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RIGORÓZNÍ PRÁCE

Absorpce xenobiotik ve vztahu k patofyziologii gastrointestinálního traktu u experimentálních zvířat

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ANOTACE

Fyziologický význam gastrointestinálního traktu (GIT) spočívá především v absorpci, biotransformaci a exsorpci eobiotik a xenobiotik. Míra transintestinálního transportu je klíčový faktor určující biologickou dostupnost perorálně podaných xenobiotik. O tom, jakým způsobem (míra a rozsah) bude molekula absorbována z lumen GIT, rozhoduje schopnost této molekuly překonávat biologické bariéry (Lüllman et al., 2002). Obecně pohyb molekul přes biologické membrány podléhá fyzikálně-chemickým zákonitostem a je závislý na funkčním stavu GIT (náplň žaludku, pH v jednotlivých částech, místní prokrvení apod.). Je známo, že kromě rozdílů v míře absorpce, i biotransformace ve střevě může přispívat ke značné inter- a intra-individuální variabilitě v biologické dostupnosti mnohých orálně podaných látek (Doherty et al., 2002).

Významnou úlohu v absorpci látek a jejich diferencí hraje pato-fyziologický stav stěny GIT. Patologický stav, ve smyslu celé škály morfologických (léze) či funkčních atypií (motilita, produkce biochemických působků) je/může být vlivem jednoho faktoru (dlouhodobá terapie nesteroidními antiflogistiky-NSA, stres, stravovací návyky) nebo je multifaktoriální podstaty (v případě idiopatických střevních zánětů). Právě nežádoucí vedlejší účinek NSA (např. indometacin), experimentálně navozující gastroenteropatie (Rainsford et al., 2003; Suleyman et al., 2009; Mehrabani et al., 2009), byl využit v našich pokusech. Mechanizmem způsobujícím poškození sliznice pod vlivem NSA je přímý toxický efekt na enterocyty, mající za následek snížení bariérové funkce střevního epitelu a průnik bakterií, makromolekul, trávicích enzymů a dalších intraluminálních toxinů do hlubších vrstev sliznice vedoucí ke stimulaci produkce prozánětlivých cytokinů (IL-6, IL-8, TNFα.), infiltraci neutrofily a k rozvoji zánětu. Druhým mechanizmem uplatňujícím se při poškození sliznice GIT je nespecifická inhibice cyklooxygenáz (COX-1 i COX-2) (Takeuchi et al., 2010). Dalším látkovým modelem vyvolávajícím enteropatie byl dextran sulfát (DS), který způsobuje léze především v ileu, céku a kolon, modelující patologický stav podobný klinickému obrazu ulcerativní kolitidy či Crohnovy choroby.

Experimentálním navozením patologického stavu žaludeční a/nebo střevní sliznice byly sledovány změny v absorpci a biotransformaci modelového léčiva 5-aminosalicylové kyseliny (indikace v léčbě nespecifických střevních zánětů), na

základě detekce plazmatických hladin parentní látky včetně jejího hlavního metabolitu N-acetyl-5-aminosalicylové kyseliny. Tyto základní experimenty byly prováděny jak na laboratorních potkanech (Rattus norvegicus, samci, kmen Wistar Han II) tak i na prasatech (Sus scrofa f . domestica, samice, hybridní plemeno České bílé a Landrace). Dále jej bylo v experimentech využito pro verifikaci aplikovatelnosti dostupných diagnostických přístupů (na prasatech). Tím byly např. elektogastrografický (EGG) záznam myoelektrické aktivity žaludku, poskytující informace o četnosti a relativní amplitudě kontrakcí antra žaludku pomocí elektrod umístěných na kůži. EGG nám pak sloužilo k odhalení možných změn v motilitě žaludku indukované objemovou zátěží nebo navozenou prokineticky působícími látkami (itopridem, erytromycinem).

Další použitou diagnostickou metodou byla kapslová endoskopie. Jde o minimálně invazivní endoskopickou vyšetřovací metodu, využívající bezdrátového přenosu snímaných obrazů sliznice GIT pomocí diagnostické mikrokamery, která byla endoskopicky zavedena za pylorus. Mikrokamera při průchodu trávicím ústrojím snímá 2 obrázky/min, životnost baterie je cca 8-9 hod. Kapslová endoskopická technika byla v našich experimentech vůbec poprvé využita pro snímaní osudu lékové formy (způsob a rychlost desintegrace) při průchodu GIT, korelovanou s absorbovanými hladinami účinné látky detekovanými v krevní plazmě (Květina et al., in press). Tento metodický postup pak může odhalit případné „absorpční okno“ účinné látky ve střevě a být informačním zdrojem pro následnou modifikaci technologického postupu vývoje lékové formy.

Konfokální laserová mikroskopická technika využívá možnosti skenování jednotlivých vrstev stěny GIT již v průběhu endoskopického vyšetření a napomáhá tak odhalit míru (hloubku) slizničních patologických změn. Horizontálním snímáním vyšetřované tkáně (a vícenásobným zvětšením) se tak liší od klasické optické mikroskopie, která hodnotí změny tkáňové architektury především v rovině vertikální. Tato vyšetřovací technika byla taktéž využita v našich experimentech.

Součástí dílčích výzkumných etap bylo i sledování vlivu probiotické medikace (*Escherichia coli* Nissle 1917) na procesy určující farmakokinetiku enterálně podaného modelového xenobiotika (5-aminosalicylová kyselina; 5-ASA) jak za fyziologických, tak i za patologických podmínek (míra vzniku a lokalizace slizničních lézí) u potkanů i prasat. Hodnocení spočívalo ve stanovení plazmatických hladin daného léčiva, změnách v morfometrických ukazatelích střevní sliznice, produkci

bakteriocinů (peptidy produkované bakteriemi), histologickou a histochemickou diagnostikou jednotlivých částí GIT.

Téměř všemi prácemi se prolíná modelové léčivo 5-ASA, které je v současné době nejpoužívanější (nejúčinější) léčivou látkou v terapii zánětlivých onemocnění střevní stěny. Na našem pracovišti byla vyvinuta analytická detekční metoda vysokoúčinné kapalinové chromatografie (Nobilis et al., 2006) pro stanovení jejích koncentrací v biologických vzorcích. Prezentované experimentální práce měly za cíl především stanovit farmakokinetické profily 5-ASA a jejího metabolitu N-acetyl-5-ASA u potkanů a prasat. Krevní vzorky byly odebírány z kanyly, chirurgicky (za aseptických podmínek) zavedené subkutánně do vena jugularis (potkan) či vena cava cranialis (prase). Zdůvodnění volby prasete, jako experimentálního zvířete používaného v našich in vivo pokusech, je několikeré. Z hlediska biotransformační enzymové kapacity a fyziologických charakteristik je do jisté míry velmi podobné člověku a rovněž z hlediska přenositelnosti získaných dat se zdá být vhodným zvířecím druhem. To dokládá i příklad mezidruhového srovnání základních farmakokinetických ukazatelů 5-ASA (Tab. 1), kde biotransformační (N-acetylační) aktivita u prasete je kvalitativně i kvantitativně nejblíže člověku, v porovnání s ostatními experimentálními zvířaty (údaje získané rovněž z našich vlastních experimentů). U opice (*Macacus rhesus*) jsou rozdíly kvantitativního rázu, u psa (bígl) k biotransformaci 5-ASA na metabolit N-acetyl-5-ASA vůbec nedochází. Dalším aspektem pak byla aplikovatelnost uváděných gastroenterologických vyšetřovacích metod, které by v podstatě nešlo využít u jiného experimentálního druhu.

Tab. 1: Mezidruhové srovnání farmakokinetických profilů perorálně podané modelové látky (střevního antiflogistika) 5-aminosalicylové kyseliny (5-ASA) a jejího hlavního metabolitu N-acetyl-5-ASA.

	člověk	prase	opice	pes
5-ASA				
AUC (µg/ml/h)	10,6 ± 5,6	7,6 ± 2,8	2,8 ± 0,3	73,9 ± 20,3
C_{max} (µg/ml)	3,3 ± 1,7	4,9 ± 3,2	1,7 ± 0,1	21,1 ± 11,8
t_{max} (h)	3,6 ± 1,2	2,4 ± 0,8	15,5 ± 12,0	1,7 ± 0,5
N-acetyl-5-ASA				
AUC (µg/ml/h)	37,1 ± 7,9	31,1 ± 8,8	9,0 ± 6,8	0
C_{max} (µg/ml)	7,8 ± 2,8	13,8 ± 5,3	5,2 ± 3,1	0
t_{max} (h)	4,3 ± 1,2	2,8 ± 0,8	15,5 ± 12,0	0

(Zadák, Květina et al., 2011)

Probiotická medikace je masivně propagována jako vhodná nejen jako preventivní, ale především i jako podpůrná (vedle konvenční lékové) terapie při různých onemocněních GIT. Dosud ovšem nebylo literárně dokumentováno zda (případně jakým způsobem) probiotická „léčba“ neovlivňuje farmako-kinetiku/dynamiku užívaných léčivých přípravků. Tato problematika se pak následně stala jedním z našich výzkumných zadání. Výsledky našich experimentů však nemohou výše uvedenou komerční invenci jednoznačně podpořit. Medikace probiotikem *E. coli* Nissle 1917 v případě fyziologických podmínek měla pozitivní (trofický efekt na střevní mukózu) nebo neutrální (produkce bakteriocinů, EGG záznam) vliv. Za patologického stavu pak zvyšuje absorpci 5-ASA, což je z pohledu terapeutického efektu nežádoucí jev, jelikož jde o topicky působící antiflogistikum. V případě kombinatorní medikace (probiotikum+indometacin) pak byly rovněž zjištěny relativně nejhorší výsledky v měřených morfometrických parametrech střevní stěny (výška glandulární sliznice, poměr výšky a šířky klků, výška a délka enterocytů na bázi Lieberkühnových krypt). Zřetel je třeba brát na kmen užívaného probiotika, míru a typ funkčního či morfologického poškození GIT.

Byly zjištěny i mezidruhové rozdíly ve vnímavosti (indukce lézí) vůči indometacinu. U potkanů stačí jednorázová dávka k vyvolání GIT lézí, které jsou v žaludku zjistitelné již za 6h po podání a s postupem času lze pozorovat i reparativní fázi indukovaných změn. Ve střevní sliznici lze léze diagnostikovat za 24h s následnou výraznou progresí patologického stavu, bez reparativních změn. U prasat bylo třeba k vyvolání gastroenteropatie opakované (10 denní) podávání indometacinu..

Uvedené diagnostické přístupy v experimentech na prasatech umožnily ohraničit využitelnost těchto vyšetřovacích technik pro rutinní aplikovatelnost v klinické praxi.

Metodický popis jednotlivých experimentů včetně dosažených výsledků je detailně popsán v přiložených publikačních výstupech.

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Přiložené publikační výstupy

Absorption kinetics of 5-aminosalicylic acid in rat: influence of indomethacin-induced gastrointestinal lesions and *Escherichia Coli* Nissle 1917 medication

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Abstract

OBJECTIVES: The therapeutic effect of probiotics has been studied in many clinical and experimental studies but no data exist concerning the influence of probiotics on pharmacokinetics of contemporary administered drugs. In this paper, we describe the influence of indomethacin-induced gastrointestinal lesions and *Escherichia Coli* Nissle 1917 medication on absorption of 5-aminosalicylic acid and its metabolite N-acetyl-5-aminosalicylic acid in rat.
METHODS: 5-aminosalicylic acid (5-ASA) was given orally to rat using gastric probe as a suspension (25 mg/kg). The plasma time profiles of 5-ASA and its metabolite were compared between Group A (animals medicated with a suspension of Escherichia coli Nissle 1917 [EcN] in dose of 3.5 × 1010 CFUs/day for 14 consecutive days), Group B (animals with indomethacin [IND]-induced gastrointestinal lesions; single dose of 25 mg/kg of IND), Group C (simultaneous administration of EcN and IND), and Group D (control animals without any medication). The blood samples for HPLC analysis has been taken from incannulated vena jugularis in time 30, 60, 90, 120, 180, 240, 360 min after 5-ASA administration to rat.
RESULTS: The pharmacokinetics of 5-ASA was not significantly changed by EcN medication (Group A) in comparison to control animals (Group D). The significantly elevated absorption (AUC and cmax) of 5-ASA was found in animals with induced gastro-enteropathy with concurrently medicated with EcN (Group C) when compred to controls. In the case of metabolite N-acetyl-5-ASA, statistically no-significant differences were found between groups.
CONCLUSIONS: Simultaneous probiotics (EcN) medication did not affect absorption 5-ASA from intestinal tract (the main site of ASAs action).

ORIGINAL ARTICLE

Abreviations:

ASAs	- aminosalicylates
AUC	- area under the curve
5-ASA	- 5-aminosalicylic acid
CFU	- colony-forming unit
c_{max}	- peak concentration
EcN	- *Escherichia coli* Nissle 1917
GI	- gastrointestinal
IBD	- inflammatory bowel disease
HPLC	- high-performance liquid chromatography
IFN-γ	- interferon-gamma
IL-2	- interleukin-2
IND	- indomethacin
LLOQ	- lower limit of quantification
LPS	- lipopolysaccharide
N-acetyl-5-ASA	- N-acetyl-5-aminosalicylic acid
N-propionyl-5-ASA	- N-propionyl-5-aminosalicylic acid
NSAIDs	- non-steroidal anti-inflammatory drugs
T_{max}	- time to peak concentration
TLRs	- toll-like receptors
TNF-α	- tumor necrosis factor-alpha
UC	- ulcerative colitis
UV	- ultraviolet

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used agents in clinical practice today. Indometahacin, besides its antiphlogistic effect, is known to produce erosins, ulcerative lesions, and petechial bleeding in the mucosa of all parts of the gastrointestinal tract, both in humans and in animal experiments (Kuneš *et al.* 2009; Kim *et al.* 2011; Heeba *et al.* 2009; Kamil *et al.* 2007; Tachecí *et al.* 2010; Hawkey & Langman 2003). Recent evidence has suggested the potential therapeutic role of probiotics in the prevention or treatment of gastrointestinal (GI) disorders (Mach 2006). Efficacy of EcN against inflammatory states in GI tract has been shown in numerous trials (Kruis *et al.* 1997, 2004). There is also an evidence for the efficacy of EcN in Crohn's disease (Malchow 1997), pouchitis (Kuzela *et al.* 2001), collagenous colitis (Tromm *et al.* 2004), antibiotic-associated colitis (Goerg & Schlorer 1998), irritable bowel syndrome (Krammer *et al.* 2006) and diverticular disease of the colon (Fric & Zavoral 2003). Supportive probiotic therapy has seen the biggest progress in inflammatory bowel disease in the last twenty years although there are not currently regulated. In 2004, based on the clinical efficacy and documented low-side effect profile the guidelines for diagnosis and treatment of ulcerative colitis as issued by the German Society of Gastroenterology and Digestive Diseases. EcN was recommended as an alternative to standard mesalazine treatment to maintain remission (Hoffmann *et al.* 2004).

Currently, no studies exist addressing the issue of influence of probiotics on pharmacokinetics of concomitant conventional drug administration.

In our study, we aimed to evaluate the pharmacokinetics 5-aminosalicylic acid (5-ASA) and its metabolite N-acetyl-5-aminosalicylic acid (N-acetyl-5-ASA) in rats medicated with probiotic strain *Escherichia coli* Nissle 1917 (EcN) and in rats with experimentally indomethacin-induced gastrointestinal lesions.

MATERIAL & METHODS

Animals

21 males of laboratory rat (Wistar Han II from breeding facility Konárovice nad Labem), weighing 287 ± 21 g, entered the study. They were kept in plastic breeding containers in air-conditioned room allowed access to water and food ad libitum. The animals were fasted 12 hours before pharmacokinetic study.

Study design

The rats were divided into four groups. Group A –the animals were medicated with a suspension of probiotic strain *Escherichia coli* Nissle 1917 (obtained from laboratories of Microbiological Institute of the Czech Academy of Sciences, Prague), serotype O6:K5:H1 (5×10^8 CFUs/day) for 14 consecutive days (using gastric probe). Group B – the rats were probed for 14 days with a saline (as a "sham manipulation"). Fourteenth day (one hour after the last dose of probiotics), indomethacin was administered (25 mg/kg as a single dose using gastric probe) to rat to induce of gastrointestinal lesions. Group C – rats were administered with *Escherichia coli* Nissle 1917 (as in group A) and indomethacin (as in group B). Group D (control group of animals) – animals probed with a saline (see group B) only.

Pharmacokinetics

The pharmacokinetic study of 5-aminosalicylic acid (5-ASA) was made next day (15^{th} day) after the last dose of medication according to the scheme of study design. The cannulation of vena jugularis (in general inhalation anaesthesia; mixture of nitrous oxide, oxygen and halothane) was performed in order to blood samples taken. The cannula was led out subcutaneously on the dorsal side of neck. The blood sampling was done in time 30, 60, 90, 120, 180, 240, 360 min after 5-ASA (mesalazine substance obtained from PRO.MED.CS Praha a.s. in dose of 25 mg/kg in 40% polyethylene glycol using gastric probe) administration from animals with free movement in breeding container. Blood samples were centrifuged (3 000 t./min, 10 min). The blood plasma was frozen at –30 °C until analysis.

Analytical procedure

HPLC bioanalytical method for the determination of 5-ASA and its metabolites in blood plasma was developed and validated in our laboratory (Nobilis *et al.* 2006). The sample preparation step consists of the deproteination of plasma by $HClO_4$ and the derivatization of ASAs followed by liquid-liquid extraction of all N-acyl-ASA-derivatives. Chromatographic analyses were performed on a 250-4 mm column containing Purospher RP-18 e, 5 microm (Merck, Darmstadt,

Germany) with a precolumn (4-4 mm). The column effluent was monitored using both UV photodiode-array (lambda = 313 nm) and fluorescence detectors (lambda(exc.) = 300 nm/lambda(emiss.) = 406 nm) in tandem. The identity of individual N-acyl-ASAs in the extracts from biomatrices was verified by characteristic UV-spectra and by HPLC/MS experiments. The whole analysis lasted 23 min at the flow rate of 1 ml.min^{-1}. LLOQ (LOD) was estimated 126(20) pmol.ml^{-1} of plasma for N-acetyl-5-ASA and 318(50) pmol.ml^{-1} of plasma for N-propionyl-5-ASA.

Statistical analysis

All data were compared using analysis of variance (ANOVA) followed by multiple-comparison tests as post hoc analysis or a Student's *t*-test for group comparison of parametric data. The differences were considered significant when $p<0.05$.

Ethics

The study was approved by the Institutional Review Board of the Animal Care Committee from the Institute of Experimental Biopharmaceutics, Czech Academy of Sciences. Animals were held and treated in accordance with the European Convention for The Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

RESULTS

The pharmacokinetics of 5-ASA was not significantly changed by EcN medication (Group A) in comparison to control animals (Group D) as seen from plasma time profiles (Figure 1) and evaluated basic pharmacokinetic parameters (Figures 3–5). The elevated (but no statistically significant) absorption (AUC and c_{max}) of 5-ASA was found in animals after indomethacin (Group B), whereas the levels of 5-ASA were significantly higher in rats medicated with EcN and with indomethacin (Group C) in comparison to controls (Group D) (Figures 1, 3 and 5).

The concentrations of metabolite N-acetyl-5-ASA in blood were lowest in EcN medicated rats (Group A), Overall, however, plasma time profiles did not differ significantly between groups (Figure 2) as well as seen from parameters AUC, C_{max} and T_{max} (Figures 6–8).

DISCUSSION

The therapeutic effect of probiotics has been studied in many clinical and experimental studies. Selective probiotics such as *Lactobacillus GG* (Kalliomäki *et al.* 2001, 2003), *Saccharomyces boulardi* (McFarland *et al.*, 1995) and *Escherichia coli* Nissle 1917 (Kruis *et al.* 1997, 2004; Rembacken *et al.* 1999) have been proven to be clinically effective, the mode of action by which they achieve their beneficial effects remained unclear. Particular probiotic strains have been successfully used for prophylaxis of intestinal infection also in livestock animals (Vanbelle *et al.* 1990; Alexopoulos *et al.* 2004). In piglets, an efficient prophylactic effect of orally administered EcN strain against the epidemic pathogenic action of the porcine enterotoxigenic *E. coli* strain – fatal in pork livestocks – was found (Schroeder *et al.* 2006). The probiotic strain *E. coli* Nissle 1917 used in this study is of the serotype O6:K5:H1 and was isolated for the first time in 1916 by the German physician Alfred Nissle (Loew 2000). Since then this bacterial strain has been used as a probiotic drug and is considered to be safe (Blum *et al.* 1995; Grozdanov *et al.* 2002, 2004; Westendorf *et al.* 2005; Duncker *et al.* 2006). EcN has been characterized extensively at the phenotypic level as well as the molecular genetic level (Blum *et al.* 1995; Blum-Oehler *et al.* 2003; Grozdanov *et al.* 2004; Sun *et al.* 2005).

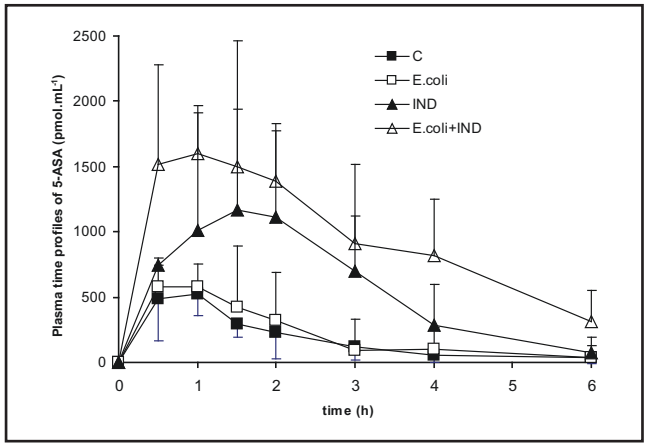

Fig. 1. Pharmacokinetics of 5–ASA in particular groups of rats after its intragastric administration (25 mg/kg). No differences were found in rats pre-medicated with probiotics E.coli in comparison to controls. Significantly higher absorption was found in animals with the combinatory treatment (E. coli + IND). Higher absorption, but statistically no-significant was in animals given indomethacin (IND). Average values ± standard deviation.

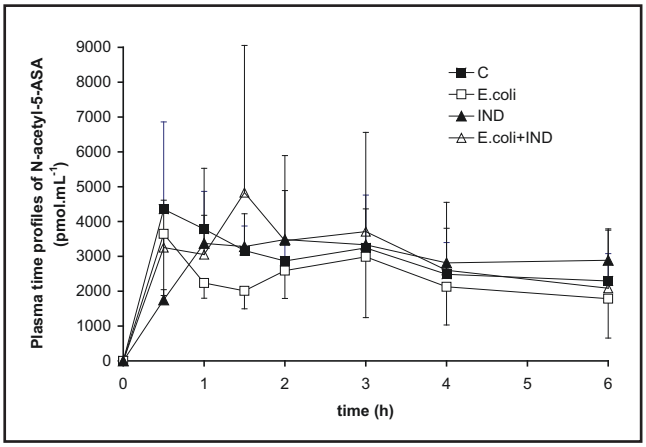

Fig. 2. Pharmacokinetics of N-acetyl-5-ASA in particular groups of rats after intragastric administration of 5-ASA (25 mg/kg). Statistically no-significant differences were found between particular groups, Average values ± standard deviation.

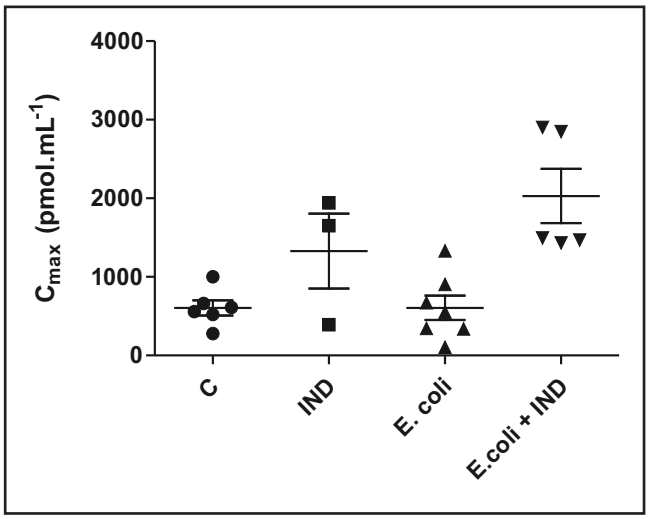

Fig. 3. 5-ASA: parameter C_{max} in each experimental group of animals (horizontal lines means average value ± SEM). Statistically significant differences were found in animals with combinatory treatment (E. coli + IND) when compared to controls (C) and to probiotics medicated rats (E. coli) – $p<0.05$ – Tukey's Multiple Comparison Test.

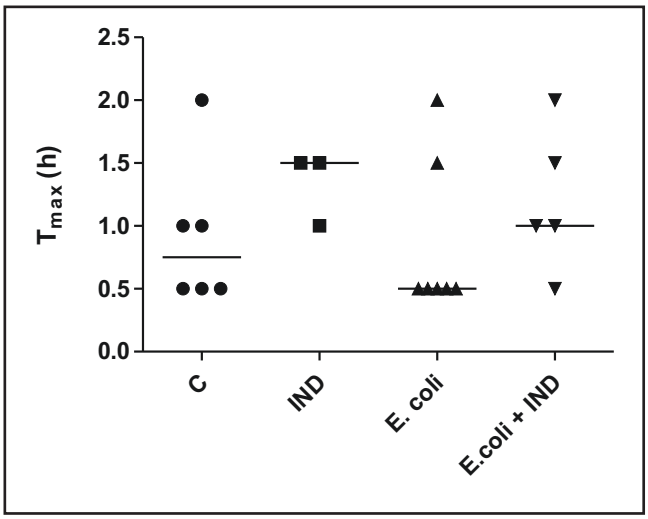

Fig. 4. 5-ASA: parameter T_{max} in each experimental group of animals (horizontal lines are medians). Statistically no-significant differences were found between groups – Dunn's Multiple Comparison Test.

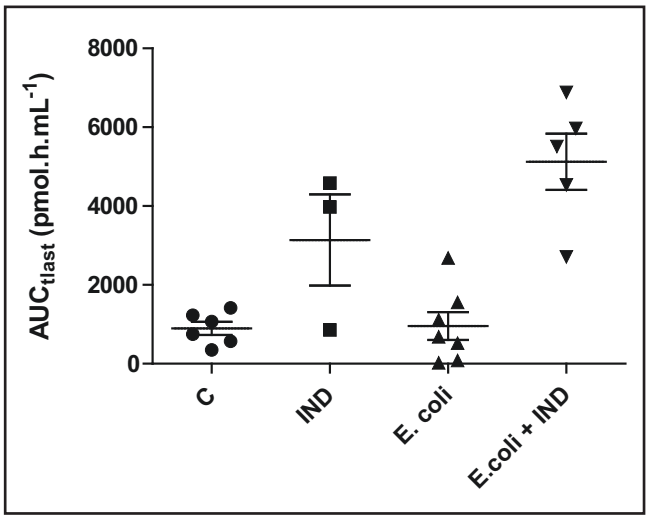

Fig. 5. 5-ASA: parameter AUC in each experimental group of animals (horizontal lines means average value ± SEM). Statistically significant differences were found in animals with combinatory treatment (E. coli + IND) when compared to controls (C) and probiotics medicated rats (E. coli) – $p<0.05$ – Tukey's Multiple Comparison Test.

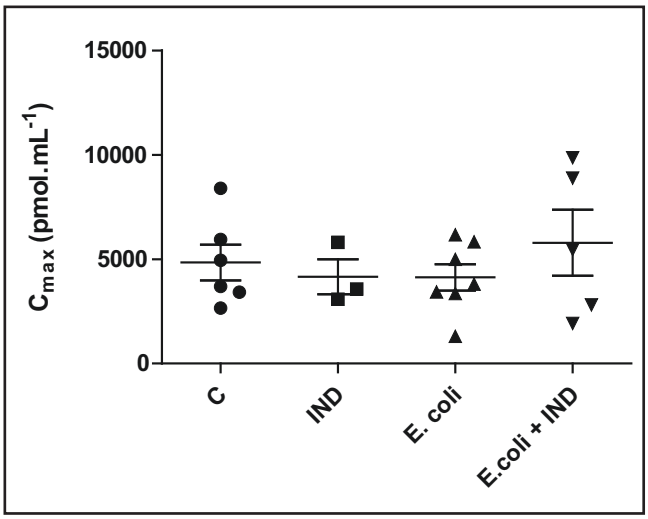

Fig. 6. N-acetyl-5-ASA: parameter C_{max} in each experimental group of animals (horizontal lines means average value ± SEM). Statistically no-significant differences were found between groups – $p<0.05$ – Tukey's Multiple Comparison Test.

EcN, an active component of Mutaflor ®, have been evaluated in the last few years as an alternative and safe treatment modality for inflammatory bowel diseases (IBD). Several randomized, placebo controlled studies have clearly demonstarted the beneficial effects of probiotics in the treatment of ulcerative colitis and pouchitis (Gionchetti *et al.* 2000, 2002; Lammers *et al.* 2005), and showing equivalent effectiveness as 5-aminosalicylic acid in maintaining remission in ulcerative colitis (UC) in humans (Malchow *et al.* 1997; Kruis *et al.* 1997, 2004; Rembacken *et al.* 1999). Furthermore, antibiotic as well as probiotic therapy attenuates both experimental colitis and human IBD (Greenberg *et al.* 2004; Kruis 2004; Sartor 2004; Schultz *et al.* 2003).

Despite the demonstrated benefit, the underlying modes of action in intestinal inflammation have yet to be elucidated at the cellular and molecular level (Grabig *et al.*, 2006). Although the probiotic medication is highly recomended as a supportive therapy in various gastrointestinal inflammatory disorders but no data in literature sources exist concerning the influence of probiotics on pharmacokinetics of contemporary administered drugs.

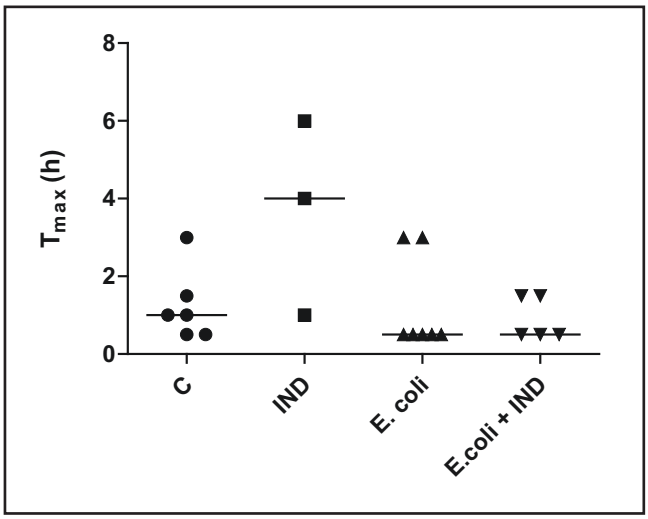

Fig. 7. N-acetyl-5-ASA: parameter T_{max} in each experimental group of animals (horizontal lines are medians). Statistically no-significant differences were found between groups – Dunn's Multiple Comparison Test.

