematical approach to these relationships is described elsewhere (Plock and Kloft 2005, de Lange *et al.* 2000, Wallgren *et al.* 1995).

Probe calibration (recovery measurements)

A microdialysis probe can be calibrated *in vitro* and *in vivo*. The former, being simple and easy to perform, provides only a rough idea of the probe´s function *in vivo*. For obvious reasons, the latter calibration techniques are more demanding and may give considerably different (ordinarily lower) results. However, these reflect the probe´s function much more precisely. The knowledge of (changes in) RR enables researchers not only to calculate the actual ECF concentrations of analytes but what is often more important sheds light on the results, as these do not depend solely on local metabolic processes but to a large extent on analyte diffusibility given by a number of factors including tissue blood perfusion.

The oldest and practically simplest way to determine the extracellular concentration of solutes (and hence the probe´s recovery) is „**zero-flow**" method by Jacobson (Jacobson *et al.* 1985). This so called „direct" method is based on a negative relationship between RR and perfusion rate. Using mathematical extrapolation, 100% recovery (i.e. when the concentration in the dialysate is equal to the concentration in the ECF) may be estimated from theoretical zero perfusion rate. However, this method requires rather long sampling intervals (at low perfusion rates) and may not be practicable at the beginning of experiments. On the other hand, when low sample volumes are not a challenge, very low perfusion rates (~ 0.1-0.3 μl min^{-1}) ensure practically 100% recovery and eliminate equilibration (with associated interpretation) problems.

The drawback of the simple Jacobson´s zero-flow method of using very low perfusion rates was partially solved by Lönnroth, who added the analyte in question into the perfusate at different concentrations and studied its recovery by the microdialysis probe (Lönnroth *et al.* 1987). The resulting linear relationship betwen the analyte´s concentration in the perfusate and the dialysate – perfusate difference indicated the stability of the probe´s function over the range of concentrations used. While the gradient of the slope defined the probe´s recovery, the x-intercept (here obtained from interpolation) was indicative of the ECF concentration, i.e. during the „**zero-net-flux**" conditions, when the concentration of the solute in the perfusate theoretically reached its concentration in the surrounding tissue fluid (hence the term „equilibration dialysis"). Due to its convenience and so far widespread acceptance the method of Lönnroth was employed for *in vitro* and *in vivo* probe calibrations also in the present thesis.

Since the aforementioned approaches require stable levels of studied analytes in the course of measurements – an assumption that may not necessarily be true *in vivo* – Olson and Justice proposed a modified no-net flux method. The **dynamic no-net-flux** method differed from its original counterpart in the fact that all probes were perfused with one concentration of the studied substance for one probe only. This procedure allowed to unmask possible dynamic alterations in the probe´s recovery and hence provide more accurate estimations of the solutes´ interstitial concentrations, however at the expense of more animals used (Olson and Justice 1993).

Nonetheless, these techniques provide mere estimations of real recoveries, which may – depending on the particular implantation with subsequent local tissue microtrauma – differ quite considerably from the expected value(s). Therefore, in order to exactly calculate the interstitial concentrations of studied substances, it is important to monitor the individual recovery of each implanted probe during the whole measuring process. This was made possible by developing reverse- or **retrodialysis** (also called delivery) methods, which were further elaborated and optimized for continuous measurements by using internal standards that are not normally present in the tissue. The RR of the probe for the internal standard and the studied substance is determined *in vitro*, and the ratio (assumed to be identical *in*

vivo) is used to calculate the actual probe´s recovery at any time point *in vivo* (Larsson *et al.* 1991). More recently, as internal standards isotope-labelled molecules are used, which share with the studied substance many of its physical and chemical characteristics whereby bringing the calculations closer to real tissue situation and enabling continuous *in vivo* probe calibration (Edwards *et al.* 2002).

7.2. SUBSTANCES USED

As microdialysis perfusion medium, room temperature flame photometer serum standard solution (Eppendorf, Hamburg, Germany) with Li^+ concentration 2 mmol l^{-1} (study I), ethanol-enriched normal 0.9 % saline with final concentration 50 mmol l^{-1} (study II) or commercially available Ringer´s solution (containing Na^+ 147.1 mmol l^{-1}, K^+ 4.0 mmol l^{-1}, Ca^{2+} 2.3 mmol l^{-1}, Cl^- 155.6 mmol l^{-1}, 310 mOsm l^{-1}, InMediec s.r.o., Luhačovice, Czech Republic, study III) was utilized.
Caffeine (Sigma-Aldrich, St. Louis, MO, USA) for i.p. application was dissolved in saline to obtain solutions with concentrations 0.5, 5 and 25 mg ml^{-1} for groups 2, 3 and 4, respectively (study II).
As luminal perfusate in study III, a purchased solution of ^{51}Cr-EDTA in 0.005 mol l^{-1} EDTA, 433.64 MBq (11.72 mCi) ml^{-1}, pH=7.0 (Perkin Elmer, Boston, MA, USA), dissolved either in R1/1 (1: 1666.7 by volume), or in the same manner in a mixture of R1/1 and 96 % ethanol (to obtain 20 % ethanol solution), was used. The former formula was followed to prepare a vehicle or control medium (CM), whereas the latter produced an ethanol medium (EM). Both media had the same ^{51}Cr-EDTA concentrations and hence also ^{51}Cr activities given by counts per minute (cpm) per volume unit. The radioactive solutions were prepared after delivery according to this protocol and were employed without modifications in the course of the study regardless of their actual activities.

7.3. ANIMAL MODELS

Animals
In all studies, adult male Wistar rats weighing 250 to 450 g were used. The animals were housed in the animal quarters under controlled environmental conditions. They had free access to standard rat chow except 16 – 18 hours before experiments, when they were fasted. Tap water was provided *ad libitum*. All animals were anesthesized with single i.p. dose of pentobarbital (50 mg kg^{-1}, Nembutal®, Abbott Laboratories, North Chicago, USA) and placed in a supine position on an unheated bed. They were kept under general anesthesia until the end of experiments by cyclic i.p. administration of Nembutal® (15 mg kg^{-1} h^{-1}). Body temperature was monitored using a rectal thermometer probe (Ama-digit ad 15th, Aprecision, Germany) and maintained at 37.5 – 38.5 °C by means of a heating lamp. The trachea was carefully exposed, opened between rings by a short incision and cannulated with 3 cm polyethylene catheter (outer diameter 2.5 mm) to ensure patent airways. For all surgical procedures, clean, but not sterile instruments/materials were used. At the conclusion of experimental procedures the animals were sacrificed by blood withdrawal from abdominal aorta and the removed serum or plasma samples were aliquoted and stored at -20 or -70 °C for ensuing biochemical analyses.

Ethical issues
All rats received humane care in accordance with the guidelines set by the Institutional Animal Use and Care Committee of the Charles University in Prague, Czech Republic. All protocols and experimental procedures were approved by a specialized Council for the Prevention of Animal Mistreatment of the Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic.

Rat model of gastric and colonic submucosal microdialysis
In studies I and II a modified technique of gastric submucosal microdialysis adopted from Kitano *et al.* was employed (Kitano *et al.* 2000). Following 3 – 4 cm long midline laparotomy performed with scissors, stomach was exteriorized and kept moist with saline. Respecting the course of blood vessels, a 6 mm (study I) or 15 mm (study II) long tunnel was made from serosal aspect in the submucosal layer of its glandular part from greater to lesser curvature by means of a 26 G (study I) or 21 G (study II) needle with care neither to penetrate through the mucosa into the lumen nor to make an additional opening in the serosa. Into the preformed tunnel a microdialysis probe CMA/20, active length 4 mm,

outer diameter 0.5 mm, cut-off 20 kDa, CMA/Microdialysis, Solna, Sweden, (study I) or MAB 11.8.10 with 6 kDa cut-off polyethylene suplhone membrane, active length 10 mm; outer diameter 0.5 mm; Microbiotech/se AB, Stockholm, Sweden (study II) were carefully inserted and fixed in place with an atraumatic suture (Fig. 2). The implantation techniques were trained in advance on other animals and the exact localisations of the probes in the submucosal region were verified by histology (Fig. 1, study I and II).

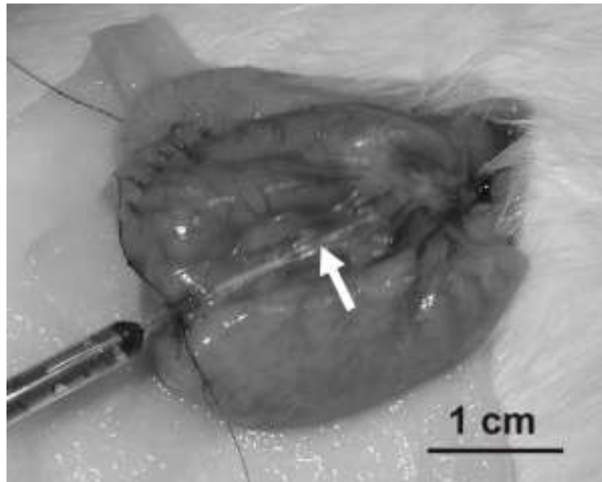

Fig. 2.
Depicted is a microdialysis probe MAB 11.8.10 Microbiotech/se AB, Stockholm, Sweden implanted in the submucosal region of rat gastric corpus. The arrow points to the dialysis part, which is positioned in between the major blood vessels perpendicular to the long axis of the organ from its greater to its lesser curvature. Training is required to master the implantation technique.

Regarding the descending colon, it was exposed, kept moist with saline and, when necessary, the region in question made free of formed stercus by gentle manipulation. Thereafter, a procedure similar to that in stomach was followed parallel to its long axis at a distance of 5 cm from the anus, where 5 – 6 mm long tunnel was created from serosal aspect in its submucosal layer by means of a 28 G needle. Probe position was selected so as to avoid interference with blood vessels. Subsequently, a microdialysis probe CMA/20, active length 4 mm, outer diameter 0.5 mm, cut-off 20 kDa, CMA/Microdialysis, Solna, Sweden (study I) or MAB 1.2.4. with 6 kDa cut-off polyethylene sulphone membrane, active length 4 mm; outer diameter 0.24 mm; Microbiotech/se AB, Stockholm, Sweden (study III) was cautiously inserted into the preformed tunnel and fixed to the serosa at the tunnel entrance with suture. Again, the implantation techniques were trained beforehand and probe positions were histologically verified.
After surgery, the abdominal opening was closed to avoid fluid losses. Microdialysis catheters were perfused at 1.2 μl min^{-1} (study I), 2 μl min^{-1} (study II) or 1.5 μl min^{-1} (study III) using a perfusion pump LD 20, Tesla Přelouč, Czech Republic (study I) or CMA 102, CMA Microdialysis AB, Solna, Sweden (studies II, III). Initial 40 (study III) or 60 min (studies I, II) stabilisation periods without specimen collection were allowed. These equilibration times were succeeded by a 30 min period to yield one (studies I and II) or three (study III) baseline samples to obtain a reference level(s). Thereafter, continuous dialysate sampling respecting the probes′ individual lag times ensued for the next 4 h (study I), 2.5 h (study II) or 1.5 h (study III) in 30 min (studies I and II) or 10 min (study III) intervals into microvials. The specimens (aliquoted for ethanol and NO in study II) were stored at -12 °C (^{51}Cr), -20 °C (Li^{+}, ethanol) or -70 °C (NO) until analysis.

Rat model of celiac ischemia
After midline laparotomy, the celiac artery was disclosed and underlaid by smooth rubber tubing (1 mm in diameter) to assist later clamping. Gastric IR model was adopted from Pajdo *et al.* (Pajdo *et al.* 2001) and was accomplished by placement/removal of a microbulldog clamp (Medin a.s., Nové Město na Moravě, Czech Republic) at the level of celiac artery origination from abdominal aorta. The success of each intervention was verified visually (assessment of blood flow distal from the site of CAO).

Rat model of colon luminal perfusion
After successful implantation of a microdialysis probe, the oral part of the descending colon was ligated with a silk thread in a distance of 1 – 2 cm from the probe as close to the colonic wall as

possible to avoid ischemisation. Thereafter, a double-lumen cannula was inserted via anal route to permit continuous perfusion of the colonic lumen by means of a syringe pump LD 20, Tesla Přelouč, Czech Republic. The inlet (inner) tubing was close to the oral colonic ligature whereas the outlet (outer) tubing was ligated to the opposite, aboral portion of colon to separate 3.0 – 3.5 cm long colonic tube for single pass perfusion. The temperature of the perfusion medium was maintained close to body temperature using a thermostatic water bath (see Fig. 1, study III). After the initial 30 min lavage at 25 ml h^{-1}, the luminal perfusion rate was maintained throughout the experiment at 6 ml h^{-1} excluding flush periods which separated the corrosive 30 min ethanol stage and consisted of a fast (25 ml h^{-1} for 8 min) and a succeeding slow (6 ml h^{-1}, 2 min) preparation phase. Prior to ethanol application (using a three-way flow switch), CM was run for 30 min to harvest the reference dialysate. The experiment was completed by final 60 min of CM perfusion. During the whole procedure, care was taken not to allow air bubbles to enter into the perfusion system. To avoid fluid losses and to ensure convenient i.p. application of Nembutal®, the skin layer of the abdominal opening was closed using microbulldog clamps.

7.4. EXPERIMENTAL PROTOCOLS

Study I

The animals were randomly assigned to three groups denoted as S, IS, and ISP (6 – 10 in each group). The first group (S) was sham operated. The second group (IS) underwent a 30 min period of complete CAO with subsequent 2.5 h of reperfusion. The rats in the third group (ISP) were preconditioned by exposure to a short IR period (5 and 25 min, respectively), followed by prolonged IR (30 min and 2.5 h, respectively).

Study II

The animals were randomly allocated to four groups (6 in each). After the collection of baseline microdialysis sample, the first (sham operated) group received by intraperitoneal injection an adequate volume of normal saline, whereas the second, third and fourth groups were intraperitoneally administered caffeine solutions with concentrations 0.5, 5 and 25 mg ml^{-1} (caffeine dose of 1, 10 and 50 mg kg^{-1} b. wt.) respectively.

Study III

The animals were allocated to two groups (6 rats in each) – the first (C, control) group was examined as a sham group, i.e. without corrosive ethanol intervention, whereas the second (E, ethanol) group was exposed to a 30 min period of EM perfusion.

7.5. PROBE PERFORMANCE STABILITY AND CALIBRATION PROCEDURES

Study I

Probe performance stability was concluded *in vivo* on the basis of an assumption of stable gastric and colonic submucosal blood perfusion in the control group in association with statistically insignificant fluctuations of lithium efflux from the probe. *In vitro* the probe was calibrated at standard laboratory temperature using zero net flux method as follows – the probe CMA 20, CMA Microdialysis AB, Solna, Sweden was immersed in 20 ml of sterile saline and successively perfused with eight saline solutions of increasing LiCl concentrations (0.4, 0.9, 1.4, 1.9, 2.4, 3, 3.5 and 4 mmol l^{-1}) at three perfusion rates (0.3, 2 and 3.3 µl min^{-1}). Sampling was performed in adequate intervals giving 100 µl of dialysate. For each Li^{+} concentration and perfusion rate the medium in the flask was freshly prepared. Following chemical analysis and calculation of lithium efflux (perfusate – dialysate concentration) the results were plotted on a graph and probe recoveries read from the slope gradients (Fig. 3).

Study II

The measurement of NO using microdialysis technique was validated in two consecutive steps employing other two groups (A and B) of pentobarbital-anesthesized rats. First, probe performance stability for nitrate was tested continuously for 7 h (measurements during equilibration period

inclusive, group A, n=5) in one experiment based on an assumption of stable NO production throughout the study period. Microdialysis sampling in gastric submucosa was realized in 30 min intervals at a perfusion rate of 2 μmol l^{-1}. As perfusate, normal saline was utilized. Second, *in vivo* recovery of the same probe type was estimated (group B, n=3) using zero-net flux method originally proposed by Lönnroth *et al.* (Lönnroth *et al.* 1987). Four perfusion media of increasing concentrations of sodium nitrate in sterile saline were consecutively applied as follows. After the initial tissue equilibration (1 h) with 10 μmol l^{-1} $NaNO_3$, a 30 min sample was collected. The perfusion medium was changed for 15 μmol l^{-1} nitrate and following 30 min equilibration, another sample harvest (30 min) ensued respecting the probe´s lag time (3 min). The experiment was completed with 50 and finally 120 μmol l^{-1} nitrate solutions. The results were plotted on a graph and probe recovery was read from the regression equation (slope gradient). Besides microdialysis, these two groups of animals underwent no further experimental treatment. All general steps (anesthesia, surgery, gastric submucosal microdialysis technique including probes but excluding perfusion media, sacrifice and analytical techniques) were equal to the experimental groups of the present study.

Study III
Probe performance stability was concluded *in vivo* on the basis of an assumption of stable colonic barrier function in the control group in association with statistically insignificant fluctuations of ^{51}Cr-EDTA recovery. *In vitro* probe recovery was determined at standard laboratory temperature by the zero-net flux method as follows. Probe was immersed in 20 ml of CM with specific activity of 21.53 cpm μl^{-1} and perfused with three consecutive solutions of increasing activities (0, 9.73 and 21.53 cpm μl^{-1}). For equilibration, initial 30 min period was allowed, which was succeeded by sampling in 10 min intervals into microvials. In each experiment, the surrounding medium in the flask was freshly prepared, perfusion rate set at 1.5 μl min^{-1} and six samples taken. The results were plotted on a graph and probe recovery was read from the regression equation (slope gradient).

7.6. NUTRITIVE BLOOD FLOW MEASUREMENTS

In study I, lithium (2 mmol l^{-1}) was employed as a convenient qualitative blood flow indicator and the level of submucosal blood perfusion was expressed as lithium inflow – outflow concentration difference, i.e. Li^+ efflux as reported previously (Hrubá *et al.* 2004). This parameter is further referred to as LM. In study II, ethanol dilution technique represented by dialysate / perfusate ratio of ethanol concentrations was utilized (Hickner *et al.* 1995). The perfusate´s concentration was 50 mmol l^{-1}.

7.7. LABORATORY ANALYSES

Microdialysate lithium
Lithium was quantified in perfusate and microdialysate solutions to estimate the probe´s function *in vitro* and the level of tissue blood perfusion *in vivo*. Li^+ was determined using EFOX 5053 flame photometer (Eppendorf, Hamburg, Germany) according to manufacturer´s instructions.

Serum nitric oxide
NO was measured as a sum of nitrate and nitrite using methods described elsewhere (Jedlickova *et al.* 2002). Briefly, NO_3^- was determined by HPLC. Prior to determination, the samples were diluted in the ratio 1:3. For the detection, UV–VIS at 212 nm for 7 minutes was utilized. As mobile phase, 0.02 mol l^{-1} $NaClO_4$ at pH 3.9 was used. NO_2^- was determined by fluorimetry. Prior to determination, the serum samples were treated as follows: to a prepared mixture (100 μl of serum with 200 μl of H_2O MilliQ) 30 μl of 2,3-diaminonaphtalene was added. After 20 min standard laboratory temperature incubation, and adding 15 μl of 2.8 mol l^{-1} NaOH, fluorescence was measured at excitation 365, and emmission 430 nm.

Serum enzyme activities
Hitachi 917 autoanalyser (Boehringer, Mannheim, Germany) with commercially available reagent kits (Roche Diagnostics GmbH, Mannheim, Germany) were utilized. For the study of the extent of liver injury, alaninaminotransferase (ALT), aspartataminotransferase (AST), lactatdehydrogenase (LDH)

and cholinesterase (CHE) serum activities were investigated. To evaluate the level of pancreatic damage, lipase (LIP) and amylase (AMYL) activities were determined. All analyses were performed in accordance with manufacturer´s instructions.

Microdialysate nitric oxide

NO was quantitatively determined as the sum of its stable metabolites (nitrite plus nitrate) in an enzymatic colorimetric assay, which involves an enzymatic conversion of nitrate to nitrite by nitrate reductase. Resulting nitrite is detected colorimetrically as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. In the experiments commercially available enzyme-linked immunosorbent assay (ELISA) kits (Assay Designs, Ann Arbor, MI, USA) were utilized only. All procedure steps were performed according to the manufacturer´s instructions.

Microdialysate ethanol

Ethanol was quantified in microdialysates to estimate the level of tissue blood perfusion. The analyses were carried out using GC-MS apparatus consisting of Varian 3300 GC coupled to Finnigan MAT Magnum MS (Thermo Fisher Scientific, formerly Finnigan). Supelcowax™-10 (30 m x 0.25 mm x 0.25 μm film thickness) GC Capillary column with helium as a carrier gas was employed. Injector and transferline temperatures were set to 230 °C for both. GC oven was programmed as follows: 55 °C, 2 min, 25 °C min^{-1} to 150 °C, hold for 1 min. The specified MS parameters were 70eV for electron ionization mode and 209 °C for ion trap temperature. The resulted MS spectra were scanned in 15 – 70 a.m.u. mass range under 4 microscans s^{-1} of detection speed regimen.

Plasma caffeine

Caffeine concentrations were determined using a modified HPLC method by Biederbick *et al.* (Biederbick *et al.* 1997). Briefly, caffeine and internal standard hydroxyethyltheophylline (aqueous solution 40 mg l^{-1}) from serum samples (100 μl + 50 μl) were extracted using 2.5 ml of dichlormethane. The organic phase was evaporated under nitrogen at 40 ºC and the samples were reconstituted in 150 μl of mobile phase, and 30 μl were injected into the HPLC column. Analysis was performed on a 2695 Waters Separations Module equipped with 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). The mobile phase was made up of an aqueous solution of sodiumdihydrogenphosphate (0.5 mmol l^{-1}, pH 5) to acetonitrile (ratio 88:12) and was pumped isocratically at a flow rate of 0.9 ml min^{-1}. Temperature of the column was set at 40 °C. The quantification of caffeine was performed at 270 nm.

Plasma malondialdehyde

MDA was determined as a secondary product of lipid peroxidation in an attempt to evaluate the level of oxidative stress produced by caffeine. The analysis was based on the reaction of MDA with thiobarbituric acid (TBA) producing a red MDA-TBA complex measured photometrically at three distinct wavelengths (485, 532 and 560 nm) and the absorbance corrected according to Allen´s formula $A_{corr} = A_{532} - [(A_{560} - A_{485}) \times 0.63 + A_{485}]$ for enhanced specificity (Hendrix and Assman 1990).

Radioactivity (gamma decay)

A commercially available multi-crystal gamma counter LB 2111 (EG&G Berthold, Germany) was utilized. The instrument was standardised for ^{51}Cr (gamma ray, 320 keV) and adjusted for energy levels ranging within 50-450 keV. Counts per minute (cpm) were calculated from 10 min duration of measurements. For counting, the original plastic vials with collected samples (15 μl) were used. To eliminate background counts, 24 identical vials with 15 μl of R1/1 solution were assessed and the obtained mean was subtracted from all measurements.

7.8. BARRIER INTEGRITY DETERMINATION

To assess the function of colon mucosal permeability, an updated ratio of activities was calculated using the following formula: [(probe dialysate cpm – background cpm) / (luminal perfusate cpm – background cpm)] x 100, where probe dialysate cpm is the activity obtained from 15 μl microdialysis

samples and luminal perfusate cpm is the mean number of counts given by six 15 μl samples of luminal perfusate stock solution (CM or EM) short before entry into the animal. The results were expressed in % of activity recovered from the perfusate passed through the animal´s gut.