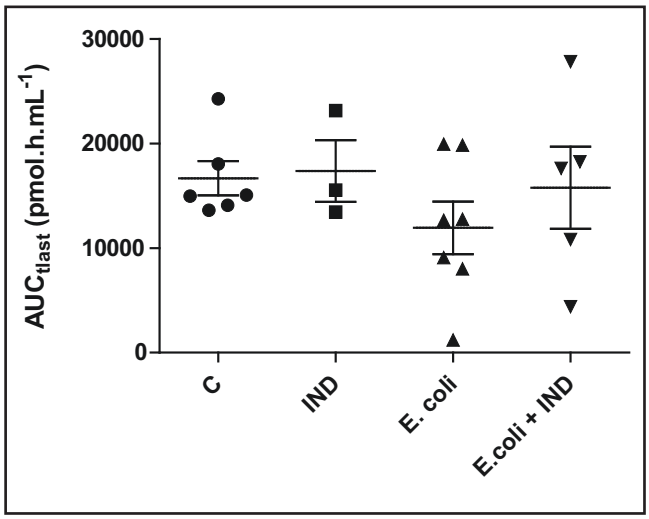

Fig. 8. N-acetyl-5-ASA: parameter AUC in each experimental group of animals (horizontal lines means average value ± SEM). Statistically no-significant differences were found between groups – $p<0.05$ – Tukey's Multiple Comparison Test.

In this study we evaluated the effect of EcN premedication on pharmacokinetics of 5-ASA in rat. At the same time, we studied the effect of EcN under the pathological condition (after the induction of gastrointestinal lesions). Indomethacin, a representative of NSAIDs family, was used as an inducer of gastrointetsinal lesions. It is a model drug commonly used to induce gastroenteropathy in the experimenatal animals, in the rats (Suleyman *et al.* 2009; Obadasoglu *et al.* 2006; Mehrabani *et al.* 2009), mice (Ettarh & Carr 1993, 1996) and pigs (Kvetina *et al.* 2008; Bures *et al.* 2011, Rainsford *et al.* 2003). In our previous experiments (Kunes *et al., 2009*), we also demonstrated its kinetic effect in the creation of lesions in various parts of rat's gastrointestinal tract.

These results document that the pre-medication (simultaneous medication) with probiotic strain *Escherichia coli* Nissle 1917 (EcN) did not affect the absorption of 5-aminosalicylic acid from gastrointestinal tract under the physiological conditions (Group A vs D) and slightly elevated in animals with induced GI lesions (Group B vs C). On the other side, the absorption of 5-ASA (without medication with EcN) was elevated in animals with indomethacin-induced gastro-enteropathy in comparison to controls. This increase in transintestinal transport of 5-ASA may indicate the predominance of its transport via mechanism of diffusion. Its elevation can be interpretated by changes (reducing of cellularity) in intestinal barrier after indomethacin-induction of GI-lesions, which are also documented by inducing other intestinal malabsorption syndroms (by methotrexat, irradiation, etc.) (Kvetina & Parizek 1966; Kunes *et al.* 2005).

The mechanism by which EcN might ameliorates the indomethacin-induced injury can be explained via TLRs signaling. EcN demonstrates potent immunomodulatory properties. In different cell culture models a differential effect on distinct T-cell populations by EcN was observed that might be the basis for immunoregulatory properties, allowing a potent but limited inflammatory response on the mucosal level. These results in reduced secretion of proinflammatory cytokines (IL-2, IFN-γ, and TNF-α) and an up-regulation of the secretion of regulatory IL-10, IL-8, and IL-1β (Sturm *et al.* 2005; Helwig *et al.* 2006; Otte & Podolsky 2004). These effects are mediated by Toll-like receptor-2 (TLR-2) signaling, expressed on activated T-cells (Sturm *et al.* 2005). The concept of recognition of EcN by TLRs was tested in TLR-2 and TLR-4 knockout mice with a significantly ameliorated dextran sulphate sodium-induced colitis in wild-type animals but no effect in either knockout (Grabig *et al.* 2006). Further study of Watanabe *et al.* (2009) describe that the inflammatory responses triggered by activation of the lipopolysaccharide (LPS)/TLR-4 signaling pathway are a key mechanism in non-steroidal anti-inflammatory drug-induced enteropathy. Earlier literature data also note that the generation of oxygen free radicals and lipid peroxidation play an important role in the development of gastric mucosal lesions (Del Soldato *et al.* 1985; Takeuchi *et al.* 1991; Vaananen *et al.* 1991).

On the base of above mentioned facts we hypothetised that EcN medication will be reduced (or will be the same) the elevated absorption of 5-ASA in animals with induced GI lesions and not that it will further increase. The statistically significantly higher absorption of 5-ASA was found in the animals with gastrointestinal lesions and concurrently pre-treated with probiotic EcN (Group C) when compared to control animals without any medication (Group D). This unex-

pected result is not easy to interpret. On the other hand, these findings are consistent with our previous experiments in pigs. The morphometric analysis of gastrointestinal tract proved deteriorating conjunctive effect of indomethacin and EcN combinatory medication (Bures *et al.* 2011). Another experiments also documented that indomethacin and EcN administered together comprised the worst impact on bacteriocinogeny in the porcine gastrointestinal tract (compared to indomethacin alone or probiotics alone) (Bures *et al.* 2011).

It is also interesting to compare the kinetics of 5-ASA and its metabolite N-acetyl-5-ASA in animals treated with EcN. It seems that EcN medication has certain (but no statistically significant) effect on N-acetylation process of 5-ASA in the intestine.

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Pharmacokinetics and organ distribution of fluorescein in experimental pigs: an input study for confocal laser endomicroscopy of the gastrointestinal tract

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Abstract

OBJECTIVE: Confocal laser scanning endomicroscopy (CLSE) is a diagnostic technology that produces virtual histology of the mucosal layer using fluorescence technique. Fluorescein (FSC) is the most commonly used fluorescence agent. Fluorescence light coming from a horizontal special focal plane is detected during confocal laser endomicroscopy of the gastrointestinal tract. FSC causes intensive yellowish discoloration of tissues, including skin and mucous membranes. This pre-clinical study was aimed to evaluate the tissue distribution and pharmacokinetics of FSC after its intravenous administration.

METHODS: The study was performed in an adult experimental pig. A reversed-phase high-performance liquid chromatographic method with fluorescence detection was used for the determination of fluorescein in blood plasma and tissue samples.

RESULTS AND CONCLUSION: The pharmacokinetic study of fluorescein determined the optimum time interval for diagnostic scanning (5–10 min.) The biodistribution study of fluorescein (aimed on the potential organ accumulation) proved the high concentration in the renal system followed by levels in bile > lung > adipose tissue > all other organs (including gastrointestinal wall) and these were relatively similar to each other. Fluorescein has a significantly low distribution in the brain (contrast with the level in adipose tissue indicates the low ability to penetrate the blood-brain barrier).

ORIGINAL ARTICLE

INTRODUCTION

Confocal laser scanning endomicroscopy (CLSE) is a key endoscopic technique which allows subsurface in vivo microscopic analysis during ongoing endoscopy, using a systemically or topically administered fluorescent agent (Gheorghe *et al.* 2008). CLSE may make in vivo histological diagnosis by virtual histology possible (Inoue *et al.* 2005). The principle of CLSE: a laser light source delivers blue excitation light at a wavelength of 488 nm. The fluorescence substance (fluorescein - FSC), being administered systemically, absorbs this light in the tissue and emits a green-yellowish light at a longer wavelength of 510–580 nm by itself. Only fluorescence light coming from a specific focal plane is detected afterwards by the endomicroscopy system. Our reserch group has successfully used CLSE in previous pre-clinical experiments in pigs. CLSE images corresponded well with those of classic haematoxylin-eosin staining (Kopáčová *et al.* 2009).

FSC hardly permeate through cell membranes. In humans, a dose of 7–30 mg/kg given intavenously as a bolus produces yellow-brown discoloration of the skin and ocular fundus (O'goshi & Serup 2006). Despite its increasing use, there are rare data on the kinetics and dynamics of this substance (Becker *et al.* 2008).

From the toxicological aspect, several cases of adverse reactions are cited in the older literature after intravenous administration of FSC. For example, nausea, pruritus, hoarseness and partial respiratory obstruction as an anaphylactic reaction (LaPiana & Penner 1968), abdominal discomfort and severe retrostemal chest pain radiating into the jaw as an acute myocardial infarction (Deglin *et al.* 1977), collapse and cardiac arrest, tachycardia and noncardiac pulmonary edema followed by death (Cunningham & Balu 1979; Heffner 1980) were observed in human after intravenous administration. Also yellow discoloration of the skin and mucous membranes may be for the patient, from a psychological point of view, certain complication.

The aim of our study was to specify the optimum diagnostic level of FSC in the tissue of particular gastrointestinal segments in pigs and to delimitate the potential toxicological risks following the relations of its organ distribution. The study was performed on experimental pigs due to their relatively very similar gastrointestinal and metabolical functions in comparison to man (Kararli 1995; Boes & Helwigh 2000).

MATERIAL AND METHODS

Animals

Five mature female pigs (Sus scrofa f. domestica), hybrids of Czech White and Landrace breeds, weighing 32.6 ± 2.3 kg (4–5 months old), entered the study. They were kept in air-conditioned rooms, fed twice a day (standard food A1; Cerea a.s., Czech republic) and allowed access to water *ad libitum*.

Pharmacokinetics

The pigs were intravenously administered with FSC (15 mg/kg; Fluorescite 10%, Alcon Lab., Texas, USA). Blood samples were withdrawn from the cannulated vena cava cranialis (cannulation one day before blood collection) using a permanent central catheter in the following time intervals: 5, 10, 20, 30, 45, 60, 90 and 120 min. after FSC administration. Blood sampling proceeded in pen with free animal movement. Blood samples were centrifuged (3 000 t./min, 10 min). The blood plasma was frozen at –30 °C until chromatographic detection.

Cannulation of vena cava cranialis via vena jugularis externa was performed (24 h before the pharmacokinetic study) under general anaesthesia: intramuscular injection of ketamine (20 mg/kg; Narkamon, Spofa, Prague, Czech Republic) and azaperone (2 mg/kg; Stresnil, Janssen-Pharmaceutica, Beerse, Belgium) was used as an introduction, continued by inhalation of nitrous oxide and oxygen in mixture and halothane (during 30 min of surgical procedure).

The pharmacokinetic parameters were evaluated using software Kinetica™ version 4.4.1 (Thermo Electron Corporation, U.S.A.).

Organ distribution

FSC (15 mg/kg) was administered intravenously to the same animals used in the previous pharmacokinetic experiment (7th day after the end of pharmacokinetic study). The animals were sacrificed with an i.v. injection of thiopental and exsanguinated ten minutes after FSC administration. Subsequently, the following tissue samples were collected: brain, thymus, heart, lung, liver, pancreas, kidney, adrenal gland, spleen, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, bladder, lymph-node, skin, adipose tissue, muscle, blood plasma and bile. Tissue samples were frozen at –30 °C until analysis.

Fluorescein detection

A reversed-phase high-performance liquid chromatographic method with fluorescence detection was used for the determination of total FSC (free and bound to plasma proteins) in blood plasma and other tissues. Plasma samples and tissue homogenates (homogenisation of the tissues in a phosphate buffer 0.05 mol/L pH 7.4) were purified using protein precipitation with an acetonitrile and zinc sulphate solution. FSC and internal standard 5-(bromomethyl) fluorescein were separated on a Zorbax Eclipse XDB-C_{18} column (Agilent, 250 mm × 4.6 mm I.D.) at a flow rate of 0.8 ml/min at 30 °C. The mobile phase consists of 25% acetonitrile and 75% of aqueous ammonium acetate (10 mmol/L, pH 6.8). HPLC analysis was performed on a 2695 Waters Separations Module equipped with Waters 2475 fluorescence detector operated at excitation and emission wavelengths of 485 nm and 535 nm.

Ethics

The study was approved by the Institutional Review Board of the Animal Care Committee of the Institute of Experimental Biopharmaceutics, Czech Academy of Sciences. Animals were held and treated in accordance with the European Convention for The Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

RESULTS

The elimination of FSC from blood is rapid (mean $t_{1/2}$ = 38 min) (Figure 1). On that account, the following distribution study was carried out at 10 min after intravenous FSC administration. The values of clearance and volume of distribution are high as well as variances in the parameter of areas under the curve (Tab). Quite monotonous decrease (monoexponencial) can be seen in the interval of blood sampling, without significantly faster phase.

In the distribution study, the levels of FSC (μg/g of tissue) were several times higher in the kidneys and slightly increased in the lungs and liver compared to other organs (heart, pancreas, spleen, thymus) and the digestion tract tissues (oesophagus, stomach, intestinal wall) (Figure 2). Results indicate that FSC was poorly distributed into the brain.

DISCUSSION

CLSE is a diagnostic technique allows a unique look at cellular structures and functions at and below the surface of the gut that has recently been introduced into live endoscopy (Hoffman *et al*. 2006; Kiesslich *et al*. 2006; Kopácová *et al*. 2007).

The intensive yellow marking of the skin after system FSC administration for CLSE of the digestive tract has a significant negative impact on the psycho-social interactions between medical staff and the patient. FSC is a small molecule that is highly water soluble, rapidly diffuses out of capillaries and into the extravascular tissue. The uptake and distribution pattern of FSC reflects both local blood flow and capillary permeability (Perbeck *et al*. 1987; Jager *et al*. 1997). The experimental proof of the caducity of these attributes and eventual selective organ FSC accumulation was the reason for argumentation for use of experimental animal species metabolically close to man in this pre-clinical study.

Besides FSC, there are other several agents that can be used in order to obtain the confocal endomicroscopy images. For example, acriflavine (administered topically) stains only the superficial layers of the mucosa, including the cell nuclei. On the other hand, the advantage of FSC is its distribution from the capillaries through the entire mucosa, showing the microvascular network and the connective tissue architecture (Hoffman *et al*. 2006). Becker *et al.* (2008) aimed their investigation to determine the ideal time period for the best CLSE imaging in pig when using FSC. They concluded the best contrast and image quality within first 8–10 minutes after injection of FSC. We obtained high quality CLSE images within the first 30 min in our previous experiments (Kopácová *et al*. 2009).

Proof of the dominant and fast FSC transportion from blood circulation into the kidney (rapid bioelimination) results from its hydrophilic properties in a

Tab. 1. Pharmacokinetic parameters of fluoresceine after its intravenous administration. Results are expressed as the mean ± S.D.

Parameters	Fluoresceine (15mg/kg)
C_{max} (μg/L)	115.3 ± 13.5
AUC_{0-120} (min.μg/L)	4881.6 ± 1736.5
$AUC_{0-\infty}$ (min.μg/L)	5687.7 ± 2597.8
$T_{½}$ (min)	37.8 ± 14.9
MRT (min)	52.1 ± 22.9
CL_{tot} (l/min/kg)	3.15 ± 1.45
Vd (L/kg)	149.8 ± 25.56

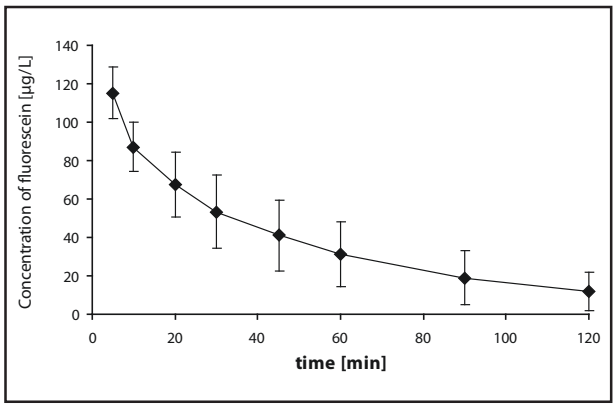

Fig. 1. Plasma time profile of fluorescein in pig after its intravenous (15 mg/kg) administration. Average values ± S.D.

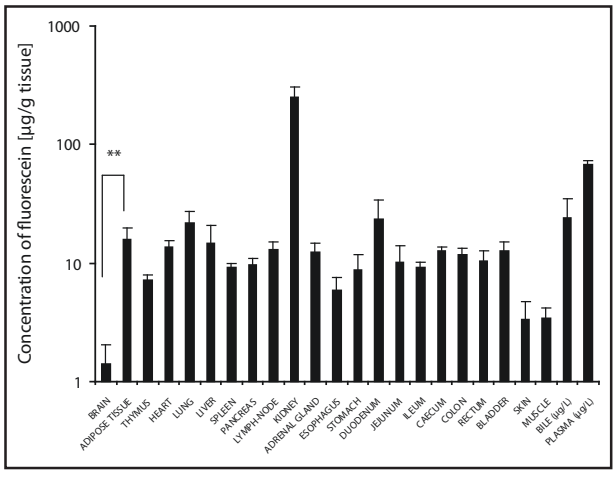

Fig. 2. Tissue distribution of fluorescein in pig (15 mg/kg) 10 min after intravenous administration administration. Average values ± S.D. (** p>0.01).

slightly basic organism environment. In humans, Dollery (1999) describes that peak plasma concentration occurs immediately after intravenous injection of FSC. Thereafter, the concentration declines rapidly. The fast decline in plasma FSC suggests a multicompartmental model of distribution, with a rapid drop in concentration within the first 10 min because of equilibration with extravascular fluid compartments. Subsequently, levels decline slowly because of elimination of FSC from the circulation predominantly determined by the kidneys. The excretion rate is more than the glomerular filtration rate, and active secretion in the renal convoluted tubules is likely to occur. That agrees with previous data (Conway 1985; Blair *et al.* 1986; Knudsen *et al.* 1992) described biexponencial decline of blood FSC concentrations after administration of similar dose (14 mg/kg). On the other hand, our results in pigs suggest that pharmacokinetics of FSC has mono-exponential character. According to these findings, it is the linear pharmacokinetics and elimination appears to be consistent with first-order kinetics.

In the liver, FSC is metabolised to fluorescein glucuronide (Blair *et al.* 1986). This metabolite also contributes substantially to the plasma fluorescence after intravenous or oral fluorescein administration (Grotte *et al.* 1985). Glucuronidation is active already 2 min after intravenous injection and at 60min., over 80% is glucuronated (Dollery 1999; Chahal *et al.* 1985). On the other hand, many studies have called attention to the necessity of measuring free plasma fluorescein (Conway 1985; Mota & Cunha-Vaz 1985; Blair *et al.* 1986), which is available for transport across the barrier. Palestine and Brubaker (1981) describe that only unbound FSC highly permeate blood-retinal barrier and thus plasma binding must be considered as a variable that may significantly affect the level of fluorescence. In our study, only total FSC (free FSC and FSC bound to plasma protein together) were measured (not glucuronide metabolite of FSC). It is therefore not possible to unambiguously interpret the essence of a high volume of distribution. A mono-exponential concentration/time dependence of FSC corresponding to the one-compartment model of distribution. High value of distribution volume (149.8 L/kg) suggests an important role in binding or biotransformation processes. In the case of FSC is it probably a combination of both. In human, FSC is highly bound to plasma proteins (about 80–90%) (Dollery 1999).

The FSC elimination liver first-pass effect is apparently about one position value lower in comparison to bile concentration. Nevertheless, the bile FSC level is high and exceeds the concentrations in other tisues and corresponds with FSC levels in the duodenal wall (in which the accumulation is higher compared the other gastrointestinal segments). Although, selective FSC uptake in diagnosed tissues (in gastrointestinal wall) will be desirable, the FSC levels are comparable with most of the evaluated tissues. The highest FSC levels were found in the lungs in comparison to other parenchymatic tissues. In spite of the fact that we macroscopically observed yellow marking of the skin, the detected concentration of FSC in tissue samples was very low and was comparable with the level in muscle (probably due to the same extent of vascularisation). To some extent there are discrepancies (differences) in the indicated fluorescein ability to migrate through the various barriers in the body. It was observed that FSC crosses into mother's milk in human (Mattern & Mayer 1990). In the toxicity study on rats, it has been found (Salem *et al.* 1979) that FSC freely crosses the placental barrier and is distributed throughout the amniotic fluid and the fetus within 15 min after intravenous injection. In spite of that fact, FSC is considered relatively non-toxic, with high doses for LD50 (mice 4738 mg/kg, rat 6721 mg/kg) (Yankell & Loux 1977) and does not appear to pose any significant risk when administered during pregnancy. FSC does not produce embryotoxic, teratogenic (Salem *et al.* 1979) or carcinogenic effect (Dollery 1999).

Martinez & Koda (1988) states that FSC penetrate into the brain in rat and that exists the sex differences in measured concentrations. Our findings in pigs revealed low FSC concentration in the brain. It is interesting, not only from the kinetic-distribution perspective but also with regards to toxicologic aspects. When comparing the relatively high FSC concentration in subcutaneous adipose tissue we can judge its handhold in the blood-brain barrier. It corresponds with the findings Malmgren & Olsson (1980) which indicates that sodium fluorescein does not cross the blood-brain barrier. On the other hand, they describe very rapid penetration of FSC into peripheral ganglia and into the epineurium and perineurium of large peripheral nerves.

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ORIGINAL ARTICLE

Type and distribution of indomethacin-induced lesions in the gastrointestinal tract of rat

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Abstract

OBJECTIVES: The therapy with non-steroidal anti-inflammatory drugs (e.g. indomethacin) is often accompanied with adverse effects in gastrointestinal tract. Aim of this experimental study was to define the time range of the creation of indomethacin-induced gastrointestinal lesions in rat (for prospective study of potential probiotic therapy). The paper follows our previous experiments where the different gastrointestinal lesions were described in the pig (Kvetina *et al.* 2008)
METHODS: Indomethacin (25mg/kg) was administered orally by a single application to rat (Wistar Han II, 200-250g). Six, 24, 48 and 72 hours after the indomethacin administration all parts of the gastrointestinal tract of six rats in each time interval were macroscopically and histologically examined.
RESULTS AND CONCLUSION: The gradual development of lesions was observed 6 hours in stomach and 24-72 hours in the intestine after the indomethacin administration. Not only the gradual development of pathophysiological alterations was observed but also the reparative phase (in stomach). 24 hours seem to be advisable time suitable for the evaluation of the probiotics effect as a potential therapy) on the indomethacin-induced gastrointestinal lesions in rats. Sensitivity of the gastrointestinal tract to the pathological lesions development seems to be higher in rats in comparison to findings described in our previous experiments in pig (Kvetina *et al.* 2008). This adverts to interspecies differences in the manifestation and in the dynamics of the development of gastrointestinal lesions.

INTRODUCTION

One of the causes of the gastrointestinal lesions genesis may be adverse reactions during the therapy with non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs treatment is the second most common aetiological factor for peptic ulcer disease and a major factor for peptic ulcer complications in human beings (Voutilainen *et al.* 2001). Conventional NSAIDs have been reported to change permeability and cause inflammation in the intestinal segments (Halter *et al.* 1996). This view is supported by observations of NSAIDs' caused mucosal lesions in the jejuno-ileal region of rats and dogs (Anthony *et al.* 1994; Billingham

& Tucker, 1979; Nygard *et al.* 1994; Rainsford, 1988). In our previous experiments, experimentally indomethacin-induced gastrointestinal lesions were also found in pig in the stomach, in terminal ileum and in the caecum (Kvetina *et al.* 2008). Aim of this experimental study was to define the dynamics of the development of the gastrointestinal lesion in rat, its distribution along the digestion tract and to compare the findings in rats and pigs. Subsequently, the study was aimed to determine the time range for the evaluation of the potential influence of probiotics (as a potential therapy) on the gastrointestinal lesions formation (in the prospective experimental studies).

MATERIAL AND METHODS

Animals and gastrointestinal lesions induction. Male Wistar Han II rats weighing 280±39g (conventional breed Biotest Ltd., Konárovice nad Labem) kept in air-conditioned rooms in plastic boxes with free access to water. Indomethacin was administered orally in one dose of 25 mg/kg to four groups of six animals. The animals were not given solid food for 12 hours before euthanasia and organ samples collection. The samples from all parts of gastrointestinal tract were macroscopically evaluated and collected for histological examination after 6 (group 1), 24 (group 2), 48 (group 3) and 72 (group 4) hours after indomethacin application. The animals were exposed to halothane overdosing, sacrificed and exsanguinated.

Diagnostic techniques:

Macroscopic examination. The images were taken using a digital camera (Konica Minolta, Dimage Z5, Tokyo, Japan) and saved as .jpg files in the computer. The type and the number of lesions distributed along the whole small and large intestine were noted.

Light microscopy. Samples were fixed in 10% neutral buffered formalin, material was processed by the common paraffin technique and histological sections 5μm-thick were stained with haematoxylin and eosin and with PAS reaction.

Evaluative criteria (the scale of damage):

I. gastritis, hyperaemia, haemorrhages, erosion II. ulceration, 0. normal (without pathological lesions).

ETHICS

The study was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, the Czech Academy of Sciences. Animals were held and treated in accordance with the European Convention for The Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

RESULTS

The formation of lesions was found in the stomach already after 6 hours (***Fig. 1 and 8***) but not in the intestine. In the intestine, the lesions were found within 24–72 hours (***Fig. 3, 4, 5 and 10***) while they became extinct during this time in stomach (***Fig. 2***). The very strong ulcerations were found in the small intestine after the 72 hours (***Fig. 6***). The damage was observed in the whole small intestine, especially in the terminal segments (jejunum, ileum) and in caecum (***Fig. 12***). Colon was not damaged by indomethacin. The microscopic evaluation of lesions was compared with tissue samples of control animals (***Figs. 7, 9, 11***). The frequency and severity of indomethacin-induced lesions are showed in the ***Table 1***.

DISCUSSION

NSAIDs are one of the most commonly used agents in the clinical practice today. All these drugs are known to produce gastro-intestinal lesions (Misra *et al.* 1990). NSAIDs cause dyspeptic complaints and lesions in the upper gastrointestinal tract (Hollenz *et al.* 2006). These effects are attributed to mechanisms such as drug-induced cyclooxygenase inhibition, oxidative stress, mitochondrial dysfunction, changes in cell membrane lipids

Table 1. The frequency any severity of indomethacin-induced lessions in rat (numbers of animals with lesions/animals investigated).
The scale of damage:
I gastritis, hyperaemia, haemorrhages, erosions
II ulceration 0 normal

Tissue	Lesion	6h	24h	48h	72h
Stomach	I	6/6	5/6	2/6	1/6
	II	6/6	3/6	1/6	1/6
	0	0/6	0/6	3/6	4/6
Duodenum	I	0/6	1/6	2/6	3/6
	II	0/6	0/6	0/6	3/6
	0	6/6	5/6	4/6	0/6
Jejunum	I	1/6	3/6	6/6	6/6
	II	0/6	3/6	6/6	6/6
	0	5/6	0/6	0/6	0/6
Ileum	I	1/6	4/6	6/6	6/6
	II	0/6	5/6	6/6	6/6
	0	5/6	0/6	0/6	0/6
Caecum	I	0/6	1/6	4/6	6/6
	II	0/6	0/6	3/6	3/6
	0	6/6	5/6	1/6	0/6
Colon	I	0/6	0/6	0/6	1/6
	II	0/6	0/6	0/6	0/6
	0	6/6	6/6	6/6	5/6

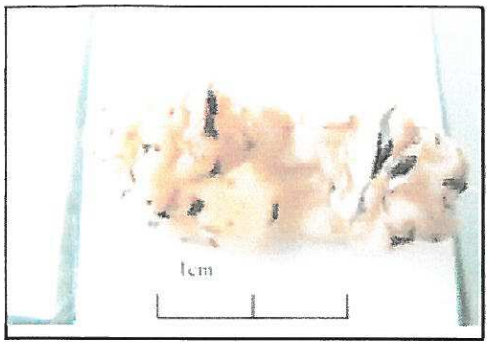

Fig. 1. Stomach-ulcers (black spots). 6 hours after indomethacin administration.

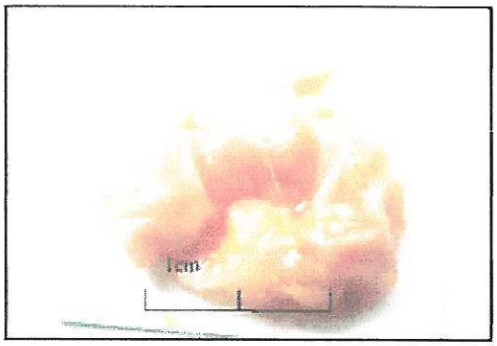

Fig. 2. Stomach without pathological lesions. 48 hours after indomethacin administration.

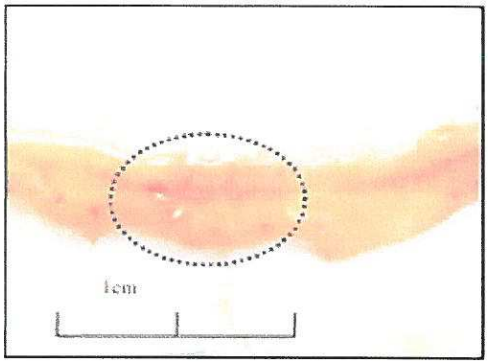

Fig. 3. Jejunum-erosions and ulcers. 24 hours after indomethacin administration.

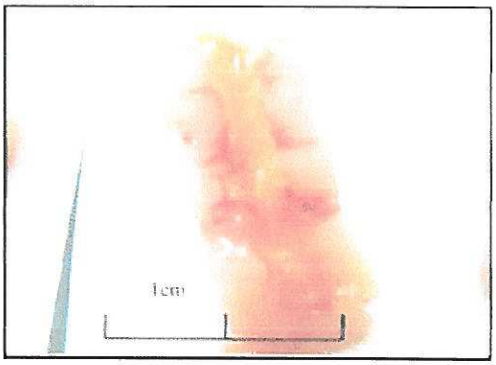

Fig. 4. Jejunum-extensive ulceration. 72 hours after indomethacin administration.

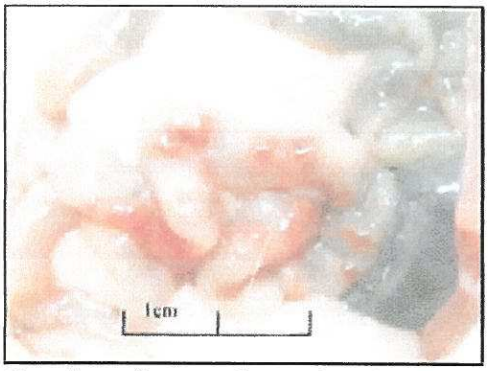

Fig. 5. Ileum-ulceration. The view from the serosal side. 48h after indomethacin administration.

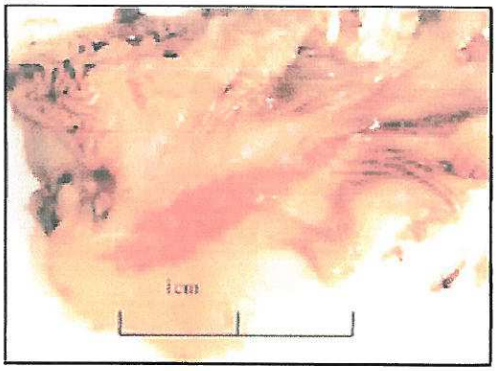

Fig. 6. Caecum-mucosal erythema. 72h after indomethacin administration.

and by an increased production of pro-inflammatory cytokines (Sivalingam *et al.* 2009; Maes, 2008). We used indomethacin (commonly used NSAIDs drug) as an experimental inducer of gastrointestinal lesions according to Nygard *et al.* (1994). Other authors also used the model of indomethacin to induce the gastroenteropathy in the rat (Suleyman *et al.* 2009; Odabasoglu *et al.* 2006; Mehrabani *et al.* 2009). We have found certain dynamics in the formation of the indomethacin-induced lesions in the gastrointestinal tract of rat. While the lesions were observed in stomach already 6 hours after the administration of indomethacin, no lesions were found in the intestine after that time. It is interesting that although the large-scale pathological lesions were found in the whole small intestine after 48-72h, stomach was practically without any pathological lesions at those times. Thus, we observed not only the progressive time-dependent genesis of gastrointestinal lesions but also reparative phase in stomach. Mehrabani *et al.* (2009) described the occurrence of multiple ulcers in stomach 24 hours after the administration of the same (25mg/kg) dose of indomethacin to rat.

We can see inter-species differences while comparing the promotion of the indomethacin-induced lesions between rat (this paper) and pig (Rainsford *et al.* 2003). In our previous paper, the most significant findings were found in stomach (petechia, erosions, single ulcers and ulcers chain) and in caecal segment (erosions, filiform ulceration, enlarged lymph-node) after the 10 consecutive days of the indomethacin medica-

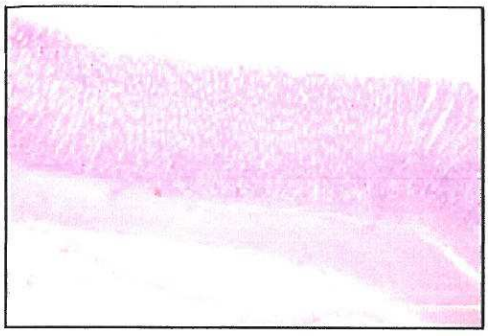

Fig. 7. Stomach – control. Magnification 100x. Staining HE/PAS. Normal gastric mucosa without pathological lesions.

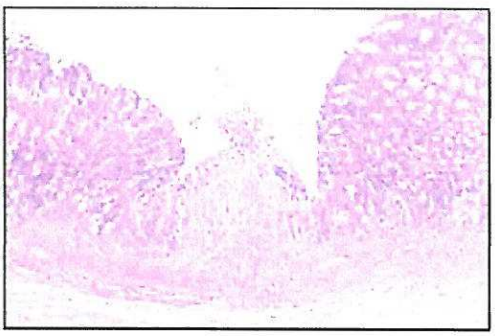

Fig. 8. Stomach – erosion. Magnification 100x. Staining HE/PAS. Erosion no exceed the layer of muscularis mucosae.

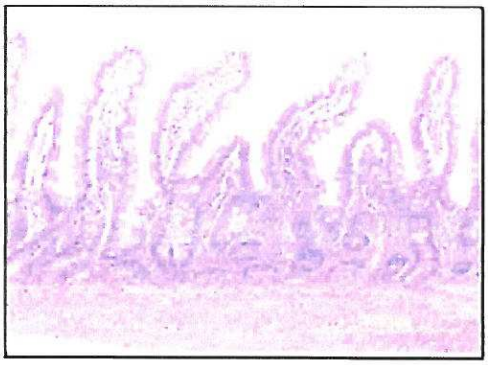

Fig. 9. Jejunum – control. Magnification 100x. Staining HE/PAS. Normal intestinal mucosa, slight and high villi.

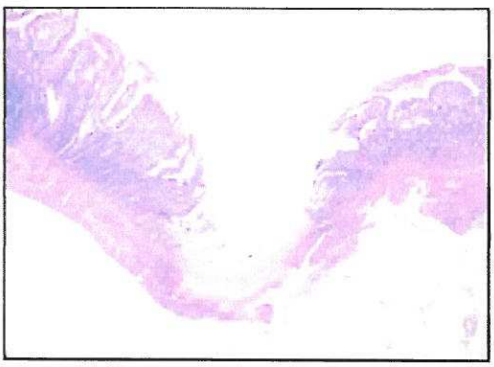

Fig. 10. Jejunum – ulcer. Magnification 100x. Staining HE/PAS. Deep ulcer, base is covered with fibrin.

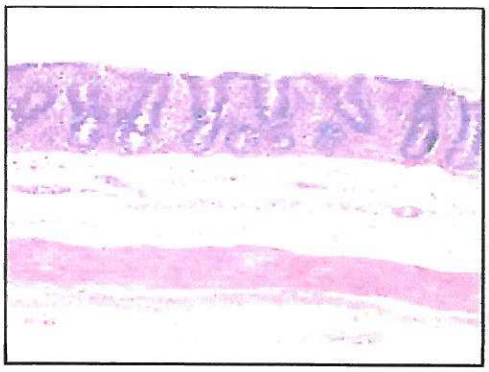

Fig. 11. Caecum – control. Magnification 100x. Staining HE/PAS. Normal mucosa in caecum.

Fig. 12. Caecum – erosion. Magnification 100x. Staining HE/PAS. Erosion of the mucosa surface.

tion to pig (Kvetina *et al.* 2008). Although we can not compare the dynamics of the origin of the lesions in pig and rat, we can conclude that the sensitivity of gastrointestinal tract in pig seems to be lower in comparison to rat in the course of relatively comparable dose of orally administered indomethacin.

We also found out that the time of 24 hours after administration (erosions and the beginning formation of ulcers) seems to be suitable for the evaluation of the influence of the probiotics on the creation of the indomethacin-induced gastrointestinal lesions in the rat. It is known that probiotics supply has beneficial effect on the gastrointestinal epithelium (Sullivan & Nord, 2005). Our further experiments will be aimed on the evaluation of the effect of nonpathogenic strain of Escherichia coli (Nissle, 1917) on indomethacin-induced small intestinal injury in rat. Watanabe *et al.* (2009) already performed similar investigation with the Lactobacillus casei strain Shirota (LcS) using the same model of induced enteropathy. Inflammatory responses triggered by activation of the lipopolysaccharide (LPS)/Toll-like receptor (TLR) 4 signalling pathway are a key mechanism in non-steroidal anti-inflammatory drug-induced enteropathy. Their findings suggest that LcS exhibits a prophylactic effect on indomethacin-induced enteropathy by suppressing the LPS/TLR4 signaling pathway, and that this probiotic effect of LcS may be mediated by L-lactic acid.

The results of our experiments documented significant degree of toxicity (already after one single dose administration) of the commonly used drug from the class of the non-steroidal anti-inflammatory acting substances. It is important to emphasize that these drugs belong among over-the-counter medicinal products.

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The use of wireless capsule enteroscopy in a preclinical study: a novel diagnostic tool for indomethacin-induced gastrointestinal injury in experimental pigs

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Abstract

OBJECTIVES: The aim was the utilization of capsule microscopy and other diagnostic techniques for prospective pre-clinical research of absorption and biotransformation mechanisms of xenobiotics in the intestinal wall after induction of gastrointestinal dysfunction. Consequently, there is a demonstration of the extrems of gastrointestinal lesions development induced with indomethacin as a representative of non-steroidal anti-inflammatory drug.

METHODS: The experimental animal species were small adult pigs (n=10; body weight 30–35 kg; 4–5 months old) used for their relative physiological and metabolic resemblance to man. The following experimental methods were used for diagnostic verification of gastrointestinal lesions (damage scale: 1 – erosions, red spots, inflammatory infiltration, 2 – single ulcers, 3 – strings of ulcers): endoscopic image for the diagnostics of gastro-duodenal segment (*in vivo* conditions), confocal laser microscopy (*ex vivo* imaging) and optical light microscopy (*in vitro*), small intestinal imaging by means of wireless capsule enteroscopy (*in vivo*), macroscopic findings and optical light microscopy (after animal sacrifice).

RESULTS: The mutual confrontation of used methodological approaches proved the conformity in the frequency and extent of damage in the gastric wall and caecum, partly in the duodenal wall and terminal ileum. The signs of first-degree damage were discovered in the jejunal-ileal segment.

CONCLUSIONS: The scale of lesions in particular gastrointestinal segments was verified using the combination of five diagnostic techniques for prospective utilisation of non-invasive capsule enteroscopy for the through-control of mucosal state.

ORIGINAL ARTICLE

INTRODUCTION

Gastrointestinal permeability changes, inflammation and ulceration are frequently associated with ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) (Vane & Botting, 1995). The lesions induced this way (ulceration and haemorrhage in concrete) occurs namely in the gastroduodenal region. Conventional NSAIDs have been reported to change permeability and cause inflammation also in the intestinal segments (Halter *et al.* 1996). This view is supported by observations of NSAIDs caused mucosal lesions in the jejunoileal region of rats (Anthony *et al.* 1994; Billingham & Tucker, 1979; Nygard *et al.* 1994; Rainsford, 1988). This fact may influence the intestinal absorption and the enterohepatic circulation of orally administered drugs. There are not many reports about the influence of injured intestinal wall on the transport of xenobiotics. For our experiments pigs were chosen since they closely resemble to humans in respect to anatomy and physiological functions in the gastrointestinal (GI) tract (Rainsford *et al.* 2003). The next open question is whether different degrees of GI damage are expressed uniformly on the quality of transintestinal transport of drugs or differently in case of drugs transported by mechanism of lipoid diffusion or active carrier mechanism. For example, the papers published previously (Hradil *et al.* 1978; Fendrich *et al.* 1970; Fendrich & Květina, 1979; Květina *et al.* 1984) demonstrated that malabsorption syndrome (induced with whole-body irradiation or with methotrexate administration) increased the transport in case of diffusion in the rat. On the other hand, in active carrier transport the absorption was decreased. There was also a decrease in cellularity of the intestinal mucosa in rats altered in such a way (Květina & Pařízek, 1966). Further a systematic study should elucidate the influences of intestinal dysfunctions on biotransformation activity of the intestinal wall.

The set designed pre-clinical studies (directed on pig as an animal representative metabolically close to man) is aimed on the prediction of changes in transintestinal transport of xenobiotics with different absorption mechanisms and with different metabolism in the intestine. The first experimental phase (this paper) is standardisation of induced GI pathological changes using combined morphological techniques (Rey *et al.* 2006; Goetz *et al.* 2007; Goetz *et al.* 2008).

MATERIAL AND METHODS

Animals

Ten mature female pigs (Sus scrofa f. domestica), hybrids of Czech White and Landrace breeds, weighing 30–35 kg of body weight (4–5 months old), were used in the study. They were kept in air-conditioned rooms (22±2 °C and 50±10% relative humidity, with lights from 7 a.m. to 7 p.m.), fed twice a day (standard food A1; Cerea a.s., Czech Republic) and with access to water *ad libitum*.

Gastrointestinal lesions induction

Gastrointestinal lesions were induced by the utilisation of "indomethacin adverse effect" (resulting in blockade of the oxygenous transformation of arachidonic acid on prostanoids):

10-day indomethacin administration (10 mg/kg/day) as one-shot dietary bolus to greedy pigs.

Diagnostic conditions

Eight transmission antennae were fixed on the skin in abdominal parts of animals kept in general anaesthesia using intramuscular injection of 20 mg ketamine (Narkamon, Spofa, Praha, Czech Republic) and 20 mg azaperone (Stresnil, Janssen-Pharmaceutica, Beerse, Belgium) as an introduction, continued by infusion of 1% thiopental (Thiopental Valeant, Valeant Czech Pharma, Czech Republic). Infusions of 0.9% saline solu-

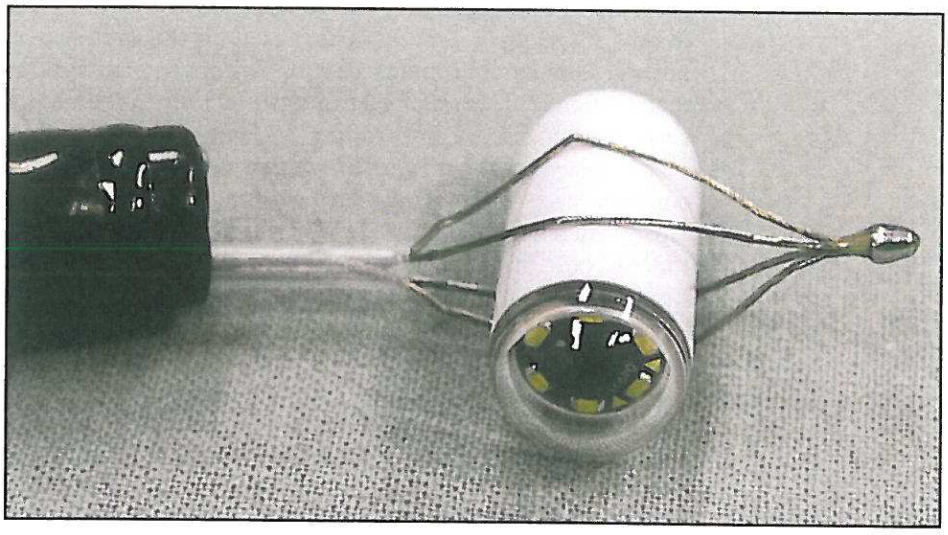

Figure 1. Endoscopy capsule grasped into a basket. The basket is inserted through the endoscopic working channel.

tion were given to secure basal hydration (1000 mL per 8 hours). All animals were covered by blankets to prevent hypothermia during capsule enteroscopy.

Diagnostic techniques

A) Video capsule enteroscopy (Keuchel et al. 2006):
The wireless capsule endoscope is a disposable capsule with an outer diameter of 11 mm, length of 26 mm and weighing 3.8 g (Figure 1). It moves through the small bowel, propelled by peristalsis and transmits data to a portable data recorder. It provides direct colour video images of the intestinal mucosa at a rate of 2 images per second for approximately 8 hours. Wireless capsule endoscopes were introduced into the duodenum by means of standard video-gastroscope. The system of EndoCapsule (Olympus Optical Co, Tokyo, Japan) was used for video-capsule enteroscopies in all animals. The system provides direct colour video images of the GI mucosa and consists of four components: (a) capsule endoscope, (b) recorder unit, (c) real time viewer, and (d) integrated workstation containing the proprietary application software. The capsule is naturally passed through the intestine and subsequently is excreted in faeces.

B) Endoscopic scanning of the gastric surface through the endoscope during the microcamera introducing. Endoscopy procedures were performed using video-gastroscope GIF-Q130 (Olympus Optical Co, Tokyo, Japan) designed for animal use only. All endoscopies were video-recorded.

C) Confocal laser microscopy of the gastric wall samples collected at the end of the microcamera scanning; 10 min. after the intravenous injection of the diagnostic fluoresceine (500 mg/animal) and following pharmacological euthanasia (an intravenous injection of embutramide, mebezonium iodide and tetracaine hydrochloride in mixture; T61, Intervet Int. BV, Boxmeer, the Netherlands; dose of 2 mL per kg) and exsanguination. Confocal laser endomicroscopy on an *ex vivo in vitro* basis was carried out within 20 minutes after the tissue samples collection. Methods of this imaging were described elsewhere (Kopáčová *et al.* in press), briefly: video endomicroscopy was performed by means of Confocal laser endomicroscopy system Pentax (Tokyo, Japan)/Optiscan (Notting Hill, Australia). It consists of a standard video colonoscope (EC3870K) with miniaturised confocal microscope on its tip, processor (EPK-100) and laser system (ISC OU1000). To prevent direct contact of the tip of video-endoscope and confocal microscope with porcine tissue we used a semipermeable membrane Dialysis tubing visking (Carl Roth, Karlsruhe, Germany). Both tissue and membrane were flushed by a saline solution during investigation.

D) Gross observation of the gastrointestinal wall during tissue collection and preparation. The photos were taken using a camera (Konica Minolta, Dimage Z5, Tokyo, Japan) and saved as ".jpg" file in the computer.

E) Optical light microscopy of the gastrointestinal wall samples collected post-mortem. The specimens were then fixed in 10% neutral buffered formaldehyde, embedded in paraffin, sectioned (5μm) and stained with hematoxylin eosin and periodic acid Schiff. The histological preparations were evaluated using a calibrated micrometer (optical zoom 010 x M10).

Evaluative criteria (scale of damage)

I – erosions, red spots, inflammatory infiltration;
II – single ulcers;
III – strings of ulcers

Ethics

The study was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, the Czech Academy of Sciences. Animals were held and treated in accordance with the European Convention for The Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

RESULTS

After 10-day indomethacin administration differently graded – morphologically detected – damage was observed in different segments of the gastrointestinal tract in all animals . The findings using the diagnostic methods described are summarised and documented in Table 1 and in Figures 2–6.

A)
Small intestinal imaging by means of wireless capsule enteroscopy show the small erosions, erythema and petechie in the jejunum and small ulcer in the pylorus [Figures 2A), 2B), 2C), 2D)].

B)
Erosions and ulcers observed with endoscope in the stomach [Figures 3A), 3B)]

C)
The confocal laser microscopy display the ulcerative tissue in the stomach in comparison to normal gastric wall [Figures 4A), 4B)].

D)
Erosions and ulcers in stomach and ulceration in caecum after gross tissue observations [Figures 5A), 5B), 5C), 5D)].

E)
Mucosal abnormalities, especially in the stomach and in distal parts of the small intestine using optical light microscopy [Figs. 6A), 6B), 6C), 6D)].

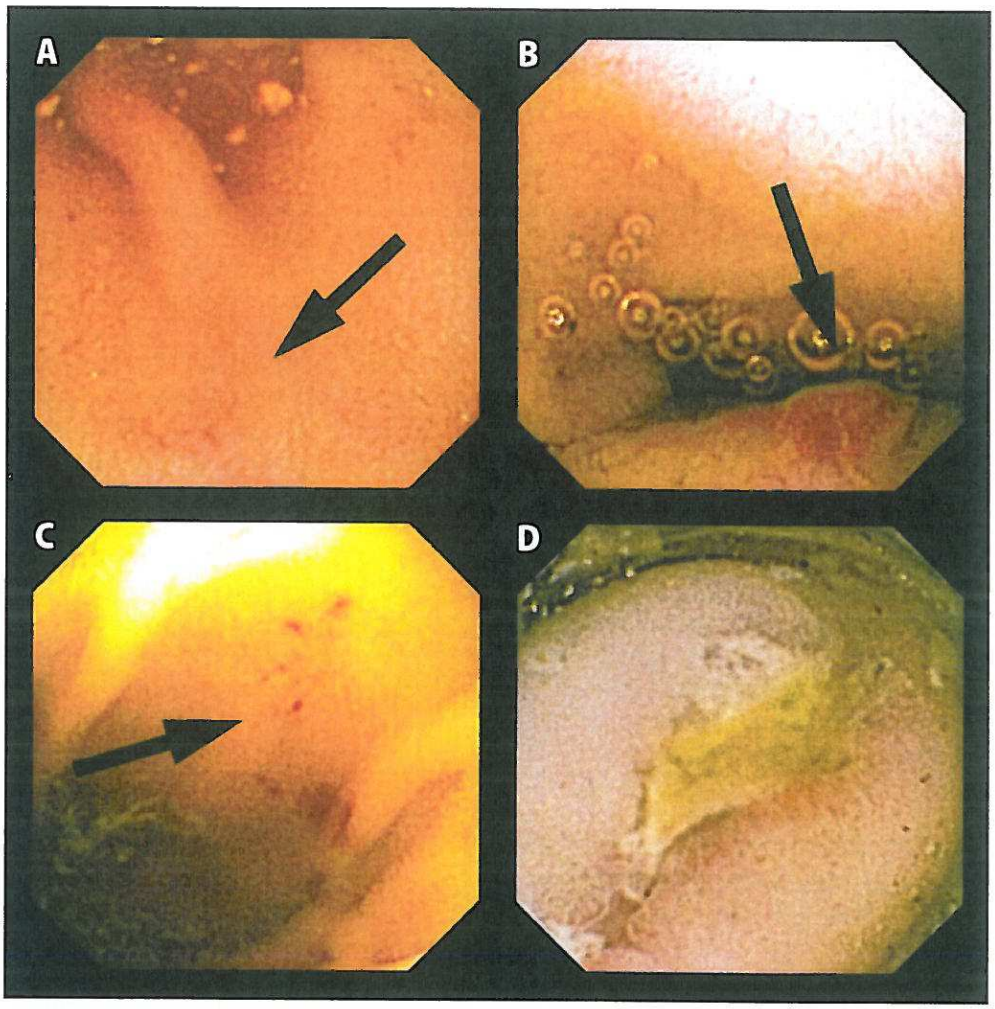

Figure 2. A) Small erosion in the proximal jejunum – arrow. Capsule endoscopy. (Diameter of image 2 cm).
B) Focal mucosal erythema in the jejunum-arrow. Capsule endoscopy. (Diameter of image 2 cm).
C) Multiple petechie of the jejunum - arrow. Capsule endoscopy. (Diameter of image 2 cm).
D) Small ulcer of the pylorus, base is covered with fibrin. Capsule endoscopy. (Diameter of image 2 cm).

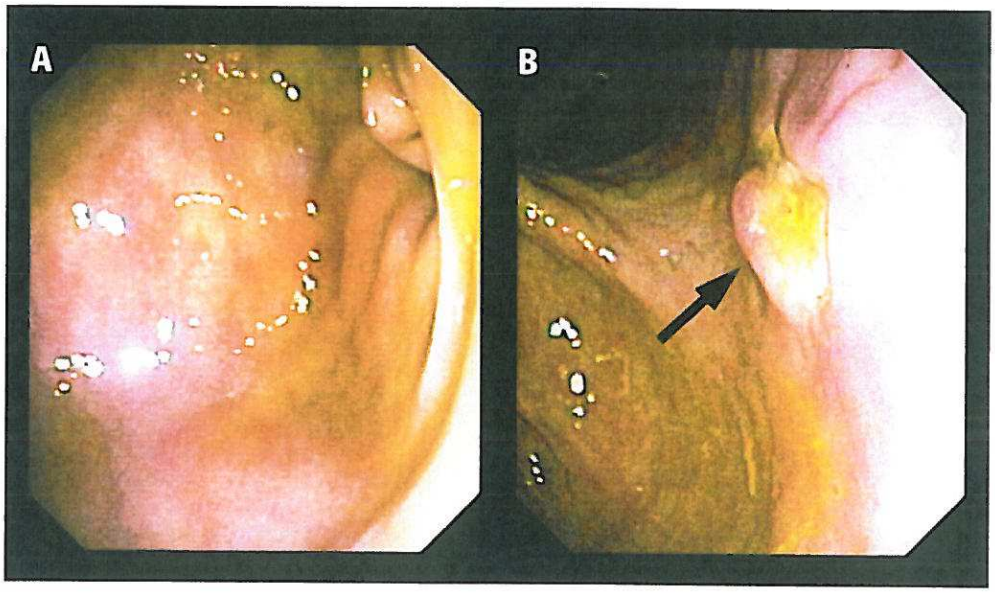

Figure 3. A) Normal gastric mucosa in the antrum. Gastroscopy.
B) Deep ulcer in the distal part of gastric body, base is covered with fibrin Gastroscopy.

The most pronounced pathological findings were observed in the gastric wall and caecum. The gastric mucosal lesions were demonstrated in all animals, covering the range from erosions to ulcer string. The erosions in approximately 50% of animals and single ulcers in approximately 20–30% of animals were found in the duodenal segment, while the variously fusing erosions in the proximal parts of the jejunum. No changes were found in the jejunal-ileal part, the erosions and solitary ulcers were manifested in several animals in the terminal ileum; the changes (erosions or ulcers, even a perforating ulcer – in one pig) were observed in all animals.

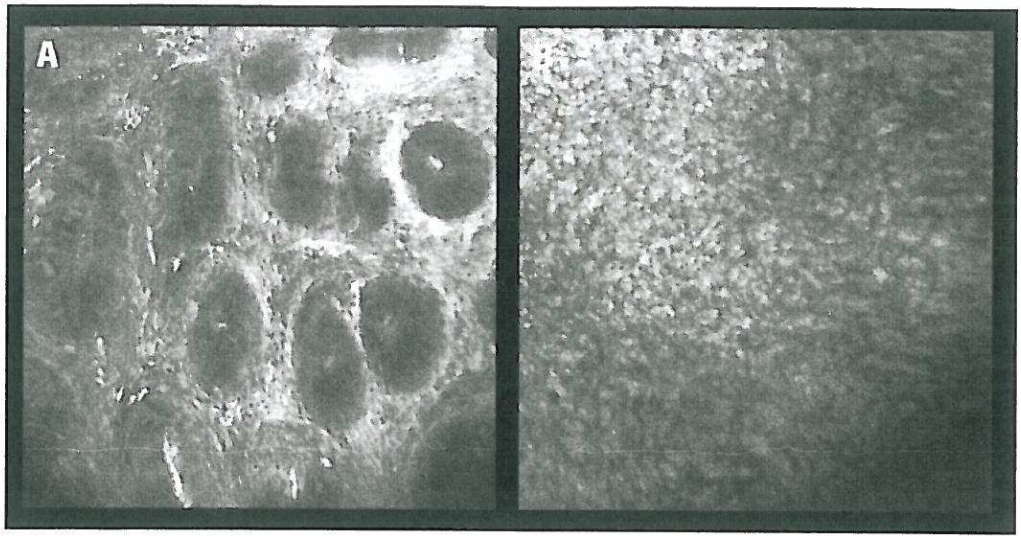

Figure 4. A) Confocal laser endomicroscopy of the normal gastric mucosa. Foveolae gastricae are clearly visible. (Magnification 1000×). **B)** Confocal laser endomicroscopy of the gastric ulcer. (Magnification 1000×).

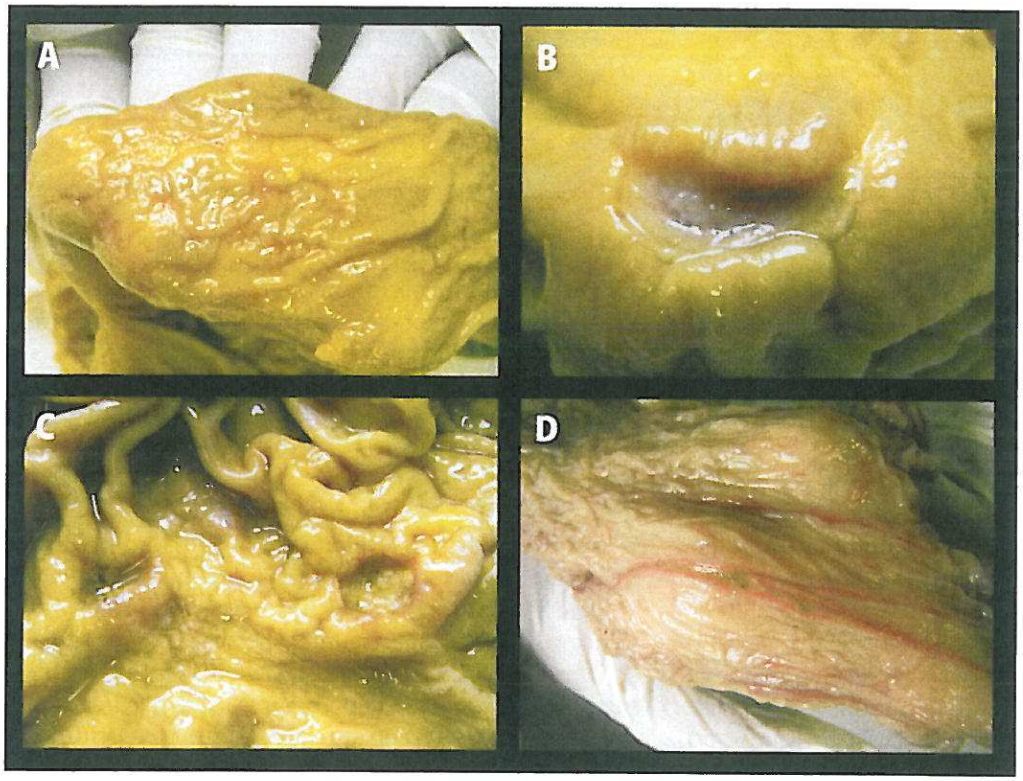

Figure 5. A) Erosions in the stomach. Gross observation. **B)** Large and deep ulcer in the stomach. Gross observation. **C)** Several ulcers. Gross observation. **D)** Filiform ulceration in the caecum. Gross observation.

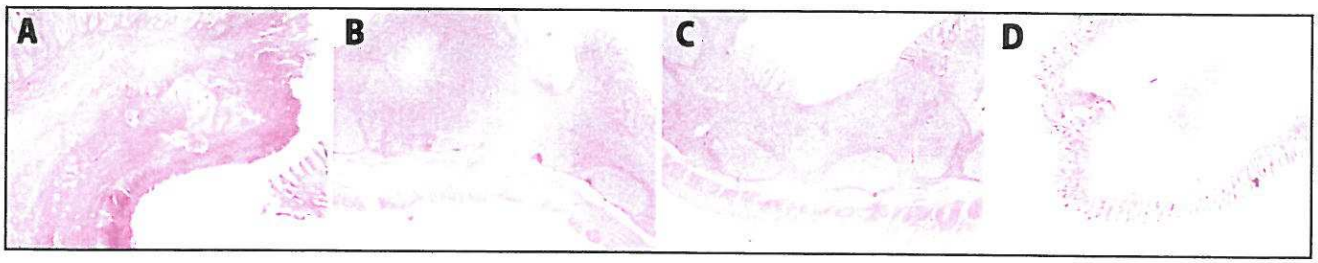

Figure 6. A) Ulcer in the stomach. **B)** Ulcer in the ileum. **C)** Erosion in the caecum. **D)** Ulcer in the caecum. Optical light microscopy. (Magnification 100×).

Table 1. The occurence and severity of indomethacin-induced lesions

Organ	Grade	Gross observation	Gastroscopy	Capsule enteroscopy	Microscopy
Stomach	I	8/10	6/7	–	10/10
	II	10/10	7/7	–	8/10
	III	6/10	5/7	–	3/10
Duodenum	I	5/10	–	5/7	5/10
	II	3/10	–	1/7	2/10
	III	0/10	–	0/7	0/10
Jejunum	I	5/10	–	5/7	8/10
	II	0/10	–	0/7	0/10
	III	0/10	–	0/7	0/10
Ileum	I	2/10	–	3/7	5/10
	II	2/10	–	0/7	0/10
	III	0/10	–	0/7	0/10
Caecum	I	8/10	–	2/7	5/10
	II	3/10	–	2/7	2/10
	III	2/10	–	0/7	1/10

(Optical microscopy and Gros observation, n=10; Gastroscopy and Capsule enteroscopy, n=7)
Data are numbers of animals with lesions / animals investigated.
Severity of lesions: Grade I – erosions, red spots, inflammatory infiltration; Grade II – individual ulcers; Grade III – strings of ulcers.