7.9. LIGHT MICROSCOPY

Stomach biopsies were taken for probe position verification (studies I and II) and to evaluate possible effects of caffeine on tissue morphology at the microscopic level (study II). Following the removal of the microdialysis catheter (study I), cleaning of the mucosal surface in cold tap water and close inspection in search for macroscopic lesions, the samples (0.5 x 1.5 cm) were taken from glandular segment surrounding the site of the probe (the implanted part of the probe was left *in situ* in study II). The tissues were fixed in 10 % neutral buffered formalin, embedded in paraffin and further treated according to standard procedures for hematoxylin–eosin (HE) stain. The slides were photographed at 125, 250 and 500-fold magnification.
Concerning the effects of caffeine, the sections were evaluated by a blinded professional observer. The grading criteria were adopted from Natale *et al.* and were as follows: grade 0 for normal mucosa, grade I for lysis and segregation of cells on the luminal surface (with intact pit cells), grade II for damage confined to gastric pits with detachment of the surface epithelium and grade III which involves injured gastric glands (whole-thickness mucosal necrosis with swelling and possible disconnection of mucosal layers, Natale *et al.* 2001).
Descending colon biopsies were taken to verify the positions of microdialysis probes (studies I and III) and to evaluate the effect of luminal perfusion with ethanol at the microscopic level (study III). After the the removal of microdialysis catheter (study I) or luminal tubings (study III) and cleaning of the luminal surface with water, samples (0.5 x 0.5 cm) were taken from the site of the probe. In study III, the specimens were taken from both perfused and proximal (unperfused) segments. The harvested biological material was immersed in 10 % neutral buffered formalin, embedded in paraffin and further treated according to standard procedures for HE stain. The slides were examined and photographed at 100-fold magnification.

7.10. DATA ANALYSIS

Unless otherwise noted, data are expressed as means ± standard error of mean (SEM) of absolute or relative values. In case of the latter, each individual´s first baseline sample (collected after equilibration period) was considered 100 %. For statistical evaluation, descriptive measures, normality tests, repeated measures analysis of variance (ANOVA, all studies) with Fisher's LSD multiple comparison *post hoc* test (in study I only) were employed using programs NCSS 2004 and Statistica. The selected level of significance was α=0.05.

8. RESULTS

8.1. STUDY I

The in vitro probe calibration

The results of *in vitro* calibration of the probe CMA 20, CMA Microdialysis AB, Solna, Sweden, is depicted in Fig. 3.

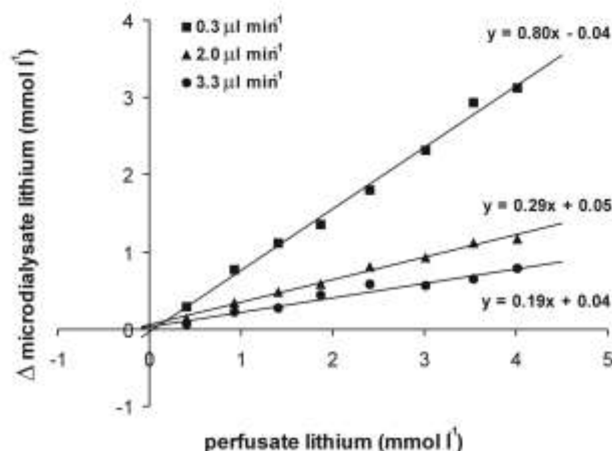

Fig. 3.
In vitro probe CMA 20 calibration ("zero net-flux" method). The Δ microdialysate (perfusate – dialysate) lithium plotted against the perfusate lithium gives a series of gradients representing the probe´s recoveries (80, 29 and 19 % for 0.3, 2 and 3.3 µl min^{-1}, respectively). 8 measurements (displayed as dots) were conducted in each experiment.

Microdialysis data

Fig. 2 (study I) summarizes the time course of LM in all groups in the submucosa of stomach (a) and colon (b). The zero interval of microdialysate collection (obtained within 60 – 90 min after commencement) provided basal values, where no statistically significant difference was found between the stomach and colon. Likewise, between-group comparisons showed comparable baseline values in stomach as well as in colon. As to the blood perfusion in stomach, in the S group, absolute values of LM showed no variation in time. Brief (5 min) ischemia in the ISP group caused a significant drop in LM from its baseline ($p<0.05$). Within the IS and ISP groups, 30 min interval of CAO produced significant depressions in LM when compared with corresponding basal levels ($p<0.01$). Immediately after onset of reperfusion, the values of LM raised in both groups, reaching levels not significantly different from their baselines. In the next 2 hours, LM within the IS group continued to elevate slowly, while the opposite holds true for ISP group, where we observed a steadily falling trend ($p<0.001$). However, none of the changes observed within the ligated groups was sufficient to statistically manifest also in between-group comparisons with controls. Regarding nutritive blood flow in the descending colon, similarly to gastric perfusion, the S group provided constant LM values over the entire experiment. Neither within-group, nor between-group comparisons in parallel time intervals reached the level of significance.

Serum NO and enzymatic activities

The extent of potential organ impairment and modulation of the body´s metabolism was evaluated by determination of selected serum analytes. Fig. 3 (study I) depicts the grade of NO production modulation by ischemia and ISP. Even though the mean serum concentration of NO in IS group was 165% of that in control group (S), the difference was only marginally insignificant ($p=0.06$). The ISP group gave results similar to controls. Likewise, the activities of serum enzymes in question were not significantly different (Fig. 4, study I).

8.2. STUDY II

Histology

Microdialysis probes were positioned correctly within the submucosal layer of gastric wall without penetration into the organ´s lumen. The histological picture was comparable to previous reports (Kitano *et al.* 2000, Cibiček *et al.* 2004) as indicated in Fig. 1a, b (study II). Lack of macroscopically

measurable whole organ mucosal alterations due to caffeine treatment was mirrored in standard microscopy, which depicted similar grades of tissue damage ranging within grades 0-II around the probe in slides from all experimental groups including sham-operated animals (Fig. 1c-e, study II). The severity of mucosal injury was unrelated to the administered caffeine dose. No grade III lesions were observed.

Microdialysis data

Results of probe performance stability are displayed in Fig. 2 (study II). In this experiment, the calculated average outflow concentration of total NO (within 5 h-time frame corresponding with the probe calibration study) was 11 μmol l^{-1}. This is in agreement with the expected value (~ 9 μmol l^{-1}) calculated using extrapolation of data obtained from the probe calibration study, i.e. in case the concentration of total NO in the perfusate was close to zero (Fig. 3, study II). The outcomes of these experiments were consistent with one another indicating stable function of the microdialysis probe and NO production over time. The *in vivo* recovery of the used probe type under given experimental conditions was determined by the gradient of the regression line and found to be 38 %. The x-intercept corresponds to the concentration of nitrite + nitrate in the surrounding extracellular medium (~ 23 μmol l^{-1}, Fig. 3, study II). This level of extracellular NO was not affected by the i.p. administration of caffeine ($p = 0.9$, ANOVA, Fig. 4a, study II). Microcirculation as represented by ethanol out/in ratio showed a tendency for dose-related alterations after caffeine instillation, yet these, as a sum, failed to reach statistical significance ($p = 0.1$, ANOVA, Fig. 4b, study II).

Serum analytes

Along with caffeine, serum MDA was measured as general oxidative stress-related marker at the conclusion of the experiments. Even here, no statistically significant dose-related trend was observed ($p = 0.8$, ANOVA, Tab. 1, study II).

8.3. STUDY III

Histology

Microdialysis probes were positioned correctly within the submucosal layer of colonic wall, as indicated in Fig. 1a (study III). Application of R1/1 and vehicle (CM) had no material effect on the microscopical picture of the perfused part of gut (Fig. 1b, study III). However, after administration of ethanol, marked mucosal impairment was confirmed by histology (Fig. 1c, study III).

Microdialysis data

During the administration of 20 % ethanol, mucosal tracer permeability showed significant elevations (2.354±0.298 % for ethanol as opposed to 0.209±0.102 % for control group, time interval 60 min, $p<0.01$), which persisted or were even more pronounced after the cessation of ethanol instillation until the end of experiment, i.e. for at least 60 min (3.352±0.188 % for ethanol compared to 0.140±0.0838 % for control group, time interval 120 min, $p<0.001$, Fig. 2, study III).

9. GENERAL DISCUSSION

9.1. METHODOLOGICAL ASPECTS

Catheter implantation technique

The implantation of the catheter is a crucial step in microdialysis experiments. Obviously, it is virtually impossible to guarantee equal degrees of microtrauma produced by individual probe insertions into the subjects. Thus, to equilibrate the ECF composition and thereby reduce intraindividual variability, the initial stabilisation episode (~ 30-90 min) is normally allowed. This time frame, however, is not able to minimize the major source of interindividual variability – cells, platelets, fibrin and other materials accumulated during (various degrees of) blood leakage around the probe and avoid the consequences thereof (see below). Taking this risk into consideration, pretermission of major blood vessels was endeavored during tunnel formation. However, due to vascular anatomy, this precaution had only limited actual successs particularly in colon (study I), leading to a greater scatter in the results from this location (Fig 2b, study I). Therefore, with awareness that the reduction of membrane dimensions and diffusion area will bring about lower recovery, study III employed shorter, thinner and more flexible probes (MAB 1.2.4 instead of CMA/20) with better outcomes. Concerning the probes implanted in the stomach, the MAB 11´s, being somewhat thicker (outer diameter exceeding 0.5 mm), are a good compromise between robustness and flexibility, which is why they proved to be a good substitute for CMA 20 in study II. Providing the results confounded by surrounding hematoma are discarded, the *in vivo* recovery measurements document stable and reliable function of these probes in the mentioned location (Fig. 2 and 3, study II).

A swedish-japanese group has solved the "microtrauma problem" by using a "postponed" microdialysis, where probe implantation surgery (lasting approximately 10 min) takes place on day one, and the experiment involving microdialysis sampling is commenced on day four (Kitano *et al.* 2000). By this time, the signs of minor hematoma are mostly resolved producing comparable interindividual baseline conditions and seemingly the most reliable results. However, this approach necessitates animals awake during microdialysis with all resulting restrictions of possible interventions, or – if animals need to be anesthesized – it requires a reoperation. Nonetheless, we intend to follow this methodology in the future.

The easiest way to circumvent the obstacle with varying levels of tissue injury is the expression of results as % of respective baseline values (each animal serves as its own control) as is ordinarily done in microdialysis experiments. (We did so in study II.) However, our latest experience with ^3H_2O (employed as a flow marker) indicate that extensive peri-probe hemorrhage make the relative changes seen during gastric ischemia considerably less pronounced than are their conterparts detected with well-implanted catheters (unpublished observations). The major reason behind this observation lies in impaired tissue diffusion characteristics resulting in reduced probe function (i.e. recovery). These effects may not just mask possible blood flow alterations and cause high interindividual variability of data (diminishing statistical significance), but even lead to the contamination of interstitial environment with undesired (intra)cellular material. Therefore, in case of hemorrhage during tunnel formation, probe reimplantation is recommended. Otherwise the inclusion of the results is discouraged.

Lithium as a flow marker

In study I, Li^+ ion was employed as a convenient blood perfusion indicator due to its nonvolatility (in contrast to ethanol), small size (7 Da), excellent water solubility resulting in good tissue diffusion characteristics with practically no attachment to probe or membrane components (as is the case with lipophilic compounds). Li^+ is inexpensive, easily available, has apyrogenic character and might hence be added into sterile perfusion solutions. Since it is administered and monitored as a drug in psychiatry, it is also well described from the pharmacological point of view and with respect to medical safety. For these reasons possible addition of Li^+ into human microdialysis perfusates would be advantageous in future and moreover, clinical trials using this ion might obtain ethical approval more easily (than e.g. radioactive tracers).

The drawback of lithium lies in its determination. As a small metal ion, it may be analysed either by flame-photometry or MS. The former is more convenient, but requires a relatively large sample

volume (≥ 100 μl due to dead space). The latter demands sophisticated and not readily available instruments. Therefore, a flame-photometer was utilised for Li^+ determinations with sampling intervals and sample dilutions set accordingly. If optimal sensitivity of the method to detect local blood flow changes is to be accomplished, lower perfusion rates and longer sampling periods should be adjusted. This follows also from the *in vitro* probe calibration, which revealed 30 % recovery at 2 μl min^{-1} (Fig. 3). When enhanced time resolution is needed, the use of MS (instead of flame photometry) is strongly suggested. Concerning the ability of LM to detect changes in blood perfusion, lithium as a flow marker proved promising. When compared to their respective basal values, the experimental groups exhibited significant dynamic changes in LM due to IPC, ischemia and reperfusion phases in contrast to control animals (Fig. 2a, study I). However, it must be critically acknowledged, that no statistically significant differences were observed among the three groups throughout the study. The lack of difference among the groups may be attributed to uneven catheter implantations, which is probably the principal reason why microdialysis remains in most instances a qualitative measure of blood perfusion.

Colon luminal perfusion and intestinal permeability assessment

Study III employed ^{51}Cr-EDTA as a suitable IP indicator because of its low molecular weight (352 g mol^{-1}) and diameter (6.8 Å), i.e. characteristics, that account for its free and rapid movement via vascular or endothelial barriers making epithelial lining integrity (function of tight junctions) the only determinant of ^{51}Cr-EDTA mucosal permeability (Nylander *et al.* 1989). In this context, microdialysis probes imitate blood vessels (Fig. 1). Providing an adequate molecular diameter / pore cut-off ratio, perfusion rate and good function, probes represent a moderate and determinable obstacle to tracer diffusion, as was the case also in our experiment (Fig. 2, study III). Employment of ^{51}Cr-EDTA was also advantageous, since it contains a radiolabelled atom. Measurements of gamma-activity demand no biochemical assays with sample losses, whereby they enable serial determination of other substances, e.g. metabolic markers or pharmaceuticals. In this way, more information can be yielded from sampling in general and from microdialysis, as a method struggling with low sample volumes, in particular. Because, for convenience, radioactive luminal perfusion solutions were prepared once and used throughout the whole experiment, the effect of decay and loss of ^{51}Cr activity (half-life = 27.7 days) on the results of intestinal mucosal permeability was eliminated by using a ratio of cpm values (probe dialysate / luminal perfusate activity). Ethanol (20 %) proved to be a strong irritant by eliciting mucosal changes of such a degree that could easily be observed macro- and microscopically in association with significant elevations in tracer permeability. These findings are in agreement with previous reports using validated methods (Wallace *et al.* 1992, Stein *et al.* 1998). Regarding the variability of IP results, moderate elevations in colon mucosal permeability in control group (at time intervals 40 and 70 min) might be ascribed to previous transient pressure changes due to brief luminal perfusion rate modifications. This apparent variability, however, was lacking statistical significance and was without histological correlate (Fig. 2, study III). Nonetheless, lower and more stable luminal perfusion rate adjustment or intraluminal pressure control inclusion should be considered in the future.

Measurement of gastric intersitial NO production

Very limited data discussing gastric NO measurement with *in vivo* microdialysis is available. Iversen *et al.* measured nerve-induced release of NO in the wall of rabbit stomach. They utilized different probe types (CMA/10, CMA Medical AB, Stockholm, Sweden, 3 x 0.7 mm dialysis membrane with 20 kDa cut-off) and perfusion rate (1 μl min^{-1}) and estimated interstitial concentrations of nitrite and nitrate to approximate 10 and 70 μmol l^{-1}, respectively. The calculated *in vitro* recovery of the probes (for given perfusion rate) equaled 31 - 33 % for nitrate and nitrite, respectively. The authors did not determine the recovery *in vivo*, but assumed that it would be in the range 10 - 40 % (Iversen *et al.* 1997). Suzuki *et al.* have recently employed similar probes to our catheters (MAB 7.8.10 with 15 kDa cut-off dialysis membrane, active length 10 mm; outer diameter 0.5 mm; Microbiotech/se AB, Stockholm, Sweden) and validated them for studying nitrosative chemistry in the lumen of human stomach. The assessed *in vitro* recovery for nitrite at pH 1.5 and perfusion rate 0.15 ml h^{-1} was 71 % (Suzuki *et al.* 2003). Our results of *in vivo* recovery may be comparable to these studies since the dialysis function of membranes *in vivo* generally diminishes (Lönnroth *et al.* 1987). The total interstitial NO concentration found in study II (~ 23 μmol l^{-1}) was lower than reported by Iversen *et al.* in rabbits. It is a matter of debate to what extent this difference may be attributable to species and/or technique used.

9.2. ISCHEMIC PRECONDITIONING

The definition of time periods for IR injury and effective IPC

In order to detect marked changes in the IR-affected gut and/or its adjacent organs, 45, 60 or even 90 min of arterial clamping are usually included (Serracino-Inglott *et al.* 2002, Koti *et al.* 2002, Cavalieri *et al.* 2002, Hotter *et al.* 1996). We worked with 30 min ischemia, since this time period was shown to be sufficiently long to eventuate in remarkable gastric lesions (Pajdo *et al.* 2001, Brzozowski *et al.* 2004). Peralta *et al.* advocated that for IPC to be effective (in the liver), specific time window between short and long ischemia be allowed, where the lower limit (being 10 minutes) is defined by the amount of accumulated adenosine sufficient to induce NO generation (Peralta *et al.* 1998) and the upper limit is determined by tissue build-up of xanthine. However, NO generation and organ protection could be reached as soon as 5 min after 10 min ischemic episode, as was shown in the small bowel by Hotter *et al.* (Hotter *et al.* 1996). Therefore, to keep the sampling intervals constant without risking loss of effect of IPC, we could safely reduce the 30 min interval between short and long CAO originally proposed by Pajdo *et al.* to 25 min (with brief 5 min ischemia preserved).

The mechanisms behind IP and rIPC – elevated blood flow and NO

The exact sequelae of molecular events taking place as a result of IPC are complex and still require investigation. In general terms, the mechanism of IPC is dependent on blood flow modulation, which may be partly due to local (Pajdo *et al.* 2001, Serracino-Inglott *et al.* 2002) and/or systemic increase in NO production (Koti *et al.* 2002). It is probable, that rIPC also acts *via* a rise in blood perfusion of the target organ, a notion supported by studies that showed rIPC to involve NO-dependent pathways (Peralta *et al.* 1999) and accompanying blood flow elevation (Brzozowski *et al.* 2004). In the liver, enhanced NO generation as a consequence of tissue adenosine accumulation (acting *via* A2 receptors) was described as playing the central "trigger" role in the protective mechanisms of IPC (Peralta *et al.* 2003). Limited data are available on the issue of specific NOS isoform involvement. A study by Serracino-Inglott *et al.* indicates that adenosine, normally released from macroergic phosphates during ischemic conditions, may play a role in preventing the down-regulation of eNOS that occurs during IR and that is responsible for the no-reflow phenomenon associated therewith. The authors suggest that adenosine may presumably exert its protective effect by preventing the cascade of pro-inflammatory events mediated by IR-induced shortage of a potent anti-inflammatory agent NO (Serracino-Inglott *et al.* 2002).

Even though majority of authors based their conclusions regarding NO on the effects of (predominantly non-selective) NOS inhibitors, some measured NO metabolite (nitrite and nitrate) production directly in the blood or tissue. Gong *et al.* studied IPC on rat orthotopic liver transplantation model and demonstrated a significant increase in recipient serum NO 2 h after the transplantation in the ischemically preconditioned donor livers vs. controls (Gong *et al.* 2004). The interruption of blood supply into the donor liver was realized by portal vein as well as hepatic artery clamping (10 min ischemia plus 10 min reperfusion with the unhepatic phase of recipients lasting 25 min). The authors also employed a non-selective NOS inhibitor to support the notion of dependency of IPC on endogenous production of NO. A rat model of selective liver lobular IR injury as studied by Koti et al. realized the long ischemic phase by clamping the corresponding vascular pedicles for 45 min and IPC by 5 min ischemia followed by 10 min reperfusion. Compared with sham-operated animals, 2 h after the reperfusion the authors observed a significant increment in plasma NO metabolites in preconditioned subjects, whereas the rats exposed to IR presented with decreased NO levels. Similar, although less pronounced positive shift resulted from NO-donor L-arginine pretreatment prior to long ischemia. On the other hand, N-ω-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, completely abolished the protection by IPC. Notably, the shifts detected in NO metabolites were accompanied by dynamic trends in hepatic microcirculation (as measured by a laser doppler flowmeter probe) showing significantly elevated flow in preconditioned livers or livers with L-arginine in contrast to IR with/out L-NAME (Koti *et al.* 2002). In a similar IR model, Peralta et al. studied the changes in tissue NO metabolites in association with blood flow using IPC and adenosine A2 receptor antagonist. The group documented increased hepatic blood flow combined with a reduction in organ injury resulting from NO released immediately after IPC – an effect ascribed to accumulated adenosine (Peralta *et al.* 1999).

The (basal and induced) serum levels of NO metabolites in study I are comparable with the previous findings. As regards nutritive blood flow in the control animals, stable values of lithium outflow indicate good reproducibility of results obtained during reference conditions. Furthermore, ischemic periods produced expected falls in this parameter regardless of their length. However, after 2.5 h the ischemic group displayed a tendency towards increased serum NO and IPC seemed to counteract this trend (Fig. 3, study I). Analogically, microcirculation as indicated by lithium microdialysis was not positively affected by IPC, but gave impression of no-reflow conditions typical for complicated IR with failed restoration of blood perfusion in contrast to ischemic group, where blood perfusion returned to the initial levels (Fig. 2a, study I). These observations might to some extent be explained by the adenosine/xanthine theory (Peralta *et al.* 1998), which would suggest an inadequate reperfusion phase during preconditioning in the third group. Under such circumstances, IPC – instead of microcirculatory improvement – would have resulted in longer and more severe (1 h) ischemia with accumulation of disproportional amounts of xanthine leading to production of excessive superoxide anion able to remove the generated NO. This scenario, however, was quite unlikely as reperfusion after clamp removal was in each case verified visually. Another possible explanation of this unexpected phenomenon could lie in (gastrointestinal) bleeding producing a fall especially in splanchnic perfusion. This condition, however, was also improbable since no heparin was administered into the animals and excessive blood loss was not confirmed at autopsy. For these reasons, inclusion of invasive arterial tonometry may prove advantageous in future. Concerning colon, neither celiac ischemia nor preconditioning produced any significant change in this organ´s blood perfusion. Again, such finding is interesting since beneficial relations due to IPC were found to exist in many distant organs with virtually no difference between local and remote effects as to potency (Brzozowski *et al.* 2004, Gho *et al.* 1996).

The extent of organ damage/protection by ischemia/IPC

In the aforementioned study, Koti *et al.* registered a significant attenuation of microcirculatory impairment and hepatocellular injury as indicated by plasma ALT and AST levels (Koti *et al.* 2002). Similarly, Gong *et al.* have demonstrated that IPC-pretreatment of donor livers results in improved liver function (lower recipient serum ALT level) and reduced degree of serum TNF (Gong *et al.* 2004). The beneficial action of IPC (as demonstrated by lowering serum ALT and AST activities) could alternatively be attained by a local pretreatment with exogenous adenosine (Serracino-Inglott *et al.* 2002). With respect to pancreas, Obermaier *et al.* presented the beneficial effect 10 min ischemia with 10 min reperfusion had on microcirculatory derangement due to postischemic pancreatitis induced by 2 h clamping of splenic vessels (Obermaier *et al.* 2004). Along these lines, Dembinski *et al.* observed a potency of IPC to reduce the severity of IR-induced hemorrhagic pancreatitis as given by plasma lipase activity (Dembinski *et al.* 2003). To induce organ injury, 30 min splenic inferior artery clamping followed by 1 h reperfusion was used, while IPC was effectuated by two brief (5 min) periods of CAO (5 minutes apart) 30 min in advance of prolonged ischemia.

Study I indicates that CAO lacks the ability to produce and modify significant liver or pancreatic damage (given by selected enzyme activities) demonstrated by others. As to the liver, this finding can be attributed to the clamping site (celiac artery vs. hepatoduodenal ligament or vascular pedicles), since hepatic artery alone is responsible for a minor part (approximately 5 – 10 %) of liver blood perfusion. Concerning pancreas, however, splenic artery (clamped in other studies) normally originates from celiac trunk, so inadequate blood flow reduction in this organ by CAO is unlikely. It is therefore a matter of debate, what could be responsible for our failure to observe significant changes in serum lipase activity (Fig. 4, study I). Nevertheless, ligations at the closest vicinity to the examined tissue are encouraged in the future.

9.3. EFFECTS OF CAFFEINE

Even though coffee is a complex mixture of substances with effects depending also on the type of coffee bean processing, study II focused only on one ingredient - caffeine. The suspected role of caffeine in the detrimental effect of coffee on gastroduodenal mucosal integrity of young asymptomatic individuals observed previously (Cibickova *et al.* 2004) was tested by measuring three parameters relevant in mucosal barrier maintenance – topical NO release, gastric submucosal

(micro)vascular function and systemic oxidative stress. Possible morphological alterations were also studied.

Caffeine and NO production

Besides vascular tone regulation, NO produced by eNOS as well as iNOS plays numerous (patho)physiological roles many of which may be related to mucosal defence and injury (Elliott and Wallace 1998). It is therefore of interest whether caffeine interferes with the release of this reactive pluripotent radical. Bruce *et al.* report of a significant decrease in exhaled NO levels in humans one hour after 100-200 mg caffeine intake (Bruce *et al.* 2002). More recent findings indicate that caffeine (16 mg kg^{-1} i.v.) may decrease NOS expression in rat skeletal muscles (Corsetti *et al.* 2007) and attenuate glutamate-induced NO synthesis in murine spinal cord *in vitro* (Godfrey *et al.* 2007). Moreover, caffeine negates the protective effect of IPC, i.e. eliminates reactive hyperemia due to the hypoperfusion-induced accumulation of adenosine and enhanced NO production (Riksen *et al.* 2006). On the contrary, endothelium of isolated rat aorta responds to caffeine by promotion of NO synthesis (Hatano *et al.* 1995). In study II, we failed to detect significant shifts in NO production up to 2.5 h after the application of increasing doses of caffeine. However, eNOS releases NO in nanomolar quantities, changes in which we may have been unable to detect using mentioned analytical procedure, while micromolar amounts of NO are produced by activated iNOS as soon as 2 h after application of inducing substance (Huang *et al.* 2005). Therefore, the presented results indicate no effect of caffeine on Ca^{2+}-independent NO production in resting gastric submucosa of anesthetized rats.

Caffeine and blood flow

Ozturkcan *et al.* showed that single i.p. injection of 7.5 – 30 mg kg^{-1} caffeine leads to elevations in rat gastric mucosal blood flow 90 min after drug application (Ozturkcan *et al.* 1974). Moreover, Koyama *et al.* found that *ex vivo* intraluminal administration of caffeine doses as high as 50 – 100 mg kg^{-1} result in a dose-dependent increase in mucosal blood flow lasting up to 90 min (Koyama *et al.* 1999). Although some tendencies of rather decreased nutritive blood flow may be observed 30-90 min after caffeine administration, the results of study II show a lack of statistically significant change in this variable throughout the experiment (Fig. 4b, study II). This would be consistent with other studies showing no direct effect of caffeine on resting blood flow (Umemura *et al.* 2006, Wierema *et al.* 2005, Daniels *et al.* 1998) and/or induced vascular contractility (Barton and Kleinert 1994). Along these lines, literature brings conflicting data concerning the effect of caffeine on endothelial function. Papamichael *et al.* document an acute detrimental effect of caffeine up to 90 min after oral intake of 80 mg of caffeine on endothelium-dependent flow-mediated dilatation of the brachial artery (Papamichael *et al.* 2005) whereas Umemura *et al.* bring evidence of its favorable role on (acetylcholine-induced) forearm vasodilation one hour after oral administration of 300 mg of caffeine, whereas baseline blood flow remained unaltered (Umemura *et al.* 2006). Interestingly, the latter authors ascribe these findings to enhanced endothelial NO synthesis caused by released Ca^{2+} from endoplasmic reticulum through activation of ryanodine-sensitive Ca^{2+} channels and the suppression of cGMP degradation (Hatano *et al.* 1995), while the former investigators account for their observation by the inhibitory effect of caffeine on soluble guanylate cyclase with eventual suppression of cGMP formation. Unfortunately, neither group measured real *in situ* NO production. Nevertheless, these explanatory discrepancies may have some rationale as the differential pharmacologic actions of caffeine depend on the plasma caffeine levels (Fredholm *et al.* 1999). Hence, the final vasoactive action of caffeine may result from a balance between its vasoconstrictive (adenosine-antagonizing) and possibly vasodilating (NO-releasing) ability.

Caffeine and systemic oxidative stress

There are data suggesting multifarious mechanisms whereby caffeine might play a role in augmenting oxidative stress (Papamichael *et al.* 2005). However, the acute unfavourable consequence of caffeine could also follow from its antagonism with adenosine – a substance producing NO and thus preventing mitochondrial oxidant damage in rat cardiomyocytes (Xu *et al.* 2005). In rats that underwent concussive head injury, Al Moutaery *et al.* demonstrated an increase in the level of inflammation and oxidative stress (significant increase in neutrophil infiltration and brain MDA) associated with a dose-dependent increase in mortality of caffeine-pretreated animals (Al Moutaery *et al.* 2003). In the present experiment, the plasma concentrations of caffeine were consonant with previous findings

(Wang and Lau 1998) and indicate good bioavailability of caffeine after i.p. application. However, 2.5 h after drug administration, only a slightly decreasing trend of plasma MDA levels lacking statistical significance was noted. This indicates no acute unfavourable effect of caffeine on whole body´s oxidative stress as far as systemically manifested effects are concerned (Tab. 1, study II).

Caffeine and gastric mucosal morphology

Study II also attempted to investigate possible effects of caffeine on macro- and microscopical levels. Even though these pilot results indicate no acute unfavourable outcomes, the microscopic findings could not be evaluated statistically and thus need to be verified with a more sophisticated histomorphometrical approach in the future.

10. CONCLUSIONS

Study I demonstrates a new alternative of gastrointestinal intersticial nutritional blood flow measurement by lithium microdialysis. Under given experimental conditions, the technique allowed detection of blood perfusion modulation in rat stomach, but failed to detect the protective changes due to ischemic preconditioning in this location. These findings together with unaltered serum parameters require further studies.

The results of study II indicate that i.p. administration of caffeine in given dosages does not produce acute macroscopic changes to gastric mucosa and is unlikely to alter gastric submucosal nutritive blood flow and nitric oxide production or aggravate systemic oxidative stress level. Additional studies (using wider range of dosages) are needed – firstly, to describe caffeine´s impact on gastric mucosa and explore possible mechanisms behind the effects of this substance on gastric physiology in relation to irritant effects of coffee and secondly, to reveal possible contributions of other substances contained in this beverage.

The methodology proposed in study III was capable of detecting barrier injury without necessitating organ detachments, blood withdrawals or even venous catheterisation. This pilot work provides a solid base for development and introduction of a novel method for a wide range of experimental settings involving mucosal permeability measurements. Moreover, it suggests that submucosal microdialysis can be considered a feasible and advantageous alternative of gut barrier function estimation and provides theoretical opportunity to observe parallel local tissue chemistry or pharmacology.

11. FUTURE PERSPECTIVES

With hundreds to thousands articles published annually on pubmed, microdialysis as a technique experiences rapid progress. Employing new technical background, probe designs and perfusion fluids, new fields of applications (e.g. tissue recovery of macromolecules like amyloid beta in the brain) are discovered.

The present thesis added its part to widening our knowledge of possibilities and limitations of microdialysis in rat gastrointestinal tract. Hence, a few lines of future research emerge; firstly, lithium should be validated against well-established markers of blood perfusion like ethanol or ^3H_2O (parallel invasive blood pressure monitoring seems appropriate). In a similar manner, a comparison of gut barrier assessment using microdialysis with lumen-to-blood or blood-to-lumen methods (ideally with the aid of intraluminal pressure control) is highly recommended. Secondly, consideration of approaches that minimize interindividual variability of data, i.e. exploration and inclusion of more accurate and convenient methods of continuous *in vivo* recovery measurements (e.g. using radioactive calibrators) and the use of posponed microdialysis, is encouraged. Finally, attempts to yield multiple variable data (e.g. recovery-, perfusion-, biochemical- and barrier function indicators plus pharmaceuticals) in parallel would bring considerable advances in understanding tissue processes on one hand and the (perspectives and limitations of) microdialysis technique on the other. We have already started walking on this avenue in subsequent experiments.

12. ACKNOWLEDGEMENTS

It is my pleasure and duty to recognize a few people without whom this work would never have been done or who considerably contributed to my postgraduate study.

Greatly appreciated is generous support, willingness and affability of the heads of institutes where I worked and/or studied, namely (without titles): *Vladimír Palička*, *Helena Živná, Jaroslav Cerman*, *Jan Wernerman and Rolf Håkanson*, further tutors and other experts, who motivated me and devoted their precious time for the sake of enlarging my knowledge and experience: *Pavel Živný*, *Zdeněk Zadák*, *Stanislav Mičuda*, *Jaroslav Chládek*, *Olav Rooyackers, Maria Klaude, Eva Čermáková, Per Norlén*, *Maria Bernsand* and *Elizabeth Dungner*, then cooperators and colleagues who kindly provided technical support: *Jana Maláková*, *Lenka Kriesfalusyová*, *Vendula Menclová*, *Jiří Kulíř*, *Hana Malá (†)*, *Jana Prokopová*, and of course numerous colleagues and friends in Hradec Králové, Stockholm and Lund, who helped otherwise and made the time during my studies more funny. My special thanks belongs to *Dana Kakrdová* and *Dagmar Ježková*, who assisted me in everyday laboratory work, arranged countless things and were always at hand with practical advice. I would also like to express my sincere gratitude to my dear family – wife *Ľubica*, son *Albert* and little daughter *Elena* for their existence, love, kindness and tolerance of my absence during experimental work and preparation of manuscripts. I am also obliged to my wife´s parents *Antonín* and *Ľubica* for taking care of my family during writing this thesis. Last but definitely not least, it is my greatest honor and deepest pleasure to acknowledge the measureless help, grace and undeserved care of *God*, my heavenly Father and imperishable foundation, meaning and hope of my life.

Concerning the financial support, this project was made possible by generous funds principally from the Research Project of the University Hospital in Hradec Králové, Czech Republic (MZO 00179906), and in part also from the grant MŠMT COST B25. My study stays in Sweden were kindly sponsored by Hospimed, s.r.o, Prague, Czech Republic.

13. References

AL MOUTAERY K, AL DEEB S, KHAN HA, TARIQ M. Caffeine impairs short-term neurological outcome after concussive head injury in rats. *Neurosurgery* **2003**;53(3):704-12.

ALICAN I, KUBES P. A critical role for nitric oxide in intestinal barrier function and dysfunction. *Am J Physiol* **1996**;270(2):225-37.

ALONSO D, HARRIS JB. Effect of xanthines and histamine on ion transport and respiration by frog gastric mucosa. *Am J Physiol* **1965**;208:18-23.

BARRACHINA MD, CALATAYUD S, CANET A, BELLO R, DIAZ DE ROJAS F, GUTH PH ET AL. Transdermal nitroglycerin prevents nonsteroidal anti-inflammatory drug gastropathy. *Eur J Pharmacol* **1995**;81(2):3-4.

BARTON B, KLEINERT JM. The effect of caffeine on digital haemodynamics. *J Hand Surg [Br]* **1994**;19(3):301-2.

BIEDERBICK W, JOSEPH G, RUMP A, THEISOHN M, KLAUS W. Caffeine in Saliva After Peroral Intake: Early Sample Collection as a Possible Source of Error. *Ther Drug Monitor* **1997**;19:521-4.

BITO L, DAVSON H, LEVIN E, MURRAY M, SNIDER N. The concentrations of free amino acids and other electrolytes in cerebrospinal fluid, in vivo dialysate of brain, and blood plasma of the dog. *J Neurochem* **1966**;33(11):1057-67.

BOTTCHER M, CZERNIN J, SUN KT, PHELPS ME, SCHELBERT HR. Effect of caffeine on myocardial blood flow at rest and during pharmacological vasodilation. *J Nucl Med* **1995**;36(11):2016-21.

BROWN JF, HANSON PJ, WHITTLE BJ. Nitric oxide donors increase mucus gel thickness in rat stomach. *Eur J Pharmacol* **1992**;223(1):103-4.

BRUCE C, YATES DH, THOMAS PS. Caffeine decreases exhaled nitric oxide. *Thorax* **2002**;57:361-3.

BRZOZOWSKI T, KONTUREK PC, KONTUREK SJ, PAJDO R, KWIECIEN S, PAWLIK M ET AL. Ischemic preconditioning of remote organs attenuates gastric ischemia-reperfusion injury through involvement of prostaglandins and sensory nerves. *Eur J Pharmacol* **2004**;499:201-13.

CARDEN DL, GRANGER DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* **2000**;190:255-66.

CASIGLIA E, BONGIOVI S, PALEARI CD, PETUCCO S, BONI M, COLANGELI G ET AL. Haemodynamic effects of coffee and caffeine in normal volunteers: a placebo-controlled clinical study. *J Intern Med* **1991**;29(6):501-4.

CAVALIERI B, PERRELLI MG, ARAGNO M, MASTROCOLA R, CORVETTI G, DURAZZO M ET AL. Ischemic preconditioning attenuates the oxidant-dependent mechanisms of reperfusion cell damage and death in rat liver. *Liver Transpl* **2002**;8:990-9.

CERVINKOVA Z, RADVAKOVA D, KOHOUT P. Liver response to indomethacin-induced intestinal injury. *Acta Medica (Hradec Králové)* **2002**;45:13-8.

CHAMULITRAT W. Nitric oxide inhibited peroxyl and alkoxyl radical formation with concomitant protection against oxidant injury in intestinal epithelial cells. *Arch Biochem Biophys* **1998**;355(2):206-14.

CIBICEK N, CIBICKOVA L, KOHOUT P, ZDANSKY P. [The use of sucrose absorption test (SaLM) for detection of upper gastrointestinal tract mucosa affections in upper dyspepsia patients – a pilot study]. *Acta Medica (Hradec Králové) Suppl* **2004**;47(1):23-8.

CIBICKOVA L, CIBICEK N, ZDANSKY P, KOHOUT P. The impairment of gastroduodenal mucosal barrier by coffee. *Acta Medica (Hradec Králové)* **2004**;47(4):275-8.

CORSETTI G, PASINI E, ASSANELLI D, SALIGARI E, ADOBATI M, BIANCHI R. Acute caffeine administration decreased NOS and Bcl2 expression in rat skeletal muscles. *Pharmacol Res* **2007**;55(2):96-103.

COUTURIER EG, LAMAN DM, VAN DUIJN MA, VAN DUIJN H. Influence of caffeine and caffeine withdrawal on headache and cerebral blood flow velocities. *Cephalalgia* **1997**;17(3):188-90.

CURWAIN BP, HOLTON P. The measurement of dog gastric mucosal blood flow by radioactive aniline clearance compared with amidopyrine clearance. *J Physiol* **1973**;229:115-31.

DANIELS JW, MOLE PA, SHAFFRATH JD, STEBBINS CL. Effects of caffeine on blood pressure, heart rate, and forearm blood flow during dynamic leg exercise. *J Appl Physiol* **1998**;85(1):154-9.

DE LANGE ECM, DE BOER AG, BREIMER DD. Methodological issues in microdialysis sampling for pharmacokinetic studies. *Adv Drug Deliv Rev* **2000**;45:125-48.

DELGADO JM, DEFEUDIS FV, ROTH RH, RYUGO DK, MITRUKA BM. Dialytrode for long term intracerebral perfusion in awake monkeys. *Arch Int Pharmacodyn Ther* **1972**;198(1):9-21.

DEMBINSKI A, WARZECHA Z, CERANOWICZ P, TOMASZEWSKA R, DEMBINSKI M, PABIANCZYK M ET AL. Ischemic preconditioning reduces the severity of ischemia/reperfusion-induced pancreatitis. *Eur J Pharmacol* **2003**;473:207-16.

DOIG CJ, SUTHERLAND LR, SANDHAM JD, FICK GH, VERHOEF M, MEDDINGS JB. Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am J Respir Crit Care Med* **1998**;158:444-51.

DREGELID E, HAUKAAS S, AMUNDSEN S, EIDE GE, SOREIDE O, LEKVEN J ET AL. Microsphere method in measurement of blood flow to wall layers of small intestine. *Am J Physiol* **1986**;250:670-8.

DZIADUŚ-SOKOŁOWSKA A, ORLEF A, BILSKI R, MROCZKA J. The effect of ethanol-caffeine interaction on the gastric mucosal barrier. *Pol J Pharmacol Pharm* **1989**;41(3):253-8.

EDWARDS JE, BROUWER KR, MCNAMARA PJ. GF120918, a P-glycoprotein modulator, increases the concentration of unbound amphenavir in the central nervous system in rats. *Antimicrob Agents Chemother* **2002**;46:2284-6.

ELLIOTT SN, WALLACE JL. Nitric oxide: a regulator of mucosal defence and injury. *J Gastroenterol* **1998**; 3:792-803.

FERRAZ JG, SHARKEY KA, REUTER BK, ASFAHA S, TIGLEY AW, BROWN ML ET AL. Induction of cyclooxygenase 1 and 2 in the rat stomach during endotoxemia: role in resistance to damage. *Gastroenterology* **1997**;113(1):195-204.

FIHN BM, SJOQVIST A, JODAL M. Involvement of enteric nerves in permeability changes due to deoxycholic acid in rat jejunum in vivo. *Acta Physiol Scand* **2003**;178:241-50.

FINK M. Cytopathic hypoxia in sepsis. *Acta Anaesthesiol Scand Suppl* **1997**;110:87-95.

FOX RH, HILTON SM. Bradykinin formation in human skin as a factor in heat vasodilatation. *J Physiol (Lond)* **1958**;142:219-32.

FRANCO L, DORIA D. Nitric oxide enhances prostaglandin production in ethanol-induced gastric mucosal injury in rats. *Eur J Pharmacol* **1998**;348(2-3):247-56.

FREDHOLM BB, BATTIG K, HOLMEN J, NEHLIG A, ZVARTAU EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* **1999**;51:83-133.

GHO BC, SCHOEMAKER RG, VAN DEN DOEL MA, DUNCKER DJ, VERDOUW PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* **1996**;94:2193-200.

GODFREY L, BAILEY I, TOMS NJ, CLARKE GD, KITCHEN I, HOURANI SM. Paracetamol inhibits nitric oxide synthesis in murine spinal cord slices. *Eur J Pharmacol* **2007**;562(1-2):68-71.

GONG JP, TU B, WANG W, PENG Y, LI SB, YAN LN. Protective effect of nitric oxide induced by ischemic preconditioning on reperfusion injury of rat liver graft. *World J Gastroenterol* **2004**;10:73-6.

GRANGER DN. Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* **1999**;6(3):167-78.

GRISHAM MB, GRANGER DN, LEFER DJ. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. *Free Radic Biol Med* **1998**;25(4-5):404-33.

GROSS SS, WOLIN MS. Nitric oxide: pathophysiological mechanisms. *Annu Rev Physiol* **1995**;57:737-69.

HAMADA E, NAKAJIMA T, HATA Y, HAZAMA H, IWASAWA K, TAKAHASHI M ET AL. Effect of caffeine on mucus secretion and agonist-dependent Ca2+ mobilization in human gastric mucus secreting cells. *Biochim Biophys Acta* **1997**;1356(2):198-206.

HAN X, FINK MP, DELUDE RL. Proinflammatory cytokines cause NO*-dependent and -independent changes in expression and localization of tight junction proteins in intestinal epithelial cells. *Shock* **2003**;9(3):229-37.

HATANO Y, MIZUMOTO K, YOSHIYAMA T, YAMAMOTO M, IRANAI H. Endothelial-dependent and –independent vasodilatation of isolated rat aorta induced by caffeine. *Am J Physiol* **1995**;269:1679-84.

HELMER KS, WEST SD, SHIPLEY GL, CHANG L, CUI Y, MAILMAN D ET AL. Gastric nitric oxide synthase expression during endotoxemia: implications in mucosal defense in rats. *Gastroenterology* **2002**;23(1):173-86.

HENDRIX T, ASSMAN R. Spectrophotometric correction for bile pigments in the thiobarbituric test for malondialdehyde-like substances in plasma. *Med Lab Sci* **1990**;47:10-16.

HICKNER RC, BONE D, UNGERSTEDT U, JORFELDT L, HENRIKSSON J. Muscle blood flow during intermittent exercise: comparison of the microdialysis ethanol technique and 133Xe clearance. *Clin Sci (Lond)* **1994**;86(1):15-25.

HICKNER RC, EKELUND U, MELLANDER S, UNGERSTEDT U, HENRIKSSON J. Muscle blood flow in cats: comparison of microdialysis ethanol technique with direct measurement. *J Appl Physiol* **1995**;9(2):638-47.

HICKNER RC, ROSDAHL H, BORG I, UNGERSTEDT U, JORFELDT L, HENRIKSSON J. The ethanol technique of monitoring local blood flow changes in rat skeletal muscle: implications for microdialysis. *Acta Physiol Scand* **1992**;146(1):87-97.

HOECKER CH, NELLE M, POESCHL J, BEEDGEN B, LINDERKAMP O. Caffeine impairs cerebral and intestinal blood flow velocity in preterm infants. *Pediatrics* **2002**;109(5):784-7.

HOTTER G, CLOSA D, PRADOS M, FERNANDEZ-CRUZ L, PRATS N, GELPI E ET AL. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun* **1996**;222:27-32.

HRUBÁ P, ŽIVNÝ P, ŽIVNÁ H, PALIČKA V. Muscle, liver and kidney interstitium blood flow changes in rats measured by microdialysis with flow marker added. *Klin Biochem Metab* **2004**;12:9-13.

HUANG CJ, TSAI PS, PAN WHT, SKIMMING JW. Microdialysis for measurement of hepatic and systemic nitric oxide biosynthesis in septic rats. *Acta Anaesthesiol Scand* **2005**;49:28-34.

HUIE RE, PADMAJA S. The reaction of no with superoxide. *Free Radic Res Commun* **1993**;18(4):195-9.

HUTCHESON IR, WHITTLE BJ, BOUGHTON-SMITH NK. Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br J Pharmacol* **1990**;101(4):815-20.

IVERSEN HH, CELSING F, LEONE AM, GUSTAFSSON LE, WIKLUND NP. Nerve-induced release of nitric oxide in the rabbit gastrointestinal tract as measured by in vivo microdialysis. *Br J Pharmacol* **1997**;120:702-6.

JACOBSON ED, LINFORD RH, GROSSMAN MI. Gastric secretion in relation to mucosal blood flow studied by a clearance technic. *J Clin Invest* **1966**;45(1):1-13.

JACOBSON I, SANDBERG M, HAMBERGER A. Mass transfer in brain dialysis devices – a new method for the estimation of extracellular amino acids concentration. *J Neurosci Meth* **1985**;15:263-8.