DISCUSSION

Despite numerous clinical reports about GI lesions induced with NSAIDs, there are rare similar findings in experimental species (especially in pigs). The minipig or pig (without overweight) as an omnivorous representative has been pharmacologically declared as a suitable experimental animal species to obtain this piece of knowledge relevant for man. The results of the presented work can be confronted with similar experimental design of the paper published by Rainsford *et al.* (2003). The aim of their research was to compare the gastrointestinal impact after three NSAIDs representatives medication (oxygenase non-selective inhibitors: aspirine, naproxen, indomethacin). The macroscopic and optical-macroscopic image of the presence and size of GI lesions and of myeloperoxidase activity in the damaged areas were the main criteria evaluated. Results of this cited paper showed that relatively the most significant GI-toxic effects were found after indomethacin medication. Similar findings from experiments performed in rats (Nygard *et al.* 1994), confirmed the justification of using indomethacin as an inducer of GI lesions with the most frequent and thus the most reliable GI injury. Ten-day exposure with NSAID was used in our paper similarly as in that of Rainsford *et al.* (2003). In the case of indomethacin, the doses of 5 mg/kg/day (n=3) and 10 mg/kg/day (n=6) were used. The body weight of pigs in the paper cited above was 13–20 kg only, while the pigs used in our experiments were in the period of new-maturity without excessive content of subcutaneous adipose tissue. The body weight range (30–35 kg) of pigs in our experiments represented approximately 50% average body weight of an adult man. Thus, the daily dose of indomethacin (10 mg/kg) was closer to the daily recommended therapeutic dose in man (3 mg/kg) than the dose in the study of Rainsford *et al.* (2003) (although is still more than threefold higher). The findings of the most important morphological changes in the stomach, pyloric area, duodenal part and in caecum are in accordance with both experimental studies.

The methodological preparation, as the declared purpose of the presented research phase, consisted in the induction of reproducible GI tract dysfunction, usable in further experimental studies aimed at systematic survey of lesions influence affecting pharmacokinetics of xenobiotics after their oral administration. For this purpose, various morphological techniques were used to characterise frequency, intensity, depth of pathological findings and their localisation. The described results and their mutual comparison helped to create the diagnostic scale for changes in particular GI segments. The application of this scale in the perspective research phase will make it possible to determine the stage of lesions according to the particular GI segments in conditions *in vivo* by means of wireless capsule enteroscopy as a relatively least invasive technique. Wireless capsule enteroscopy can be carried out simultaneously

with the tested xenobiotics without mutual interference. The confrontation between the extent of pathological findings in the particular sections of digestion tract and the intensity of absorption of model drugs can be helpful towards the character of absorption, exsorption and biotransformation changes revealed. At the same time, it can contribute to the identification of "absorption window" or "absorptive deaf sections" in GI tract for various model drugs (according to their physical-chemical and structural characteristics).

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Wireless Video Capsule Enteroscopy in Preclinical Studies: Methodical Design of Its Applicability in Experimental Pigs

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Abstract The aim of this project was to develop a methodology to introduce wireless video capsule endoscopy in preclinical research. Five mature female pigs (*Sus scrofa domestica*) were selected for the study. Capsule endoscopes (the EndoCapsule system; Olympus) were introduced into the duodenum endoscopically in each of the animals. The life span of batteries (i.e., total time of endoscopy recording) was 487–540 min (median 492 min). The capsule endoscope reached the cecum during enteroscopy once (after 7 h 57 min), in the remaining cases, endoscopy recordings terminated in the distal or terminal ileum. All capsule enteroscopies found a normal pattern of the small intestine. The intestinal lumen is narrower, transverse folds are sparse or even absent, villi are wider but less prominent in pigs compared to humans. Capsule endoscopy in experimental pigs will be helpful for future trials on injury of different drugs and xenobiotics to the small bowel.

Keywords Capsule endoscopy · Enteroscopy · Experimental pigs · Small bowel

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Introduction

Wireless video capsule endoscopy represents fundamental progress in non-invasive imaging of the gastrointestinal tract, particularly the small bowel. The method was introduced into clinical practice in 2001 and more than 500,000 examinations have been performed in humans worldwide up to the present time [1–5]. At a very early stage, an initial study on experimental use of capsule endoscopy in dogs was published [6]. However, other papers dealing with the use of capsule endoscopy in a different experimental setting are still missing. The aim of this project was to develop a methodology to introduce wireless video capsule endoscopy in experimental pigs. The small adult pig can be used in experiments as an omnivorous representative due to its relatively very similar gastrointestinal functions in comparison to humans [7].

Animals and Methods

Preparation for the Project

In a pilot test on experimental pigs, we proved that endoscopic insertion of video capsules into the duodenum is feasible. A transverse semi ball-shaped pyloric fold (torus

pyloricus) was found in the porcine stomach of all animals, serving as a "gatekeeper" and thus making the endoscopic insertion technically difficult. The position of capsule endoscopes was set in three animals: in the proximal jejunum (330 cm) after 90 min, in the proximal ileum after 180 min, and in the cecum after 9 h. In another four animals, the time until natural excretion of capsule endoscopes in the feces was measured, this was 72, 48, 40, and 65 h (mean 56 ± 15 h). The length of the small intestine of 4-month-old pigs was found to be highly variable. This was measured at immediate autopsy in 13 animals, ranging from 11.2 to 16.8 m (mean 13.4 ± 1.8 m).

Methodical Design

Five mature female pigs (*Sus scrofa f. domestica*), hybrids of Czech White and Landrace breeds, weighing 36.6 ± 3.0 kg (4–5 months old) were entered into the study. An EndoCapsule system (Olympus Optical Co, Tokyo, Japan) was used for video capsule enteroscopies in all animals. The wireless capsule endoscope has an outer diameter of 11 mm, length of 26 mm, and weighing 3.8 g. Capsule endoscopes were introduced into the duodenum endoscopically in all animals. Endoscopy procedures were performed using video-gastroscopes GIF-Q130 (Olympus Optical Co, Tokyo, Japan) dedicated for animal use only. All endoscopies were recorded on DVD. Four capsule endoscopes were placed into the duodenum by means of a G25 retrieval basket (Sun, Vikýřovice, Czech Republic) and the remaining one using the AdvanCE delivery system (US Endoscopy, Mentor, USA).

Capsule enteroscopies were carried out in the stable phase of general anesthesia (intramuscular bolus of 20 mg/kg ketamine "Narkamon—Spofa, Praha, Czech Republic" +2 mg/kg azaperone "Stresnil—Jansen Pharmaceutica, Beerse, Belgium") on supine position in all animals (Fig. 1). Repeated doses of 1% thiopental (Thiopental, Valeant Czech Pharma, Prague, Czech Republic) were administrated when appropriate to the lateral auricle vein. Syntostigmine (0.5 mg i.v.—Hoechst-Biotika, Martin, Slovakia) was administrated immediately after successful placement of the capsule endoscope into the duodenum (to eliminate the influence of general anesthesia on the gastrointestinal passage). Infusions of 0.9% saline solution were chosen to secure basal hydration (1,000 ml/8 h). All animals were covered with blankets to prevent hypothermia during capsule enteroscopy. The procedure was finished after discharge of the batteries.

Data obtained during capsule enteroscopy were downloaded from the recorder unit to the workstation. It contains proprietary application software "Endo Capsule Software" (Olympus Optical Co, Tokyo, Japan), which enables the smooth management of capsule endoscope examination data. All video images were evaluated by a single physician, using usually auto speed adjustment (the review speed is automatically increased when there is little movement in consecutive images) with the lower limit for 12-frames/s of review speed in most recordings.

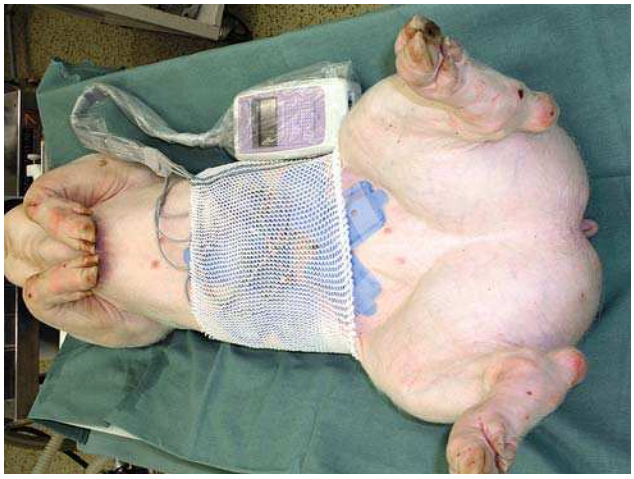

Fig. 1 Capsule endoscopy in an experimental pig. General view of the arrangement of investigation. The antenna system is affixed to skin of the abdomen; the data recorder is next to it

The next morning the pigs were sacrificed by means of pharmacological euthanasia (T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 ml/kg). Immediate autopsy was performed and samples collected of the pyloric transverse fold, and specimens of gastric and intestinal segments for light microscopy.

Ethics

The project was approved by the Institutional Review Board of the Animal Care Committee at the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Protocol Number 149/2006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [8].

Results

Capsule endoscopy was successful in all animals. Endoscopical placement of the capsule endoscope into the duodenum took 1.5 to 8 min (mean 5.5 min, median 6 min) using a basket and 42 min using a special delivery system. Endoscopic introduction of the capsule endoscope into the duodenum was difficult because of the pyloric transverse fold (see Fig. 2) that makes the pylorus relatively stenotic for endoscopic accessories. This fold (called

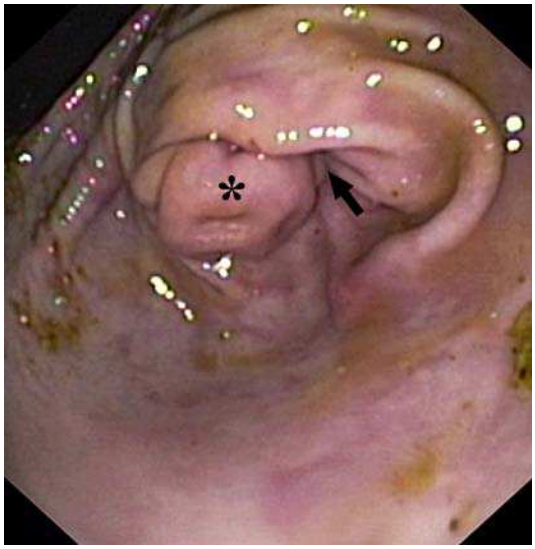

Fig. 2 Endoscopic view of the transverse pyloric fold—torus pyloricus (*asterisk*) in a porcine stomach. This fold makes the pylorus (*arrow*) relatively stenotic for endoscopic accessories

"torus pyloricus") is constituted by the ovoid-shaped muscularis propria and enlarged mucosa (Fig. 3).

The life span of batteries (i.e., total time of the endoscopy recording) was 487–540 min (mean 505 ± 22 min, median 492 min). The capsule endoscope reached the cecum during enteroscopy once (at 7 h 57 min), in the remaining cases, endoscopy recordings terminated in the distal or terminal ileum. Upon autopsy the next day after capsule enteroscopy, the capsule endoscopes were found in the cecum in all cases.

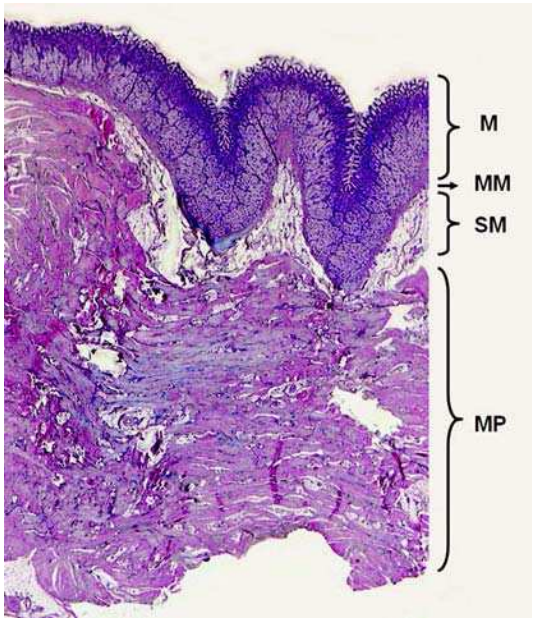

Fig. 3 Histology of the transverse pyloric fold in a porcine stomach. This fold is created mostly by the ovoid-shaped capacious muscularis propria (MP) and enlarged mucosa (M). The normal submucosa is marked as SM, the muscularis mucosa is lettered as MM. Hematoxylin-eosin, magnification 10×

Total length of the entire small intestine, including the duodenum (at immediate autopsy) was 11.2–13.7 m (mean 12.2 ± 1.1; median 11.7 m). It was not possible to set the exact borderline between the jejunum and ileum either endoscopically (during capsule enteroscopy), or macroscopically (at autopsy) or histologically. Approximately half comprises the jejunum and the other half the ileum. Movement of the capsule endoscope had less regional transit abnormalities in the duodenum and jejunum as compared to the ileum. In our pilot testing in one animal before this trial, the mean speed of the capsule endoscope in the proximal and middle jejunum was 4 cm/s. In the present study, there were several periods of persistence of capsule endoscope at one location without any propulsive movement (5–60 min). A rough estimation of the approximate location of the capsule endoscope (after its introduction into the duodenum) at the time of recording was as follows: the proximal jejunum at 10 min, middle jejunum at 60 min, borderline of the jejunum and ileum at approximately 150 min, middle ileum at 270 min, distal ileum at 450 min, terminal ileum at >480 min. In case of persistence of the capsule endoscope in one place without any propulsive movement, this time is subtracted (from the time line) to estimate the approximate position of the capsule endoscope.

The quality of the small-intestinal images ranged from very good to excellent in the jejunum in all animals (Fig. 4). Due to the large volume of intestinal content in the ileum, the visibility of some ileal segments was a little bit worse. There were no technical complications (e.g., early discharge of the battery, loss of data recording etc.). All capsule enteroscopies found a normal pattern of the small intestine in all experimental animals. A larger volume of intestinal juice with a marked content of bile was constantly seen in the small intestinal lumen in all enteroscopies. The endoscopic pattern of the small bowel in experimental pigs seems to be slightly different from that seen in humans (compare Figs. 4, 5). The intestinal lumen is narrower, transverse folds are sparse or even absent, villi are wider but less prominent in experimental pigs compared to humans.

Discussion

The aim of this paper was to work out a methodology and to set up capsule enteroscopy in experimental pigs (as an omnivorous representative metabolically close to humans). Our study produced new data on capsule endoscopy in experimental pigs. Capsule enteroscopy was accomplished

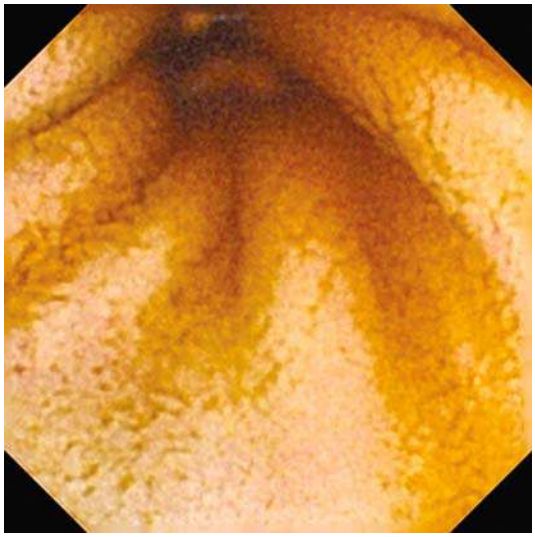

Fig. 4 Enteroscopy picture of the normal porcine jejunum (capsule endoscopy by means of Olympus EndoCapsule System). Transverse folds are absent, villi are wider but less prominent. A large volume of intestinal juice with a marked content of bile is seen

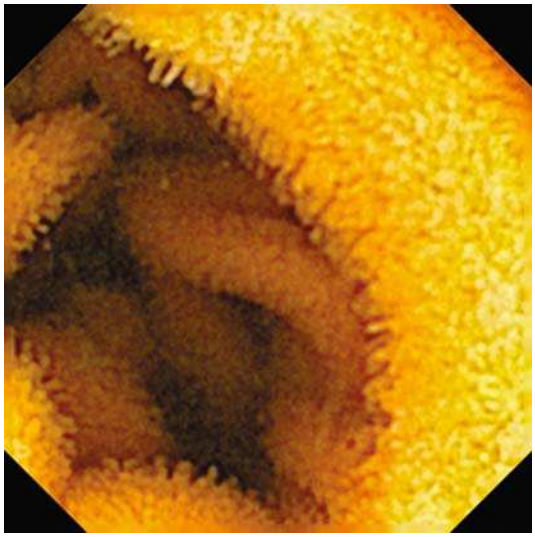

Fig. 5 Immersion enteroscopy picture of the normal human jejunum (capsule endoscopy by means of EndoCapsule System Olympus). Transverse folds (plicae circulares Kerkringi) are regular and dense, villi are clearly visible, being high and spiky. The intestinal lumen is usually empty. Picture reprinted from Tachecí et al. [10], reproduced with permission

in all animals. Thus, this method is feasible in an experimental setting in pigs. However, it was necessary to overcome several difficulties. Flexible upper endoscopy is more demanding in pigs (compared to humans). The mean distance from incisors to the gastro-esophageal junction is about 60 cm in these pigs. The porcine stomach is pouch-shaped and gastric cardia is close to the pylorus, so the endoscopic approach to the duodenum is rather hooked. To prevent a delay due to persistence of the video capsule in the stomach, we introduced all capsules directly into the duodenum by means of a standard flexible video-gastroscope. Pigs have an epipharyngeal diverticulum so it was necessary to introduce the gastroscope with a grasped capsule very carefully so as not to lose it during insertion and to prevent aspiration of the capsule. There was a distinct difference in the time necessary for gastroscopic introduction of capsule into the duodenum, using a basket and special delivery system. There is a transverse pyloric fold (torus pyloricus) in the porcine stomach that makes the pylorus relatively stenotic for endoscopic accessories. The outer diameter of the special delivery system is 13 mm and it must be introduced in a prograde way. We succeeded only after several repeated attempts using this system. The capsule grasped into a basket was introduced sideways and proved much easier and shorter (outer diameter of the capsule is 11 mm).

The entire small intestine of a young adult pig (weighing 35 kg) measures about 12 m in length immediately after pharmacological euthanasia. This is twice as long as in an adult human (weighting 70 kg) despite the fact that the pig is an omnivore like humans. The movement of capsule endoscope was more rapid in the jejunum compared to the ileum. This fact reflects the natural motility character of the jejunum. Furthermore, syntostigmine was administrated at the beginning of the experiment to enhance the propulsive work of the small intestine and to forestall any negative effect of general anesthesia on gastrointestinal motility. Half-time of syntostigmine is only 1–2 h so it cannot assure stimulated motility during the whole investigation. IV infusions to secure proper hydration and covering animals with blankets to forestall hypothermia during capsule enteroscopy are mandatory to prevent further negative effects on the small intestinal motility.

The mean life span of the batteries used in the experiment was about 500 min. Capsule enteroscopy (similar to other methods) is not able to recognize the exact borderline between the jejunum and ileum. We have proposed a rough estimation of the approximate location of the capsule endoscope, being merely a conservative estimate. We are fully aware of the limits of this proposal (inter-individual variability in the motility pattern, absence of reliable landmarks, the cecum was reached only in one animal during capsule enteroscopy recording etc.). In clinical studies in humans (having the small bowel twice shorter than a pig), the cecum is reached; that means the entire small intestine is investigated in only about 75%. In up to 25% of cases, the batteries also run out before the capsule passes the ileo-cecal valve in humans [9].

In summary, wireless video capsule endoscopy is feasible in experimental pigs. This paper concerns methodological

preparation for further experimental studies on (1) the effect of different drugs and xenobiotics on the small intestine and (2) the mechanism of pharmacokinetics of different pharmaceutical formulations of drugs in the small bowel. Our study provided several new data, differentiating capsule endoscopy in experimental pigs from those performed in humans.

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ORIGINAL ARTICLE

Bacteriocinogeny in experimental pigs treated with indomethacin and *Escherichia coli Nissle*

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Abstract

AIM: To evaluate bacteriocinogeny in short-term high-dose indomethacin administration with or without probiotic *Escherichia coli Nissle 1917* (EcN) in experimental pigs.

METHODS: Twenty-four pigs entered the study: Group A (controls), Group B (probiotics alone), Group C (indomethacin alone) and Group D (probiotics and indomethacin). EcN (3.5×10^{10} bacteria/d for 14 d) and/or indomethacin (15 mg/kg per day for 10 d) were administrated orally. Anal smears before and smears from the small and large intestine were taken from all animals. Bacteriocin production was determined with 6 different indicator strains; all strains were polymerase chain reaction tested for the presence of 29 individual bacteriocin-encoding determinants.

RESULTS: The general microbiota profile was rather uniform in all animals but there was a broad diversity in coliform bacteria (parallel genotypes A, B1, B2 and D found). In total, 637 bacterial strains were tested, mostly *Escherichia coli* (*E. coli*). There was a higher incidence of non-*E. coli* strains among samples taken from the jejunum and ileum compared to that of the colon and rectum indicating predominance of *E. coli* strains in the large intestine. Bacteriocinogeny was found in 24/77 (31%) before and in 155/560 (28%) isolated bacteria at the end of the study. Altogether, 13 individual bacteriocin types (out of 29 tested) were identified among investigated strains. Incidence of four *E. coli* genotypes was equally distributed in all groups of *E. coli* strains, with majority of genotype A (ranging from 81% to 88%). The following types of bacteriocins were most commonly revealed: colicins Ia/Ib (44%), microcin V (18%), colicin E1 (16%) and microcin H47 (6%). There was a difference in bacteriocinogeny between control group A (52/149, 35%) and groups with treatment at the end of the study: B: 31/122 (25%, $P = 0.120$); C: 43/155 (28%, $P = 0.222$); D: 29/134 (22%, $P = 0.020$). There was a significantly lower prevalence of colicin Ib, microcins H47 and V (probiotics group, $P < 0.001$), colicin E1 and microcin H47 (indomethacin group, $P < 0.001$) and microcins H47 and V (probiotics and indomethacin group, $P = 0.025$) compared to controls. *Escherichia fergusonii* (*E. fergusonii*) was identi-

fied in 6 animals (6/11 isolates from the rectum). One strain was non-colicinogenic, while all other strains of *E. fergusonii* solely produced colicin E1. All animals started and remained methanogenic despite the fact that EcN is a substantial hydrogen producer. There was an increase in breath methane (after the treatment) in 5/6 pigs from the indomethacin group (C).

CONCLUSION: EcN did not exert long-term liveability in the porcine intestine. All experimental pigs remained methanogenic. Indomethacin and EcN administered together might produce the worst impact on bacteriocinogeny.

Key words: Bacteriocinogeny; *Escherichia coli Nissle 1917*; Experimental pigs; Indomethacin

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) represent the group of most commonly used drugs worldwide. NSAIDs may cause severe injury to all parts of the gastrointestinal tract. The pathogenesis of NSAID-induced entero- and colopathy is more multifactorial and complex than formerly assumed but is not yet fully understood. A combination of local and systemic effects plays an important role in pathogenesis. NSAID-induced entero- and colopathy is a stepwise process involving direct mucosal toxicity, mitochondrial damage, breakdown of intercellular integrity, enterohepatic recirculation and neutrophil activation by luminal contents including bacteria. Unlike upper gastrointestinal toxicity, cyclo-oxygenase-mediated mechanisms are probably less important[1-3]. Intestinal bacteria play a significant role in the pathogenesis of NSAID-induced entero- and colopathy. In experimental studies, NSAIDs cannot induce enteropathy in germ-free rats[4].

Probiotic bacteria are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host[5]. Probiotics likely function through enhancement of the barrier function of the gut, immunomodulation, and competitive adherence to the mucus and epithelium[6]. Probiotic bacteria may exert a systemic anti-inflammatory effect[7] and modulate apoptosis[8]. Probiotics have been suggested for amelioration or prevention of various diseases including antibiotic-associated diarrhoea, irritable bowel syndrome and inflammatory bowel disease. Further possible beneficial effects are being studied (including anti-cancer potential, lowering of serum cholesterol levels and blood pressure reduction, *etc.*)[9-11]. It has been hypothesised that probiotic bacteria might reduce the adverse effects of NSAIDs on the small and large intestine. However, initial studies provided controversial results, both with ameliorating and deteriorating outcomes[12-15]. NSAID-induced small intestinal injury is Toll-like receptor 4 dependent[14]. Probiotic *Escherichia coli Nissle 1917* (EcN) might ameliorate experimental colitis (induced by dextran sodium sulphate) *via* Toll-like receptor 2 and 4 pathways[16,17].

Colicins and microcins, members of the bacteriocin family, are produced by bacteriocinogenic strains of *Escherichia coli* (*E. coli*) and some related species of *Enterobacteriaceae*. They are toxic to susceptible bacterial strains of the same family[18-20]. However, some bacteriocins also exert an inhibitory effect on eukaryotic cells, including observed antineoplastic action *in vitro* and *in vivo*[21-25]. Bacteriocins might induce apoptosis[26] as some regulators of apoptosis (e.g. Bcl family with pro- and anti-apoptotic members) share similar structures with pore-forming colicins[27]. The possible role of bacteriocins was also investigated in clinical studies on bacillary dysentery[28], inflammatory bowel disease[29] and colorectal cancer[30]. Bacteriocins might have a dual role: they may act as both antibiotics and probiotics[31]. One of the most commonly used probiotic bacterial strains, EcN, is a producer of microcins H47 and M[32-34].

The aim of this study was to evaluate bacteriocinogeny in short-term high-dose indomethacin administration with or without probiotic bacteria EcN in an experimental porcine model. A small adult pig can be used in experiments as a representative of an omnivore due to its relatively similar gastrointestinal functions in comparison with man[35-38].

MATERIALS AND METHODS

Ethics

The Project was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Record Number 1492006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes[39].

Animals

Twenty-four healthy mature (4-5 mo old) female pigs (*Sus scrofa* f. *domestica*, hybrids of Czech White and Landrace breeds) weighing 33.0 ± 1.7 kg, were included in our study. The animals were divided into four groups: Group A (controls, 6 animals), Group B (probiotics alone, n = 6), Group C (indomethacin alone, n = 6) and Group D (probiotics and indomethacin, n = 6). All animals were fed twice a day (standard assorted food A1 of equal amounts).

Drug and probiotic bacteria administration

EcN (3.5×10^{10} live bacteria/d for 14 d) and/or indomethacin (Indomethacin suppositories, Berlin-Chemie, Germany; 15 mg/kg per day for 10 d) were administrated as one-shot dietary bolus to hungry pigs.

Autopsy

Twenty-four hours after the last drug and/or probiotic bacteria administration (groups B to D) or after 14 d of stabling (Group A), the pigs were sacrificed (after 24 h of fasting) by means of pharmacological euthanasia (iv administration of embutramide, mebezonium iodide and tetracaine hydrochloride - T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 mL per kg) and exsanguinated. Immediate autopsy was performed and smears for bacterial cultures were taken.

Bacterial culture, isolation and identification

Before the experiment anal smears were taken from all animals. At autopsy, smears from mucosa of the jejunum, ileum, caecum, transverse colon and rectum were taken from each animal and immediately inserted into a transport liver-enriched broth. Standard primary cultures were inoculated on blood and MacConkey agars (24 h at 37℃), followed by standard clone isolation. Up to 9 different colonies of coliform bacteria were isolated from each sample (on blood, MacConkey and deoxycholate agars). Particular bacteria were precisely identified by the Vitek2 system (BioMérieux, Marcy l'Etoile, France). All bacterial strains were frozen in cryotube vials at minus 90℃ until bacteriocin genotyping.

Analysis of bacteriocin production

The bacteriocin production of all strains was tested in parallel on 4 different agar plates containing (1) TY medium; (2) nutrient broth; (3) TY medium supplemented with mitomycin C; and (4) TY medium supplemented with trypsin. The TY medium consisted of yeast extract (Hi-Media, Mumbai, India) 5 g/L, tryptone (Hi-Media) 8 g/L, sodium chloride 5 g/L; the TY agar consisted of a base layer (1.5%, w/v, solid agar) and a top layer (0.7%, w/v, soft agar). A Difco™ nutrient broth (Difco Laboratories, Sparks, MD) 8 g/L, NaCl 5 g/L, was used for production of relatively unenriched 1.5% (w/v) agar plates. Mitomycin C (0.01%, w/v) and trypsin (0.1%, w/v) were used for induction of colicin production and for protease sensitivity tests, respectively. The previously described set of *E. coli* indicator strains including *E. coli* K12-Row, C6 (φ), B1, P400, and S40 was used to identify the producer strains together with *Shigella sonnei* 17 indicator[40,41]. To test bacteriocin production, the agar plates were inoculated by needle stab and the plates were incubated at 37℃ for 48 h. The tested macrocolonies were then killed with chloroform vapours and each plate was then overlaid with a thin layer of soft agar containing 10^7 cells/mL of an indicator strain and the plates were incubated at 37℃ overnight. All investigated *E. coli* strains were tested on four parallel plates against 6 indicator strains stated.

Table 1 DNA primers used for polymerase chain reaction detection of colicin encoding genes

Bacteriocin type	Primer name	5'-sequence-3'	Length of PCR product (nt)
A	ColA-F	CGTGGGGAAAAGTCATCATC	
	ColA-R	GCTTTGCTCTTTCCTGATGC	475
B	colicinB-F	AAGAAAATGACGAGAAGACG	
	colicinB-R	GAAAGACCAAAGGCTATAAGG	493
D	ColD-F	CTGGACTGCTGCTGGTGATA	
	ColD-R	GAAGGTGCGCCTACTACTGC	420
E1	colicinE1-F	TGTGGCATCGGGCGAGAATA	
	colicinE1-R	CTGCTTCCTGAAAAGCCTTTT	650
E2	ColE2-F	TGATGCTGCTGCAAAAGAG	
	ColE2-R	TTCAAAGCGTTCCCTACCAC	409
E3	ColE3-F	TAAGCAGGCTGCATTTGATG	
	ColE3-R	TCGGATCTGGACCTTTCAAC	413
E4	ColE4-F	GAAGGCTGCATTTGATGCT	
	ColE4-R	CGGATCCGGACCTTTAATTT	409
E5	ColE3-F	TAAGCAGGCTGCATTTGATG	
	ColE5-R	TTGAATTCTCGAATCGTCCA	430
E6	ColE6-F	ACCGAACGTCCAGGTGTT	
	ColE6-R	TTTAGCCTGTCGCTCCTGAT	399
E7	ColE7-F	GCATTCTGCCATCTGAAAT	
	ColE7-R	CTTCTGCCCACTTTCTTTCG	431
E8	ColE3-F	TAAGCAGGCTGCATTTGATG	
	ColE8-R	GACTGATTGGCTTGTCGTGA	449
E9	ColE3-F	TAAGCAGGCTGCATTTGATG	
	ColE9-R	GACTTTTCTCCCTCCGACCT	418
Ia	ColIa-F	GCATGCAAATGACGCTCTTA	
	ColIa-R	GAGGACGCCAGTTCTCTGTC	473
Ib	ColIb-F	AACGAGTGGGTCGATGATTC	
	ColIb-R	CCTTTTCTGCGCTCGTATTC	464
Js	ColJs-F	TCAAAATGTTTGGGCTCCTC	
	ColJs-R	TAATCTGCCCTGTCCCACTG	254
K	ColK-F	CAGAGGTCGCTGAACATGAA	
	ColK-R	TCCGCTAAATCCTGAGCAAT	469
M	ColM-F	GCTTACCACTTCGCAAAACC	
	ColM-R	GAGCGACTCTCCGATAATGC	429
N	ColN-F	AGCTTGGCGAGTATCTTGGA	
	ColN-R	CAACACAGCCCCGAATAAAC	401
S4	ColS4-F	TATATGGCCCAACTGCTGGT	
	ColS4-R	CGTAAGGACGGACACCTGTT	456
U	ColU-F	TGATTGCTGCGAGAAAAATG	
	ColU-R	TCTGACAGCCTCTCCCTGTT	485
Y	ColY-F	GCAGGCAGAAAAGAACAAGG	
	ColY-R	CGGACGTTATTTGCCTTCAT	477
5	Col5-F	CATTGGCAAAAGCGAAATCT	
	Col5-R	TGCAACTCTGGAAACAATCG	443
10	Col10-F	GGTTACCGGATTTCCTGGAT	
	Col10-R	TTCTAGATGCTTGGCCCACT	448

PCR: Polymerase chain reaction.