JANSSON K, REDLER B, TRUEDSSON L, MAGNUSSON A, UNGERSTEDT U, NORGREN L. Postoperative on-line monitoring with intraperitoneal microdialysis is a sensitive clinical method for measuring increased anaerobic metabolism that correlates to the cytokine response. *Scand J Gastroenterol* **2004**;39:434-9.

JEDLICKOVA V, PALUCH Z, ALUSIK S. Determination of nitrate and nitrite by high-performance liquid chromatography in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **2002**;780:193-7.

KITANO M, NORLÉN P, HÅKANSON R. Gastric submucosal microdialysis: a method to study gastrin- and food-evoked mobilization of ECL-cell histamine in conscious rats. *Regul Pept* **2000**;86:113-23.

KOHOUT P: [Small intestinal barrier function]. In: [Modes of non-invasive small bowel investigation]. KOHOUT P (ED), Galén, Praha, **2002**;28.

KOIKE K, MOORE FA, MOORE EE, READ RA, CARL VS, BANERJEE A. Gut ischemia mediates lung injury by a xanthine oxidase-dependent neutrophil mechanism. *J Surg Res* **1993**;54(5):469-73.

KONTUREK PC, BRZOZOWSKI T, SLIWOWSKI Z, PAJDO R, STACHURA J, HAHN EG ET AL. Involvement of nitric oxide and prostaglandins in gastroprotection induced by bacterial lipopolysaccharide. *Scand J Gastroenterol* **1998**;33(7):691-700.

KOTI RS, YANG W, DASHWOOD MR, DAVIDSON BR, SEIFALIAN AM. Effect of ischemic preconditioning on hepatic microcirculation and function in a rat model of ischemia reperfusion injury. *Liver Transpl* **2002**;8:1182-91.

KOYAMA R, KATAOKA H, TANAKA Y, NAKATSUGI S, FURUKAWA M. Effect of caffeine on ibuprofen-induced gastric mucosal damage in rats. *J Pharm Pharmacol* **1999**;51(7):817-24.

KUBES P, KANWAR S, NIU XF, GABOURY JP. Nitric oxide synthesis inhibition induces leukocyte adhesion via superoxide and mast cells. *FASEB J* **1993**;7(13):1293-9.

KUBES P. Ischemia-reperfusion in feline small intestine: a role for nitric oxide. *Am J Physiol* **1993**;264(1):143-9.

LARSSON CI. The use of an "internal standard" for control of the recovery in microdialysis. *Life Sci* **1991**;49:73-8.

LIEM DA, VERDOUW PD, PLOEG H, KAZIM S, DUNCKER DJ. Sites of action of adenosine in interorgan preconditioning of the heart. *Am J Physiol Heart Circ Physiol* **2002**;283:29-37.

LÖNNROTH P, JANSSON PA, SMITH U. A microdialysis method allowing characterization of intercellular water space in humans. *Am J Physiol* **1987**;253(2):228-31.

LOPEZ-BELMONTE J, WHITTLE BJ, MONCADA S. The actions of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa. *Br J Pharmacol* **1993**;108(1):73-8.

MACKENDRICK W, CAPLAN M, HSUEH W. Endogenous nitric oxide protects against platelet-activating factor-induced bowel injury in the rat. *Pediatr Res* **1993**;34(2):222-8.

MALLICK IH, YANG W, WINSLET MC, SEIFALIAN AM. Ischaemic preconditioning improves microvascular perfusion and oxygenation following reperfusion injury of the intestine. *Br J Surg* **2005**;92:1169-76.

MAROTTA RB, FLOCH MH. Diet and nutrition in ulcer disease. *Med Clin North Am* **1991**;75(4):967-79.

MCCALLION K, WATTANASIRICHAIGOON S, GARDINER KR, FINK MP. Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. *Shock* **2000**;14:429-34.

MENCONI MJ, UNNO N, SMITH M, AGUIRRE DE, FINK MP. Nitric oxide donor-induced hyperpermeability of cultured intestinal epithelial monolayers: role of superoxide radical, hydroxyl radical, and peroxynitrite. *Biochim Biophys Acta* **1998**;1425(1):189-203.

MERCER DW, CASTANEDA AA, DENNING JW, CHANG L, RUSSELL DH. Effects of endotoxin on gastric injury from luminal irritants in rats: potential roles of nitric oxide. *Am J Physiol* **1998**;275(3):449-59.

MURAKAMI M, MORIGA M, MIYAKE T, UCHINO H. New device for the measurement of hydrogen clearance rate from the gastric mucosa. *Gastroenterol Jpn* **1980**;15:385-9.

MURRY CE, JENNINGS RB, REIMER KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **1986**;74:1124-36.

NATALE G, LAZZERI G, BLANDIZZI C, GHERARDI G, LENZI P, PELLEGRINI A ET AL. Seriate histomorphometry of whole rat stomach: an accurate and reliable method for quantitative analysis of mucosal damage. *Toxicol Appl Pharmacol* **2001**;174:17-26.

NISHIDA K, OHTA Y, ISHIGURO I. Role of gastric mucosal constitutive and inducible nitric oxide synthases in the development of stress-induced gastric mucosal lesions in rats. *Biochem Biophys Res Commun* **1997**;236(2):275-9.

NIU XF, SMITH CW, KUBES P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ Res* **1994**;74(6):1133-40.

NYLANDER O, KVIETYS P, GRANGER DN. Effects of hydrochloric acid on duodenal and jejunal mucosal permeability in the rat. *Am J Physiol* **1989**;257:653-60.

OBERMAIER R, VON DOBSCHUETZ E, DROGNITZ O, HOPT UT, BENZ S. Ischemic preconditioning attenuates capillary no-reflow and leukocyte adherence in postischemic pancreatitis. *Langenbecks Arch Surg* **2004**;389:51-6.

OLSON RJ, JUSTICE JR JB. Quantitative microdialysis under transient conditions. *Anal Chem* **1993**;65:1017-22.

OZTURKCAN O, DE SAINT BLANQUAT G, DERACHE R. Effet de la cafféïne sur le flux sanguin de la muqueuse gastrique chez le Rat. *Thérapie* **1974**;29:941-4.

PAJDO R, BRZOZOWSKI T, KONTUREK PC, KWIECIEN S, KONTUREK SJ, SLIWOWSKI Z ET AL. Ischemic preconditioning, the most effective gastroprotective intervention: involvement of prostaglandins, nitric oxide, adenosine and sensory nerves. *Eur J Pharmacol* **2001**;427:263-6.

PAPAMICHAEL CM, AZNAOURIDIS KA, KARATZIS EN, KARATZI KN, STAMATELOPOULOS KS, VAMVAKOU G ET AL. Effect of coffee on endothelial function in healthy subjects: the role of caffeine. *Clin Sci (Lond)* **2005**;109(1):55-60.

PARMAR NS, TARIQ M, AGEEL AM. Effect of nicotine, alcohol and caffeine pretreatment on the gastric mucosal damage induced by aspirin, phenylbutazone and reserpine in rats. *Jpn J Pharmacol* **1985**;39(1):1-6.

PAYNE D, KUBES P. Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. *Am J Physiol* **1993**;265(1):189-95.

PERALTA C, CLOSA D, XAUS C, GELPI E, ROSELLO-CATAFAU J, HOTTER G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. *Hepatology* **1998**;28:768-73.

PERALTA C, HOTTER G, CLOSA D, PRATS N, XAUS C, GELPI E ET AL. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. *Hepatology* **1999**;29:126-32.

PERALTA C, SERAFIN A, FERNANDEZ-ZABALEGUI L, WU ZY, ROSELLO-CATAFAU J. Liver ischemic preconditioning: a new strategy for the prevention of ischemia-reperfusion injury. *Transplant Proc* **2003**;35:1800-2.

PFEIFFER CJ, ROTH JLA. Studies on the secretory and cytotoxic actions of caffeine on the ferret gastric mucosa. *Exp Mol Pathol* **1970**;13:66-78.

PLOCK N, KLOFT CH. Microdialysis – theoretical background and recent implementation in applied life-sciences. *Eur J Pharm Sci* **2005**;25:1-24.

POST H, HEUSCH G. Ischemic preconditioning. Experimental facts and clinical perspective. *Minerva Cardioangiol* **2002**;50:569-605.

PRICE KJ, HANSON PJ, WHITTLE BJ. Localization of constitutive isoforms of nitric oxide synthase in the gastric glandular mucosa of the rat. *Cell Tissue Res* **1996**;285(1):157-63.

PRICE KJ, HANSON PJ. Constitutive nitric oxide synthases in rat gastric mucosa: subcellular distribution, relative activity and different carboxyl-terminal antigenicity of the neuronal form compared with cerebellum. *Digestion* **1998**;59(4):308-13.

PRZYKLENK K, BAUER B, OVIZE M, KLONER RA, WHITTAKER P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* **1993**;87:893-9.

RIKSEN NP, ZHOU Z, OYEN WJG, JASPERS R, RAMAKERS BP, BROUWER RMHJ ET AL. Caffeine prevents protection in two human models of ischemic preconditioning. *J Am Coll Cardiol* **2006**;48:700-7.

ROTH JA, IVY AC. The pathogenesis of caffeine-induced ulcers. *Surgery* **1945**;17:644-9.

SALZMAN AL, MENCONI MJ, UNNO N, EZZELL RM, CASEY DM, GONZALEZ PK ET AL. Nitric oxide dilates tight junctions and depletes ATP in cultured Caco-2BBe intestinal epithelial monolayers. *Am J Physiol* **1995**;268(2):361-73.

SERRACINO-INGLOTT F, VIRLOS IT, HABIB NA, WILLIAMSON RC, MATHIE RT. Adenosine preconditioning attenuates hepatic reperfusion injury in the rat by preventing the down-regulation of endothelial nitric oxide synthase. *BMC Gastroenterol* **2002**;2:22-7.

SOLLIGÅRD E, JUEL IS, BAKKELUND K, JOHNSEN H, SAETHER OD, GRONBECH JE ET AL. Gut barrier dysfunction as detected by intestinal luminal microdialysis. *Intensive Care Med* **2004**;30:1188-94.

SOMMER T. Microdialysis in the assessment of regional intestinal ischemia. Thesis, Aalborg University **2004**:10-3.

STALLKNECHT B, DONSMARK M, ENEVOLDSEN LH, FLUCKEY JD, GALBO H. Estimation of rat muscle blood flow by microdialysis probes perfused with ethanol, [14C]ethanol, and 3H2O. *J Appl Physiol* **1999**;86:1054-61.

STEIN J, RIES J, BARRETT KE. Disruption of intestinal barrier function associated with experimental colitis: possible role of mast cells. *Am J Physiol* **1998**;274:203-9.

STUEHR DJ, GRIFFITH OW. Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol* **1992**;65:287-346.

SUGI K, MUSCH MW, FIELD M, CHANG EB. Inhibition of Na+,K+-ATPase by interferon gamma down-regulates intestinal epithelial transport and barrier function. *Gastroenterology* **2001**;120(6):1393-403.

SUZUKI H, MORIYA A, IIJIMA K, MCELROY K, FYFE VE, MCCOLL KEL. Validation of microdialysis probes for studying nitrosative chemistry within localized regions of the human upper gastrointestinal tract. *Scand J Gastroenterol* **2003**;38:856-63.

SZELENYI I. [Neutral-red clearance technique for measuring gastric mucosal blood flow in the rat] *Arzneimittelforschung* **1981**;31:675-7.

SZLACHCIC A, BILSKI R, DZIADUS-SOKOLOWSKA A, MICHALSKI J, MROCZKA J. The effect of nitric oxide donors and L-arginine on the gastric electrolyte barrier. *J Physiol Pharmacol* **2001**;52(2):211-20.

TANAKA A, KUNIKATA T, MIZOGUCHI H, KATO S, TAKEUCHI K. Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. *J Physiol Pharmacol* **1999**;50(3):405-17.

TEPPERMAN BL, BROWN JF, KOROLKIEWICZ R, WHITTLE BJ. Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: effect of endotoxin challenge. *J Pharmacol Exp Ther* **1994**;271(3):1477-82.

TEPPERMAN BL, BROWN JF, WHITTLE BJ. Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am J Physiol* **1993**;265(2):214-8.

TEPPERMAN BL, SOPER BD. Nitric oxide synthase induction and cytoprotection of rat gastric mucosa from injury by ethanol. *Can J Physiol Pharmacol* **1994**;72(11):1308-12.

TERRY P, LAGERGREN J, WOLK A, NYREN O. Reflux-inducing dietary factors and risk of adenocarcinoma of the esophagus and gastric cardia. *Nutr Cancer* **2000**;38:186-91.

THOREN A, JAKOB SM, PRADL R, ELAM M, RICKSTEN SE, TAKALA J. Jejunal and gastric mucosal perfusion versus splanchnic blood flow and metabolism: an observational study on postcardiac surgical patients. *Crit Care Med* **2000**;28:3649-54.

TISDALL MM, SMITH M. Cerebral microdialysis: research technique or clinical tool. *BJA* **2006**;97(1):18-25.

TOSSMAN U, UNGERSTEDT U. Microdialysis in the study of extracellular levels of amino acids in the brain. *Acta Physiol Scand* **1986**;128:9-14.

TRIPP MA, TEPPERMAN BL. Role of calcium in nitric oxide-mediated injury to rat gastric mucosal cells. *Gastroenterology* **1996**;111(1):65-72.

UIL JJ. Clinical implications of the sugar absorption test. Thesis, Utrecht University **1996**:2-14.

UMEMURA T, UEDA K, NISHIOKA K, HIDAKA T, TAKEMOTO H, NAKAMURA S ET AL. Effects of acute administration of caffeine on vascular function. *Am J Cardiol* **2006**;98:1538-41.

UNGERSTEDT U, PYCOCK C. Functional correlates of dopamine neurotransmission. *Bull Schweiz Akad Med Wiss* **1974**;30(1-3):44-55.

WALLACE JL, HIGA A, MCKNIGHT GW, MACINTYRE DE. Prevention and reversal of experimental colitis by a monoclonal antibody which inhibits leukocyte adherence. *Inflammation* **1992**;16:343-54.

WALLGREN F, AMBERG G, HICKNER RC, EKELUND U, JORFELDT L, HENRIKSSON J. A mathematical model for measuring blood flow in skeletal muscle with the microdialysis ethanol technique. *J Appl Physiol* **1995**;79(2):648-59.

WANG Y, LAU CHE. Caffeine has similar pharmakokinetics and behavioral effects via the IP and PO routes of administration. *Pharmacol Biochem Behav* **1998**;60(1):271-8.

WHITTLE BJ, LOPEZ-BELMONTE J, MONCADA S. Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br J Pharmacol* **1990**;99(3):607-11.

WIEREMA TK, HOUBEN AJ, KROON AA, POSTMA CT, KOSTER D, VAN ENGELSHOVEN JM ET AL. Mechanisms of adenosine-induced renal vasodilatation in hypertensive patients. *J Hypertens* **2005**;23(9):1731-6.

WITTMERS LE, ALICH A, QUIRK DR. Effect of caffeine on the Gastric Potential Difference (GPD). *FASEB Journal* **1998**;12(5):737.

XU Z, PARK SS, MUELLER RA, BAGNELL RC, PATTERSON C, BOYSEN PG. Adenosine produces nitric oxide and prevents mitochondrial oxidant damage in rat cardiomyocytes. *Cardiovasc Res* **2005**;65(4):803-12.

YANO S, ISOBE Y, HARADA M. The etiology of caffeine-induced aggravation of gastric lesions in rats exposed to restraint plus water-immersion stress. *J Pharmacobiodyn* **1982**;5(7):485-94.

14. APPENDIX

14.1. FULLTEXTS OF ORIGINAL STUDIES I-III

The reprints were made with kind permission from the publishers.

study I

ORIGINAL ARTICLE

LITHIUM MICRODIALYSIS AND ITS USE FOR MONITORING OF STOMACH AND COLON SUBMUCOSAL BLOOD PERFUSION - A PILOT STUDY USING ISCHEMIC PRECONDITIONING IN RATS

Norbert Cibiček[1], Stanislav Mičuda[2], Jaroslav Chládek[2], Pavel Živný[1], Zdeněk Zadák[3], Eva Čermáková[4], Vladimír Palička[1]

Charles University in Prague, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic: Institute of Clinical Biochemistry and Diagnostics[1]; Department of Pharmacology[2]; Department of Gerontology and Metabolism, Research Laboratory[3]; Department of Biophysics and Biostatistics[4]

Summary: During shock, exposure of gut to ischemia determines patient's survival. Ischemic preconditioning (ISP) elevates nitric oxide and blood perfusion, whereby it protects organs against subsequent severe ischemia/reperfusion. Using appropriate flow marker, microdialysis may serve to monitor interstitial microcirculation. Hence, our aim was to test the reliability of lithium as a flow marker (lithium microdialysis, LM) on an ISP model. Rats were divided into three groups. Two (ischemic and preconditioned) groups underwent 30 min celiac artery occlusion (CAO) with 2.5 h reperfusion. 25 min before CAO, the latter experienced 5 min ischemia. Sham-operated animals served as controls. LM in stomach and colon submucosa, serum nitric oxide, hepatic and pancreatic enzymes were measured. In stomach, LM indicated a decrease in blood perfusion evoked by CAO ($p<0.01$) in both experimental groups. During reperfusion, the ischemic animals showed a restoration of microcirculation, unlike the preconditioned ones, whose blood perfusion failed to regenerate ($p<0.001$). For any group, LM showed no microcirculation modification in colon. Serum analytes remained unchanged. We conclude that LM appears to be a potentially suitable indicator of gastrointestinal interstitial microcirculation. However, we failed to demonstrate any beneficial effect of ISP on pancreas, systemic nitric oxide and local/remote microcirculation within studied organs.

Key words: *Microdialysis; Microcirculation; Gastrointestinal tract; Ischemic preconditioning*

Introduction

It is well recognized that in critical situations, the prognosis of patients depends largely on maintenance of adequate blood perfusion in order to preserve the barrier integrity and metabolic functions of gastrointestinal tract and its adjacent organs. Recent evidence has shown that the viability of gut exposed to ischemic insult can be enhanced by ischemic preconditioning (ISP) taking place prior to sustained ischemia (1, 3). Because of deleterious impact ischemia in splanchnic organs may exert on the entire organism via bacterial- and toxin translocation and cytokine production, ISP offers an interesting therapeutic approach for clinical practice (14).

ISP refers to a process by which a (series of) brief ischemic episode(s) confers protection against subsequent prolonged ischemia/reperfusion. Regarding the mechanism, a number of neurohumoral mediator pathways have been proposed, where elevated nitric oxide followed by enhanced blood perfusion plays a central role (14). ISP can be examined with respect to one specific organ, or considering other organs at a distance. The former differentiates between local and regional ISP, whereas the latter defines an inter-organ or remote ISP (rISP). With respect to abdominal organs, ISP has been exemplified in the liver (14), pancreas (3), small intestine (10) or stomach (1). However, rISP in colon has not attracted much interest, yet.

In many organs including the gut, *in vivo* microdialysis is a well-established miniinvasive means of local tissue metabolism monitoring (2). Using appropriate flow marker (also called tracer), this method offers opportunity to assess tissue microcirculation based on an assumption that the washout of tracer from the probe is related to extracellular fluid kinetics, which depends primarily on microcirculatory conditions. However, the most frequent tracer molecule (ethanol) is volatile, requires a relatively sophisticated analytical determination, or needs to be labeled radioactively (15). Recently, a novel, apyrogenic, non-radioactive and freely diffusible flow marker with well-defined toxicity in humans - lithium, has successfully been applied to describe blood perfusion modifications in rat liver, kidney and muscle interstitium (7). Nevertheless,

further information on the usefulness of this promising tracer are needed.

Hence, the aim of this study was twofold: first, to verify the hypothesis that lithium could be used for monitoring of microcirculation in the alimentary tube and second, to evaluate the influence of celiac ISP on celiac artery occlusion (CAO)-induced microcirculatory changes in stomach and descending colon. Both hypotheses were tested on a rat model of gastric ischemia and ISP performed at the level of celiac artery trunk (1). Furthermore, the systemic effects of ISP and CAO were assessed using selected serum enzyme activities and nitric oxide concentration.

Material and Methods

Materials. Flame photometer serum-standard solution (Eppendorf, Hamburg, Germany) with Li^+ concentration of 2.00 mmol $l^{-1}$was used as microdialysis perfusion medium.

Animals. Adult male Wistar rats weighing 260 to 330 g, were used. The animals were housed in the animal quarters under controlled environmental conditions. They had free access to standard rat chow except 16–18 hours before experiments, when they were fasted. Tap water was provided *ad libitum.* The rats received care in accordance with the guidelines set by the Institutional Animal Use and Care Committee of the Charles University in Prague, Czech Republic. All experimental procedures were approved by local ethics committee.

Experimental protocol. The animals were randomly assigned to three groups denoted as S, IS, and ISP (n = 6–10 in each group). The first group (S) was sham-operated. The second group (IS) underwent a 30 min period of complete CAO with subsequent 2.5 h of reperfusion. The rats in the third group (ISP) were preconditioned by exposure to a short ischemia/reperfusion period (5 and 25 min, respectively), followed by prolonged ischemia/reperfusion (30 min and 2.5 h, respectively). During all procedures, the animals were under general i.p. pentobarbital anesthesia (50 mg kg^{-1} followed by 15 mg kg^{-1} h^{-1}) and operated in supine position. The rectal temperature was monitored and maintained at 38.5 °C with a heating lamp. The trachea was cannulated to ensure patent airways. After midline laparotomy, the celiac artery was disclosed and underlaid by smooth rubber tubing to assist later clamping. Ischemia/reperfusion was accomplished by placement/removal of a micro-bulldog clamp at the level of celiac artery origination from abdominal aorta. The success of each intervention was verified visually (assessment of blood flow distal from the site of occlusion).

Gastric and colon submucosal microdialysis technique. After laparotomy, stomach was exteriorized and moistened continuously. Avoiding blood vessels, a 6 mm long tunnel was made from serosal aspect in the submucosal layer of its glandular part from greater to lesser curvature by means of a 26 G needle. For the descending colon, similar procedure was followed along with its long axis at a distance of 5 cm from the anus. Into the tunnels, microdialysis probes (CMA/20, active length 4 mm, outer diameter 0.5 mm, cut-off 20 kDa, CMA/Microdialysis, Solna, Sweden) were carefully inserted and fixed in place with sutures. After each experiment, probes were tested for leakage and their positions were verified by histology (Fig. 1). After surgery, the abdominal opening was closed to avoid fluid losses. Microdialysis catheters were perfused at 1.2 µl min^{-1} using a perfusion pump. An initial 60 min stabilisation period was followed by 5 h of experimental procedure (see above) with continuous dialysate sampling in 30 min intervals into microvials. Samples were stored at -20 °C until lithium determination. At the end of experiment, arterial blood was withdrawn and the removed serum was stored at -20°C until analysis.

Blood perfusion measurement. The extent of submucosal blood perfusion was expressed as lithium inflow - outflow concentration (Li_{in-out}) difference, i.e. Li efflux as reported previously (7). Dialysate Li^+ was determined using EFOX 5053 flame photometer (Eppendorf, Hamburg, Germany) according to manufacturer's instructions.

Determination of nitric oxide in serum. Nitric oxide was measured indirectly (and expressed) as nitrate and nitrite (the stable nitric oxide metabolites) using HPLC with UV-VIS detection and fluorimetry methods described elsewhere (8).