Identification of individual colicin types

All investigated strains were tested with colony polymerase chain reaction (PCR). A bacterial colony was resuspended in 100 μL of sterile water and 1 μL of this suspension was added to the PCR reaction. Individual colicin types (colicins A, D, E2-E9, Ia, Ib, Js, K, M, N, S4, U, Y, 5 and 10) were detected using PCR with primers designed using the Primer3 program[42]. The list of primer pairs and the corresponding length of PCR products are listed in Table 1. Control bacterial producers stemmed from our stock and comprised *E. coli* BZB2101pColA - CA31, BZB2102 pColB - K260, BZB2103 pColD -

Table 2 Bacteriocinogeny of particular strains isolated at the end of experiment

Parameter	Small intestine			Colon and rectum		
	Bacterio-cinogeny	Types of bacteriocin producers (No. of strains)	No. of unique bacteriocin producers	Bacterio-cinogeny	Types of bacteriocin producers (No. of strains)	No. of unique bacteriocin producers
Group A	22/55 (40%)	E1 (1); E1, Ia, V (1); E1, V (2); Ia (2); Ia, B, K, M, H47 (2); Ia, H47, V (2); Ia, V (6); Ib (3); J25 (1); S4, U (1); S4, V (1)	11	30/94 (32%)	B (1); B, H47, Ib, K, M (2); B, M (1); C7, E1, Ib, V (1); E1 (1); E1, Ia (1); E1, Ia, V (1); E1, V (2); E7 (1); H47, Ia, V (1); H47, S4 (1); H47, V (2); Ia (6); Ia, V (4); Ib (3); M (1); S4, V (1)	17
Group B	11/43 (26%)	E1 (7); Ia (2); Ia, V, H47 (1); Ib (1)	4	20/79 (25%)	B, H47, K, M, Ia (1); B, M (1); E1 (4); E1, V (1); Ia (11); Ia, V (1); Ib (1)	7
Group C	17/58 (29%)	B, M, V (1); Ia (1); Ia, V (7); Ib (6); S4 (2)	5	26/97 (27%)	B, Ia, (1); E1, Ib (1); J25, Ia (1); Ia (5); Ia, E7, V (1); Ia, H47 (1); Ia, M (1); Ia, V (8); Ib (7)	9
Group D	9/45 (20%)	E1 (3); E1, Ia (1); E1, Ia, V (1); E1, Ib (1); Ia (1); Ia, V (1); Ib (1)	7	20/89 (22%)	E1 (4); E1, Ia (1); E1, Ia, V (1); E1, Ib (2); H47, V (1); Ia (7); Ia, V (1); Ib (2); Ib, V (1)	9

Small intestine: Bacterial strains isolated from mucosa of the jejunum and ileum; Colon and rectum: Bacterial strains isolated from mucosa of the caecum, transverse colon and rectum; Group A: Control animals with no treatment; Group B: Probiotics alone (see text for details); Group C: Indomethacin alone (see text for details); Group D: Probiotics and indomethacin (see text for details); Bacteriocinogeny: Number of bacteriocinogenic strains out of all tested; Types: Particular bacteriocin types found in single isolates; M: Colicin M, not for microcin M.

CA23, BZB2107 pColE4 - CT9, BZB2108 pColE5 - 099, BZB2150 pColE6 - CT14, BZB2120 pColE7 - K317, BZB2279 pColIa - CA53, BZB2202 ColIb - P9, BZB2116 pColK - K235, PAP1 pCol101M - BZBNC22, BZB2123 pColN - 284, *E.* 189BM pColE2 - P9, *E. coli* 385/80 pColE1, pColV, *E. coli* 185M4 pColE3 - CA38, *E. coli* W3110 pColE8, W3110 pColE9, *E. coli* K-12 pColS4, *Shigella boydii* M592 (serovar 8) pColU, *E. coli* K339 pColY, *Sh. sonnei* pColJs, *E. coli* pCol5, *E. coli* pCol10, *E. coli* 449/82 pColX (microcin B17), *E. coli* 313/66 pColG (microcin H47), *E. coli* 363/79 pColV (microcin V), *E. coli* TOP10F' pDS601 (microcin C7), *E. coli* D55/1 (microcin J25), and *E. coli* B1239 (microcin L). Sequentially related colicin genes (colicins E2-E9, Ia-Ib, U-Y, and 5-10, respectively) often yielded PCR products with primer pairs of related colicin types and therefore all these PCR products were sequenced. The PCR detection primers for colicins B and E1 and for 6 microcin types including B17, C7, H47, J25, L, and V, were taken from Gordon *et al*[43]. The phylogenetic group of each *E. coli* strain was determined using the triplex PCR protocol according to Clermont *et al*[44]. Sequence analysis was performed using Lasergene software (DNASTAR, Inc., Madison, WI, USA).

Hydrogen and methane breath testing

Hydrogen and methane breath tests were performed before and the morning following completion of the treatment, carried out under general anaesthesia in spontaneously breathing animals. Alveolar air was aspirated by means of percutaneous puncture of the trachea. Immediate measurement of hydrogen and methane was accomplished in triplicate by means of gas chromatography (Microlyzer DP Plus Quintron, Milwaukee, WI, USA). Results were expressed as parts per million (ppm).

Statistical analysis

Data were statistically analysed with χ^2 with Yates correction and by Mann-Whitney rank sum test. Statistical software was used for this analysis (SigmaStat version 3.1, Jandel Co., Erkrath, Germany).

RESULTS

The general microbiota profile was rather uniform in all animals but there was a broad diversity in coliform bacteria (parallel genotypes A, B1, B2 and D found). In total, 637 bacterial strains were tested, mostly *E. coli*. The remaining isolates comprised *Salmonella enterica* ssp *Arizonae* (21 isolates), *Pasteurella aerogenes* (20), *Escherichia fergusonii* (*E. fergusonii*) (11), *Aeromonas hydrophila/caviae* (9), *Klebsiella pneumoniae* (8), *Enterobacter cloacae* (4), *Morganella morganii* (4), *Citrobacter braakii* (2), *Citrobacter youngae* (2), *Citrobacter freundii* (1), *Acinetobacter lwoffii* (1) and *Pseudomonas aeruginosa* (1). There was a higher incidence of non-*E. coli* strains among samples taken from the jejunum and ileum compared to that of the colon and rectum indicating predominance of *E. coli* strains in the large intestine (data not shown).

Bacteriocinogeny was found in 24/77 (31%) before and in 155/560 (28%) isolated bacteria at the end of the study. Altogether, 13 individual bacteriocin types (out of 29 tested) were identified among investigated strains. Incidence of four *E. coli* genotypes was equally distributed in all groups of *E. coli* strains, with majority of genotype A (ranging from 81% to 88%). The following types of bacteriocins were most commonly revealed: colicins Ia/Ib (44%), microcin V (18%), colicin E1 (16%) and microcin H47 (6%). There was a difference in bacteriocinogeny between control group A (52/149, 35%) and groups with treatment at the end of the study: B: 31/122 (25%, P = 0.120); C: 43/155 (28%, P = 0.222); D: 29/134 (22%, P = 0.020). See Table 2 for details. There was a significantly lower prevalence of colicin Ib, microcins H47 and V (probiotics group, $P < 0.001$), colicin E1 and microcin H47 (indomethacin group, $P < 0.001$) and microcins H47 and V (probiotics and indomethacin group, P = 0.025) com-

Table 3 Analysis of porcine alveolar breath for hydrogen and methane (in ppm - parts per million) before and after the treatment

Group	Hydrogen before	Hydrogen after	Statistical significance	Methane before	Methane after	Statistical significance
A	N/A	3.50 ± 2.81	N/A	N/A	69.33 ± 56.64	N/A
B	6.0 ± 2.82	2.0 ± 0	NS	106.50 ± 94.05	80.00 ± 48.02	NS
C	1.17 ± 0.41	5.0 ± 3.29	NS	34.67 ± 25.65	66.17 ± 38.83	NS
D	2.0 ± 1.16	6.0 ± 6.0	NS	60.75 ± 34.77	62.00 ± 27.71	NS

Group A (controls with no treatment, n = 6), Group B (probiotics alone, n = 6), Group C (indomethacin alone, n = 6) and Group D (probiotics plus indomethacin, n = 6). N/A: Not applicable; NS: Not significant.

pared to controls (Table 2). *E. fergusonii* was identified in 6 animals (6/11 isolates from the rectum). One strain was non-colicinogenic, while all other strains of *E. fergusonii* solely produced colicin E1.

Data on porcine alveolar breath analysis of hydrogen and methane are given in Table 3. All animals started and remained methanogenic. Differences between groups were not statistically significant. There was an increase in breath methane (after the treatment) in 5/6 pigs from the indomethacin group (C).

DISCUSSION

Probiotic bacteria might act in three different ways: they are able to modulate the host's defence mechanisms, they have a direct impact on other micro-organisms and finally probiotic effects may be based on actions affecting microbial products like toxins, host products (e.g. bile salts) and food ingredients[45].

Our hypothesis for this study was that (1) indomethacin would suppress bacteriocin production of *Enterobacteriaceae*; (2) probiotic bacteria EcN would colonise the porcine gastrointestinal tract permanently; (3) they would protect intestinal microbiota from suppressive action of indomethacin; and (4) EcN would convert the starting methanogenic phenotype of pigs to a hydrogenic one. Surprisingly, most of our presumptions were not proved.

There is no simple way to explain this. The first question that should be addressed is a possible role of human probiotic bacteria in the porcine gastrointestinal tract. It is necessary to consider whether human probiotics can be also assumed to act as probiotic microbiota for domestic pigs. Criteria for probiotics of human origin were proposed[46], however, potential probiotic bacteria isolated from porcine faeces are usually tested *in vitro* to be active against two or three common porcine pathogens only[47-50].

Genotype B2 and production of microcin H47 were considered as markers of EcN in our study. None of our 637 isolates comprised these bacteria. Viability and sufficient amount of bacteria were ensured before their administration in our project. According to our results, it is unlikely that EcN could exert long-term viability in the porcine intestinal tract. Other swine studies by several authors[51-54] were able to identify intestinal colonisation by EcN in pigs and piglets but not by all of them[55]. There is no final proof of long-term colonisation of the gastrointestinal tract by EcN in healthy humans. In an interesting study by Schierack *et al*[56], probiotic *Enterococcus faecium* supplementation showed no significant effect on the numbers and diversity of *Enterobacteriaceae* species, or on the total counts, diversity and distribution of virulence gene-positive *E. coli* strains in healthy domestic pigs.

Aspirin and some NSAIDs, including indomethacin, influence intestinal bacteria[57-60]. Indomethacin might exert some impact on intestinal microbiota in our study, as there was an increase in breath methane after the treatment in 5/6 pigs from the indomethacin group. Another interesting result from our current study showed a marked lower prevalence of colicin Ib, microcins H47 and V (probiotics group), colicin E1 and microcin H47 (indomethacin group) and microcins H47 and V (EcN and indomethacin group) compared to controls. We interpret this difference as a sign of adverse effects of probiotics and/or indomethacin on porcine microbiota. Bacteriocinogeny in controls (35%) was higher compared to the indomethacin (28%), probiotic (25%) and indomethacin and probiotic groups (22%). This evident trend did not reach statistical significance for the probiotic group (B) and indomethacin group (C). However, there was a statistically significant difference between controls and indomethacin and probiotic group (D). We can assume that indomethacin and EcN comprise the worst impact on bacteriocinogeny in the porcine gastrointestinal tract. This would be consistent with other studies showing that other probiotics might deteriorate NSAID-induced injury to the intestine[13].

Composition of food, especially supplements with prebiotics, might influence the probiotic effect of intestinal bacteria[61,62]. This factor is unlikely to play an important role in our study. All animals received identical assorted food with cereals, animal fat, soya oil and a mix of supplements (lysine, threonine, methionine, lactic acid).

In our current study, the microbiota profile was rather uniform in all animals due to identical breed and feed. However, there was a broad diversity in coliform bacteria; the main four genotypes A, B1, B2 and D were identified in parallel. Similar diversity was also found in other porcine studies, with prevailing group A[56,63]. Dixit *et al*[63] showed that differences among individual pigs accounted for 6% of the observed genetic diversity, whilst 27% of the genetic variation could be explained by clonal composition differences among gut regions (isolates obtained from the duodenum, ileum, colon and faeces of 8 pigs). Finally, the absence of virulence genes in these com-

mensals indicates that they may be suitable as a probiotic consortium, particularly if they also display increased adherence to enterocytes and antagonistic activity against pathogenic strains of *E. coli*[63].

E. fergusonii was identified as a new species of *Enterobacteriaceae* in 1985[64]. This is considered to be an opportunistic pathogen of farm animals including domestic pigs[65]. We identified *E. fergusonii* in 6 animals, all pigs were healthy without any sign of infective disease. Interestingly 10/11 isolated bacteria solely produced colicin E1. Colicins produced by *E. fergusonii* strains closely resemble colicins encoded by *E. coli*[66]. In a previous series of human isolates, only 6/50 (12%) strains were bacteriocinogenic, 3 of which produced colicin E1[67].

In humans, all intestinal hydrogen and methane are produced by so called "hydrogenic and methanogenic" bacteria[68-70]. However, most authors do not usually specify which particular bacteria constitute these producers. Hydrogen is produced by bacterial fermentation of saccharides in the intestinal lumen. Concurrently, hydrogen is consumed by other intestinal bacteria to synthesise methane, acetate and hydrogen sulphide. Methane is synthesised solely by bacteria in the intestine (four mols of hydrogen and one mol of carbon dioxide create one mol of methane and water). This reaction reduces the volume of gas that would otherwise be present in the colon[71-76]. The question of intestinal methane producers has not been definitely solved yet. We hypothesised that common coliform bacteria could also synthesise methane[77], however, this assumption was not proved by our further studies[78,79]. McKay *et al*[80] found that several anaerobes (*Bacteroides*, *Clostridium* and others) produced hydrogen but rarely methane. Hydrogen is also produced by *Enterobacteriaceae*[81]. In adult Caucasians, only 30%-50% of people produce methane while hydrogen is produced by 90%-98% of people[69]. Kien *et al*[82] found low breath hydrogen and higher methane in piglets (even in a subgroup supplemented with lactulose). In our current study, all animals revealed a solely methanogenic phenotype (by the analysis of their alveolar breath). This fact could be explained as they came from an identical breed and received the same assorted food. All animals remained methanogenic despite the fact that EcN is a substantial hydrogen producer[77]. This further supports our finding that EcN *1917* did not have major impact on porcine intestinal microbiota.

In conclusion, it is unlikely that probiotic EcN could exert long-term liveability in the porcine intestine. All experimental pigs remained methanogenic, despite the fact that EcN is a substantial hydrogen producer. The indomethacin and probiotic group had a significantly lower rate of bacteriocinogeny compared to controls with no treatment. These control pigs revealed higher bacteriocinogeny with simultaneous production of up to five different bacteriocins per single strain. Indomethacin and probiotics administered together might provide the worst impact on bacteriocinogeny in the porcine gastrointestinal tract.

COMMENTS

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) represent the group of most commonly used drugs worldwide. NSAIDs may cause severe injury to all parts of the gastrointestinal tract. The pathogenesis of NSAID-induced entero- and colopathy is more multifactorial and complex than formerly assumed but has still not been fully understood. A combination of local and systemic effects plays an important role in pathogenesis. NSAID-induced entero- and colopathy is a stepwise process involving direct mucosal toxicity, mitochondrial damage, breakdown of intercellular integrity, enterohepatic recirculation and neutrophil activation by luminal contents including bacteria. Unlike upper gastrointestinal toxicity, cyclo-oxygenase-mediated mechanisms are probably less important. Intestinal bacteria play a significant role in the pathogenesis of NSAID-induced entero- and colopathy. In experimental studies, NSAIDs cannot induce enteropathy in germ-free rats.

Research frontiers

Probiotic bacteria are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. Probiotics likely function through enhancement of the barrier function of the gut, immunomodulation, and competitive adherence to the mucus and epithelium. Probiotic bacteria might exert a systemic anti-inflammatory effect and modulate apoptosis. Probiotics have been suggested for amelioration or prevention of various diseases including antibiotic-associated diarrhoea, irritable bowel syndrome and inflammatory bowel disease. Further possible beneficial effects are being studied (including anti-cancer potential, lowering serum cholesterol levels and blood pressure reduction, *etc.*). It has been hypothesised that probiotic bacteria might reduce the adverse effects of NSAIDs on the small and large intestine. However, initial studies provided controversial results, both with ameliorating and deteriorating outcomes.

Innovations and breakthroughs

Based on the current study, it is unlikely that probiotic *Escherichia coli Nissle 1917* (EcN) could exert long-term liveability in the porcine intestine. Genotype B2 and production of microcin H47 were considered as markers of EcN in the study. The authors did not find such bacteria among any of the 637 isolates. All experimental pigs remained methanogenic, despite the fact that EcN is a substantial hydrogen producer. The indomethacin and probiotic group had a significantly lower rate of bacteriocinogeny compared to controls with no treatment. These control pigs revealed higher bacteriocinogeny with simultaneous production of up to five different bacteriocins per single strain. Indomethacin and probiotics administered together might produce the worst impact on bacteriocinogeny in the porcine gastrointestinal tract.

Applications

Bacteriocins might induce apoptosis as some regulators of apoptosis (e.g. Bcl family with pro- and anti-apoptotic members) share similar structures with pore-forming colicins. Bacteriocins might have a dual role: they may act as both antibiotics and probiotics. One of the most commonly used probiotic bacterial strains, EcN, is a producer of microcins H47 and M.

Terminology

Colicins and microcins, members of the bacteriocin family, are produced by bacteriocinogenic strains of *Escherichia coli* and some related species of *Enterobacteriaceae*. They are toxic to susceptible bacterial strains of the same family. However, some bacteriocins also exert an inhibitory effect on eukaryotic cells, including observed antineoplastic action *in vitro* and *in vivo*.

Peer review

This is an innovative manuscript in basic research, which adequately addresses the ethics of the experiment. Its presentation is accurate but very complex in the reading and interpretation of many variables.

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ORIGINAL ARTICLE

Electrogastrography in experimental pigs: the influence of gastrointestinal injury induced by dextran sodium sulphate on porcine gastric erythromycin-stimulated myoelectric activity

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Abstract

OBJECTIVES: Electrogastrography (EGG) is a non-invasive investigation of gastric myoelectrical activity. The aim of study was to evaluate the impact of erythromycin on EGG in gastrointestinal toxic injury induced by dextran sodium sulphate (DSS) in experimental pigs. **METHODS:** The experiments were carried out on 12 adult pigs (weighing 30–35 kg). EGG was recorded using Digitrapper equipment (Synectics Medical AB, Stockholm). Running spectrum activity was used for EGG evaluation. There were two groups of animals: Group I: 6 controls with erythromycin administration (1,600 mg intragastrically); Group II: 6 animals treated with DSS (for 5 days, 0.25 g/kg per day in a dietary bolus) followed by erythromycin administration. Baseline and subsequent six separate 30-minute EGG-recordings (from time 0 to 360 min) were accomplished in each animal. **RESULTS AND CONCLUSION:** A total of 84 records were analysed. Baseline dominant frequency of slow waves was fully comparable in both groups. In Group I, there was a significant increase in dominant frequency after erythromycin administration (maximum between 240–360 min). There was a flat non-significant and delayed increase in dominant frequency after erythromycin administration in Group II. The difference between Group I and II at particular time intervals was not significant but a diverse trend was evident. EGG recording enables us to register a gastric myoelectrical effect of prokinetic drugs. Erythromycin induced a significant increase in the dominant frequency of slow waves. DSS caused toxic injury to the porcine gastrointestinal tract responsible for the delayed and weaker myoelectrical effect of erythromycin in experimental animals.

INTRODUCTION

Gastrointestinal motility is controlled by a complex multifactorial system based on electromechanical and neuro-endocrine function. The myoelectric activity of the gastrointestinal tract consists of two kinds of potentials: slow waves and spike activity organised in myoelectric migrating complex (Thor *et al.* 2007). The slow wave of the gastrointestinal tract mainly functions to trigger the onset of spike to elicit smooth muscle contraction, which provides the essential power of motility. Smooth muscle myogenic control activity or slow wave is believed to originate in the interstitial cells of Cajal. The electrical coupling promotes interaction between muscle cells and the interstitial cells of Cajal additionally contribute to slow wave rhythmicity (Chang 2005; Chen *et al.* 1994).

Surface electrogastrography (EGG) is a non-invasive method for clinical assessment of gastric myoelectrical activity. Our group demonstrated for the first time in our previous studies that EGG is reliable and feasible in experimental pigs too (Varayil *et al.* 2009; Květina *et al.* 2010; Ali *et al.* in press). Porcine EGG is fully comparable with that recorded in healthy humans (Varayil *et al.* 2009; Parkman *et al.* 2003).

Macrolides with lactone ring containing 14 atoms as erythromycin and clarithromycin have prokinetic effect on the gastrointestinal tract by acting as motilin receptor agonists (Hawkyard *et al.* 2007). Erythromycin, currently the most potent prokinetic drug, has been shown to initiate gastric interdigestive migrating motor complexes which are the motor events responsible for gastric emptying (Prather *et al.* 1993; Keshavarzian *et al.* 1993; Curry *et al.* 2001).

In humans, inflammatory bowel disease is associated with altered myoelectric activity and gastric emptying, both in Crohn's disease and ulcerative colitis (Gryboski *et al.* 1992; Bracci *et al.* 2003; Kohno *et al.* 2006). Dextran sodium sulphate (DSS) has been widely used for experimental models of colitis, including porcine ones (Mackenzie *et al.* 2003; Bassaganya-Riera *et al.* 2006).

The aim of this study was to assess the influence of gastrointestinal injury induced by DSS on porcine gastric myoelectric activity stimulated by intragastrically administrated erythromycin. The results allow us to organize further experimental studies concentrated on the gastric motility disorders and its treatment in the inflammatory bowel disease patients.

MATERIAL AND METHODS

Animals

Twelve healthy mature female pigs (*Sus scrofa f. domestica*, hybrids of Czech White and Landrace breeds; 4–5 months old, weighing 30–35 kg) were included into the study. The animals were fed twice a day (standard assorted food A1) and had allowed free access to water.

Experimental design

All EGG recordings were accomplished in the morning after 24-hour fasting. The animals were divided into the two groups. The 6 control animals (Group I) received no pre-treatment. A 30-minute baseline EGG was recorded. Afterwards, 1,600 mg of erythromycin was administrated intragastrically. Subsequent six separate 30-minute EGG-recordings (from time 0 to 360 min) were accomplished in each animal. Another six animals (Group II) were treated with DSS (molecular weight 36–50 kDa; MP Biomedicals, Solon, OH, USA) for 5 days: 0.25 g/kg per day in a dietary bolus every morning. The next day after the last dose of DSS, a 30-minute baseline EGG was recorded. Afterwards, 1,600 mg of erythromycin was administrated intragastrically. Subsequent six separate 30-minute EGG-recordings (from time 0 to 360 min) were accomplished in each animal in the same manner as in Group I.

After accomplishment of EGG recording, the pigs were sacrificed by means of pharmacological euthanasia (T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 mL/kg). Immediate autopsy was performed to exclude direct toxic injury to the porcine stomach by DSS.

Electrogastrography

Surface cutaneous EGG was recorded using a Digitrapper (Synectics Medical AB, Stockholm, Sweden). All EGGs were carried out under general anaesthesia. Intramuscular injection of ketamine (20 mg per kg; Narkamon, Spofa, Prague, Czech Republic) was used as an introduction. Repeated doses of thiopental were administrated intravenously when appropriate. Intravenous infusions of 0.9% saline solution were chosen to secure basal hydration (1,000 mL/8 hours).

All animals were lying in a right lateral position during EGG recording. The epigastric area was shaved before application of electrodes to decrease impedance in signal conduction through the skin. Electrode placement always began with placing the first electrode within 5 cm of the xiphoid process in the centre and then subsequently placing the other two at a distance of 15 cm from the central electrode in the left and right hypochondrium respectively.

Running spectral analysis (based on Fourier transform) was used for the evaluation of the EGG. The results were expressed as running spectrum percent activity and dominant frequency of slow waves was set at all intervals of EGG recordings (according to Varayil *et al.* 2009).

Ethics

The Project was approved by the Institutional Review Board of the Animal Care Committee of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Protocol Number 149/2006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate

Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

Statistical analysis

Data were statistically treated by means of descriptive statistics, non-paired t-test and Mann-Whitney rank sum test using SigmaStat software (Version 3.1, Jandel Corp., Erkrath, Germany).

RESULTS

EGG recording was successfully accomplished in all animals. A total of 84 records were analysed. Baseline dominant frequency of slow waves was fully comparable in both groups (see Table 1). In Group I, there was a significant increase in dominant frequency after erythromycin administration from basal mean values 4.57 ± 1.28 to its maximum between 240–360 min with mean values 6.61 ± 0.56 and 6.63 ± 1.16 cycles per minute, respectively, p=0.005 and p=0.015 (see Table 1 and Figure 1 for details). There was a flat non-significant and delayed increase in dominant frequency after erythromycin administration in Group II (see Figure 2). The difference between Group I and II at particular time intervals was not significant but a diverse trend was evident (see Table 1 for details).

Autopsy found a normal gastrointestinal tract in all animals in Group I. In Group II, severe colitis was found in all animals especially in the caecum and right hemi-colon (with ulcers, bleeding erosions and mucosal erythema). The stomach and small intestine were macroscopically and histologically normal in all animals in Group II.

DISCUSSION

Our current study set two major aims, firstly to evaluate the gastric myoelectric effect of erythromycin, a potent prokinetic drug; and secondly to assess the consequences of toxic DSS on the functioning of the porcine stomach. Both parts were successfully accomplished. Nevertheless, our initial results must be evaluated and interpreted with caution, we are fully aware of the possible limits of this study.

Pigs can be used in various preclinical experiments (Květina *et al.* 2008; Kuneš *et al.* 2010) as a representative of the omnivore due to their relatively very similar gastrointestinal functions in comparison to man (Kararli 1995). However, there are some distinct differences in the anatomy and physiology of the stomach between humans and pigs (Bureš *et al.* 2009; Kopáčová *et al.* 2010; Tachecí *et al.* 2010). The porcine stomach is pouch-shaped, gastric cardia is close to the pylorus, and a special transverse pyloric fold serves as a "gatekeeper". Gastric emptying of pigs is much slower, there are significant remnants of food in the porcine stomach even after 36–48 hours of fasting found at gastroscopy (Kopáčová *et al.* 2010; Tachecí *et al.* 2010).

Erythromycin induced a significant increase in the dominant frequency of slow waves in our current

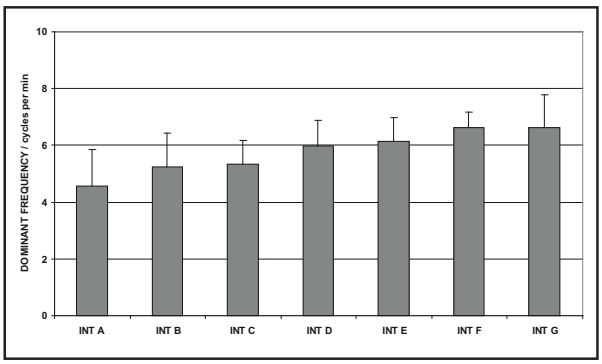

Fig. 1. Electrogastrography in control experimental pigs (Group I.). Mean dominant frequency of slow waves before and after erythromycin administration. See text for details of the study design.
INT A: a 30-minute basal EGG recording before the administration of erythromycin
INT B: EGG recording after erythromycin administration in time 0 to 30 min
INT C: time interval 30–60 min after erythromycin administration
INT D: time interval 60–90 min after erythromycin administration
INT E: time interval 90–120 min after erythromycin administration
INT F: time interval 240–270 min after erythromycin administration
INT G: time interval 330–360 min after erythromycin administration
Statistically significant difference: INT A vs. INT D: p=0.048; INT A vs. INT E: p=0.031; INT A vs. INT F: p=0.005; INT A vs. INT G: p=0.015; INT B vs. INT F: p=0.029.

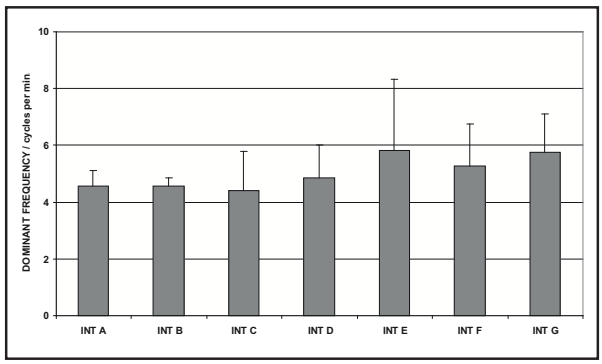

Fig. 2. Electrogastrography in experimental pigs pre-treated with dextran sodium sulphate for five days (Group II.). Mean dominant frequency of slow waves before and after erythromycin administration. See text for details of the study design.
INT A: a 30-minute basal EGG recording before administration of erythromycin
INT B: EGG recording after erythromycin administration in time 0 to 30 min
INT C: time interval 30–60 min after erythromycin administration
INT D: time interval 60–90 min after erythromycin administration
INT E: time interval 90–120 min after erythromycin administration
INT F: time interval 240–270 min after erythromycin administration
INT G: time interval 330–360 min after erythromycin administration
There was no statistically significant difference between particular intervals.

Tab. 1. Electrogastrography in experimental pigs. Dominant frequency of slow waves (cycles per minute). See text for details of the study design.

Interval	Group I.		Group II.		*p*-value
	Mean ± Std Dev	Median IQR	Mean ± Std Dev	Median IQR	
A	4.57 ±1.28	4.46 3.39–5.16	4.57 ±0.55	4.32 4.21–5.23	NS (*p*=0.991)
B	5.23 ±1.20	5.40 4.69–5.70	4.56 ±0.30	4.48 4.44–4.54	NS (*p*=0.215)
C	5.33 ±0.83	5.23 4.63–5.83	4.41 ±1.39	4.19 3.53–4.72	NS (*p*=0.195)
D	5.99 ±0.88	6.21 5.79–6.62	4.85 ±1.15	4.46 4.04–5.23	NS (*p*=0.081)
E	6.14 ±0.84	6.33 5.90–6.77	5.83 ±2.50	4.85 4.68–5.63	NS (*p*=0.240)
F	6.61 ±0.56	6.49 6.21–6.90	5.28 ±1.46	5.30 4.01–6.70	NS (*p*=0.180)
G	6.63 ±1.16	6.42 5.70–6.84	5.76 ±1.36	6.24 4.53–6.90	NS (*p*=0.258)

Interval A: a 30-minute basal EGG recording before administration of erythromycin
Interval B: EGG recording after erythromycin administration in time 0 to 30 min.
Interval C: time interval 30–60 min. after erythromycin administration
Interval D: time interval 60–90 min. after erythromycin administration
Interval E: time interval 90–120 min. after erythromycin administration
Interval F: time interval 240–270 min. after erythromycin administration
Interval G: time interval 330–360 min. after erythromycin administration
Std Dev: standard deviation
IQR: inter-quartile range
NS: statistically non-significant difference

study. These findings were consistent in all animals. To the best of our knowledge there are no available published data so far to evaluate the impact of erythromycin on porcine electrogastrography. In humans, erythromycin was studied both in healthy volunteers and patients with gastroparesis (characterised by tachygastria in EGG). DiBaise *et al.* evaluated the effect of low dose erythromycin (50 mg or 100 mg i.v. infusion in adult healthy volunteers). A two-hour EGG recording showed a decrease of three cycles per minute rhythm and corresponding increase of tachygastria after 100 mg infusion (DiBaise *et al.* 2001). Faure *et al.* administered erythromycin (3 mg/kg i.v.) to ill children and found no correlation between increased antral motor index (number of waves × sum of amplitudes) and running total spectrum power ratio, regardless of diagnosis (chronic intestinal pseudo-obstruction, chronic vomiting or abdominal distension) (Faure *et al.* 2000). Chen *et al.* studied the effect of erythromycin (100 mg i.v.) in gastroparesis. A 30-minute EGG recording revealed a significant increase in the dominant power of EGG together with an improvement in the regularity of gastric slow waves (Chen *et al.* 1998). We used the intragastric delivery of erythromycin in pigs as an equivalent of the oral route in humans. The oral route may be preferred because erythromycin-related, fatal cardiac complications (published formerly) have been associated with parenteral administration only (Farrar *et al.* 1993; Gouyon *et al.* 1994).

The doses of erythromycin used in our study were relatively high (1,600 mg) for several reasons. Oral administration of a low dose might result in inadequate serum concentrations with no prokinetic effect and the peak serum drug concentration after this type of administration is 4–10 times lower than when the drug is administered by the intravenous route in humans (Parsons *et al.* 1980; Houin *et al.* 1980). And last but not least, there is still a lack of data on erythromycin pharmacokinetics in experimental pigs.

All our porcine EGGs were acquired under general anaesthesia just for practical reasons, although both general and epidural anaesthesia may affect the myoelectrical activity of the stomach in humans (Cheng *et al.* 1999; Lombardo *et al.* 2009; Oshima 2009). Delayed gastric emptying and start of intestinal absorption of erythromycin might participated on delayed effect of erythromycin observed during EGG recording (see below).

However, the most important factor is the time of the EGG recording. We accomplished a 6-hour EGG in all animals and revealed an increase in dominant frequency with its maximum between 240–360 min after erythromycin administration. These changes might be missed in case of shorter recording. Most human studies found erythromycin-induced changes of dominant power in particular. Our previous porcine study demonstrated that the running spectrum percent activity is superior to the power analysis in an experimental setting (Ali *et al.*, in press). Andreis *et al.* evaluated EGG in conscious and anaesthetised dogs. Erythromycin induced an increased power ratio, the amplitude increased whereas frequency decreased (Andreis *et al.* 2008).

DSS, a sulphated polysaccharide, reproducibly induces experimental acute and chronic colitis. DSS is thought to induce mucosal injury and inflammation initially through a direct toxic effect on epithelial cells with subsequent activation of macrophages and T lymphocytes resulting in cytokine mediated cytotoxicity (Ni *et al.* 1996; Leung *et al.* 2000). There are several ways to explain the impact of DSS on porcine erythromycin-induced myoelectric changes. Despite the normal gross appearance of the stomach at autopsy, DSS might exert its direct toxic effect on the porcine myoelectric function. In our current study, the baseline dominant frequency of slow waves was fully comparable in both groups, with and without pre-treatment with DSS. However, the DSS group failed to increase myoelectric activity after erythromycin stimulation. The possible explanation is an analogy with inflammatory bowel

disease, human colitis could be associated with altered myoelectric activity of the uninvolved stomach (Gryboski *et al.* 1992; Bracci *et al.* 2003; Kohno *et al.* 2006). This possible explanation has been supported by an experimental study by Aube *et al.* They found altered myoelectrical activity in the non-inflamed ileum of rats with experimental colitis induced by trinitrobenzene sulphonic acid (Aube *et al.* 1999).

In conclusion, EGG recording enables us to register a significant gastric myoelectric effect of prokinetic drugs in experimental pigs. Erythromycin induced a significant increase in the dominant frequency of slow waves in our setting. DSS caused toxic injury to the porcine gastrointestinal tract that was responsible for delayed and weak myoelectric effect of erythromycin in experimental pigs. Additional studies are warranted to further clarify this phenomenon. Our settings allow set up further experimental studies on gastric motility disorders in inflammatory bowel diseases.

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Conflicts of interests

The authors disclose no conflicts.

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Morphometric analysis of the porcine gastrointestinal tract in a 10-day high-dose indomethacin administration with or without probiotic bacteria *Escherichia coli Nissle 1917*

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Abstract

Background: Nonsteroidal anti-inflammatory drugs may cause severe injury to all parts of the gastrointestinal tract. It has been hypothesised that probiotic bacteria might reduce this adverse effect. The aim of this study was to perform a morphometric evaluation of the gastrointestinal tract in experimental pigs treated using a 10-day high-dose of indomethacin with or without *Escherichia coli Nissle 1917* (EcN). Methods: Twenty-four healthy mature pigs were included: Group A (controls; 6 animals), Group B (EcN; $n = 6$), Group C (indomethacin; $n = 6$) and Group D (EcN & indomethacin; $n = 6$). EcN (3.5×10^{10} live bacteria/day for 14 days) and/or indomethacin (15 mg/kg/day for 10 days) were administered. Specimens of the stomach, small and large bowel were routinely processed for microscopic examination. The height of glandular mucosa, height and width of interfoveolar spaces and villi and basement size of epithelial cells were evaluated. Results: Different effects of indomethacin and EcN on particular parts of the gastrointestinal tract were shown. The indomethacin and probiotics group demonstrated a significantly lower height of cryptal mucosa and colonocytes and widening of the basement size of colonocytes compared to controls ($p = 0.004$; $p < 0.001$; $p = 0.025$). The height of cryptal mucosa was significantly higher in the EcN group compared to controls ($p = 0.001$). Conclusions: Indomethacin alone induced marked adaptation of the gastric mucosa. EcN alone provided a significant favourable trophic effect on the colonic mucosa. However, indomethacin and probiotics administered together comprise the worst impact on all porcine stomach, small and large bowel.

Keywords

morphometric analysis, porcine gastrointestinal tract, indomethacin, probiotics, *Escherichia coli 1917 Nissle*

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent the group of most commonly used drugs worldwide. NSAIDs may cause severe injury to all parts of the gastrointestinal tract, both in humans and in an experimental setting.[1–5] Gastric damage by NSAIDs is primarily a consequence of inhibition of cyclooxygenase-1 (COX-1) in the upper GI tract. COX-1 inhibition reduces mucosal generation of protective prostaglandins such as prostaglandin E2. While inhibition of COX-1 is the major mechanism

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by which NSAIDs produce gastric injury, mediators besides prostaglandins and nitric oxide may also be involved.[2] The pathogenesis of NSAID-induced entero- and colopathy is more multifactorial and complex than formerly assumed but has still not been fully understood. A combination of local and systemic effects plays an important role in pathogenesis. NSAID-induced entero- and colopathy is a stepwise process involving direct mucosal toxicity, mitochondrial damage, breakdown of intercellular integrity, enterohepatic recirculation and neutrophil activation by luminal contents including bacteria.[5] Aspirin and some NSAIDs, including indomethacin, influence intestinal bacteria.[6–10] Metronidazol can reduce indomethacin-induced intestinal toxicity.[11]

Probiotic bacteria are live microorganisms that when administered in adequate amounts confer a health benefit on the host.[12] Probiotics likely function through enhancement of the barrier function of the gut, immunomodulation and competitive adherence to the mucus and epithelium.[13] Furthermore, probiotic bacteria might exert a systemic anti-inflammatory effect[14] and modulate apoptosis.[15] Probiotics have been suggested for amelioration or prevention of various diseases including antibiotic-associated diarrhoea, irritable bowel syndrome and inflammatory bowel disease.[16]

It has been hypothesised that probiotic bacteria might reduce the adverse effects of NSAIDs on the gastrointestinal tract. However, initial studies provided controversial results, both with ameliorating and deteriorating outcomes.[17–20]

The aim of this project was to perform a morphometric study of the gastrointestinal tract in experimental pigs treated using short-term high-dose indomethacin with or without probiotic bacteria *Escherichia coli Nissle 1917* (EcN).

Methods

Animals

Twenty-four healthy young mature (4–5 months old) female pigs (*Sus scrofa* f. *domestica*, hybrids of Czech White and Landrace breeds) weighing 33.0 $\pm$ 1.7 kg, were included in our study. The animals were divided into four groups: Group A (controls; 6 animals), Group B (probiotics alone; $n = 6$), Group C (indomethacin alone; $n = 6$) and Group D (indomethacin & probiotics; $n = 6$). All animals were fed twice a day (standard assorted A1 food of equal amounts).

Drug and probiotic bacteria administration

EcN (3.5×10^{10} live bacteria/day for 14 days) and/or indomethacin (Indomethacin suppositories, Berlin-Chemie, Germany; 15 mg/kg/day for 10 days) were administrated as one-shot dietary bolus to hungry pigs. EcN was provided by the Institute of Microbiology, Academy of Sciences of the Czech Republic.

Autopsy

Twenty-four hours after the last drug and/or probiotic bacteria administration (Groups B to D) or after 14 days of stabling (Group A), the pigs were killed (after 24 hours of fasting) by means of pharmacological euthanasia (i.v. administration of embutramide, mebezonium iodide and tetracaine hydrochloride—T61, Intervet International BV, Boxmeer, the Netherlands; dose of 2 mL per kg) and exsanguinated. Immediate autopsy was performed and specimens for structural and morphometric analysis were taken.

Histological examination

Only samples of macroscopically normal appearance were extirpated. Specimens of the stomach, jejunum, ileum and colon were routinely processed for microscopical examination. The samples were fixed with 10% neutral buffered formalin for 24 hours and subsequently embedded into paraffin. Five-micrometers-thick tissue sections of each part were cut and stained with hematoxylin eosin for morphometric evaluation and Gram staining to exclude bacterial invasion.

Measurement of morphometric parameters

Stained samples were evaluated using a BX-51 microscope (Olympus Optical Co, Tokyo, Japan) and computer image analysis ImagePro 5.1 (Media Cybernetics, Bethesda, MD, USA). Ten microscopic fields at a 100-, 200- and 800-fold original magnification were taken from each pig. The following parameters were evaluated:

- In the stomach, the height of glandular mucosa at 100-fold original magnification (see Figure 1), height and width of interfoveolar spaces at 200-fold original magnification (Figure 2) and length of the basement membrane of 10 epithelial cells together with height of epithelial cells localised at the base of the interfoveolar space were assessed at 800-fold original magnification (Figure 3).

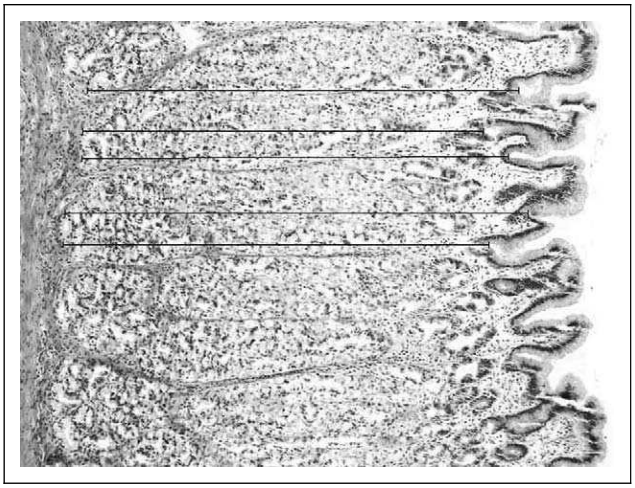

Figure 1. A sample of the porcine stomach at 100-fold original magnification. The distance between foveolar bottom and the bottom of crypts leading vertically towards the muscularis mucosae was measured for evaluation of the height of glandular mucosa in the stomach. Five measurements per microscopic field were performed. Hematoxylin-eosin staining.

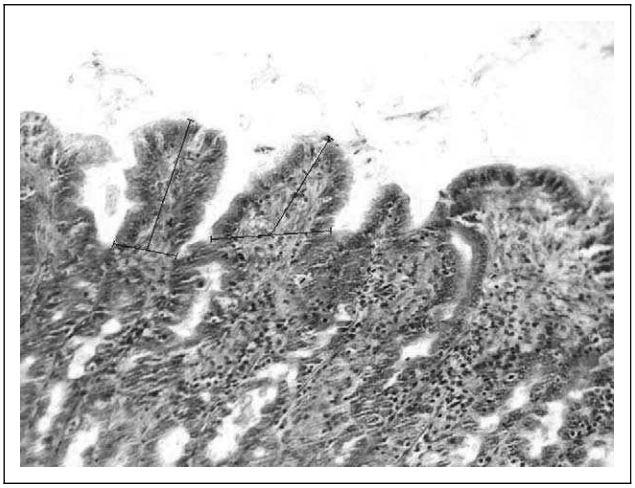

Figure 2. The porcine stomach at 200-fold original magnification. The width of the interfoveolar space at its base and height of the space represented by the axis line between the centre of the width line and the top of the interfoveolar space were measured. Two measurements per microscopic field were performed. Hematoxylin-eosin staining.

- In the jejunum and ileum, the height of cryptal mucosa at 100-fold original magnification (Figure 4), height and width of villi at 200-fold original magnification (Figure 5) and length of the lamina basalis of 10 epithelial cells together with height of enterocytes localised at the base of villi were measured at 800-fold original magnification (Figure 6).

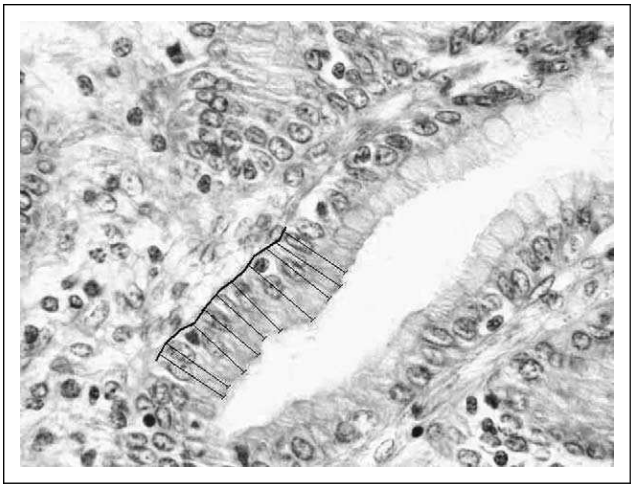

Figure 3. The length of the basement membrane of 10 epithelial cells localised at the base of the interfoveolar space and the height of the 10 cells were evaluated in the porcine stomach at 800-fold original magnification. Hematoxylin-eosin staining.

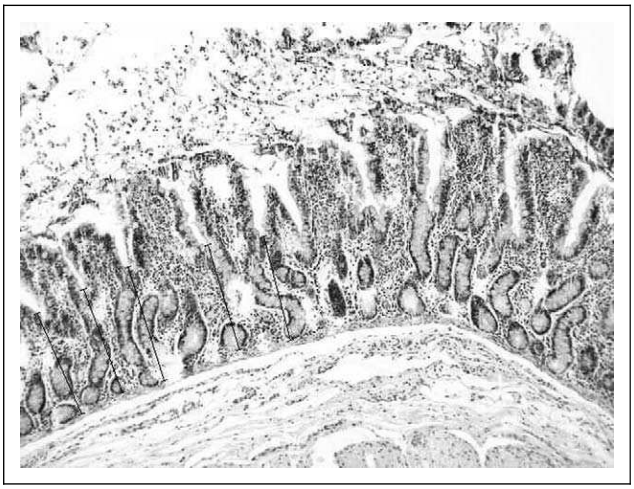

Figure 4. A sample of the control porcine ileum at 100-fold original magnification. For evaluation of the height of cryptal mucosa in the ileum and jejunum, only microphotographs of longitudinal crypt sections were taken. The height of cryptal mucosa was determined as the distance between the villus base and the end of Lieberkühn crypts leading vertically from the villus base towards the muscularis mucosae. Five measurements per microscopic field were performed. Hematoxylin-eosin staining.

- In the colon, the height of cryptal mucosa was evaluated at 100-fold original magnification (Figure 7). The length of the basement membrane of 10 epithelial cells together with height of colonocytes localised at the lumen space were measured at 800-fold original magnification (Figure 8).

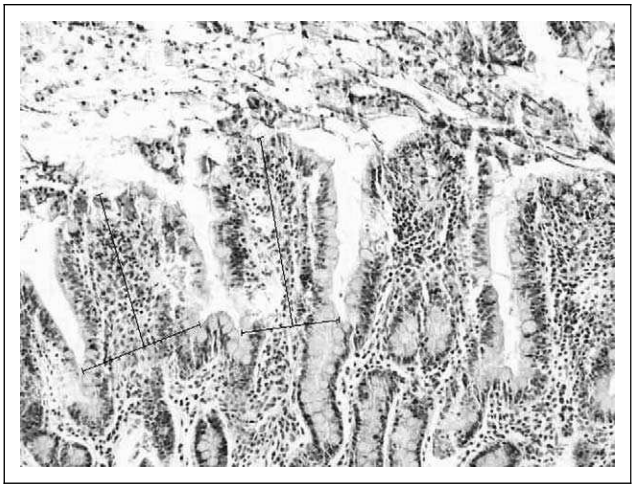

Figure 5. The porcine ileum at 200-fold original magnification. The width of a villus was measured at its base, while its length was represented by the axis distance between the centre of the width line and the top of the villus. Two measurements per microscopic field were performed. Hematoxylin-eosin staining.

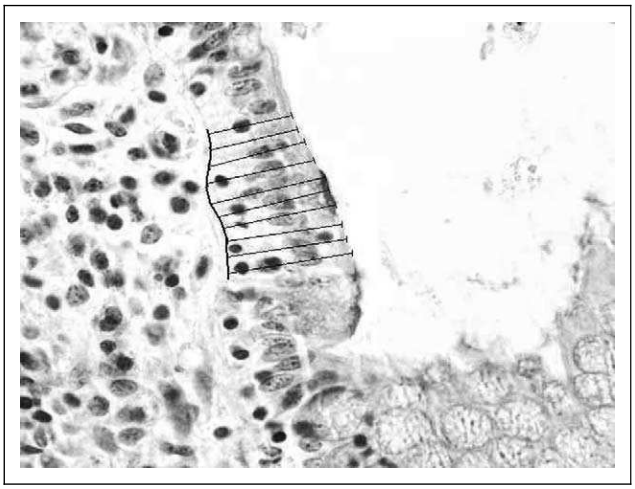

Figure 6. A sample of the porcine ileum at 800-fold original magnification. In the ileum and jejunum, the length of the basement membrane of 10 epithelial cells localised the base of villi and the heights of the 10 enterocytes were measured. Hematoxylin-eosin staining.

Ethics

The project was approved by the Institutional Review Board of Animal Care Committee of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Record Number 1492006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.[21]

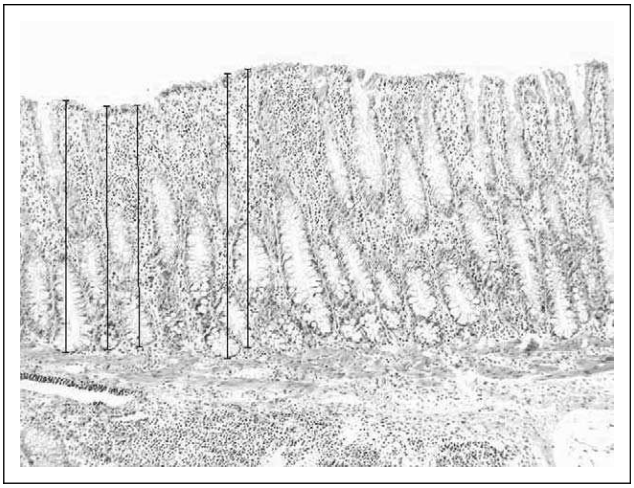

Figure 7. The porcine transverse colon at 100-fold original magnification. Microphotographs of longitudinal crypt sections were taken for evaluation of the height of cryptal mucosa in the colon transversum. The height of cryptal mucosa was determined as the distance between the bottom of Lieberkühn crypts and surface leading vertically from the crypts towards the surface. Five measurements per microscopic slide were performed. Hematoxylin-eosin staining.

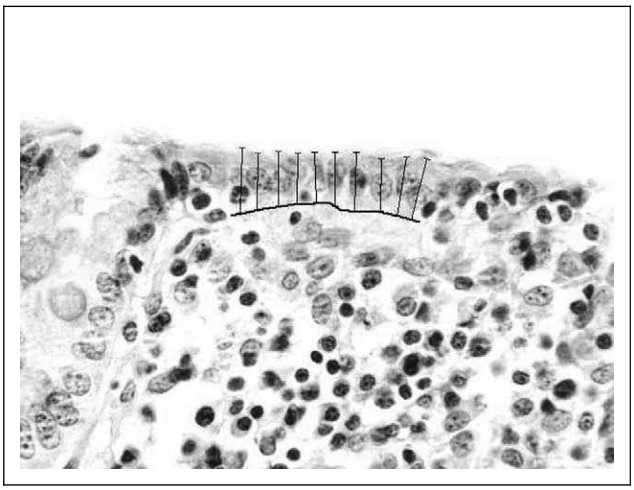

Figure 8. A sample of the porcine transverse colon at 800-fold original magnification. The length of the basement membrane of 10 epithelial cells localised at the intestine lumen and the heights of the 10 colonocytes were measured. Hematoxylin-eosin staining.

Statistical analysis

The results are expressed as mean $\pm$ SEM (standard error of the mean). Data were statistically analysed using a Mann-Whitney rank sum test. Statistical software was used for this analysis (SigmaStat version 3.1; Jandel Corporation, Erkrath, Germany). Statistical significance was set at $p < 0.05$.

Table 1. Morphometric analysis of the porcine stomach

Parameter mean 2× SEM (μm)	Group A	Group B	Group C	Group D	Statistical significance
Height of glandular part of the mucosa	1010.3 27.9	1036.1 35.9	979.1 42.7	1095.1 56.1	A vs D $p = 0.059$; C vs D $p < 0.001$
Height of the interfoveolar space	116.2 10.7	118.0 9.6	134.6 11.6	115.6 9.8	C vs D $p = 0.051$
Width of the interfoveolar space	108.7 13.5	115.7 8.0	123.8 9.1	127.4 13.7	NS
Height of epithelial cells	23.9 0.41	22.8 0.47	22.5 0.48	23.9 0.56	A vs B $p < 0.001$; A vs C $p < 0.001$; B vs D $p = 0.008$; C vs D $p < 0.001$
Length of lamina basalis of 10 epithelial cells	44.7 2.0	45.6 2.3	52.0 2.2	55.0 2.9	A vs D $p < 0.001$; A vs C $p < 0.001$; B vs C $p < 0.001$; B vs D $p < 0.001$

SEM: standard error of the mean.
Notes: Group A (controls with no treatment; 6 animals); Group B (probiotics alone; $n = 6$); Group C (indomethacin alone; $n = 6$); Group D (indomethacin and probiotics; $n = 6$).

Table 2. Morphometric analysis of the porcine jejunum

Parameter mean 2× SEM (μm)	Group A	Group B	Group C	Group D	Statistical significance
Height of cryptal mucosa	333.2 10.3	293.9 14.4	350.1 14.1	311.5 17.3	A vs B $p < 0.001$; A vs D $p = 0.005$; B vs D $p < 0.001$; C vs D $p < 0.001$
Height of villi	262.2 19.6	226.8 19.8	284.5 28.4	237.5 19.6	A vs B $p = 0.005$; A vs D $p = 0.053$; B vs C $p = 0.004$; C vs D $p = 0.048$
Width of villi	192.3 14.4	167.4 12.4	170.7 11.9	166.1 11.6	A vs B $p = 0.023$; A vs C $p = 0.037$; A vs D $p = 0.019$
Height of enterocytes	34.4 0.42	33.4 0.53	32.8 0.35	30.7 0.49	A vs C $p < 0.001$; A vs D $p < 0.001$; B vs D $p < 0.001$; C vs D $p < 0.001$
Length of lamina basalis of 10 enterocytes	44.2 1.5	44.7 1.6	44.3 1.7	45.8 1.6	NS

SEM: standard error of the mean.
Notes: Group A (controls with no treatment; 6 animals); Group B (probiotics alone; $n = 6$); Group C (indomethacin alone; $n = 6$); Group D (indomethacin and probiotics; $n = 6$).

Results

Altogether, 18,240 morphometric measurements were accomplished. There was no bacterial contamination in any specimen.

Morphometric analyses of the porcine stomach showed significant difference in both height of epithelial cells and their basement size (expressed as length of the lamina basalis of 10 epithelial cells) between controls and the indomethacin group ($p < 0.001$). Values of basal size of epithelial cells were significantly higher in the indomethacin and probiotics group compared to the controls ($p < 0.001$). See Table 1 for details.

In the jejunum, EcN administration was associated with significantly lower values of height of cryptal mucosa ($p < 0.001$) and height and width of villi ($p = 0.005$; $p = 0.023$) compared to the controls (Table 2).

Morphometric analyses of the ileum revealed significantly decreased height of enterocytes and widening of their basement size (assessed by length of the basement membrane of 10 epithelial cells) in the indomethacin group and indomethacin and probiotics group compared to controls ($p < 0.001$). The height of cryptal mucosa was significantly higher in the indomethacin group and indomethacin and probiotics group compared to the controls ($p = 0.003$; $p = 0.044$; Table 3).

In the porcine colon, the indomethacin and probiotics group demonstrated significantly lower height of cryptal mucosa and colonocytes and widening of the basement size colonocytes compared to the controls ($p = 0.004$; $p < 0.001$; $p = 0.025$). The height of

Table 3. Morphometric analysis of the porcine ileum

Parameter mean 2× SEM (μm)	Group A	Group B	Group C	Group D	Statistical significance
Height of cryptal mucosa	297.6 10.7	292.1 15.7	331.6 16.0	327.6 19.7	A vs C $p = 0.003$; A vs D $p = 0.044$; B vs C $p = 0.001$; B vs D $p = 0.018$
Height of villi	206.5 16.7	186.4 14.7	210.7 16.6	189.0 13.7	B vs C $p = 0.040$
Width of villi	177.7 11.1	180.3 13.4	186.4 12.8	185.2 14.3	NS
Height of enterocytes	29.1 0.38	30.2 0.44	27.8 0.53	27.0 0.52	A vs C $p < 0.001$; A vs D $p < 0.001$; B vs C $p < 0.001$; B vs D $p < 0.001$
Length of lamina basalis of 10 epithelial cells	44.9 1.8	46.9 1.9	50.6 2.3	50.8 2.1	A vs C $p < 0.001$; A vs D $p < 0.001$; B vs C $p = 0.014$; B vs D $p = 0.007$

SEM: standard error of the mean.
Notes: Group A (controls with no treatment; 6 animals); Group B (probiotics alone; $n = 6$); Group C (indomethacin alone; $n = 6$); Group D (indomethacin and probiotics; $n = 6$).

Table 4. Morphometric analysis of the porcine colon

Parameter mean 2× SEM (μm)	Group A	Group B	Group C	Group D	Statistical significance
Height of cryptal mucosa	446.5 9.1	474.2 11.9	462.4 10.8	428.5 12.6	A vs B $p = 0.001$; A vs D $p = 0.004$; B vs C $p = 0.029$; B vs D $p < 0.001$; C vs D $p < 0.001$
Height of colonocytes	23.3 0.33	24.9 0.40	20.3 0.32	20.0 0.37	A vs C $p < 0.001$; A vs B $p < 0.001$; A vs D $p < 0.001$; B vs C $p < 0.001$; B vs D $p < 0.001$
Length of lamina basalis of 10 colonocytes	52.1 3.0	49.6 3.3	59.1 2.7	56.7 2.5	A vs C $p = 0.002$; A vs D $p = 0.025$; B vs C $p < 0.001$; B vs D $p < 0.001$

SEM: standard error of the mean.
Notes: Group A (controls with no treatment; 6 animals); Group B (probiotics alone; $n = 6$); Group C (indomethacin alone; $n = 6$); Group D (indomethacin and probiotics; $n = 6$).

cryptal mucosa was significantly higher in the EcN group compared to the controls ($p = 0.001$; Table 4).

All other results are summarised in Tables 1–4.

Discussion

The primary objective of this project was to evaluate the effect of indomethacin on morphological changes of the gastrointestinal tract in experimental pigs. We decided to use a short-term high-dose of indomethacin (twice as the highest dose for humans) with or without EcN. Our hypothesis for this study was that probiotic bacteria EcN would protect the porcine stomach and intestine from any harmful effects of indomethacin. Considerably consistent data were achieved in particular groups of animals. Nevertheless, our results must be interpreted with caution. The height of the glandular part in the gastric mucosa was lower in the indomethacin group, which might be interpreted as prompt adaptation changes. The significantly enlarged height of the glandular part and widening of epithelial cells at the basement membrane of the porcine stomach (Group D) is at least partly explained by mucosal oedema and might by assessed as a deteriorating conjunctive effect of indomethacin and EcN administered together. The decreased size of villi in the small intestine in the EcN group might be interpreted as an unfavourable effect of probiotics on the porcine small bowel.

We considered the length of the basement membrane of 10 epithelial cells as the most relevant and reliable marker. Epithelial cells at the lamina basalis decreased, shrank and widened, both in the small and large intestine in the indomethacin and EcN group. These data can be interpreted as a deteriorating impact of combined indomethacin and probiotic bacteria on the porcine intestine. These findings are consistent with the results of our previous study on colicins and microcins in experimental pigs. Indomethacin and EcN administered together comprised the worst impact on bacteriocinogeny in the porcine gastrointestinal tract (compared to indomethacin

alone or probiotics alone).[22] EcN alone significantly broadened the width of the glandular part of colonic mucosa. We assess this finding as a positive trophic effect of probiotic bacteria on the porcine large bowel.

We are fully aware of possible limits of our study. Mild edema of the mucosa is difficult to assess accurately. This might influence particular measurements and may make the interpretation of acquired data difficult. We did not evaluate apoptosis. In general, its rate is low in the gastrointestinal tract but might be induced by NSAIDs[23] and modulated by probiotic bacteria.[15] Further markers of mucosal damage/proliferation/apoptosis (proliferating cell nuclear antigen, Ki67, inducible NO synthase, caspase and others) and/or effect on mucins might be considered and addressed in future studies. And last but not least, we did not focus on possible functional changes of the gastrointestinal tract due to action of indomethacin and/or probiotic bacteria.

There are only few data on morphometric analysis in the available literature.[24–27] A previously published paper focused on indomethacin-induced injury but not on the synchronous effect of NSAIDs and probiotics. Ettarh et al.[24,26] performed morphometric analyses in the indomethacin-treated mouse. They found a significant decrease in villous height,[24] decreased epithelial volume, crypt losses and increased mitotic activity in the non-ulcerated parts of the murine small intestine.[26] Driak et al.[28,29] were probably the first to quantify morphometric changes of the rat gastrointestinal tract (after gamma-irradiation). We partly adopted their methods for our experimental setting.