Determination of serum enzyme activities. Hitachi 917 autoanalyser (Boehringer, Mannheim, Germany) with commercially available reagent kits (Roche Diagnostics GmbH, Mannheim, Germany) were utilized. For the study of the extent of liver injury, alaninaminotransferase (ALT), aspartataminotransferase (AST), lactatdehydrogenase (LDH) and cholinesterase (CHE) serum activities were investigated. To evaluate the level of pancreatic damage, lipase (LIP) and amylase (AMYL) activities were determined. All analyses were performed in accordance with manufacturer's instructions.

Histology. The removed tissues were fixed in 10% formaline, embedded in paraffin and further treated according to procedures standard for hematoxylin-eosine stain.

Statistics. Data are expressed as means ± standard error of mean (SEM). For statistical evaluation, Analysis Of Variance with Fisher's LSD multiple comparison *post hoc* test (programs NCSS 2004 and Statistica) were used. The chosen level of significance was α=0.05.

Results

Blood perfusion estimation. Fig. 2 summarizes the time course of Li_{in-out} differences in all groups in the submucosa of stomach (a) and colon (b). The zero interval of microdialysate collection (obtained within 60–90 min after commencement) provided basal values, where no sta-

tistically significant difference was found between the stomach and colon. Likewise, between-group comparisons showed comparable baseline values in stomach as well as in colon.

Blood perfusion in stomach (Fig. 2a). In the S group, absolute values of Li_{in-out} difference showed no variation in time. Brief (5 min) ischemia in the ISP group caused a significant drop in Li_{in-out} difference from its baseline ($p<0.05$). Within the IS and ISP groups, 30 min interval of CAO produced significant depressions in Li_{in-out} difference when compared with corresponding basal levels ($p<0.01$). Immediately after onset of reperfusion, the values of Li_{in-out} difference raised in both groups, reaching levels not significantly different from their baselines. In the next 2 hours, Li_{in-out} difference within the IS group continued to elevate slowly, while the opposite holds true for ISP group, where we observed a steadily falling trend ($p<0.001$). However, none of the changes observed within the ligated groups was sufficient to statistically manifest also in between-group comparisons with controls.

Blood perfusion in descending colon (Fig. 2b). Similar to gastric perfusion, the S group provided constant values of Li_{in-out} difference over the entire experiment. Neither within-group, nor between-group comparisons in parallel time intervals reached the level of significance.

Serum nitric oxide and enzymatic activities. The extent of potential organ impairment and modulation of the body's metabolism was evaluated by determination of selected serum analytes. Fig. 3 depicts the grade of nitric oxide production modulation by ischemia and ISP. Even though the mean serum concentration of nitric oxide in IS group was 165% of that in control group (S), the difference was only marginally significant ($p=0.06$). The ISP group gave results similar to controls. Likewise, the activities of serum enzymes in question were not statistically different (Fig. 4).

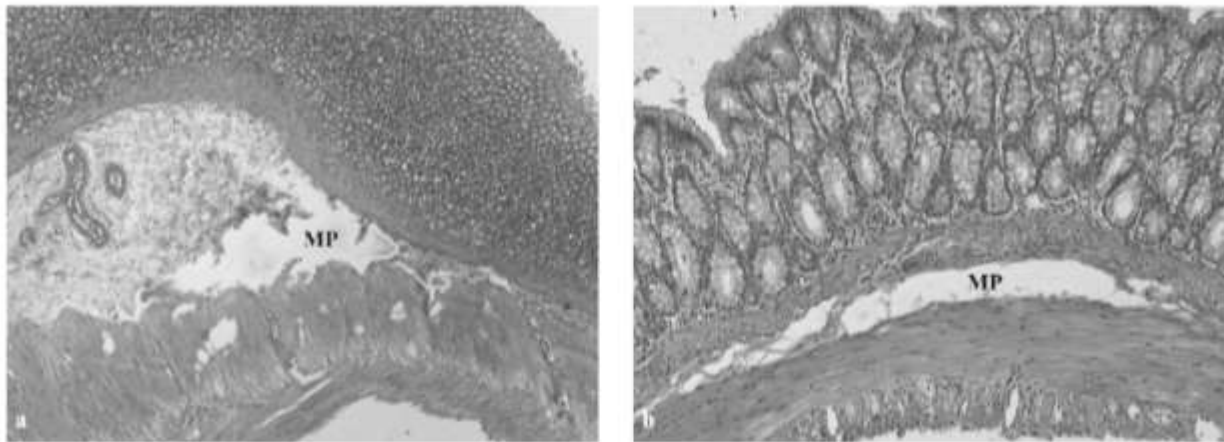

Fig. 1: Histological appearance of the wall of stomach (panel a) and descending colon (panel b) with previous probe positions marked MP ("microdialysis probe"). Sham-operated group, haematoxylin-eosine stain.

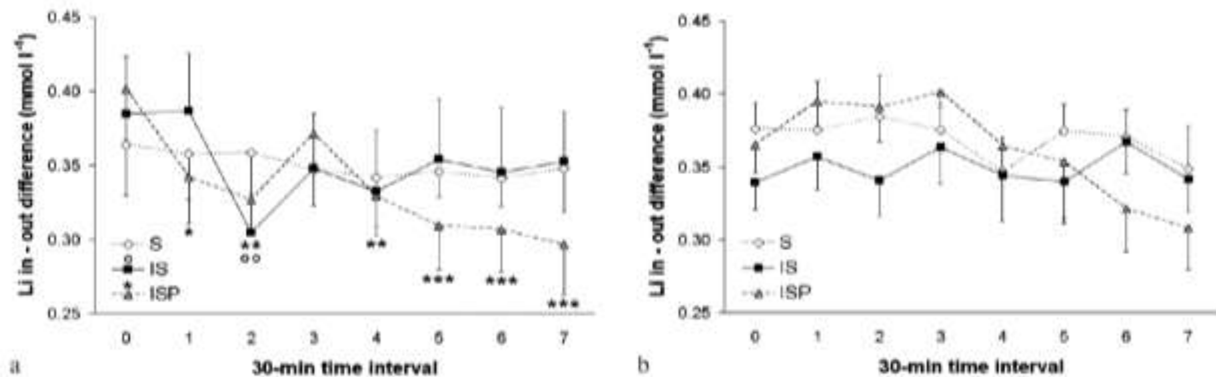

Fig. 2: A comparison of submucosal stomach (a) and colon (b) blood perfusion among the three groups (S, IS, ISP) followed in time and expressed as Li_{in-out} difference. Within the groups, markings *,°; **,°° and ***,°°° denote $p<0.05$; $p<0.01$ and $p<0.001$, respectively as compared to corresponding reference (baseline) levels. Results are mean ± SEM of 6-8 rats (2 animals excluded due to hemorrhage around the probe).

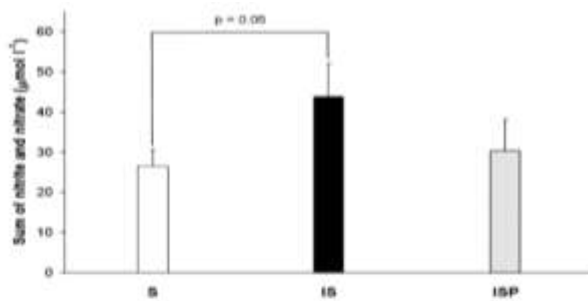

Fig. 3: Effect of ischemia (IS) and ischemic preconditioning (ISP) on serum nitric oxide (shown as the sum of nitrite and nitrate and compared with sham-operated group, S). Results are mean + SEM of 6–10 rats.

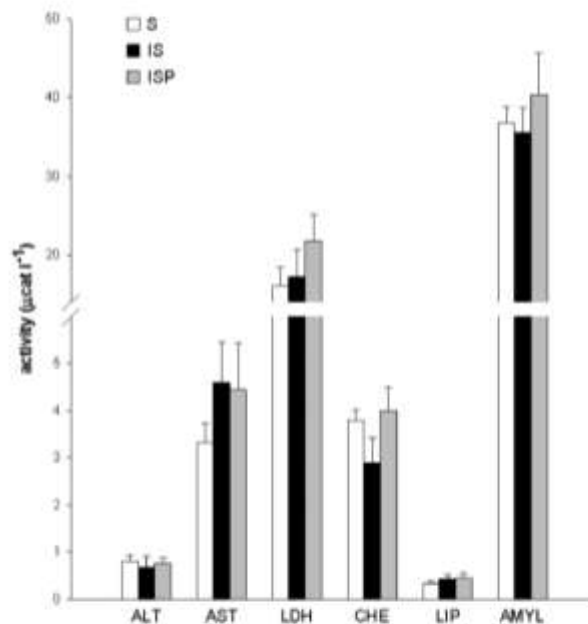

Fig. 4: Effects of ischemia (IS) and ischemic preconditioning (ISP) on serum enzyme activities (expressed as comparisons with sham-operated group, S). Results are mean + SEM of 6–10 rats. Level of significance was not reached in any of the studied parameters.

Discussion

This pilot work presents a new modification of blood perfusion measurement using microdialysis in two distinct parts of rat alimentary tube. Our most important findings were twofold: first, the anticipated stability of $Li_{in\text{-}out}$ difference in the S group and its response to ischemic periods in the experimental groups and second, the unexpected decrease in $Li_{in\text{-}out}$ difference observed during reperfusion in the ISP group as well as statistically invariable serum levels of nitric oxide and enzyme activities.

In sham-operated animals, the stability of $Li_{in\text{-}out}$ difference indicates a steady state of microcirculation throughout the experiment. The decreases seen in stomach during ischemia and/or ISP in respective groups correspond to the changes induced by CAO. In the course of reperfusion, the flow marker was indicating either a predictable tendency of stomach microcirculation to return to the initial pre-ischemic levels (IS group), or after a short-term rise displayed an unexpected deterioration of tissue microcirculation till the end of experiment (ISP group). This is contrary to studies which, using other techniques, recognized preservation of postischemic microcirculation as one of the main mechanisms of protection elicited by ISP (1, 10). In addition, applying this method, there was no detectable change in tissue perfusion of descending colon suggesting practically no effect of remote (stomach) ischemia and/or ISP in this part of alimentary tract. Such finding is interesting since beneficial relations due to ISP were found to exist in many distant organs with virtually no difference between local and remote effects as to potency (4, 1).

In general terms, the mechanism of local/remote effects of ISP is likely to be dependent on signalling pathways including systemic elevation of nitric oxide production, whereas pure sustained ischemia is associated with a decline in nitric oxide level (9). We did not observe the proclaimed hemodynamic effects of ISP, but concurrently neither did we detect a significant rise in serum nitric oxide metabolites in the ISP group. This might to some extent be explained by the adenosine/xanthine theory, which would suggest an inadequate reperfusion phase during preconditioning in the third group. Be that the case, instead of blood perfusion improvement, ISP would have resulted in longer and more severe (1 h) ischemia with accumulation of disproportional amounts of xanthine leading to production of excessive superoxide anion able to remove the generated nitric oxide (13).

With regard to the time window for ISP, it was documented, that one 5 min episode of arterial occlusion effectuated 30 min in advance of prolonged ischemia was sufficient to reduce lesion area in rat stomach (12). In small bowel, Hotter et al. (6) produced comparable effects (nitric oxide generation, organ protection) with 10 min ischemia only 5 min before prolonged ischemic period. As to the duration of injurious ischemia, marked changes in organs like stomach, pancreas or liver are acchieved after 30 to 120 min (1, 3, 9, 11). Even though our protocol met all the reported criteria for the induction of ischemia and ISP in stomach, CAO was found to lack the ability to produce (and thus also modify) significant liver or pancreatic damage demonstrated by others (9, 5, 11, 3).

Conclusions

This pilot study demonstrates a new alternative of gastrointestinal intersticial blood perfusion measurement by LM. Under given experimental conditions, the technique

allowed a detection of selective microcirculation modulation in rat stomach, but failed to detect reported protective potencies of local ISP in this organ. Lack of other splanchnic or systemic effects as indicated by absence of change in LM and selected serum biochemical parameters requires further studies.

Acknowledgements

The authors acknowledge a helpful cooperation with Assoc. prof. Živná, Ms. Ježková and Ms. Kriesfalusyová during the experimental work in the vivarium. We also thank Ms. Malá and Ms. Prokopová for skillful technical assistance with the biochemical analyses.

This work was principally supported by a Research Project from the Czech Ministry of Foreign Commerce (MZO 00179906), and in part also by a grant from the Czech Ministry of Education (MŠMT COST B25).

References

1. Brzozowski T, Konturek PC, Pajdo R, et al. Importance of brain-gut axis in the gastroprotection induced by gastric and remote preconditioning. J Physiol Pharmacol 2004;55:165–77.
2. de la Pena A, Liu P, Derendorf H. Microdialysis in peripheral tissues. Adv Drug Deliv Rev 2000;45:189–216.
3. Dembinski A, Warzecha Z, Ceranowicz P, et al. Effect of ischemic preconditioning on pancreatic regeneration and pancreatic expression of vascular endothelial growth factor and platelet-derived growth factor-A in ischemia/reperfusion-induced pancreatitis. J Physiol Pharmacol 2006;57:39–58.
4. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. Circulation 1996;94:2193–200.
5. Gong JP, Tu B, Wang W, Peng Y, Li SB, Yan LN. Protective effect of nitric oxide induced by ischemic preconditioning on reperfusion injury of rat liver graft. World J Gastroenterol 2004;10:73–6.
6. Hotter G, Closa D, Prados M, et al. Intestinal preconditioning is mediated by a transient increase in nitric oxide. Biochem Biophys Res Commun 1996; 222:27–32.
7. Hrubá P, Živný P, Živná H, Palička V. Muscle, liver and kidney interstitium blood flow changes in rats measured by microdialysis with flow marker added. Klin Biochem Metab 2004;12:9–13.
8. Jedličková V, Paluch Z, Alusik S. Determination of nitrate and nitrite by high-performance liquid chromatography in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2002;780:193–7.
9. Koti RS, Yang W, Dashwood MR, Davidson BR, Seifalian AM. Effect of ischemic preconditioning on hepatic microcirculation and function in a rat model of ischemia reperfusion injury. Liver Transpl 2002;8:1182–91.
10. Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischaemic preconditioning improves microvascular perfusion and oxygenation following reperfusion injury of the intestine. Br J Surg 2005;92:1169–76.
11. Obermaier R, von Dobschuetz E, Drognitz O, Hopt UT, Benz S. Ischemic preconditioning attenuates capillary no-reflow and leukocyte adherence in postischemic pancreatitis. Langenbecks Arch Surg 2004;389:511–6.
12. Pajdo R, Brzozowski T, Konturek PC, et al. Ischemic preconditioning, the most effective gastroprotective intervention: involvement of prostaglandins, nitric oxide, adenosine and sensory nerves. Eur J Pharmacol 2001;427:263–76.
13. Peralta C, Closa D, Xaus C, Gelpi E, Rosello-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. Hepatology 1998;28:768–73.
14. Peralta C, Serafin A, Fernandez-Zabalegui L, Wu ZY, Rosello-Catafau J. Liver ischemic preconditioning: a new strategy for the prevention of ischemia-reperfusion injury. Transplant Proc 2003;35:1800–2.
15. Stallknecht B, Donsmark M, Enevoldsen LH, Fluckey JD, Galbo H. Estimation of rat muscle blood flow by microdialysis probes perfused with ethanol, [14C]-ethanol, and 3H2O. J Appl Physiol 1999;86:1054–61.

Submitted August 2006.
Accepted September 2006.

Corresponding author:

Norbert Cibiček, MD, Institute of Clinical Biochemistry and Diagnostics, University Hospital, Sokolská 581, 500 05 Hradec Králové, Czech Republic, e-mail: cibicekn@lfhk.cuni.cz

study II

Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2008, 152(1):83–90.

© *N. Cibicek, H. Zivna, J. Cibicek, E. Cermakova, V. Vorisek, J. Malakova, S. Micuda, V. Palicka*

CAFFEINE DOES NOT MODULATE NUTRITIVE BLOOD FLOW TO RAT GASTRIC SUBMUCOSA – A MICRODIALYSIS STUDY

Norbert Cibicek[a]*, Helena Zivna[b], Jaroslav Cibicek[c], Eva Cermakova[d], Viktor Vorisek[a], Jana Malakova[a], Stanislav Micuda[e], Vladimir Palicka[a]

[a] *Institute of Clinical Biochemistry and Diagnostics,*
[b] *Radioisotope Laboratories and Vivarium,*
[d] *Computer Technology Center,*
[e] *Department of Pharmacology, Charles University, Prague, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic,*
[c] *Department of Pathology, "Forlife" General Hospital, Komárno, Slovak Republic*
e-mail: cibicek@seznam.cz

Received: February 20, 2008; Accepted (with revisions): April 15, 2008

Key words: Caffeine/Microcirculation/Nitric oxide/Microdialysis/Rat

Background and Aims: Coffee irritates the gastric mucosa disrupting its barrier and increasing the risk of peptic ulcers. However, caffeine's contribution to these effects has not yet been elucidated. In this study we looked at the local effect of caffeine on the microcirculation and nitric oxide production in rats together with systemic marker of oxidative stress malondialdehyde as possible mechanisms whereby caffeine might participate in mucosal barrier impairment.

Materials and Methods: Four groups of rats were anesthetized and administered as a bolus four different intraperitoneal doses of caffeine (0, 1, 10 and 50 mg kg^{-1} b.wt.). The gastric submucosal microcirculation and nitric oxide production were then recorded for 2.5 hours by *in situ* microdialysis using the flow marker ethanol. At the completion of the experiments, plasma caffeine and malondialdehyde levels as well as morphological mucosal injury were determined.

Results: There were no major differences in the macro- or microscopic pictures of the mucosa among the groups. Local microcirculatory (ethanol out/in ratio) and nitric oxide monitoring failed to demonstrate statistically significant changes as did measurement of plasma malondialdehyde in response to caffeine injections.

Conclusions: Caffeine *per se* seems unlikely to contribute to the gastric mucosal barrier injury associated with coffee consumption by alterations in nutritive blood flow, nitric oxide production or aggravation of systemic oxidative stress. This information is relevant for better understanding of the mechanisms involved in caffeine-mediated influences on gastric physiology in relation to the irritant effects of coffee.

INTRODUCTION

Caffeine (contained in coffee, tea, caffeinated beverages, cocoa, chocolate etc.) is the most consumed stimulant drug of abuse worldwide. With regard to coffee, epidemiological data support its irritant role in stomach and esophagus in association with gastroesophageal reflux leading to enhanced risk of ulcers and cancer in the afflicted areas[1,2]. Recently, this irritant effect was also confirmed in young asymptomatic individuals[3]. The beverage is a complex mixture of possibly antagonistic substances comprising, apart from caffeine, isoflavones, polyphenols, diterpenes etc. exhibiting effects according to the type of coffee bean processing[4,5]. To elucidate the cause of the irritant nature of coffee to the stomach, focusing on the effects of isolated and purified (pharmacologically) active components of coffee would lead to more information. Of these components caffeine is the most studied.

Maintenance of the gastric mucosal barrier integrity is dependent on the balance between aggressive and protective factors represented by hydrochloric acid on one hand and adequate mucosal blood flow with sufficient mucus production on the other. Caffeine, a known acid secretagogue, has long been suspected of causing mucosal hypoperfusion due to (micro)vascular impairment[6]. This hypothesis was later supported by electron microscopy[7]. More recent observations report suppressed acetylcholine (ACh)-induced mucus production[8] by caffeine and gastric mucosal transmembrane potential difference[9], completing the barrier-braking mosaic picture of caffeine. However, the aforementioned notion has been challenged by experimental observations showing enhanced mucosal blood flow by caffeine[10] and a protective influence of this drug on mucosal barrier integrity[11] suggesting actually a preventive role for caffeine in gastric mucosal injury[12].

Caffeine is a methylxanthine with pluripotent and possibly opposing pharmacological actions. It is a nonselective adenosine receptor antagonist, phosphodiesterase inhibitor, ryanodine-sensitive Ca^{2+} channel activator and soluble guanylate cyclase (GC) inhibitor. As a consequence, these actions may interfere with nitric oxide (NO) production and/or its second messenger cyclic guanosine monophosphate (cGMP) pathway leading to modulation of a wide spectrum of mucosal barrier-related (patho)physiological effects exerted by NO including vascular tone regulation or modulation of oxidative stress

level[13]. The literature, to the best of our knowledge, provides rather limited data on caffeine's effect on gastric (sub)mucosal microcirculation and has not included parallel monitoring of local NO release (using microdialysis), so far. Similarly, the putative effect of caffeine on oxidative stress necessitates clarification. The aim of the present study was twofold: first, to evaluate the possible impact of caffeine on gastric submucosal microcirculation, nitric oxide production and morphology, and second, to measure plasma malondialdehyde (MDA) as a marker of oxidative stress (lipid peroxidation).

MATERIALS AND METHODS

Animals

Conventionally bred adult white male Wistar rats (Biotest s.r.o., Konárovice, Czech Republic) weighing 300-400 g, were used. The animals were housed in the animal quarters for at least 7 days prior to experiments under controlled environmental conditions. The rats had free access to standard pellets (ST 1-TOP, Velas, Prague, Czech Republic) except for the 16-18 h before experiments, when they were fasted. Tap water was provided *ad libitum* until the day of the experiment. All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of the Charles University, Prague, Faculty of Medicine in Hradec Králové, Czech Republic. All experimental procedures were approved by the same committee.

Animal preparation

All animals were anesthetized with single i.p. dose of pentobarbital (50 mg kg^{-1}, Nembutal®, Abbott Laboratories, North Chicago, USA) and placed in a supine position on an unheated bed. They were kept under general anesthesia by cyclic i.p. administration of Nembutal® (15 mg kg^{-1} h^{-1}). Body temperature was monitored using a rectal thermometer probe (Ama-digit ad 15th, Aprecision, Germany) and maintained at 37.5–38.0 °C by means of a heating lamp. The trachea was cannulated with a short polyethylene catheter to ensure patent airways.

Substances

For microdialysis, ethanol-enriched normal 0.9 % saline (final concentration 50 mmol l^{-1})[14] was used as a perfusion medium. Caffeine (purchased from Sigma-Aldrich, St. Louis, MO, USA) was dissolved in normal saline to obtain solutions with concentrations 0.5, 5 and 25 mg ml^{-1} for groups 2, 3 and 4, respectively.

Gastric submucosal microdialysis technique

Modified technique originally described by Kitano *et al.*[15] was used. Briefly, following 3-4 cm long midline laparotomy, stomach was exposed. Firstly, respecting the course of blood vessels, a 21 G needle was introduced into the submucosa of gastric fundus with care neither to penetrate through the mucosa into the lumen nor to make an additional opening in the serosa. Secondly, a microdialysis probe (MAB 11.8.10 with 6 kDa cut-off polyethylene suplhone membrane, active length 10 mm; outer diameter 0.5 mm; Microbiotech/se AB, Stockholm, Sweden) was cautiously inserted into the preformed tunnel (Fig. 1a). Finally, the probe was fixed to the serosa with a suture. Continuous microdialysis was effectuated by perfusing the catheters with ethanol solution by means of CMA 102 microdialysis pump (CMA Microdialysis AB, Solna, Sweden) at a perfusion rate of 2 µl min^{-1}. For tissue equilibration, an initial 60 min period (without specimen collection) was allowed which was succeeded by a 30 min period to yield the baseline sample. Thereafter, sampling ensued for the next 2.5 h in 30 min intervals into sterile 200 µl polyethylene microvials. The specimens were aliquoted and stored at −70 °C (for nitric oxide) or −20 °C (for ethanol) until analysis.