In conclusion, detailed morphometric analysis showed a different effect of indomethacin and probiotic bacteria on particular parts of the porcine gastrointestinal tract. Indomethacin alone induced marked adaptation changes in the gastric mucosa. EcN alone provided a favourable trophic effect on the colonic mucosa. However, indomethacin and probiotics administered together might comprise the worst impact on all porcine stomach, small and large bowel. Further studies are needed to clarify this phenomenon. This should be helpful in the context of possible interaction of probiotics or EcN and intestinal transport of model drugs, xenobiotics and/or function tests.

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Declaration of conflicting interest

The authors disclose no conflicts of interest.

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Experimental Administration of the Probiotic *Escherichia coli* Strain Nissle 1917 Results in Decreased Diversity of *E. coli* Strains in Pigs

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Abstract The strain *Escherichia coli* Nissle 1917 (EcN) is widely used as an efficient probiotic in therapy and prevention of human infectious diseases, especially of the intestinal system. Concurrently, small adult pigs are being used as experimental omnivore models to study human gastrointestinal functions. EcN bacteria were applied to 6 adult healthy female pigs in a 2-week trial. 6 Control animals remained untreated. Altogether, 164 and 149 bacterial strains were isolated from smear samples taken from gastrointestinal mucosa in the experimental and control group, respectively. Each individual *E. coli* strain was then tested for the presence of 29 bacteriocin-encoding determinants as well as for DNA markers of A, B1, B2 and D phylogenetic groups. A profound reduction of *E. coli* genetic variance (from 32 variants to 13 ones, $P = 0.0006$) was found in the experimental group, accompanied by a lower incidence of bacteriocin producers in the experimental group when compared to control (21.3 and 34.9%, respectively; $P = 0.007$) and by changes in the incidence of individual bacteriocin types. The experimental administration of EcN strain was not sufficient for stable colonization of porcine gut, but induced significant changes in the enterobacterial microbiota.

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Introduction

Alfred Nissle introduced the strain *Escherichia coli* Nissle 1917 (EcN) to world literature in 1918 [16]. Immediately, Nissle started to apply it as a causative remedy in cases of chronical intestinal disorders [16]. Since then, the EcN strain, serologically typified to the serotype O6: K5: H1, has gained world-wide medical recognition and popularity, being increasingly applied as a safe probiotic, especially under the industrial denomination MUTAFLOR®. Initially, it was applied as human medicine, being effective not only in therapy [13], but also in prevention of infectious diseases of the intestinal tract [14]. Gradually, the positive probiotic role of EcN was proved in therapy of human inflammatory bowel diseases (ulcerative colitis, Crohn disease [20]), in treatment and prevention of infectious enteritis caused by various bacterial pathogens [2], in enhancement of immunity in newborn and premature infants [6], followed by the analogous positive experience of preventing both experimental and natural infectious diseases in animals—first in mice, then beef cattle and—since 2006, in pigs [21].

In piglets, an efficient prophylactic effect of orally administered EcN strain against the epidemic pathogenic action of the porcine enterotoxigenic *E. coli* strain—fatal in pork livestocks—was found [21]. Soon thereafter, it was published that the EcN strain colonizes the piglet's

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intestine, being able to survive there for at least 4 weeks following its oral administration [3]. At about the same time, this strain was found, surprisingly, as a sporadic natural component of intestine microflora of pigs in some swine herds in Germany [12]. Nevertheless, the EcN strain provokes only sporadic and minor effects on the distribution of mucosal cells of the pig's gut immunity system [7]. Moreover, the efficiency of the intestine long-term colonization of individual pigs is highly variable [3].

Since, the explanation of the generally accepted probiotic effect of the EcN strain (MUTAFLOR®) is especially in veterinary medicine burdened by too many uncertainties, gaps and contradictions, we undertook a study extending our previous study [4] and mapped the occurrence of the EcN strain in the bacterial microflora of pig intestinal tract, classifying phylogenetic groups and the presence and types of bacteriocin-encoding determinants.

Materials and Methods

Twelve healthy young adult female pigs (*Sus scrofa* f. *domestica*, hybrids of Czech White and Landrace breeds, 4–5 months old) weighing from 32 to 37 kg (median of 32.8 kg; average of 32.3 ± 0.8 kg and 33.7 ± 1.8 kg for experimental and control animals, respectively) were included in our study. The experimental group of 6 animals received one dose a day of 3.5×10^{10} live EcN bacteria for 14 days, whilst 6 control animals remained untreated. All animals were fed twice a day with an equal amount of standard food A1. Following 24 h of fasting since the last dose, the pigs were sacrificed and exsanguinated. The project was approved by the Animal Care Committee Institutional Review Board of the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic, Record Number 1492006. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Bacterial isolations and identifications were performed as described previously [4]. Following autopsy, smears from mucosa of the jejunum, ileum, caecum, transverse colon and rectum were taken in each animal and immediately inserted into a transport liver-enriched broth. Standard primary agar cultures were used for clone isolation. Up to seven different colonies of coliform bacteria were isolated from each sample. Coliform bacteria were identified by the VITEK2 system (BioMérieux, Marcy l'Etoile, France) and stored at −80°C.

The bacteriocin production of all strains was tested in parallel on four different agar plates containing (i) TY medium, (ii) nutrient broth, (iii) TY medium supplemented with mitomycin C and (iv) TY medium supplemented with trypsin as described previously [23]. The previously described set of *E. coli* indicator strains including *E. coli* K12-Row, C6 (φ), B1, P400 and S40 was used to identify the producer strains together with *Shigella sonnei* 17 indicator [1, 9, 11, 24, 25]. All investigated *E. coli* strains were tested on four parallel plates against six indicator strains stated. All investigated strains were tested with colony PCR. A bacterial colony was resuspended in 100 µl of sterile water, and 1 µl of this suspension was added to the PCR reaction. Individual colicin types (colicins A, B, D, E1-E9, Ia, Ib, Js, K, M, N, S4, U, Y, 5 and 10) were detected using previously published PCR primer sets [8, 23]. Control bacterial producers stemmed from our stock and were published recently [23]. The PCR detection primers for 5 microcin types including B17, H47, J25, L and V were taken from Gordon and O'Brien [8], primers for microcin C7 were taken from Šmajs et al. [22]. The phylogenetic group of each *E. coli* strain was determined using the triplex PCR protocol according to Clermont et al. [5]. Sequence analysis was performed using Lasergene software (DNASTAR, Inc., Madison, WI, USA).

Statistical significance analyses of genotypes and bacteriocin types in both strain groups were performed by applying standard methods derived from the binomial distribution, including the two-tailed test. *STATISTICA* version 8.0 (StatSoft, Tulsa, OK, USA) was used for statistical calculations. Alternatively, an interactive calculation tool for χ^2 tests of "goodness of fit" and independence was used for the calculation of statistical significance of obtained results [18].

Results

Amongst 149 *E. coli* strains, isolated from smears from 6 control untreated animals, we found 32 various strain-specific genetic variants with average number of identified strains of 6.8 per animal (ranging from 5 to 9). These variants resulted from combining specific *E. coli* phylogenetic groups and types of bacteriocin-encoding determinants. Our results (see Table 1) showed 17 variants of the phylogenetic group A, 6 of the group B1, 1 of group B2 and 8 of the group D. 51 isolates (i.e. 34.2%) produced colicins, 33 isolates (22.1%) produced microcins and 32 isolates (21.5%) both colicins and microcins. The most frequently produced colicin type was Ia (23 isolates, 15.4%), the most frequent microcin was V (27 isolates, 18.1%), nearly always in coproduction with colicins, most frequently with colicins Ia or E1. The only microcin produced separately from colicins was microcin J25, produced by the only strain of the phylogenetic group B2. In the control animals' intestinal and gut microflora we found production of 9 individual colicin types or combinations

thereof, along with production of 4 microcins V, H47, C7 and J25 or combination of V and H47. No type prevalence of either bacteriocin combination could be detected. The marker combination of the phylogenetic group B2 and production of microcin H47 (which were proved for the EcN strain [8, 15]) could not be found in any of the tested smears.

From smears of 6 pigs, the diet of which was enriched with 14 daily doses of the probiotic, i.e. with 3.5×10^{10} live EcN bacteria, we could isolate only 13 variants amongst 164 *E. coli* tested strains, 5 of which were additional to these of the control isolates (see Table 2 for details). The average number of identified strains was 5.5 per animal (ranging from 3 to 8). 6 Strains belonged to the phylogenetic group A, 3 strains to group B1, 1 to group B2 and 3 to group D. Only 35 isolates (21.3%) produced colicins, 4 isolates (2.4%) produced microcins, all of these in coproduction with colicins. Again, the most frequently produced colicin was Ia (18 isolates, 11.0%). The most frequently produced microcin was H47 (3 isolates, 1.8%), in coproduction with various colicins. Neither in the experimental animals, treated orally with the EcN strain, we were able to identify a single isolate with markers identical with the EcN strain in their intestine and bowel contents.

Statistical comparisons of the experimental and the control results are shown in Table 3. The proportions of the two phylogenetic groups (B2, D) were about the same in both experimental and control groups, whilst there was a significant increase of phylogroup A (92.7 and 77.2% in experimental and control group, respectively, $P < 0.0001$) and decrease in the incidence of phylogroup B1 amongst experimental strains (3.0 and 14.8% in experimental and control group, respectively, $P < 0.0001$). The incidence of bacteriocinogenic strains was significantly lower in the experimental group when compared to control group (21.3 and 34.9%, respectively, $P = 0.007$). Similarly, the incidence of microcin producers was significantly lower in experimental group (2.4 and 22.1% in experimental and control group, respectively, $P < 0.0001$). The proportion of

Table 1 Genetic variants of *E. coli* found in 149 strains isolated from control pigs gastrointestinal tract

Genetic variant serial no.	Phylogenetic group	Producer of		Number of isolates	
		Colicin	Microcin	Absolute	Percent
1	A	–	–	83	55.70
2	A	B	–	1	0.67
3	A	B, M	–	1	0.67
5	A	E1	V	5	3.36
6	A	E1, Ia	–	1	0.67
7	A	E1, Ia	V	2	1.34
8	A	E1, Ib	–	1	0.67
9	A	E2	–	1	0.67
10	A	E2, Ia	–	1	0.67
11	A	Ia	–	1	0.67
12	A	Ia	V	9	6.04
13	A	Ia	V, H47	2	1.34
14	A	Ib	–	1	0.67
15	A	Ib	V	1	0.67
16	A	Ib	V, H47	2	1.34
17	A	S4	V	1	0.67
18	A	S4	H47	1	0.67
19	B1	–	–	13	8.72
20	B1	E1, Ib	V, C7	1	0.67
21	B1	Ia	–	1	0.67
22	B1	Ia	V	1	0.67
24	B1	Ib	–	5	3.36
25	B1	S4	V	1	0.67
27	B2	–	J25	1	0.67
29	D	B, Ia, K, M	H47	2	1.34
30	D	B, Ib, K, M	H47	2	1.34
31	D	E1	–	2	1.34
32	D	Ia	–	1	0.67
33	D	Ia	V	1	0.67
34	D	Ia	V, H47	1	0.67
36	D	M	–	1	0.67
37	D	S4, U	–	1	0.67
Nissle 1917 (EcN)	B2	–	H47	–	–

Table 2 Genetic variants of *E. coli* strains found in 164 strains isolated from experimental pigs gastrointestinal tract

Genetic variant serial no.	Phylogenetic group	Producer of		Number of isolates	
		Colicin	Microcin	Absolute	Percent
1	A	–	–	123	75.00
3	A	B, M	–	1	0.61
4	A	E1	–	13	7.93
11	A	Ia	–	12	7.32
12	A	Ia	V	1	0.61
14	A	Ib	–	2	1.22
19	B1	–	–	1	0.61
21	B1	Ia	–	3	1.83
23	B1	Ia	V, H47	1	0.61
26	B2	–	–	1	0.61
28	D	–	–	4	2.44
29	D	B, Ia, K, M	H47	1	0.61
35	D	K	H47	1	0.61

Table 3 Most significant quantitative differences of markers investigated in *E. coli* isolates from gut smears of control (untreated) and of treated pigs

Marker	Incidence in strains isolated from animals		
	Control (%) $n = 149$	Treated (%) $n = 164$	Significance of difference
Number of genetic variations found amongst isolates	32 (21.5)	13 (7.9)	$P = 0.0006$
Incidence of bacteriocinogeny	52 (34.9)	35 (21.3)	$P = 0.007$
Incidence of microcinogeny	33 (22.1)	4 (2.4)	$P < 0.0001$
Incidence of producers of both colicin and microcin	32 (21.5)	4 (2.4)	$P < 0.0001$
Colicin Ib producers	13 (8.7)	2 (1.2)	$P = 0.002$
Microcin V producers	28 (18.1)	2 (1.2)	$P < 0.0001$
Incidence of phylogenetic group A	115 (77.2)	152 (92.7)	$P < 0.0001$
Incidence of phylogenetic group B1	22 (14.8)	5 (3.0)	$P < 0.0001$
Incidence of genetic variant 11	1 (0.7)	12 (7.3)	$P = 0.003$
Incidence of genetic variant 12	9 (6.0)	1 (0.6)	$P = 0.006$
Incidence of genetic variant 19	13 (8.7)	1 (0.6)	$P = 0.005$
Sole colicin E1 (no microcin) producers in phylogenetic group A	0 (0.0)	13 (7.9)	$P = 0.0004$

producers of 5 frequent colicins (Ia, E1, B, M and K) in both groups was comparable, decrease in incidence of colicin Ib was found in the experimental group (1.2 and 8.7% in experimental and control group, respectively, $P = 0.002$) as well as decrease in the incidence of microcin V producers (1.2 and 18.1% in experimental and control group, respectively, $P < 0.0001$). In the control group (of slightly less isolates), colicins S4, E2 and U were found that were not present in the experimental group. There was no significant, statistically provable relation of any colicin type to any phylogenetic group either in control or in experimental isolates. The *E. coli* strains isolated from experimental pigs contained 13 isolates of the group A producing solely colicin E1 (7.9% of all the isolates, whilst there was none in the control set; $P = 0.0004$). In the control set, the only 2 producers of the sole colicin E1 belonged to the phylogenetic group D.

Whilst 32 genetic variants (based on detection of bacteriocin-encoding determinants and *E. coli* phylogroups) appeared amongst control isolates, only 13 were present amongst experimental ones ($P = 0.0006$). From 13 genetic variants of experimental *E. coli* strains, 8 were the same as these found in the control set of and 5 were completely new.

Discussion

In this article, genetic analysis of *E. coli* strains from the intestine of experimental pigs after application of probiotic EcN bacteria (applied as industrial preparation MUTAFLOR®) was performed and these results were compared to controls. Control healthy pigs were fed with a standard diet, whilst experimental healthy animals were fed in the same manner, but with added live EcN strain during a 14-day trial. The intestinal tract of pigs, inhabited by commensal saprophytic enterobacteria in a way analogous to that of humans, is considered as a research animal model for a man.

The general incidence of bacteriocinogenic strains amongst pigs' standard *E. coli* appears to be 27.5%. This is distinctly less than amongst human standard *E. coli* strains where incidence of bacteriocinogeny varies between 43.4% [25] and 55.0% [23]. Veterinary strains isolated from pigs in many Czech Republic farms showed an incidence of bacteriocinogeny of about 70% (D. Šmajs, unpublished results). Since, all animals tested in this study were stalled together at the same place and obtained the same diet; it is likely that these factors might be one potential reason for a relatively low incidence of bacteriocinogeny. Another potential explanation focuses on the relatively low age of experimental animals and/or particular breed dependence.

We found a considerable decrease of incidence of *E. coli* clones (strains) producing bacteriocins, both colicins and microcins, accompanied by a narrower variance of colicin and types produced, as a consequence of the administered probiotic EcN strain. Also the incidence of clones (strain variants) of the phylogenetic group B1 was deeply decreased. Thus, the EcN strain distinctly interferes with the spectrum of *E. coli* strains and clones in the intestinal microflora, being able to change it quickly, although perhaps being present only in amounts undetectable by direct microbial cultivation from mucosal smears. One of the possible explanations includes EcN-mediated stimulation of the host immune-response with subsequent negative selection of some bacterial strains. However, other types of

selection mechanisms (e.g. mediated by microcins M and H47 produced by the EcN strain) should be considered. In contrast, the study performed by Prilassnig et al. [19] revealed that in humans, EcN intake induced increase in variation of isolated *E. coli* strains. Since only two selected parameters (DNA regions differentiating *E. coli* phylogroups and bacteriocin-encoding genes) were systematically tested in both experimental and control groups of strains, the question arises whether the observed decrease in genetic diversity is limited to these genetic determinants or not. Subsequent studies should test if additional markers of genetic diversity of *E. coli* strains will show the same trend and also if other bacterial species living in the porcine gut are influenced by oral administration of the EcN strain.

The EcN strain is believed to be able to colonize human intestinal tract [14, 15, 19]. In contrast, the EcN strain itself surprisingly could not be identified in any samples taken along the gastrointestinal tract of experimental pigs only 24 h after administration of more than 10 billions of living bacteria. The EcN strain is known to belong to the *E. coli* phylogenetic group B2 [10] and to produce microcins M and H47, but no colicin type [17]. The only explanation is that the dose of 3.5×10^{10} live bacteria daily, administered for 14 days, was not sufficient to reach the threshold concentration level in the porcine intestine contents to be isolated as a prevalent microbial enterobacterial strain. Thus, though being active in its probiotic function in the intestine, this strain was in no way dominant in its microflora, forming a minority of it. This unexpected result is in agreement with the experience of Bureš et al. [4] analyzing porcine alveolar breath for hydrogen and methane, just as with Duncker et al. [7] showing only minor effects of the EcN strain on the distribution of immunity cells in the gut mucosa of healthy pigs. Also, in the longitudinal study of Barth et al. [3], decreased incidence of samples positive for EcN DNA was found with increased time from EcN administration. Moreover, the study of Barth et al. [3] detected EcN strain using PCR-based detection of genetic determinants specific for the EcN strain. Therefore, positive PCR reactions were also obtained for bacterial numbers close to the detection limit of this method (i.e. 1,000 CFU g^{-1} of faeces [3]).

Taken together, the experimental administration of the probiotic *E. coli* EcN strain was not sufficient for stable colonization of porcine gut and therefore porcine model is of limited use in study of some human gastrointestinal functions. Nevertheless, EcN bacteria induced detectable and statistically significant changes in the enterobacterial microbiota in the porcine model.

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ORIGINAL ARTICLE

Wireless Capsule Endoscopy in Enteropathy Induced by Nonsteroidal Anti-inflammatory Drugs in Pigs

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Abstract
Aim The aim of this study is to evaluate the diagnostic yield of capsule endoscopy in nonsteroidal anti-inflammatory drug (NSAID)-induced enteropathy in pigs.
Materials and Methods Indomethacin (400 mg/day) was administrated orally for 10 days to eight female pigs weighing 36.3 ± 2.4 kg. Afterwards, capsule endoscopy was performed, using the EndoCapsule system (Olympus Optical Co., Tokyo, Japan). The following morning, pharmacological euthanasia and immediate autopsy were performed.
Results Small bowel injury compatible with NSAID-induced enteropathy was observed in 7/8 animals. The most common lesions were red spots and erosions. Ulcers and small intestinal bleeding were identified sporadically. Sensitivity and specificity of capsule endoscopy were 83.3% and 95.8%, respectively.
Conclusion Our results indicate that wireless capsule endoscopy is a highly accurate noninvasive method for evaluation of experimental NSAID-induced enteropathy.

Keywords Experimental pigs · Enteroscopy · Capsule endoscopy · Nonsteroidal anti-inflammatory drugs · Indomethacin · NSAID-induced enteropathy

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed drugs worldwide. These drugs can cause serious injury to any part of the gastrointestinal tract, including life-threatening complications such as bleeding or perforation [1].

The first clinical studies on use of capsule endoscopy in diagnostics of NSAID-induced enteropathy in humans have already been published [2–5]. However, there are only limited data on experimental NSAID-induced enteropathy [6]. Rainsdorf et al. [7] investigated fecal occult blood loss (using ^{59}Fe-prelabeled erythrocytes), gross pathology on autopsy, and mucosal myeloperoxidase activity of

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NSAID-induced gastropathy and enteropathy in experimental pigs, but did not deal with any endoscopy imaging.

Our group has developed a methodology of capsule endoscopy examination in experimental pigs [8]; capsule endoscopy findings from that study served as control data in the series reported herein. We previously presented our own model of indomethacin-induced gastrointestinal injury in experimental pigs, including preliminary data on the use of capsule endoscopy in its assessment [9]. The aim of this paper is to report detailed evaluation of the diagnostic yield of wireless capsule endoscopy in experimental NSAID-induced enteropathy.

Materials and Methods

Ethical Approval

The project was approved by the Institutional Review Board of the Animal Care Committee at the Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic (protocol number 149/2006).

Animals

Eight healthy mature (4–5-month-old) female pigs (*Sus scrofa* f. *domestica*, hybrids of Czech White and Landrace breeds) weighing 36.3 ± 2.4 kg were included in this study. All animals were fed twice a day (standard assorted food A1) with free access to water. Animals were held and treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [10].

Indomethacin Administration

Indomethacin was administrated as a one-shot dietary bolus to hungry animals in the morning for 10 consecutive days (400 mg/day, indomethacin suppositories; Berlin-Chemie, Germany).

Anesthesia

Capsule endoscopy was performed on day 11 of the experiment. All animals were kept under general anesthesia in supine position during the whole procedure (Fig. 1). Intramuscular injection of ketamine (20 mg/kg; Narkamon, Spofa, Praha, Czech Republic) and azaperone (2 mg/kg, Stresnil; Janssen-Pharmaceutica, Beerse, Belgium) were used to induce anesthesia, which was continued by infusion of 1% thiopental (up to 25 mg/kg; Valeant Czech Pharma, Praha, Czech Republic) into the lateral auricle vein. Syntostigmine was employed as a prokinetic agent, administrated as a bolus immediately after successful placement of the capsule endoscope into the duodenum in all animals (0.5 mg i.v.; Hoechst-Biotika, Martin, Slovakia). Infusions of 0.9% saline solution were chosen to secure basal hydration (1,000 ml/8 h). All animals were covered to prevent hypothermia during investigation.

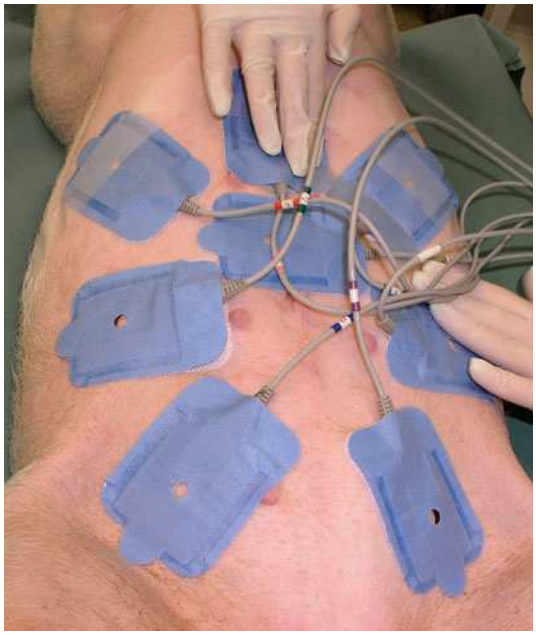

Fig. 1 Capsule endoscopy in the animal model. Notice the antennas fixed on the anterior abdominal wall

Capsule Endoscopy

The EndoCapsule system (Olympus Optical Co., Tokyo, Japan) was used in our study in all eight animals. The capsule endoscope was introduced into the duodenum by means of GIF-Q130 videogastroscope (Olympus Optical Co., Tokyo, Japan) using a retrieval basket G25 (Sun, Vikýřovice, Czech Republic) through an overtube placed inside the esophagus and stomach (Fig. 2).

After the investigation had been concluded (the main limitation being the lifetime of the batteries of 8–9 h), raw data were recorded into the computer and the endoscopy video was reconstructed. All findings were evaluated by means of Endo Capsule software (Olympus Optical Co., Tokyo, Japan) by a single gastroenterologist.

We used the method of rough estimation (using the time passed from the duodenal bulb) for capsule localization in the small bowel, because there are no clear endoscopy markers of borderlines between the jejunum and ileum (approximately one-half comprises the jejunum and the other half the ileum). The time borders between the small intestine segments were calculated from the presumptive speed of capsule endoscope advance (4 cm/s in our pilot

Fig. 2 The capsule endoscope is grasped in the basket before its endoscopy-assisted insertion into the duodenum

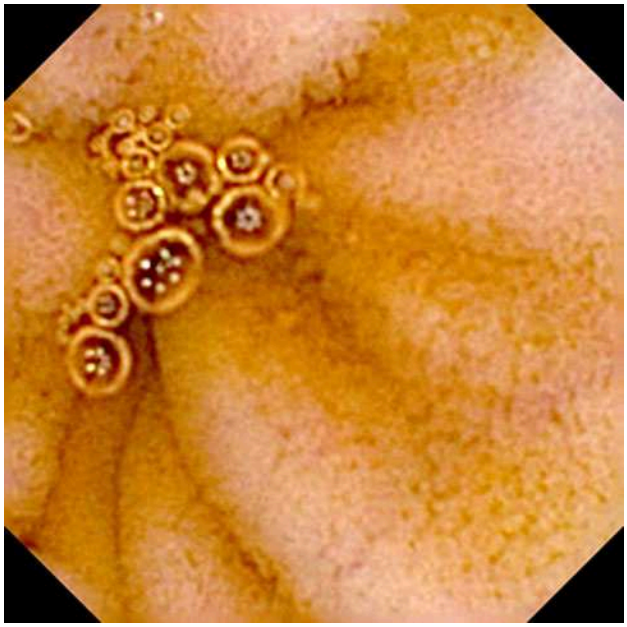

Fig. 3 Normal jejunal mucosa. Capsule endoscopy

testing set [8]) and mean length of the small bowel (ascertained during immediate autopsy), with the jejunum at time 10 min, the borderline of the jejunum and ileum at approximately 150 min, and the terminal ileum at time >480 min [8]. The regional transit abnormalities observed especially in the distal parts of ileum were excluded from the capsule position calculation.

The endoscopy findings were classified into four degrees: (1) normal small intestinal mucosa (Fig. 3); (2) mild (red spots, erosions or aphthae up to 10, mucosal erythema; Figs. 4, 5, and 6); (3) moderate (10–20 erosions or aphthae);

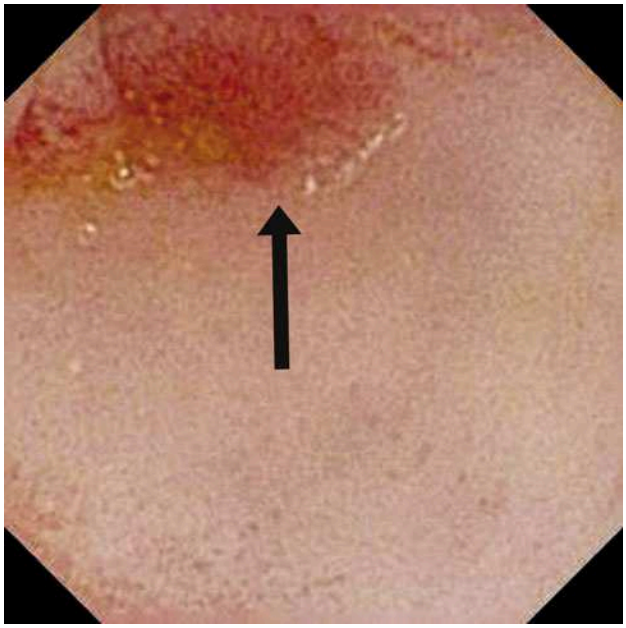

Fig. 4 *Red spot* in the proximal jejunum—*arrow* (mild enteropathy). Capsule endoscopy

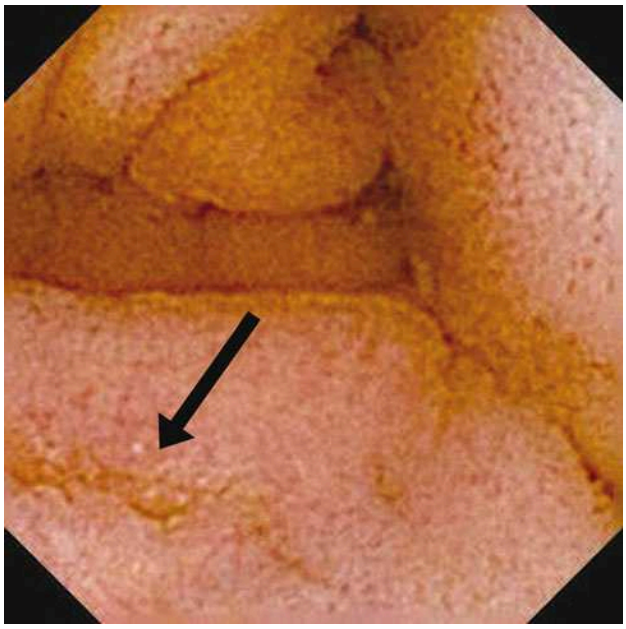

Fig. 5 Linear erosion in the proximal jejunum with normal surrounding mucosa—*arrow* (mild enteropathy). Capsule endoscopy

and (4) severe enteropathy (more than 20 erosions or aphthae and/or ulcer and/or visible fresh blood; Fig. 7).

Autopsy

Twenty-four hours after capsule endoscopy, the pigs were sacrificed by means of pharmacological euthanasia

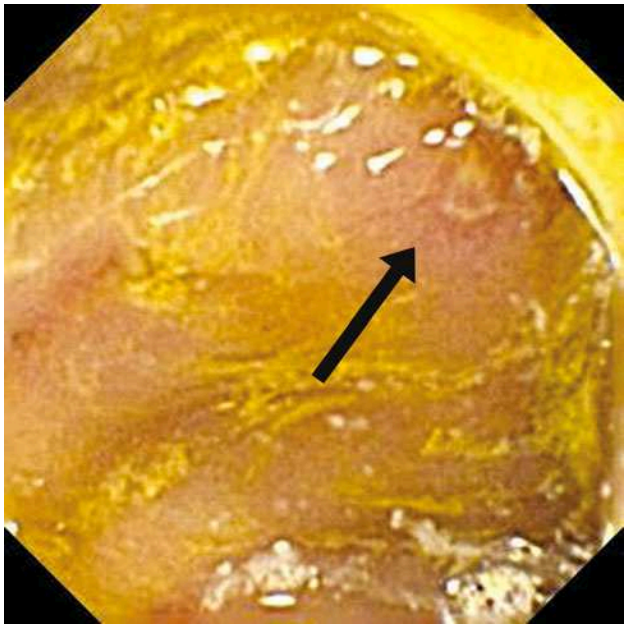

Fig. 6 Ileal erosion with mild border erythema—*arrow* (mild enteropathy). Capsule endoscopy

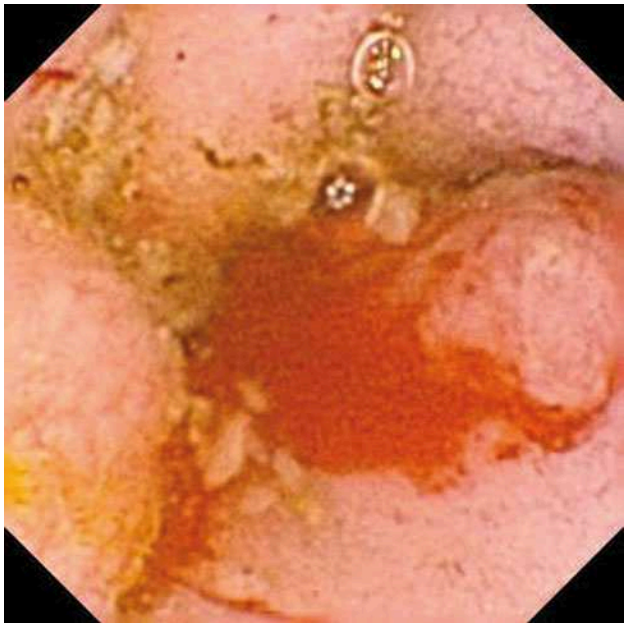

Fig. 7 Acute bleeding into the distal duodenum (severe enteropathy). Capsule endoscopy

(i.v. administration of embutramide, mebezonium iodide, and tetracaine hydrochloride—T61; Intervet International BV, Boxmeer, The Netherlands; dose of 2 ml/kg) and exsanguinated. Immediate autopsy was performed and gross pathology was recorded.