Experimental design

The animals were randomly allocated to four groups (6 in each). After the collection of baseline microdialysis sample, the first (sham-operated) group received by intraperitoneal injection an adequate volume of normal saline, whereas the second, third and fourth groups were intraperitoneally administered caffeine solutions with concentrations 0.5, 5 and 25 mg ml^{-1} (caffeine dose of 1, 10 and 50 mg kg^{-1} b. wt.) respectively. At the conclusion of experiments, the animals were sacrificed by blood withdrawal from abdominal aorta. The obtained plasma was stored at −70 °C for ensuing biochemical determination of caffeine and MDA. After the removal of stomach from the body, the mucosal surface of the glandular part was gently cleaned in cold tap water and closely inspected in search for macroscopic lesions. The tissues surrounding the probe (0.5 × 1.5 cm) with the probe itself *in situ* were then dissected and the biopsy immersed in 10% formaline for histological examination.

Probe performance stability and in vivo *recovery determination for nitrate*

The measurement of NO using microdialysis technique was validated in two consecutive steps employing other two groups (A and B) of pentobarbital-anesthetized rats. First, probe performance stability for nitrate was tested continuously for 7 h (measurements during equilibration period inclusive, group A, n = 5) in one experiment based on an assumption of stable NO production throughout the study period. Microdialysis sampling in gastric submucosa was realized in 30 min intervals at a perfusion rate of 2 µl min^{-1}. As perfusate, normal saline was utilized. Second, *in vivo* recovery of the same probe type was estimated (group B, n = 3) using zero-net flux method originally proposed by Lönnroth *et al.*[16]. Four perfusion media of increasing concentrations of sodium nitrate in sterile saline were consecutively applied as follows. After the initial tissue equilibration (1 h) with 10 µmol l^{-1} $NaNO_3$, a 30 min sample was collected. The perfusion medium was changed for 15 µmol l^{-1} nitrate and following 30 min equilibration, another sample harvest (30 min) ensued respecting the probe's lag time (3 min). The experiment

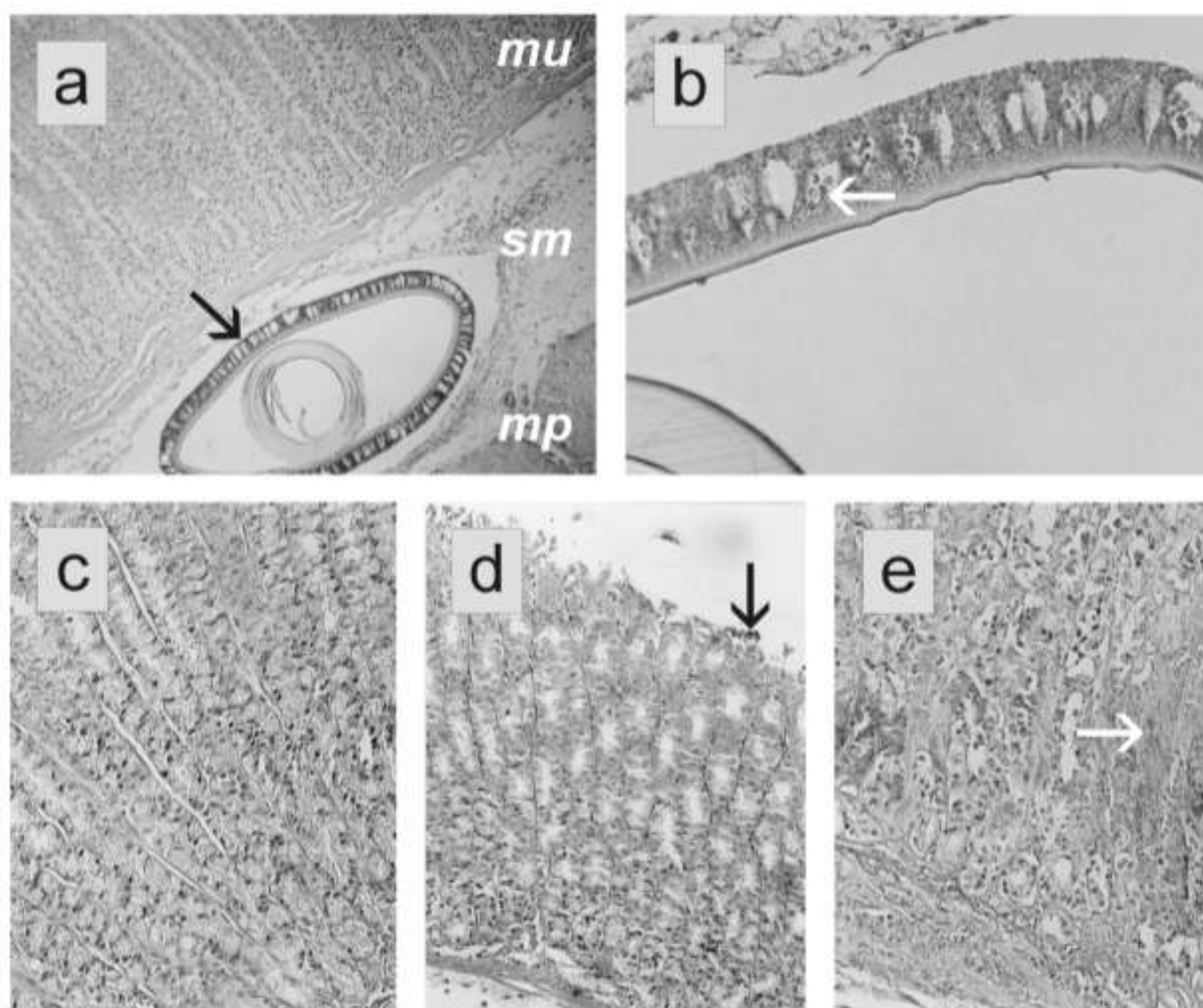

Fig. 1. Morphological studies. Panel "a" confirms the position of the microdialysis probe within the submucosal layer of gastric wall (arrow). The catheter is surrounded by noticeable edema and hyperemia with diffuse inflammatory infiltrate of mild degree penetrating at some places into the mucosa. The probe's tougher inner membrane was slightly damaged during tissue slicing (mu = mucosa, sm = submucosa, mp = muscularis propria). Panel "b" depicts the detail of the probe's outer membrane permeated by polymorphonuclear leukocytes (arrow). Pictures "c-e" show normal gastric mucosa, i. e. grade 0, and typical lesions of grade I and II - detached cells and necrosis of pits (arrows), respectively. Standard hematoxylin-eosine stain, original magnification "a" 125×, "b" 500×, "c-e" 250×.

was completed with 50 and finally 120 μmol l^{-1} nitrate solutions. The results were plotted on a graph and probe recovery was read from the regression equation (slope gradient). Besides microdialysis, these two groups of animals underwent no further experimental treatment. All general steps (anesthesia, surgery, gastric submucosal microdialysis technique including probes but excluding perfusion media, sacrifice and analytical techniques) were equal to the experimental groups of the present study.

Nutritive blood flow measurement

Using flow marker (e.g. ethanol)-enriched perfusion medium, microdialysis offers opportunity to indirectly assess tissue microcirculation (nutritive blood flow) in various organs. The principle of the so called "ethanol dilution technique" is based on the negative correlation between blood perfusion and ethanol efflux from the probe represented by out/in ratio of ethanol concentrations[14, 17, 18].

Biochemical analyses

Total NO was quantitatively determined as the sum of its stable metabolites (nitrite plus nitrate) in an enzymatic colorimetric assay, which involves an enzymatic conversion of nitrate to nitrite by nitrate reductase. Resulting nitrite is detected colorimetrically as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. In the experiments commercially available

ELISA kits (Assay Designs, Ann Arbor, MI, USA) were utilized only. All procedure steps were performed according to the manufacturer's instructions.

The analyses of ethanol were carried out using GC-MS apparatus consisting of Varian 3300 Gas Chromatograph (GC) coupled to Finnigan MAT Magnum Mass Spectrometer (MS; Thermo Fisher Scientific, formerly Finnigan). Supelcowax™-10 (30 m x 0.25 mm x 0.25 µm film thickness) GC Capillary column with helium as a carrier gas was employed. Injector and transferline temperatures were set to 230 °C for both. GC oven was programmed as follows: 55 °C, 2 min, 25 °C min^{-1} to 150 °C, hold for 1 min. The specified MS parameters were 70eV for electron ionization mode (EI) and 209 °C for ion trap temperature. The resulted MS spectra were scanned in 15–70 a.m.u. mass range under 4 microscans s^{-1} of detection speed regimen.

Plasma caffeine concentrations were determined using a modified HPLC method by Biederbick *et al.*[19]. Briefly, caffeine and internal standard hydroxyethyltheophylline (aqueous solution 40 mg l^{-1}) from plasma samples (100 µl + 50 µl) were extracted using 2.5 ml of dichlormethane. The organic phase was evaporated under nitrogen at 40 °C and the samples were reconstituted in 150 µl of mobile phase, and 30 µl were injected into the HPLC column. Analysis was performed on a 2695 Waters Separations Module equipped with 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). The mobile phase was made up of an aqueous solution of sodiumdihydrogenphosphate (0.5 mmol l^{-1}, pH 5) to acetonitrile (ratio 88:12) and was pumped isocratically at a flow rate of 0.9 ml min^{-1}. The temperature of the column was set at 40 °C. The quantification of caffeine was performed at 270 nm.

Plasma MDA was determined as a secondary product of lipid peroxidation in an attempt to evaluate the level of oxidative stress produced by caffeine. The analysis was based on the reaction of MDA with thiobarbituric acid (TBA) producing a red MDA-TBA complex measured photometrically at three distinct wavelengths (485, 532 and 560 nm) and the absorbance corrected according to Allen's formula $A_{corr} = A_{532} - [(A_{560} - A_{485}) \times 0.63 + A_{485}]$ for enhanced specificity[20].

Histological analyses

Stomach biopsies were taken to evaluate possible effects of caffeine on tissue morphology at microscopic level and for probe position verification. The samples (0.5 × 1.5 cm) were taken from glandular segment surrounding the probe. The tissues were fixed in 10 % neutral buffered formalin and further treated according to standard procedures for hematoxylin–eosin (HE) stain. The sections were evaluated by a blinded professional observer and photographed at 125, 250 and 500-fold magnification. The grading criteria were adopted from Natale *et al.* and were as follows: grade 0 for normal mucosa, grade I for lysis and segregation of cells on the luminal surface (with intact pit cells), grade II for damage confined to gastric pits with detachment of the surface epithelium and grade III

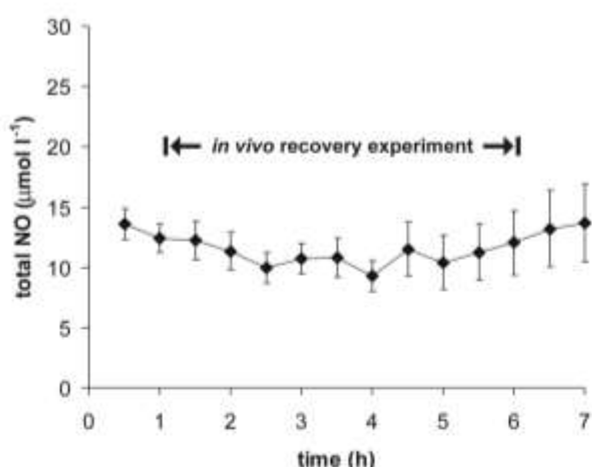

Fig. 2. The graph depicts total gastric submucosal nitric oxide (NO) production (presented as a sum of nitrite and nitrate), as monitored in anaesthetized rats by *in vivo* microdialysis and confirms its stability throughout the experiment. The arrows specify the time frame of *in vivo* recovery determination (measured in another study, see Fig. 3). Data are expressed as means ± SEM of 5 measurements.

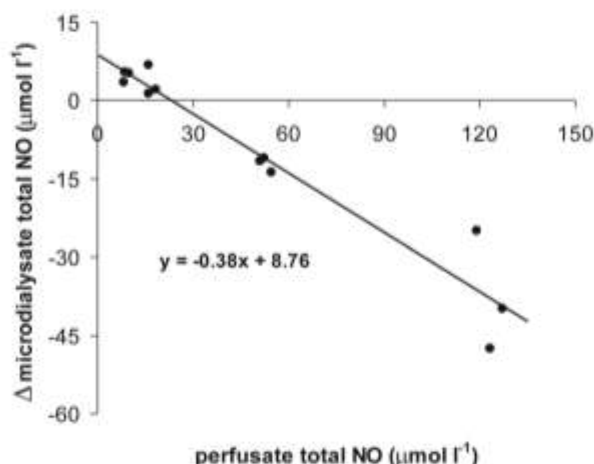

Fig. 3. *In vivo* probe calibration ("zero net-flux" method). The Δ microdialysate (dialysate – perfusate) total NO plotted against the perfusate total NO gives a gradient representing the probe's recovery (38 %). The x-intercept corresponds to the concentration of NO in the surrounding extracellular medium (~ 23 µmol l^{-1}). The y-intercept approximates the mean concentration obtained in due course from stability experiment (11 µmol l^{-1}, Fig. 2). Single results of three animals are displayed as dots.

which involves injured gastric glands (whole-thickness mucosal necrosis with swelling and possible disconnection of mucosal layers)[21].

Statistics

Data are expressed as means ± standard error of mean (SEM), unless otherwise noted. For statistical evaluation, normality tests and repeated measures ANOVA were used. The data were processed by the program NCSS 2004. The chosen level of significance was $\alpha = 0.05$.

RESULTS

Histology

Microdialysis probes were positioned correctly within the submucosal layer of gastric wall without penetration into the organ's lumen. The histological picture was comparable to previous reports[15, 22] as indicated in Fig. 1a, b. Lack of macroscopically measurable whole organ mucosal alterations due to caffeine treatment was mirrored in standard microscopy, which depicted similar grades of tissue damage ranging within grades 0-II around the probe in slides from all experimental groups including sham-operated animals (Fig. 1c-e). The severity of mucosal injury was unrelated to the administered caffeine dose. No grade III lesions were observed.

Microdialysis data

The results of probe performance stability are displayed in Fig. 2. In this experiment, the calculated average outflow concentration of total NO (within 5 h-time frame corresponding with the probe calibration study) was 11 µmol l^{-1}. This is in agreement with the expected value (~ 9 µmol l^{-1}) calculated using extrapolation of data obtained from the probe calibration study, i.e. in case the concentration of total NO in the perfusate was close to zero (Fig. 3). The outcomes of these experiments were consistent with one another indicating stable function of the microdialysis probe and NO production over time. The *in vivo* recovery of the used probe type under given experimental conditions was determined by the gradient of the regression line and found to be 38 %. The x-intercept corresponds to the concentration of nitrite + nitrate in the surrounding extracellular medium (~ 23 µmol l^{-1}, Fig. 3). This level of extracellular NO was not affected by the i.p. administration of caffeine ($p = 0.9$, ANOVA, Fig. 4a). The microcirculation as represented by the ethanol out/in ratio showed a tendency for dose-related alterations after caffeine but the difference was not statistically significant ($p = 0.1$, ANOVA, Fig. 4b).

Plasma analytes

Along with caffeine, plasma MDA was measured as general oxidative stress-related marker at the conclusion of the experiments. Even here, no statistically significant dose-related trend was observed ($p = 0.8$, ANOVA, Tab. 1).

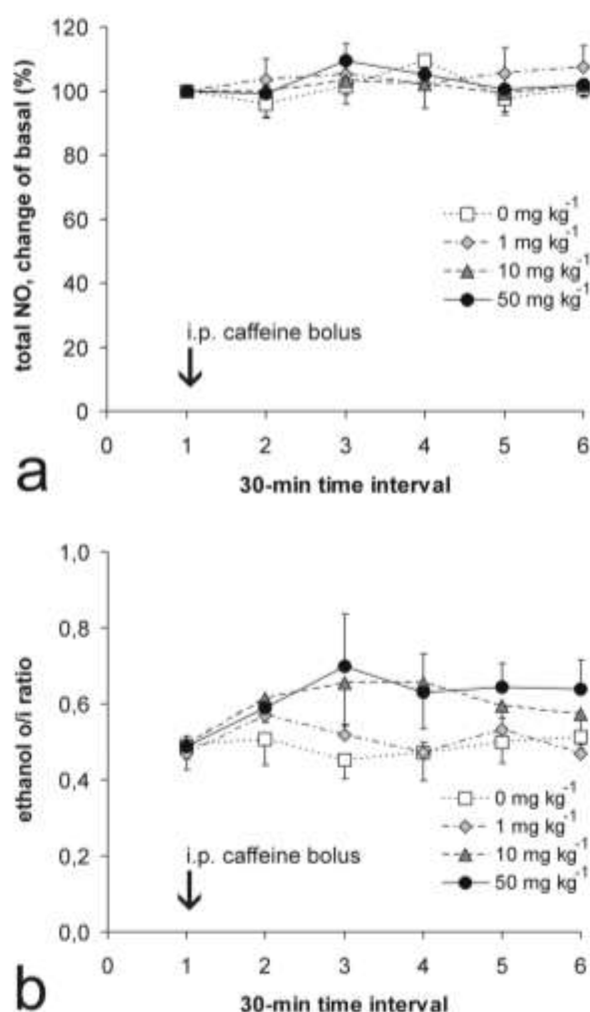

Fig. 4. Panel "a" presents the total NO production in response to increasing caffeine load. No difference was registered within 2.5 h of monitoring. Panel "b" illustrates the time course of microdialysate ethanol out/in ratio (negatively correlates with nutritive blood flow) indicating a slight tendency of microcirculation to deteriorate in a dose-dependent manner (particularly at intervals 3 and 5) following the injection of caffeine. However, this trend lacks statistical significance. Results are means ± SEM of 6 measurements.

DISCUSSION

Effect of caffeine on (sub)mucosal morphology

Even though macroscopical observations (e.g. a computerized planimetry) are a "golden standard" in the evaluation of gross gastric mucosal injury for their ability to assess the whole organ, they are inconvenient for detection and scoring of minor (visibly hardly discernible) changes. Since, in accordance with literature[23], the latter was also the case in our experiment, gastric mucosal impairment was studied microscopically using standard

histology. However, owing to the exactingness of the suggested method of whole organ histological evaluation[21], we decided for the present time to grade the most prominent cellular/tissue changes found in comparable parts of glandular stomachs (in the vicinity of probe implantation site) of experimental groups. Since this time we did not quantify the lesions, the statistical approach was not feasible. Hence, the present study provides only qualitative data on the effects of microdialysis and caffeine on rat gastric tissue morphology.

NO measurement using microdialysis

There is very limited data on gastric NO measurement with *in vivo* microdialysis. Iversen *et al.* measured nerve-induced release of NO in the wall of rabbit stomach. They utilized different probe types (CMA/10, CMA Medical AB, Stockholm, Sweden, 3 x 0.7 mm dialysis membrane with 20 kDa cut-off) and perfusion rate (1 µl min^{-1}) and estimated interstitial concentrations of nitrite and nitrate to approximate 10 and 70 µmol l^{-1}, respectively. The calculated *in vitro* recovery of the probes (for given perfusion rate) equaled 31 - 33 % for nitrate and nitrite, respectively. The authors did not determine the recovery *in vivo*, but assumed that it would be in the range 10–40 %[24]. Suzuki *et al.* recently employed similar probes to our catheters (MAB 7.8.10 with 15 kDa cut-off dialysis membrane, active length 10 mm; outer diameter 0.5 mm; Microbiotech/se AB, Stockholm, Sweden) and validated them for studying nitrosative chemistry in the lumen of human stomach. The assessed *in vitro* recovery for nitrite at pH 1.5 and perfusion rate 0.15 ml h^{-1} was 71 %[25]. Our results of *in vivo* recovery may be comparable to these studies since the dialysis function of membranes *in vivo* generally diminishes[16]. The interstitial concentration of total NO found in our study (~ 23 µmol l^{-1}) was lower than reported by Iversen *et al.* in rabbits. It is a matter of debate to what extent this difference may be attributable to species and/or technique used.

Effect of caffeine on NO production

Besides vascular tone regulation, nitric oxide released by Ca^{2+}-dependent endothelial (e)NOS as well as Ca^{2+}-independent inducible (i)NOS plays a large number of (patho)physiological roles many of which may be related to mucosal defence and injury[13]. It is therefore of interest whether caffeine interferes with the release of this reactive pluripotent radical. Bruce *et al.* report of a significant decrease in exhaled NO levels in humans one hour after 100-200 mg caffeine intake[26]. The latest findings indicate that caffeine (16 mg kg^{-1} i.v.) may decrease nitric oxide synthase (NOS) expression in rat skeletal muscles[27] and attenuate glutamate-induced NO synthesis in murine spinal cord *in vitro*[28]. Moreover, caffeine negates the protective effect of ischemic preconditioning, i.e. eliminates reactive hyperemia due to the hypoperfusion-induced accumulation of adenosine and enhanced NO production[29]. In contrast, endothelium of isolated rat aorta responds to caffeine by promotion of NO synthesis[30]. In the present study, we failed to detect significant shifts in NO production up to 2.5 h after the application of increasing doses of caffeine. However, eNOS releases NO in nanomolar quantities, changes we may have been unable to detect with the analytical procedure we used while micromolar amounts of NO are produced by activated iNOS as soon as 2 h after application of inducing substance[31]. Therefore, our results indicate no effect of caffeine on Ca^{2+}-independent NO production in resting gastric submucosa of anesthetized rats.

Effect of caffeine on blood flow

Despite the generally recognized constricting role of caffeine in the brain[32], heart[33], limb[34] or gut[35] vasculature, the literature is inconsistent as far as gastric (sub)mucosal perfusion is concerned. Understandably, the discrepancy with respect to blood flow is reflected in the contrary effects of caffeine on acute induced gastric mucosal injury[36, 23, 12]. Ozturkcan *et al.* showed that a single i.p. injection of 7.5 – 30 mg kg^{-1} caffeine leads to elevations in rat gastric mucosal blood flow 90 min after drug application[10]. Moreover, Koyama *et al.* found that *ex vivo* intraluminal administration of caffeine doses as high as 50–100 mg kg^{-1} result in a dose-dependent increase in mucosal blood flow lasting up to 90 min[12]. Although in the present study some tendencies to decreased nutritive blood flow could be observed 30-90 min after caffeine administration, the results show a lack of statistically significant change in this variable throughout the experiment (Fig. 4b). This would be consistent with other studies showing no direct effect of caffeine on resting blood flow[37–39] and/or induced vascular contractility[40]. Along these lines, there is conflict-

Table 1. The table summarizes the effect of increasing doses of caffeine on plasma caffeine and malondialdehyde (MDA) levels at the conclusion of experiments. In spite of a negative trend, no statistically significant difference or association between administered caffeine and plasma MDA was found. Data are displayed as means ± SEM of 6 measurements.

i. p. caffeine dose (mg kg^{-1} b.wt.)	0	1	10	50
plasma caffeine (mg l^{-1})	0 ± 0.00	1.43 ± 0.07	11.80 ± 0.42	56.51 ± 1.68
plasma MDA (µmol l^{-1})	0.94 ± 0.05	0.91 ± 0.12	0.84 ± 0.03	0.80 ± 0.15

ing data on the effect of caffeine on endothelial function. Papamichael *et al.* describe acute detrimental effects of caffeine up to 90 min after oral intake of 80 mg of caffeine on endothelium-dependent flow-mediated dilatation of the brachial artery[41] whereas Umemura *et al.* report its favorable role on (acetylcholine-induced) forearm vasodilation one hour after oral administration of 300 mg of caffeine, whereas baseline blood flow remained unaltered[37]. Interestingly, the latter authors ascribe these findings to enhanced endothelial NO synthesis caused by released Ca^{2+} from endoplasmic reticulum through activation of ryanodine-sensitive Ca^{2+} channels and the suppression of cGMP degradation[30], while the former investigators account for their observation as the inhibitory effect of caffeine on soluble GC with eventual suppression of cGMP formation. Unfortunately, neither group measured real *in situ* NO production. Nevertheless, these explanatory discrepancies may have some rationale as the differential pharmacologic actions of caffeine depend on the plasma caffeine levels[42]. Hence, the final vasoactive action of caffeine may result from a balance between its vasoconstrictive (adenosine-antagonizing) and possibly vasodilating (NO-releasing) ability.

Effect of caffeine on oxidative stress

There are data suggesting multifarious mechanisms whereby caffeine might play a role in augmenting oxidative stress[41]. However, the acute unfavorable consequence of caffeine could also follow from its antagonism with adenosine - a substance producing NO and thus preventing mitochondrial oxidant damage in rat cardiomyocytes[43]. In rats that underwent concussive head injury, Al Moutaery *et al.* demonstrated an increase in the level of inflammation and oxidative stress (significant increase in neutrophil infiltration and brain MDA) associated with a dose-dependent increase in mortality of caffeine-pretreated animals[44]. In the present experiment, the plasma concentrations of caffeine were consonant with previous findings[45] and indicate good bioavailability of caffeine after i.p. application. However, 2.5 h after drug administration, only a slight dose-dependent decreasing trend of plasma MDA levels lacking statistical significance was noted. This indicates no acute detrimental effect of caffeine on whole body's oxidative stress as far as systemically manifested effects are concerned (Tab. 1).

CONCLUSIONS

The results of the present study indicate that i.p. administration of caffeine in given dosages does not produce acute macroscopic changes to gastric mucosa and is unlikely to alter gastric submucosal nutritive blood flow and nitric oxide production or aggravate systemic oxidative stress level. Additional histomorphometric studies are needed to describe the effect of caffeine on gastric mucosa and further attempts need to be made to explore possible mechanisms whereby caffeine might participate in irritant effects of coffee.

ABBREVIATIONS

cGMP - cyclic guanosine monophosphate
GC - guanylate cyclase
MDA - malondialdehyde
NO - nitric oxide
e/iNOS - endothelial/inducible nitric oxide synthase

ACKNOWLEDGEMENTS

The authors acknowledge skillful technical assistance of Mrs. Ježková in the vivarium, Mrs. Menclová and Mrs. Lošonská in the biochemical and histopathological laboratories, respectively. This work was supported by Research Project from the Czech Ministry of Foreign Commerce MZO 00179906.

REFERENCES

1. Marotta RB, Floch MH. Diet and nutrition in ulcer disease. Med Clin North Am 1991; 75(4):967-79.
2. Terry P, Lagergren J, Wolk A, Nyren O. Reflux-inducing dietary factors and risk of adenocarcinoma of the esophagus and gastric cardia. Nutr Cancer 2000; 38:186-91.
3. Cibickova L, Cibicek N, Zdansky P, Kohout P. The impairment of gastroduodenal mucosal barrier by coffee. Acta Medica (Hradec Králové) 2004; 47(4):275-8.
4. Ehrlich A, Basse H, Henkel-Ernst J, Hey B, Menthe J, Lücker PW. Effect of differently processed coffee on the gastric potential difference and intragastric pH in healthy volunteers. Methods Find Exp Clin Pharmacol 1998; 20(2):155-61.
5. Fiebich BL, Valente P, Ferrer-Montiel A, Candelario-Jalil E, Menthe J, Luecker P. Effects of coffees before and after special treatment procedure on cell membrane potentials in stomach cells. Methods Find Exp Clin Pharmacol 2006; 28(6):369-72.
6. Roth JA, Ivy AC. The pathogenesis of caffeine-induced ulcers. Surgery 1945; 17:644-9.
7. Pfeiffer CJ, Roth JLA. Studies on the secretory and cytotoxic actions of caffeine on the ferret gastric mucosa. Exp Mol Pathol 1970; 13:66-78.
8. Hamada E, Nakajima T, Hata Y, Hazama H, Iwasawa K, Takahashi M et al. Effect of caffeine on mucus secretion and agonist-dependent Ca2+ mobilization in human gastric mucus secreting cells. Biochim Biophys Acta 1997; 1356(2):198-206.
9. Dziaduś-Sokołowska A, Orlef A, Bilski R, Mroczka J. The effect of ethanol-caffeine interaction on the gastric mucosal barrier. Pol J Pharmacol Pharm 1989; 41(3):253-8.
10. Ozturkcan O, de Saint Blanquat G, Derache R. Effet de la caféine sur le flux sanguin de la muqueuse gastrique chez le Rat. Thérapie 1974; 29:941-4.
11. Wittmers LE, Alich A, Quirk DR. Effect of caffeine on the Gastric Potential Difference (GPD). FASEB Journal 1998; 12(5):A737.
12. Koyama R, Kataoka H, Tanaka Y, Nakatsugi S, Furukawa M. Effect of caffeine on ibuprofen-induced gastric mucosal damage in rats. J Pharm Pharmacol 1999; 51(7):817-24.
13. Elliott S, Wallace JL. Nitric oxide: a regulator of mucosal defence and injury. J Gastroenterol 1998; 33:792-803.
14. Arner P, Hellmér J, Hagström-Toft E, Bolinder J. Effect of phosphodiesterase inhibition with amrinone or theophylline on lipolysis and blood flow in human adipose tissue in vivo as measured with microdialysis. J Lipid Res 1993; 34:1737-43.
15. Kitano M, Norlén P, Håkanson R. Gastric submucosal microdialysis: a method to study gastrin- and food-evoked mobilization of ECL-cell histamine in conscious rats. Regul Pept 2000; 86:113-23.

16. Lönnroth P, Jansson PA, Smith U. A microdialysis method allowing characterization of intercellular water space in humans. Am J Physiol 1987; 253(2):228-31.
17. Hickner RC, Ekelund U, Mellander S, Ungerstedt U, Henriksson J. Muscle blood flow in cats: comparison of microdialysis ethanol technique with direct measurement. J Appl Physiol 1995; 79(2):638-47.
18. Korth U, Krieter H, Denz Ch, Janke Ch, Ellinger K, Bertsch T et al. Intestinal ischaemia during cardiac arrest and resuscitation: comparative analysis of extracellular metabolites by microdialysis. Resuscitation 2003; 58:209-17.
19. Biederbick W, Joseph G, Rump A, Theisohn M, Klaus W. Caffeine in Saliva After Peroral Intake: Early Sample Collection as a Possible Source of Error. Therapeutic Drug Monitoring 1997; 19:521-4.
20. Hendrix T, Assman R. Spectrophotometric correction for bile pigments in the thiobarbituric test for malondialdehyde-like substances in plasma. Med Lab Sci 1990; 47:10-6.
21. Natale G, Lazzeri G, Blandizzi C, Gherardi G, Lenzi P, Pellegrini A et al. Seriate histomorphometry of whole rat stomach: an accurate and reliable method for quantitative analysis of mucosal damage. Toxicol Appl Pharmacol 2001; 174:17-26.
22. Cibíček N, Mičuda S, Chládek J, Živný P, Zadák Z, Čermáková E et al. Lithium microdialysis and its use for monitoring of stomach and colon submucosal blood perfusion – a pilot study using ischemic preconditioning in rats. Acta Medica (Hradec Králové) 2006; 49(4):227-31.
23. Parmar NS, Tariq M, Ageel AM. Effect of nicotine, alcohol and caffeine pretreatment on the gastric mucosal damage induced by aspirin, phenylbutazone and reserpine in rats. Jpn J Pharmacol 1985; 39(1):1-6.
24. Iversen HH, Celsing F, Leone AM, Gustafsson LE, Wiklund NP. Nerve-induced release of nitric oxide in the rabbit gastrointestinal tract as measured by *in vivo* microdialysis. Br J Pharmacol 1007; 120:702-6.
25. Suzuki H, Moriya A, Iijima K, McElroy K, Fyfe VE, McColl KEL. Validation of microdialysis probes for studying nitrosative chemistry within localized regions of the human upper gastrointestinal tract. Scand J Gastroenterol 2003; 38:856-63.
26. Bruce C, Yates DH, Thomas PS. Caffeine decreases exhaled nitric oxide. Thorax 2002; 57:361-3.
27. Corsetti G, Pasini E, Assanelli D, Saligari E, Adobati M, Bianchi R. Acute caffeine administration decreased NOS and Bcl2 expression in rat skeletal muscles. Pharmacol Res 2007; 55(2):96-103.
28. Godfrey L, Bailey I, Toms NJ, Clarke GD, Kitchen I, Hourani SM. Paracetamol inhibits nitric oxide synthesis in murine spinal cord slices. Eur J Pharmacol 2007; 562(1-2):68-71.
29. Riksen NP, Zhou Z, Oyen WJG, Jaspers R, Ramakers BP, Brouwer RMHJ et al. Caffeine prevents protection in two human models of ischemic preconditioning. J Am Coll Cardiol 2006; 48:700-7.
30. Hatano Y, Mizumoto K, Yoshiyama T, Yamamoto M, Iranai H. Endothelial-dependent and -independent vasodilatation of isolated rat aorta induced by caffeine. Am J Physiol 1995; 269:H1679-84.
31. Huang CJ, Tsai PS, Pan WHT, Skimming JW. Microdialysis for measurement of hepatic and systemic nitric oxide biosynthesis in septic rats. Acta Anaesthesiol Scand 2005; 49:28-34.
32. Couturier EG, Laman DM, van Duijn MA, van Duijn H. Influence of caffeine and caffeine withdrawal on headache and cerebral blood flow velocities. Cephalalgia 1997; 17(3):188-90.
33. Bottcher M, Czernin J, Sun KT, Phelps ME, Schelbert HR. Effect of caffeine on myocardial blood flow at rest and during pharmacological vasodilation. J Nucl Med 1995; 36(11):2016-21.
34. Casiglia E, Bongiovi S, Paleari CD, Petucco S, Boni M, Colangeli G et al. Haemodynamic effects of coffee and caffeine in normal volunteers: a placebo-controlled clinical study. J Intern Med 1991; 229(6):501-4.
35. Hoecker Ch, Nelle M, Poeschl J, Beedgen B, Linderkamp O. Caffeine impairs cerebral and intestinal blood flow velocity in preterm infants. Pediatrics 2002; 109(5):784-7.
36. Yano S, Isobe Y, Harada M. The etiology of caffeine-induced aggravation of gastric lesions in rats exposed to restraint plus water-immersion stress. J Pharmacobiodyn 1982; 5(7):485-94.
37. Umemura T, Ueda K, Nishioka K, Hidaka T, Takemoto H, Nakamura S et al. Effects of acute administration of caffeine on vascular function. Am J Cardiol 2006; 98:1538-41.
38. Wierema TK, Houben AJ, Kroon AA, Postma CT, Koster D, van Engelshoven JM et al. Mechanisms of adenosine-induced renal vasodilatation in hypertensive patients. J Hypertens 2005; 23(9):1731-6.
39. Daniels JW, Mole PA, Shaffrath JD, Stebbins CL. Effects of caffeine on blood pressure, heart rate, and forearm blood flow during dynamic leg exercise. J Appl Physiol 1998; 85(1):154-9.
40. Barton B, Kleinert JM. The effect of caffeine on digital haemodynamics. J Hand Surg [Br] 1994; 19(3):301-2.
41. Papamichael CM, Aznaouridis KA, Karatzis EN, Karatzi KN, Stamatelopoulos KS, Vamvakou G et al. Effect of coffee on endothelial function in healthy subjects: the role of caffeine. Clin Sci (Lond) 2005; 109(1):55-60.
42. Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widesptread use. Pharmacol Rev 1999; 51:83-133.
43. Xu Z, Park SS, Mueller RA, Bagnell RC, Patterson C, Boysen PG. Adenosine produces nitric oxide and prevents mitochondrial oxidant damage in rat cardiomyocytes. Cardiovasc Res 2005; 65(4):803-12.
44. Al Moutaery K, Al Deeb S, Khan HA, Tariq M. Caffeine impairs short-term neurological outcome after concussive head injury in rats. Neurosurgery 2003; 53(3):704-12.
45. Wang Y, Lau ChE. Caffeine has similar pharmakokinetics and behavioral effects via the IP and PO routes of administration. Pharmacol Biochem Behav 1998; 60(1):271-8.

study III

Physiol. Res. 56: 611-617, 2007

Colon Submucosal Microdialysis: A Novel *in vivo* Approach in Barrier Function Assessment - A Pilot Study in Rats

N. CIBIČEK[1], H. ŽIVNÁ[2], Z. ZADÁK[3], J. KULÍŘ[4], E. ČERMÁKOVÁ[5], V. PALIČKA[1]

[1]*Institute of Clinical Biochemistry and Diagnostics,* [2]*Radioisotope Laboratories and Vivarium,* [3]*Research Laboratory of the Department of Gerontology and Metabolism,* [4]*Department of Nuclear Medicine,* [5]*Department of Biophysics and Biostatistics, Charles University in Prague, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové, Czech Republic*

Received April 11, 2006
Accepted August 30, 2006
On-line available December 19, 2006

Summary

During shock, prognosis of a patient depends largely on intestinal barrier function. The potency of gut epithelium to represent an obstacle to toxins is determined by the blood supply. All established methods of mucosal function determination necessitate the functional involvement of bloodstream. Microdialysis allows monitoring of extracellular substances in the gut submucosa, but its potential use for gut barrier integrity assessment is unknown. Twelve rats underwent perfusion of the descending colon either with 20 % ethanol or control medium (vehicle). Both media contained equal amounts of a radioactive tracer substance (^{51}Cr-EDTA). Mucosal permeability for ^{51}Cr-EDTA was assessed by microdialysate to luminal perfusate activity ratios. Sampling was performed using the colon submucosal microdialysis technique. The group subjected to ethanol treatment had profound macro- and microscopical alterations in perfused colonic segment associated with a significant increase in tracer permeability during ethanol exposure (2.354±0.298 % for ethanol as opposed to 0.209±0.102 % for control group, $p<0.01$), which remained elevated for 60 min after cessation of ethanol administration (3.352±0.188 % for ethanol compared to 0.140±0.0838 % for the control group, $p<0.001$). Submucosal microdialysis with radioactive tracer substance can be considered a feasible and advantageous alternative of gut barrier function estimation. Parallel monitoring of local tissue chemistry with this method remains a challenge in the future.

Key words
Microdialysis • Colon barrier function • ^{51}Cr-EDTA permeability • Rat

Introduction

In critical care, the prognosis of patients depends largely on intestinal mucosal barrier function being predominantly determined by a degree of blood supply (Doig *et al.* 1998). Although an impressive array of previous studies proposes several experimental modalities of gut barrier integrity estimation (in the form of various clearance techniques), no approach has offered an indisputable capability to detect alterations in the course

 ISSN 0862-8408
© 2007 Institute of Physiology v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic
Fax +420 241 062 164
E-mail: physres@biomed.cas.cz
http://www.biomed.cas.cz/physiolres

of ischemic periods, particularly if arterial clamping lasts for a longer time (Udassin *et al.* 1998, Iwata *et al.* 1998, Kawai *et al.* 1994). The principal problem lies in the requirement of ensuring constant transport of low-molecular tracer between the lumen and the bloodstream regardless of its direction (Fihn *et al.* 2003). Although some procedures do not necessitate alterations due to repeated blood withdrawals (with or without compensatory fluid infusions), they may have other considerable impacts on the subject´s physiology (e.g. detachment of kidneys by ligatures). In addition, when studying dynamics of extracellular molecules with short half-lives, frequent simultaneous sampling is needed. To our best knowledge, literature has not as yet suggested an approach allowing monitoring of intestinal permeability and related local metabolic processes (all without involvement of the bloodstream).

In many organs including the gut submucosa, microdialysis is a well-established means of observing the kinetics of extracellular substances (de la Pena *et al.* 2000, Kitano *et al.* 2000, Solligard *et al.* 2004). It is interesting that possibilities of bowel barrier function assessment have not yet been challenged using this advantageous technique. Hence, the aim of the present work was to develop a continuous method of mucosal permeability measurement based on a simple principle of passive tracer penetration from lumen into the submucosa and thus omitting the interfering involvement of the bloodstream (or urine) during ischemia. The study was designed to verify the hypothesis that microdialysis as a method applied in this region of the gut at least qualitatively responds to barrier integrity alterations according to our expectations. Our supposition was tested on a modified example of colon barrier impairment by ethanol (Sobue *et al.* 2003). There, we expected to find an increase in mucosal permeability represented by an elevation in radioactivity of the dialysate.

Methods

Subjects

Twelve conventionally bred adult white male rats of the Wistar strain (Biotest s.r.o., Konárovice, Czech Republic) weighing 250-450 g, were used. The animals were housed in the animal quarters for at least 7 days prior to the experiments at 22-24 °C, 40-60 % relative humidity, air exchange 12-14 times per hour and 12-hour light-dark cycle periods. The rats had free access to standard laboratory rat chow pellets (ST 1-TOP, Velaz, Prague, Czech Republic) except for 16-18 h before the experiments, when they fasted. During food deprivation, the animals were stationed in plastic cages with raised mesh floors to prevent coprophagia. Tap water was provided *ad libitum* until the day of the experiment. All animals received care in accordance with the guidelines set by the institutional Animal Use and Care Committee of the Charles University in Prague, Czech Republic. All experimental procedures were approved by the Committee for Protection of Animals against Cruelty (Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic).

Substances

For microdialysis, commercially available Ringer´s solution (R1/1) (InMediec s.r.o., Luhačovice, Czech Republic) was used as a perfusion medium. It contained (in $mmol.l^{-1}$) Na^+ 147.1, K^+ 4.0, Ca^{2+} 2.3, Cl^- 155.6 (310 $mOsm.l^{-1}$, room temperature). As luminal perfusate, a purchased solution of ^{51}Cr-EDTA in 5 $mmol.l^{-1}$ EDTA, 433.64 MBq (11.72 mCi) per ml, pH 7.0 (Perkin Elmer, Boston, MA, USA), dissolved either in R1/1 (1: 1666.7 by volume), or in the same manner in a mixture of R1/1 and 96 % ethanol (to obtain 20 % ethanol solution), was employed. The former formula was followed to prepare a vehicle or control medium (CM), whereas the latter produced an ethanol medium (EM). Both media had the same ^{51}Cr-EDTA concentrations and hence also ^{51}Cr activities given as cpm per volume unit. The radioactive solutions were prepared after delivery according to this protocol and were utilized without modifications in the course of all experiments regardless of their actual activities.

Colon submucosal microdialysis technique

All animals were anesthetized with single i.p. dose of pentobarbital (50 $mg.kg^{-1}$, Nembutal®, Abbott Laboratories, North Chicago, USA) and placed in a supine position on an unheated bed. They were kept under general anesthesia by cyclic i.p. administration of Nembutal® (15 $mg.kg^{-1}.h^{-1}$). Body temperature was monitored using a rectal thermometer probe (Ama-digit ad 15th, Aprecision, Germany) and maintained at 38.2-38.8 °C by means of a heating lamp. For all surgical procedures, clean, but not sterile instruments/materials were used. The trachea was carefully prepared and cannulated with a short polyethylene catheter (outer diameter 2 mm) to ensure patent airways. 3-4 cm long midline laparotomy was performed with scissors through

linea alba. Descending colon was exteriorized and, when necessary, the region in question was made free of formed stercus by gentle manipulation. When exposed, the organ was kept moist with gauze soaked in 0.9 % saline (at room temperature). Firstly, parallel to the long axis of the organ at a distance of 5 cm from the anus, a 5-6 mm long tunnel was created from serosal aspect in its submucosal layer by means of a 28 G needle. Probe position was selected so as to avoid interference with blood vessels. The instrument was run close under the serosa with care neither to penetrate through the mucosa into the colonic lumen nor to make an additional redundant opening in the serosa. Secondly, a microdialysis probe (MAB 1.2.4. with 6 kDa cut-off polyethylenesulphone membrane, active length 4 mm; outer diameter 0.24 mm; Microbiotech/se AB, Stockholm, Sweden) was cautiously inserted into the preformed tunnel (Fig. 1a). The implantation technique was trained in advance on other animals and the exact localisation of the probe in the submucosal region of colon was verified histologically (Fig. 3a). Finally, the probe was fixed to the serosa at the tunnel entrance with an atraumatic Ethibond 5/0 suture (Ethicon ltd., U.K.). Throughout the experiment, continuous microdialysis was ensured by perfusing the catheters with R1/1 solution by means of a CMA 102 microdialysis pump (CMA Microdialysis AB, Solna, Sweden) at a perfusion rate of 1.5 μl.min^{-1}. For equilibration, an initial 40 min period (without specimen collection), followed by a yield of the first three control samples (during normal luminal perfusion) was designed. Sampling took place in 10 min intervals into 300 μl polyethylene Beckman type vials. The specimens were stored at –20 °C until analysis.

Animal mode of colon luminal perfusion

After successful implantation of a microdialysis probe, the oral part of the descending colon was ligated with a silk thread in a distance of 1-2 cm from the probe as close to the colonic wall as possible to avoid ischemization. Thereafter, a double-lumen cannula was inserted *via* anal route to permit continuous perfusion of the colonic lumen by means of a microprocessor-controlled syringe pump (LD 20, Tesla Přelouč, Czech Republic). The inlet (inner) tubing (2.5 cm in length) was situated in the vicinity of the oral colonic ligature and was nearly as long as the perfused part of the colon. On the other hand, the outlet (outer) tubing was ligated on the opposite, aboral side of colon and had only minimal protrusion into the lumen (0.5 cm). After cannulation and

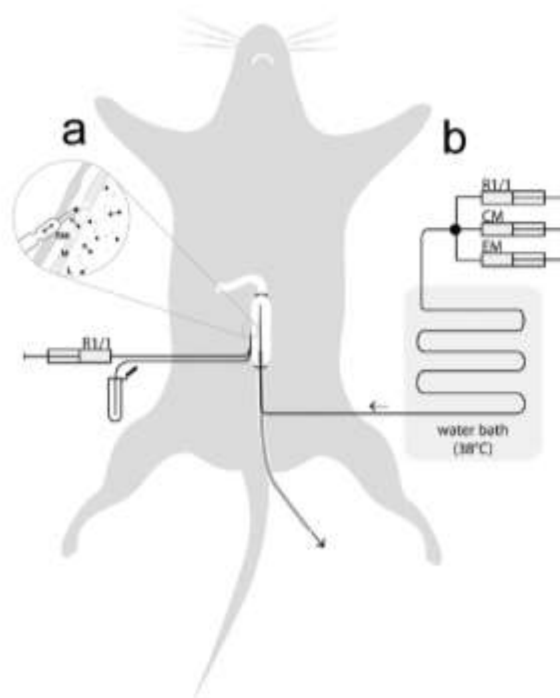

Fig. 1. A schematic view of *in vivo* submucosal microdialysis sampling (a) with parallel single-pass luminal perfusion of the descending colon (b) (details in the text). Note that this technique does not necessitate an artificial opening in the alimentary tract.

ligation was completed, a separated 3.0-3.5 cm long colonic lumen was prepared for single pass perfusion (Fig. 1b). At all times, to bring the temperature of the perfusion medium closer to body temperature, the inlet duct was passed in part through a thermostatic water bath (38 °C). For luminal washout, the flow was commenced at an initial rate of 25 ml/h for 30 min with R1/1 solution. In the next step, CM was applied using a three-way flow switch. Again, for quick washout, the same rate (25 ml.h^{-1}) was used for 8 min followed by 2 min at the rate of 6 ml.h^{-1}. Thereafter, continuous microdialysis sample collection was commenced by first three 10-min control episodes (to obtain baseline levels). When perfusion with CM was finished and baseline specimens yielded, the flow was switched again for administration of EM. After washout period (25 ml.h^{-1} for 8 min followed by 2 min at the rate of 6 ml.h^{-1}), three regular 10-min sampling intervals succeeded. When ethanol phase was completed, prior to the last, 60-min period of CM perfusion, a washout episode was carried out as described above. The total number of samples was 12. During the whole procedure, care was taken not to allow air bubbles to enter into the perfusion system. To avoid

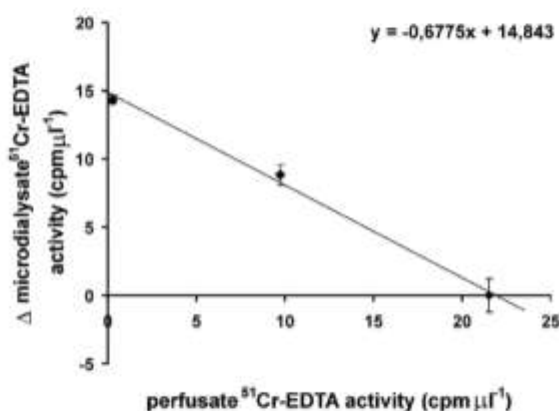

Fig. 2. *In vitro* probe calibration ("no net-flux" method). The Δ microdialysate (dialysate – perfusate) ^{51}Cr-EDTA activity plotted against the perfusate ^{51}Cr-EDTA activity gives a gradient representing the probe recovery (67.8 %). The x-intercept is in compliance with the actual activity of the surrounding medium. Results are means ± S.E.M. of 6 measurements.

fluid losses and to ensure convenient i.p. administration of Nembutal®, the skin layer of the abdominal opening was closed using microbulldog clamps.