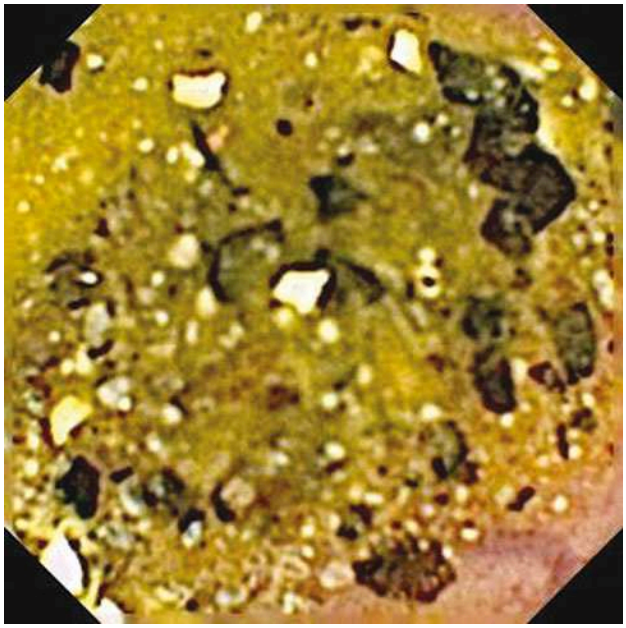

Fig. 8 Plant and food remnants in the distal ileum worsening visibility of the small bowel mucosa. Capsule endoscopy

Results

Capsule endoscopy was accomplished in all included animals, and neither technical nor clinical complications of the investigation were observed. All endoscopy capsules were introduced into the duodenum successfully by means of a gastroscope (procedure time: mean 7 min, median 3 min). The total time of capsule endoscopy (battery lifetime) was 481–549 min (mean 501 ± 21 min, median 494 min). The entire small bowel was investigated in one case only (no. 6). In five animals (nos. 1, 3, 4, 5, and 7) the terminal ileum and in two (nos. 2 and 8) the distal ileum was reached during the procedure. On immediate autopsy (the day after capsule endoscopy) the capsule was found in the cecum in all cases. Total length of the small intestine, including the duodenum, was 11.2–16.8 m (mean 13.3 ± 2.3 m; median 12.6 m) at immediate autopsy.

Evaluating capsule endoscopy, visibility of the small intestinal mucosa was limited in parts of the ileum due to intestinal content (Fig. 8) (in 6/8 animals, comprising 27 ± 7% of images recorded from the ileum).

Findings compatible with NSAID enteropathy were observed in 7/8 experimental pigs (Figs. 3, 4, 5, 6, and 7) and were confirmed in 50.0% (6/12) on gross autopsy (Table 1). Severe enteropathy represented by acute duodenal bleeding was classified on capsule endoscopy in one pig (no. 8; Fig. 7) only. In six animals, we observed erosions in the jejunum and ileum (nos. 1, 3, 5, and 6; Figs. 5 and 6) or multiple red spots in the duodenum, jejunum, and ileum (nos. 1, 2, 3, and 4; Fig. 4). In those four animals red

spots were seen on capsule endoscopy but were not confirmed on autopsy (Table 2). Although the capsule was introduced endoscopically into the duodenum, capsule endoscopy also revealed NSAID-induced injury apart from in the small bowel (pyloric ulcer when looking back at the pyloric region and gastric erosions during the capsule's return into the antrum and back in 2/8 animals).

Discussion

The purpose of this study was to determine the diagnostic yield of capsule endoscopy in noninvasive mucosal imaging of an acute model of NSAID-induced enteropathy. Small intestinal injury compatible with NSAID-induced enteropathy was observed in 7/8 animals. The most frequent findings on capsule endoscopy were mild mucosal injury (multiple red spots and erosions), in 6/8 animals. Its clinical impact is probably low. Although human capsule endoscopy studies reported such lesions in up to 40% of healthy volunteers or controls [2, 4, 11], in our pilot project (used as control group) capsule endoscopy findings were entirely normal in all healthy animals [8]. This discrepancy can be explained by markedly worse visibility of the small bowel mucosa in pigs, resulting in incomplete examination (see comments below) with risk of missing isolated red spots. In four animals, red spots seen on capsule endoscopy were not confirmed on autopsy, probably due to the etiology of these changes (mucosal congestion could hardly be revealed on gross autopsy). Clinically more important lesions such as acute small bowel bleeding were observed less frequently, in one animal only.

Most data on experimental NSAID-induced enteropathy have been published for acute or chronic studies using rat models [12–21]. We decided to use experimental pigs in our project because of similarities with human gastrointestinal anatomy and physiology [22]. We are aware of only one study on NSAID-induced intestinal injury in pigs. Rainsford et al. [7] found multiple gastric ulcers on autopsy in all nine male experimental pigs after 10-day peroral indomethacin administration (5 or 10 mg/kg/day). One duodenal ulcer and multiple superficial erosions and/or ulcers were revealed in the cecum, but surprisingly no mucosal lesions were found within the small bowel. They

Table 1 Small bowel findings on capsule endoscopy and autopsy

Animal number	Investigation method	Duodenum	Jejunum	Ileum	Terminal ileum
1.	Capsule endoscopy	Erosions	Erosion, red spots	Normal findings	Normal findings
	Autopsy	Normal findings	Erosions	Normal findings	Normal findings
2.	Capsule endoscopy	Red spots	Red spots	Red spots	x
	Autopsy	Normal findings	Normal findings	Normal findings	Normal findings
3.	Capsule endoscopy	Red spots	Erosions	Normal findings	Normal findings
	Autopsy	Normal findings	Erosions	Erosions	Normal findings
4.	Capsule endoscopy	Normal findings	Red spots	Normal findings	Normal findings
	Autopsy	Normal findings	Normal findings	Normal findings	Normal findings
5.	Capsule endoscopy	Erosions	Erosions	Normal findings	Normal findings
	Autopsy	Erosions	Erosions	Normal findings	Normal findings
6.	Capsule endoscopy	Normal findings	Normal findings	Erosions	Normal findings
	Autopsy	Normal findings	Normal findings	Erosions	Normal findings
7.	Capsule endoscopy	Normal findings	Normal findings	Normal findings	Normal findings
	Autopsy	Normal findings	Normal findings	Normal findings	Normal findings
8.	Capsule endoscopy	Bleeding	Normal findings	Normal findings	x
	Autopsy	Blood	Blood	Normal findings	Blood

Table 2 Sensitivity and specificity of capsule endoscopy

Autopsy				
Capsule endoscopy	Mucosal breaks[a]	Mucosal breaks[a]	Normal findings	
		5	1	Positive predictive value: 83.3%
	Normal findings	1	23	Negative predictive value: 95.8%
		Sensitivity: 83.3%	Specificity: 95.8%	

[a] Mucosal breaks were defined as areas of visible broken mucosal surface

did not use any endoscopic imaging in their study. We decided on the same dose of indomethacin (10–11 mg/kg/day) for 10 days in young adult experimental female pigs; however, such a dose is twice as high as the upper recommended limit for the daily dose in adult humans (200 mg). Despite this we consider our data comparable to those of human studies, because sporadic experimental data prove that plasma concentrations after high-dose indomethacin (10 mg/kg) in pigs are within the range encountered during arthritis therapy in humans [7]. The possible stronger local effect remains questionable, but the drug concentrations in the small intestinal mucosa were the same for lower (5 mg/kg) and high doses of indomethacin [7]. We are aware of the need for further pharmacological data to identify the best comparable dose of this drug in experimental pigs in relation to humans.

Our study revealed the presence of small intestinal mucosal lesions induced by oral indomethacin on capsule endoscopy in the majority of experimental pigs (87.5%). These findings were confirmed in 50.0% on autopsy. Prevalence of NSAID-induced enteropathy in humans treated with nonselective NSAIDs is 55–78% in the available literature (mostly for chronic users) [3, 5, 23, 24]. We also confirmed the utility of capsule endoscopy in this experimental model for NSAID-induced enteropathy research.

Using gross autopsy as a gold standard we were able to set the diagnostic yield of capsule enteroscopy for major small intestinal lesions, so-called mucosal breaks (erosions, aphthae, ulcers). Thus sensitivity, specificity, and positive and negative predictive values of capsule endoscopy for NSAID-induced enteropathy in experimental pigs were all relatively high (sensitivity and positive predictive value: 83.3%, specificity and negative predictive value: 95.8%). Our data are comparable to those published in clinical capsule endoscopy studies in humans [3, 5, 23, 24]. The authors admit the theoretical risk of missing some small lesions on autopsy (sensitivity is probably lower than 100%), but this risk is even higher for other remaining diagnostic in vivo methods (double-balloon enteroscopy, intraoperative enteroscopy, roentgen studies) to the best of our knowledge.

We are fully aware of possible limitations of our study. The number of animals used was limited (eight tested pigs, five controls); nevertheless, we think that such a number is comparable to similar experimental studies. Despite gastroscopic delivery of wireless capsule endoscope into the duodenum in all animals (to prevent its delay in the stomach), the majority of capsule endoscopes ran out of power in the distal ileum, so that the entire small bowel was investigated in only one animal (12.5%). There are several reasons for this problem. The total length of the small bowel, regional transit abnormalities of endoscopy capsule, and motility changes during general anesthesia can be considered the most important ones. However, no significant mucosal lesion (mucosal break) was found in the distal/terminal ileum beyond the reach of capsule endoscopy in any animal on autopsy. Most lesions were seen in the duodenum (five) and jejunum (six) in our study. In clinical studies in humans (with half the small bowel length of pigs) the entire small intestine is investigated in only about 66–75% [25–27]. Another possible limit of our study was intestinal content (with remnants of food) in the ileum. This made detailed evaluation of the mucosa difficult. Nevertheless, in only one pig were some mucosal breaks not identified on capsule endoscopy, so the negative predictive value in our study is high (95.8%).

The potential impact of other drugs used in our study on small bowel mucosa or blood flow could also represent possible problems in interpretation. To the best of our knowledge, small bowel lesions (ulcers, erosions, red spots) following one-shot administration of ketamine, azaperone, thiopental, syntostigmine, embutramide, mebezonium, and tetracaine have not been described.

In conclusion, wireless capsule endoscopy is highly accurate in noninvasive evaluation of mucosal lesions (mucosal breaks) in NSAID-induced enteropathy in experimental pigs. To the best of our knowledge, this is the first paper on capsule endoscopy in NSAID-induced small intestinal injury in experimental pigs. We think that it provides important new knowledge in two regards: (1) setting up and working out the methods for such an experimental model, and (2) providing a basis for further preclinical studies on treatment of experimental NSAID enteropathy.

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interdisciplinary toxicology

REVIEW ARTICLE

Preclinical electrogastrography in experimental pigs

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ABSTRACT
Surface electrogastrography (EGG) is a non-invasive means of recording gastric myoelectric activity or slow waves from cutaneous leads placed over the stomach. This paper provides a comprehensive review of preclinical EGG. Our group recently set up and worked out the methods for EGG in experimental pigs. We gained our initial experience in the use of EGG in assessment of porcine gastric myoelectric activity after volume challenge and after intragastric administration of itopride and erythromycin. The mean dominant frequency in pigs is comparable with that found in humans. EGG in experimental pigs is feasible. Experimental EGG is an important basis for further preclinical projects in pharmacology and toxicology.

KEY WORDS: electrogastrography; preclinical studies; experimental pig

This paper is dedicated to the memory of Prof. Helena Rašková, MD., DSc.

Introduction

Surface electrogastrography (EGG) is a non-invasive means of recording gastric myoelectrical activity or slow waves from cutaneous leads placed over the stomach. Neuromuscular activities of the stomach generate electrical phenomena termed "gastric slow waves". The gastric myoelectrical activity is made up of two types of electrical signals termed slow waves or electrical control activity and superimposed spikes also called electrical response activity. The gastric pacemaker is located at the greater curvature of stomach adjacent to the junction between the fundus and the body (Chen & McCallum, 1994; Parkman *et al.*, 2003; Chang, 2005).

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Assessment of motor function of the stomach

Normal gastrointestinal motor function is a complex series of events that requires coordination of the sympathetic and parasympathetic nervous systems, neurons within the stomach and intestine, as well as the smooth muscle cells of the gut. Several tools are available to evaluate motor function and related disorders, like EGG, gastric emptying tests (gastric scintigraphy, breath tests using ^{13}C-octanoic acid, ^{13}C-acetate or spirulina, a plant based protein), gastroduodenal manometry, electronic barostat and planimetry of the gastric antrum by abdominal ultrasonography or magnetic resonance. Simultaneous measurement of intra-luminal pH and pressure by a special capsule is a new method of investigation (Camileri *et al.*, 1998; 2010). The capsule, when swallowed, can simultaneously measure phasic pressure amplitudes and pH as it traverses different segments of the gastrointestinal tract. The characteristic change in pH between the stomach and the small intestine provides an indication of the gastric emptying time for a non-digestible solid >1 cm long (Camilleri, 2010).

Fasted and postprandial recording running spectrum percent activity and changes in the aplitude (power

analysis) are the major measures for the evaluation of EGG. According to the American Motility Society (Parkman *et al.*, 2003), normal EGG frequency (2.0–4.0 cycles per minute) should comprise ≥70% of recording time in humans. The possible relationship between EGG and gastric emptying also remains controversial (Mintchev *et al.*, 1993; Bortolotti, 1998; Sanmiguel *et al.*, 1998; Sha *et al.*, 2009). The major parameters of EGG and gastric emptying measures (*e.g.* half-life of elimination in ^{13}C-octanoic acid breath test) mutually correlate in healthy humans (Bureš *et al.*, 2007). There could probably be a difference between healthy status and disease (*i.e.* diabetes mellitus, systemic sclerosis, functional dyspepsia, eating disorders, gastrectomy etc.) (Chen & McCallum, 1994; Diamanti *et al.*, 2003; Bureš *et al.*, 2007; 2008; Camileri *et al.*, 2010).

In humans, pathological changes at EGG, *i.e.* alterations in frequency (bradygastria, tachygastria, or mixed, dysrhytmias) and reduction in the amplitude of the postprandial electrical signal are seen in patients with idiopathic and diabetic gastroparesis, functional dyspepsia, anorexia nervosa, nausea of pregnancy, vector/motor sickness, Helicobacter pylori infection, status after gastric surgery. Dysrhythmias have also been described in patients with functional dyspepsia, with or without evidence of gastric stasis (Chen & McCallum, 1994; 2006; Bureš *et al.*, 1998; 2008; Sanmiguel *et al.*, 1998; Ogawa *et al.*, 2004). In healthy people, dominant frequency varies during the day (maximum frequency at midday and minimum frequency during the night) (Lindberg *et al.*, 1996). Age, sex (including menstrual cycle in women) and body-mass index might influence EGG in humans (Real Martínez *et al.*, 2001; Tojl *et al.*, 2007). However, even more physiological and/or social events can influence EGG in humans, like meals and volume challenge, ethanol intake, listening to enjoyable music etc. (Levanon *et al.*, 1998; Lin *et al.*, 2007; Kobak & Bor, 2007).

Importance of preclinical studies

In humans, several drugs were tested to influence myoelectrical activity of the stomach, like prokinetics (cisapride, tegaserod, domperidon), muscarinic M3 receptor agonists (cevimeline), hyoscine butylbromide, fentanyl and others (Chiba *et al.*, 2007; Walldén *et al.*, 2008; Americo *et al.*, 2009). Interestingly, probiotics and prebiotics might influence EGG in humans too. Probiotic *Lactobacillus reuteri* stimulated gastric emptying and improved maturation of the EGG activity mimicking the effect of breast milk in preterm infants (Indrio *et al.*, 2009).

The precise role of EGG in the clinical evaluation of patients or monitoring of therapeutic response to medications, and how this test adds to the information obtained from a gastric emptying test, remain the subject of ongoing research.

The pig, as a representative of the omnivore, is relatively close to man in a number of metabolic and physiological indicators (Květina *et al.*, 1999; Nobilis *et al.*, 2003; Anzenbacherová *et al.*, 2003). It is not uncommon that prediction focusing on the transfer of knowledge towards human drug therapy is based on precisely this experimental species (Květina *et al.*, 2009). Regarding body mass index, in the case of use of small adult pigs in a body weight range of 30–40 kg, the proportions of passive (fat) and active (muscle) masses are comparable to an adult man. Conversion of doses of studied xenobiotics and even manner and form of their administration becomes relatively comparable (Květina *et al.*, 2008b). Contrary to clinical observations, experimental design allows for standardisation of the experiment set and identification and definition of the test conditions.

Electrogastrography in experimental pigs

The small adult pig can be used in various preclinical experiments as a representative of the omnivore due to its relatively very similar gastrointestinal functions in comparison to man (Kararli, 1995). However, there are some distinct differences in the anatomy and physiology of the stomach between humans and pigs (Bureš *et al.*, 2009; Kopáčová *et al.*, 2010; Květina *et al.*, 2008a). The porcine stomach is pouch-shaped and the gastric cardia is close to the pylorus. A special transverse pyloric fold (torus pyloricus) serves as a "gate-keeper" (Kopáčová *et al.*, 2010). Gastric emptying of pigs is much slower, put through small separated amounts. There are significant remnants of food in the porcine stomach even after 36–48 hours of fasting (Kopáčová *et al.*, 2010; Tachecí *et al.*, 2009).

To the best of our knowledge, there are no previous reports on EGG in pigs in the available literature. Our group recently set up and worked out the methods for EGG in experimental pigs (Varayil *et al.*, 2009). All EGG recordings were carried out under general anaesthesia (introduction: intramuscular administration of ketamin and azaperone, repeated doses of thiopental when appropriate).

In our setting, all animals were lying in a right lateral position during the EGG recording. The epigastric area was shaved before application of electrodes to decrease impedance in signal conduction through the skin. Electrode placement always began with placing the first electrode roughly within 5 cm of the xyphoid process in the centre and then subsequently placing the other 2 roughly at a distance of 15 cm from the central electrode in left and right hypochondrium respectively (Figure 1). After connecting the device the recording was started and the animals were closely monitored for any flinging movement. A single EGG recording always lasted 30 minutes. All possible artefacts (especially motion ones) must be removed before the final evaluation. We used a running spectral analysis (based on Fourier transform) for the evaluation of the experimental EGG. Results are expressed as running spectrum percent activity. All low, medium and high frequencies of gastric slow waves were found in particular animals (Figures 2–7). The normal dominant frequency that we found in pigs in this project

(3.3 ± 0.5 cycles per minute) is fully comparable with those in adult humans (Varayil *et al.*, 2009). Thus our original assumption was proven – the young adult pig is a suitable model for experimental EGG.

Volume challenge

The water load test is a standardised test to induce gastric distension and to evoke gastric motility responses without the complex hormonal response of a caloric test meal. EGG with water load test has been validated as being reliable and reproducible in humans (Koch *et al.*, 2000; Chen *et al.*, 2006).

In our recent EGG study in experimental pigs, we decided on volume challenge of 360 mL water that is comparable with 500 mL usually used in adult humans. The mean dominant frequency after volume challenge was significantly higher compared with the basic measurement (Varayil *et al.*, 2009). Several studies performed previously in humans have shown that volume overload after drinking water generally affects both dominant frequency (to tachygastria) and dominant power (characterised by an increase in amplitude) which has been attributed to factors such as 1) gastric distension 2) gastric displacement 3) slow wave changes and/or 4) neurohumoral mechanisms (Chen *et al.*, 2006; Friesen *et al.*, 2007; Jones *et al.*, 2003; Lin *et al.*, 2000).

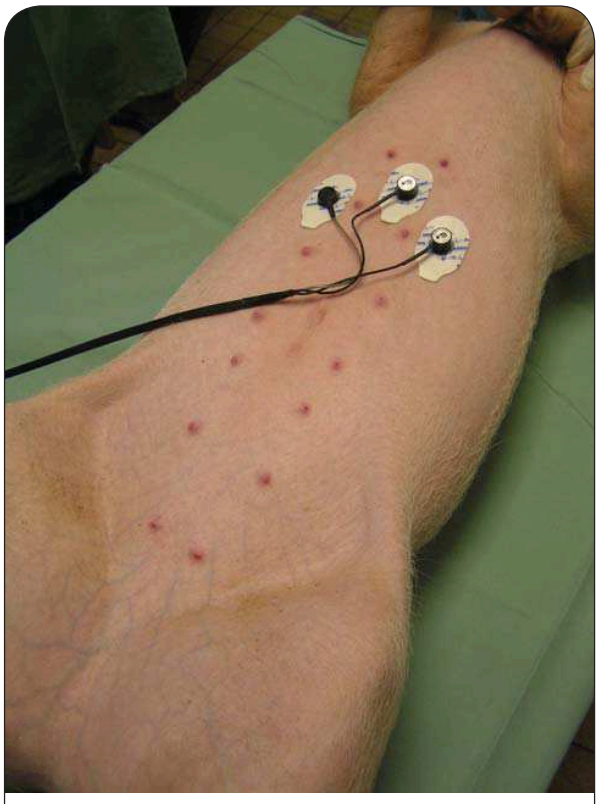

Figure 1. Electrogastrography in experimental pigs. General arrangement of the electrodes placement for EGG recording.

Itopride

Itopride is a dopamine D2 antagonist with acetylcholinesterase inhibitory activity that has prokinetic effects and probably effects on gastric accommodation and hypersensitivity (Longstreth, 2010).

We used itopride as a model prokinetic drug in experimental pigs. EGG recording was carried out immediately after intragastric administration of 100 mg itopride (*i.e.* ~3 mg/kg; corresponding to the maximum single dose for man). There was no obvious change in dominant frequency during subsequent EGG recording (Varayil *et al.*, 2009). However, it is possible that the dose was not big enough and/or there was not the required time to make it possible for itopride to exert its prokinetic effect. Itopride has linear kinetics. In humans, maximum plasmatic concentration (t-max) of itopride is reached at about 45 minutes after oral administration (half-time of elimination is 6 hours). Gastric emptying and start of intestinal absorption of itopride might be delayed under general anaesthesia (Schurizek, 1991; Umenai *et al.*, 2009). Iwanaga *et al.* (1996) studied the gastroprokinetic effect of itopride in conscious dogs. At a dose of 3 mg/kg, itopride did not affect gastrointestinal motility. With itopride 10 mg/kg the contractile force of the gastric antrum was increased (doubled) within 5 minutes after intra-duodenal administration of itopride (Iwanaga *et al.*, 1996).

Erythromycin

Macrolides are a group of closely related antibiotics characterised by a 14-, 15- or 16-membered lactone ring. Macrolides have been found to have pharmacodynamic properties beyond their antimicrobial mode of action, like anti-inflammatory, immunomodulatory and gastrointestinal prokinetic effects (Hawkyard & Koerner, 2007). Erythromycin is the most potent prokinetic drug available nowadays. Erythromycin has been shown to initiate gastric interdigestive migrating motor complexes, which are the motor events responsible for gastric emptying of indigestible solids. Erythromycin induces high amplitude gastric propulsive contractions that literally dump solid residue, including non-digestible materials, out of the stomach (Prather *et al.*, 1993; Keshavarzian, 1993; Curry *et al.*, 2001). Erythromycin also stimulates fundic contractility, or at least inhibits the accommodation response of the proximal stomach after food ingestion (Bruley des Varannes *et al.*, 1995; Fraser & Mittal, 1994; Curry *et al.*, 2001; Camilleri, 2010). Erythromycin seems to have a different mechanism of action in the stomach compared to the duodenum (Mathis & Malbert, 1995).

Erythromycin is used especially in the treatment of diabetic gastroparesis in humans (Camilleri, 2010). A systematic review identified 35 clinical trials involving erythromycin for gastroparesis of which five fulfilled inclusion criteria for the review (Maganti, 2003). All studies were small (≤13 subjects), of short duration (≤4 weeks) and had methodological weaknesses. Nevertheless, improvement

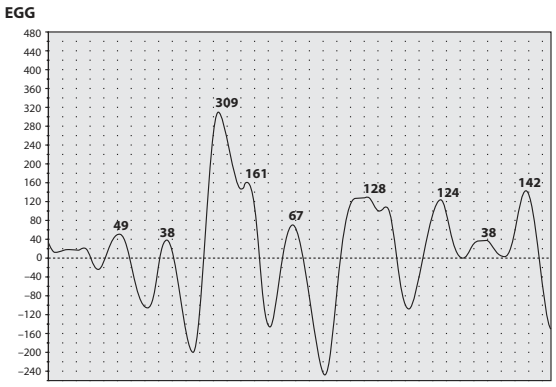

Figure 2. EGG rhythm of three cycles per minute at online recording.

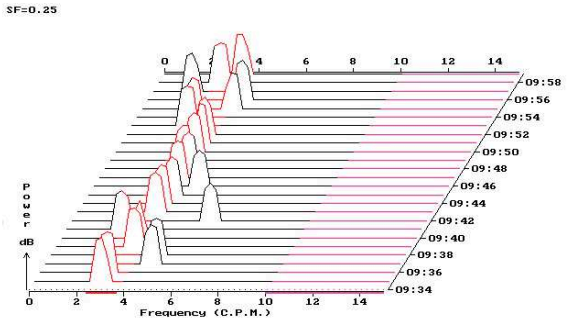

Figure 3. Protocol of EGG recording with prevailing rhythm of three cycles per minute (60% running spectrum percent activity).

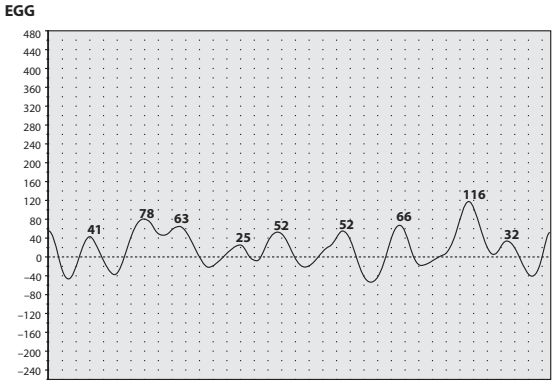

Figure 4. Pattern of bradygastria at online EGG recording.

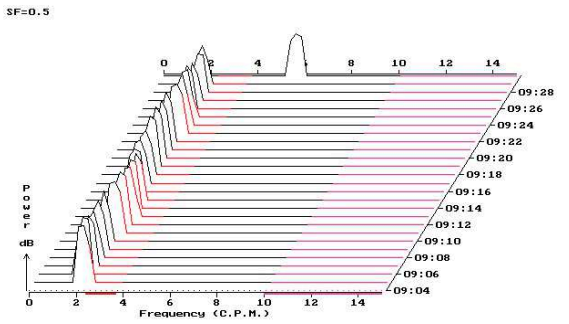

Figure 5. Protocol of EGG recording with prevailing bradygastria (96% running spectrum percent activity).

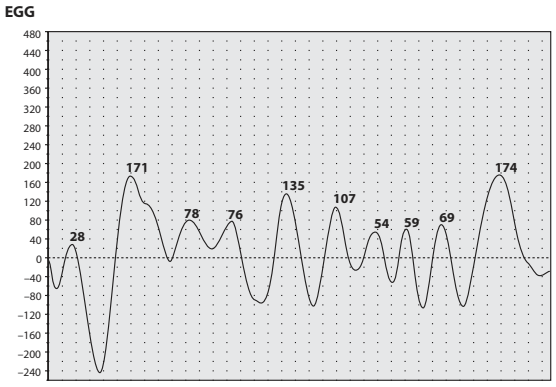

Figure 6. Pattern of tachygastria at online EGG recording.

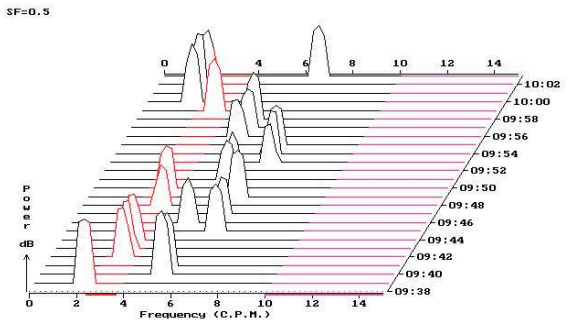

Figure 7. Protocol of EGG recording with prevailing tachygastria (60% running spectrum percent activity).

was reported in 26 of 60 patients (43%) (Maganti *et al.*, 2003). Van der Voort *et al.* (2003) investigated functional dyspepsia, irritable bowel syndrome and healthy controls. Disturbed gastric emptying correlated with a lack of postprandial increase in the EGG amplitude. Prokinetic erythromycin improved both gastric emptying and gastric electrical activity (van der Voort *et al.*, 2003).

In our latest project, we used EGG to test the effect of erythromycin on gastric myoelectrical activity in experimental pigs. Intragastric administration of a therapeutic

dose of erythromycin (1600 mg) substantially increased the gastric myoelectrical activity. There was a significant prokinetic effect of erythromycin (compared to baseline recording): a statistically significant increase in dominant frequency at recordings starting 90 minutes with maximum 330 minutes after erythromycin administration. In a human study in healthy volunteers, low dose i.v. erythromycin also achieved an increase in percent tachygastria (DiBaise *et al.*, 2001).

Tasks and perspectives for future

Further improvements in methods of EGG in experimental pigs are needed. Optimal placement of leads must be searched for before each recording (different for drug testing and volume challenge). Power analysis must become an inherent part of each evaluation. It will be also useful to verify and validate if the ratio of antral motor index is beneficial in experimental pigs. This index is calculated as the number of waves the sum of amplitudes (Faure *et al.*, 2000). And last but not least, standard protocol of EGG in experimental pigs must be completed.

Not only several drugs, but also different probiotic bacteria are to be tested by means of EGG in the near future. Experimental EGG is an optimal non-invasive method to investigate the motor effect of particular drugs.

Conclusions

EGG in experimental pigs is feasible. The mean dominant frequency in pigs is comparable with that found in humans. Experimental EGG is an important basis for further preclinical projects in pharmacology and toxicology.

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