Experimental design

The animals were allocated to two groups – the first (C, control, n = 6) group was examined as a sham group, i.e. without corrosive ethanol intervention (received only R1/1 and CM, with washouts preserved), whilst the second (E, ethanol, n = 6) group was exposed to a 30-min period of EM perfusion. All other procedures were identical for both groups. The animals were sacrificed by exsanguination from the abdominal aorta. After removal of the tubing from the descending colon and the isolation of the organ from the body, implanted probe was liberated, and colon tissue was dissected into a perfused and a proximal (oral) unperfused segment. The removed biological material was immersed in the preserving agent (10 % formalin) for ensuing histological analysis.

Radioactivity (gamma decay) measurement

A commercially available multi-crystal gamma counter LB 2111 (EG&G Berthold, Germany) was utilized. The instrument was standardized for ^{51}Cr (gamma ray, 320 keV) and adjusted for energy levels ranging within 50-450 keV. Counts per minute (cpm) were calculated from 10-min measurements. For counting, the original plastic vials with collected samples (15 μl) were used. To eliminate background counts, 24 identical vials with 15 μl of R1/1 solution were assessed and the obtained mean value was subtracted from all measurements.

Probe in vitro recovery calculation

To obtain a basic knowledge of microdialysis probe function with regard to transport of ^{51}Cr-EDTA *via* its semipermeable membrane, probe recovery was determined *in vitro* by no net-flux method as follows. Probe was immersed in 20 ml of ^{51}Cr-EDTA solution with specific activity of 21.53 cpm.μl^{-1} at standard laboratory temperature and perfused with three consecutive solutions (at equivalent temperature) of increasing activity (0, 9.73 and 21.53 cpm.μl^{-1}). For equilibration, an initial 30-min period was allowed, which was succeeded by sampling in 10-min intervals into microvials. In each experiment, the surrounding medium was freshly prepared, perfusion rate set at 1.5 μl.min^{-1} and six samples taken. The results were plotted on a graph and probe recovery was read from the regression equation (slope gradient).

Barrier integrity determination

To assess the function of colonic mucosal permeability, an updated ratio of activities was calculated using the following formula: [(probe perfusate cpm – background cpm) / (luminal perfusate cpm – background cpm)] x 100, where probe perfusate cpm is the activity obtained from 15 μl microdialysis samples and luminal perfusate cpm is the mean number of counts given by six 15 μl samples of luminal perfusate stock solution (CM or EM) shortly before administration to the animal. The results were expressed in %.

Histological analyses

Colon biopsies were taken to evaluate the effect of ethanol perfusion at the microscopic level and for probe position verification. The samples of the descending colon were taken from both perfused and proximal (unperfused) segments. The tissues were fixed in 10 % neutral buffered formalin and further treated according to standard procedures (hematoxylin-eosin stain). The sections were evaluated by light microscopic examination and photographed at 100-fold magnification.

Statistics

Data are expressed as means ± S.E.M. For statistical evaluation, parametric (repeated measures ANOVA with multiple comparison by a *post hoc* Fisher's

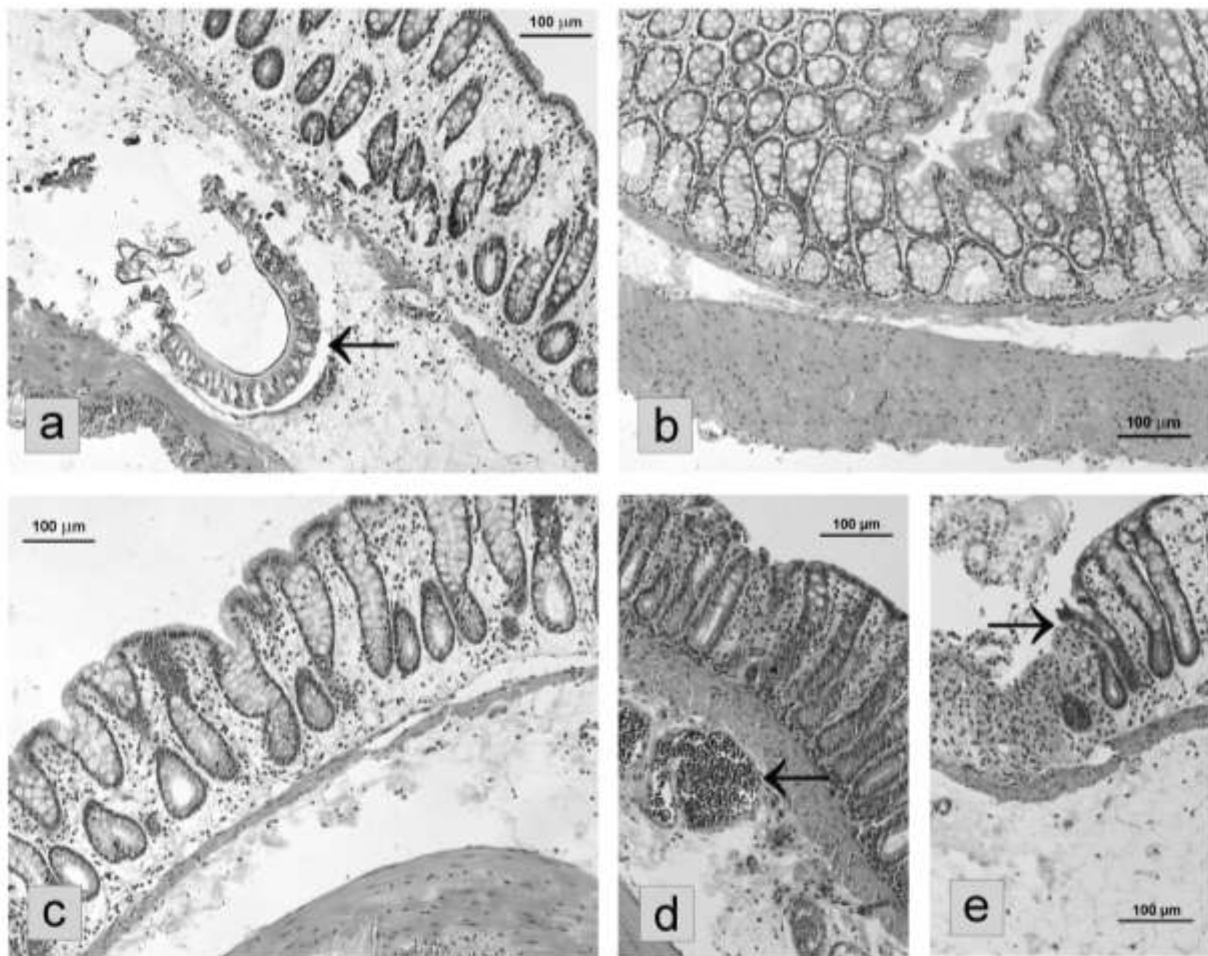

Fig. 3. Histological appearance of the wall of descending colon (HE stain, magnifications marked by scale bars). a, Probe position verification. Arrow points to probe remnants. The probe was damaged after experiment during tissue processing for slide preparation. b, Proximal (unperfused) part of colon showing intact tissue layers. c, Picture taken after the organ´s luminal perfusion with vehicle alone (control group). No marked changes observable. d, and e, Ethanol group. Leukocyte-filled dilated submucosal vessels (arrow, d) accompany the loss of mucosal architecture (arrow, e) owing to erosive action of ethanol.

LSD test) and non-parametric (Friedman´s ANOVA with Wilcoxon´s test) methods were applied. The data were processed by the programs NCSS 2004 and Statistica. The chosen level of significance was α=0.05.

Results

Histology

Microdialysis probes were positioned exactly within the submucosal layer of colonic wall, as indicated in Figure 3a. The unperfused part of the colon was unaffected by the experimental procedure (Fig. 3b). Application of R1/1 and vehicle (CM) had no material influence on the microscopical picture of the perfused part of the gut (Fig. 3c). However, after administration of ethanol, marked submucosal reaction/impairment was confirmed by histology (Fig. 3d,e).

Microdialysis data

The results of probe calibration are depicted in Figure 2. The *in vitro* recovery of the used probe type under the given conditions was estimated to be 67.8 %. With regard to barrier integrity estimation by microdialysis, there were some doubts as to the normality of data distributions given by the low number of subjects. However, non-parametric tests confirmed the findings of parametric methods. During the administration of 20 % ethanol, mucosal tracer permeability showed significant elevations (2.354±0.298 % for ethanol as opposed to 0.209±0.102 % for control group, time interval 60 min, $p<0.01$), which persisted or were even more pronounced after the ethanol instillation was discontinued until the end of the experiment, i.e. for at least 60 min (3.352±0.188 % for ethanol compared to 0.140±0.084 % for the control group, time interval 120 min, $p<0.001$) (Fig. 4).

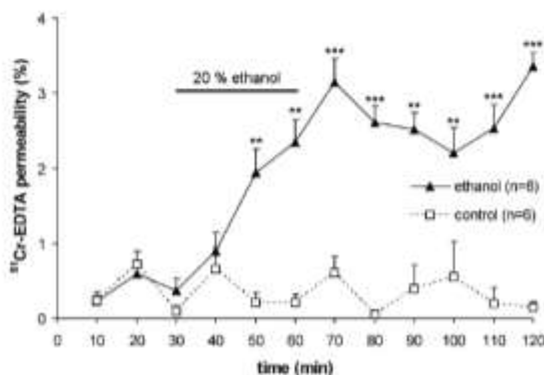

Fig. 4. Colon barrier function as assessed by lumen-to-submucosa ^{51}Cr-EDTA permeability (15 μl perfusion medium to 15 μl microdialysate activity x 100, expressed in %). Note the gradual increase in mucosal permeability in the course of ethanol perfusion in ethanol group. Results are means +S.E.M. At shown intervals, markings ** and *** denote $p < 0.01$ and $p < 0.001$, respectively.

Discussion

Methodological considerations

^{51}Cr-EDTA was employed as a suitable tracer because of its low molecular weight (352 g.mol^{-1}) and diameter (6.8 Å), i.e. characteristics that account for its free and rapid movement *via* vascular or endothelial barriers (Nylander *et al.* 1989). Hence, the only determinant of ^{51}Cr-EDTA mucosal permeability is epithelial lining integrity (function of tight junctions). In this context, microdialysis probes generally imitate blood vessels. Providing an adequate molecular diameter/pore cut-off ratio, perfusion rate and good function, probes generally represent a moderate and determinable obstacle to tracer diffusion as was also the case in our experiment. Moreover, application of ^{51}Cr-EDTA was advantageous, since it contains a radiolabeled atom. Measurements of gamma-activity do not require any biochemical assays with inevitable sample losses, whereby they may enable serial determination of other substances, e.g. metabolic markers or pharmaceuticals. In this way, twice as much information could be yielded from one approach in general and from microdialysis, as a method coping with low sample volumes, in particular. Unfortunately for present time, we were not able to directly demonstrate this potential unique capacity of microdialysis due to sample loss during secondary analytical procedure. Unlike elsewhere, our perfusion solutions were prepared once and used throughout the whole experiment. Hence, to eliminate the effect of radioactive decay and loss of ^{51}Cr activity (half-life = 27.7 days) on results of mucosal permeability, the approach of using a ratio of cpm values was chosen.

The effect of ethanol perfusion

The lack of change in the studied parameter within control group indicates that the implantation technique and presence of the probe in the submucosal region did not affect barrier integrity. The marked increase in ^{51}Cr-EDTA permeability in the ethanol group was consistent with observed macro- and microscopic mucosal injury and leukocyte infiltration (Fig. 3d and e, macroscopic picture not shown). These findings convincingly showed a profound disintegration of colonic epithelial barrier due to 30-min treatment with 20 % ethanol. It is notable that an analogous protocol applied in the stomach produced a significant, yet transient increase, followed by restitution in ^{51}Cr-EDTA blood-to-lumen clearance after 10 min perfusion with 20 % ethanol. Here, the finding of rapid barrier recovery was confirmed by minimal histological damage to the mucosa (Sobue *et al.* 2003). However, a single-shot intracolonic administration of 50 % ethanol caused elevations in ^{51}Cr-EDTA lumen-to-blood clearance, which persisted at least for 3 h, but would completely resolve by 2 days thereafter (Stein *et al.* 1998). Likewise, in a comparable model of colitis induced by trinitrobenzene sulfonic acid in 30 % ethanol, the most marked enhancement in neutrophil infiltration of colon (as estimated by myeloperoxidase activity and confirmed by histology) occurred 3-6 h after the induction of colitis. During this period, the greatest increase in colonic permeability to ^{51}Cr-EDTA was also observed (Wallace *et al.* 1992). To sum up, concerning intraluminal ethanol perfusion, the results obtained from microdialysis applied in the vicinity of tight junctions conform to the outcomes of clearance methods. Nevertheless, in order to demonstrate the suggested basic advantage of proposed technique, a parallel comparison with an established method (based on a non-radioactive tracer) should be performed on an ischemic model in the future.

Conclusions

The most important finding of our study was a significant elevation of tracer penetration into the microdialysis probe following the administration of ethanol. The proposed methodology was capable of detecting barrier injury without necessitating organ detachments, blood withdrawals or even vessel

catheterization. This pilot study provides a basement for development and introduction of a novel method for a wide range of experimental settings involving mucosal permeability measurements. The presented results indicate that submucosal microdialysis can be considered a feasible and advantageous alternative of gut barrier function estimation. Parallel monitoring of local tissue chemistry or pharmacology with this method remains a future challenge.

Acknowledgements

The authors wish to acknowledge a helpful and obliging cooperation with Ms. Kriesfalusyová in the Radioisotope Laboratory. We also thank Ms. Hollerová for skillful technical assistance with preparation of histological slides and Assoc. Prof. Rudolf for making electronical photographs. This work was principally supported by a Research Project from the Czech Ministry of Foreign Commerce MZO 00179906.

References

DE LA PENA A, LIU P, DERENDORF H. Microdialysis in peripheral tissues. *Adv Drug Deliv Rev* **45**: 189-216, 2000.

DOIG CJ, SUTHERLAND LR, SANDHAM JD, FICK GH, VERHOEF M, MEDDINGS JB: Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am J Respir Crit Care Med* **158**: 444-451, 1998.

FIHN BM, SJOQVIST A, JODAL M: Involvement of enteric nerves in permeability changes due to deoxycholic acid in rat jejunum in vivo. *Acta Physiol Scand* **178**: 241-250, 2003.

IWATA F, JOH T, UEDA F, YOKOYAMA Y, ITOH M: Role of gap junctions in inhibiting ischemia-reperfusion injury of rat gastric mucosa. *Am J Physiol* **275**: G883-G888, 1998.

KAWAI T, JOH T, IWATA F, ITOH M: Gastric epithelial damage induced by local ischemia-reperfusion with or without exogenous acid. *Am J Physiol* **266**: G263-G270, 1994.

KITANO M, NORLÉN P, HÅKANSON R: Gastric submucosal microdialysis: a method to study gastrin- and food-evoked mobilization of ECL-cell histamine in conscious rats. *Regul Pept* **86**: 113-123, 2000.

NYLANDER O, KVIETYS P, GRANGER DN: Effects of hydrochloric acid on duodenal and jejunal mucosal permeability in the rat. *Am J Physiol* **257**: G653-G660, 1989.

SOBUE M, JOH T, OSHIMA T, SUZUKI H, SENO K, KASUGAI K, NOMURA T, OHARA H, YOKOYAMA Y, ITOH M: Contribution of capsaicin-sensitive afferent nerves to rapid recovery from ethanol-induced gastric epithelial damage in rats. *J Gastroenterol Hepatol* **18**: 1188-1195, 2003.

SOLLIGÅRD E, JUEL IS, BAKKELUND K, JOHNSEN H, SAETHER OD, GRONBECH JE, AADAHL P: Gut barrier dysfunction as detected by intestinal luminal microdialysis. *Intensive Care Med* **30**: 1188-1194, 2004.

STEIN J, RIES J, BARRETT KE: Disruption of intestinal barrier function associated with experimental colitis: possible role of mast cells. *Am J Physiol* **274**: G203-G209, 1998.

UDASSIN R, HASKEL Y, SEROR D, WELBOURNE TC: Plasma-to-lumen clearance of para-aminohippurate can replace ^{51}Cr-EDTA clearance in the evaluation of intestinal mucosal injury. *Pediatr Surg Int* **13**: 112-114, 1998.

WALLACE JL, HIGA A, MCKNIGHT GW, MACINTYRE DE: Prevention and reversal of experimental colitis by a monoclonal antibody which inhibits leukocyte adherence. *Inflammation* **16**: 343-354, 1992.

Corresponding author

N. Cibiček, Institute of Clinical Biochemistry and Diagnostics (ÚKBD), University Hospital (FN), Sokolská 581, 500 05 Hradec Králové, Czech Republic. E-mail: cibicek@seznam.cz

14.2. THE AUTHOR

Norbert Cibiček, born 1978, Slovak Republic (former Czechoslovakia)

Qualifications, studies and employment

1998 – 2004 Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic (MUDr. degree)
2001 Humboldt University, Berlin, Germany (pregraduate study)
2003 Ruprecht-Karls University Hospital, Mannheim, Germany (pregraduate residency)
2004 – 2007 University Hospital Hradec Králové, Institute of Clinical Biochemistry and Diagnostics (clinical residency)
2006 Karolinska University Hospital, Huddinge, Sweden (postgraduate research stay)
2004 – 2008 Charles University in Prague, Faculty of Medicine in Hradec Králové (Ph.D. student, medical chemistry & biochemistry)

Publications (Original articles, case reports and reviews)

CIBICEK N, CIBICKOVA L, KOHOUT P, ZDANSKY P. [The use of sucrose absorption test (SaLM) for detection of upper gastrointestinal tract mucosa affections in upper dyspepsia patients – a pilot study]. *Acta Medica (Hradec Králové) Suppl* **2004**;47(1):23-28.

CIBICKOVA L, **CIBICEK N**, ZDANSKY P, KOHOUT P. The impairment of gastroduodenal mucosal barrier by coffee. *Acta Medica (Hradec Králové)* **2004**;47(4):275-8.

CIBIČEK N. [Infectious endocarditis.] *Postgraduate Medicine Czech* **2005**;6(4):99-100.

CIBICEK N, MICUDA S, CHLADEK J, ZIVNY P, ZADAK Z, CERMAKOVA E ET AL. Lithium microdialysis and its use for monitoring of stomach and colon submucosal blood perfusion – a pilot study using ischemic preconditioning in rats. *Acta Medica (Hradec Králové)* **2006**;49(4):227-231.

CIBICKOVA L, SOUKUP T, **CIBICEK N**, CHLADEK J. Nitric oxide and systemic sclerosis. *Acta Medica (Hradec Králové)* **2006**;49(4):245-246.

CIBIČEK N, ŽIVNÁ H, ZADÁK Z, KULÍŘ J, ČERMÁKOVÁ E, PALIČKA V. Colon submucosal microdialysis: a novel in vivo approach in barrier function assessment - a pilot study in rats. *Physiol Res* **2007**;56(5):611-617.

CIBIČKOVÁ L, PALIČKA V, **CIBIČEK N**, ČERMÁKOVÁ E, MIČUDA S, BARTOŠOVÁ L ET AL. Differential effects of statins and alendronate on cholinesterases in serum and brain of rats. *Physiol Res* **2007**;56(6):765-770.

MICUDA S, FUKSA L, BRCAKOVA E, OSTERREICHER J, CERMANOVA J, **CIBICEK N** ET AL. Zonation of mrp2 in rat liver after induction with dexamethasone. *J Gastroen Hepatol* **2007** [e-pub]

CIBIČKOVÁ Ľ, **CIBIČEK N**. [Will statins and bisphosphonates have common indications?] *Farmakoterapie* **2007**;6:550-551.

POJAR M, MANĎÁK J, **CIBÍČEK N**, LONSKÝ V, DOMINIK J, PALIČKA V ET AL. Peripheral tissue metabolism during off-pump versus on-pump coronary artery bypass graft surgery: the microdialysis study. *Eur J Cardiothorac Surg* **2008**;33(5):899-905.

CIBIČEK N, ŽIVNÁ H, CIBIČEK J, ČERMÁKOVÁ E, VOŘÍŠEK V, MALÁKOVÁ J ET AL. Caffeine does not modulate nutritive blood flow to rat gastric submucosa – a microdialysis study. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **2008**;152(1):83-90.

CIBICKOVA L, HYSPLER R, TICHA A, **CIBICEK N**, PALICKA V, CERMAKOVA E ET AL. Cholesterol synthesis in central nervous system of rat is affected by simvastatin as well as by atorvastatin. *Pharmazie* **2008** (in print)

CIBIČKOVÁ Ľ, PALIČKA V, HYŠPLER R, **CIBIČEK N**, ČERMÁKOVÁ E. Alendronate lowers cholesterol synthesis in the central nervous system of rats – a preliminary study. *Physiol Res* **2009**;58(3) (in print)

Publications (abstracts)

CIBICEK N, FLÄRING U, WERNERMAN J, ROOYACKERS O, KLAUDE M. The effect of endotoxemia on muscle proteasome activity in healthy volunteers. *Clin Nutr Suppl* **2007**;2(2):153-154.

CIBICKOVA L, HYSPLER R, PALICKA V, CERMAKOVA E, **CIBICEK N**. Cholesterol synthesis in central nervous system of rat is affected by simvastatin as well as by atorvastatin. *Clin Nutr Suppl* **2007**;2(2):17.

CIBIČEK N, MANĎÁK J, POJAR M, NEDVÍDKOVÁ J, ČERMÁKOVÁ E, ŽIVNÝ P ET AL. Extracorporeal circulation during cardiac surgery impairs skeletal muscle energy metabolism – a microdialysis study. *Klin Biochem Metabol Suppl* **2007**;15:62.