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

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

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

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

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

INTRODUCTION

Caffeine (contained in coffee, tea, caffeinated beverages, cocoa, chocolate etc.) is the most consumed stimulant drug of abuse worldwide. With regard to coffee, epidemiological data support its irritant role in stomach and esophagus in association with gastroesophageal reflux leading to enhanced risk of ulcers and cancer in the afflicted areas1, 2. Recently, this irritant effect was also confirmed in young asymptomatic individuals3. The beverage is a complex mixture of possibly antagonistic substances comprising, apart from caffeine, isoflavones, polyphenols, diterpenes etc. exhibiting effects according to the type of coffee bean processing4, 5. To elucidate the cause of the irritant nature of coffee to the stomach, focusing on the effects of isolated and purified (pharmacologically) active components of coffee would lead to more information. Of these components caffeine is the most studied.

Maintenance of the gastric mucosal barrier integrity is dependent on the balance between aggressive and protective factors represented by hydrochloric acid on one hand and adequate mucosal blood flow with sufficient mucus production on the other. Caffeine, a known acid secretagogue, has long been suspected of causing mucosal hypoperfusion due to (micro)vascular impairment6. This hypothesis was later supported by electron microscopy7. More recent observations report suppressed acetylcholine (ACh)-induced mucus production8 by caffeine and gastric mucosal transmembrane potential difference9, completing the barrier-braking mosaic picture of caffeine. However, the aforementioned notion has been challenged by experimental observations showing enhanced mucosal blood flow by caffeine10 and a protective influence of this drug on mucosal barrier integrity11 suggesting actually a preventive role for caffeine in gastric mucosal injury12.

Caffeine is a methylxanthine with pluripotent and possibly opposing pharmacological actions. It is a non-selective adenosine receptor antagonist, phosphodiesterase inhibitor, ryanodine-sensitive Ca²⁺ channel activator and soluble guanylate cyclase (GC) inhibitor. As a consequence, these actions may interfere with nitric oxide (NO) production and/or its second messenger cyclic guanosine monophosphate (cGMP) pathway leading to modulation of a wide spectrum of mucosal barrier-related (patho)physiological effects exerted by NO including vascular tone regulation or modulation of oxidative stress
level\textsuperscript{13}. The literature, to the best of our knowledge, provides rather limited data on caffeine’s effect on gastric (sub)mucosal microcirculation and has not included parallel monitoring of local NO release (using microdialysis), so far. Similarly, the putative effect of caffeine on oxidative stress necessitates clarification. The aim of the present study was twofold: first, to evaluate the possible impact of caffeine on gastric submucosal microcirculation, nitric oxide production and morphology, and second, to measure plasma malondialdehyde (MDA) as a marker of oxidative stress (lipid peroxidation).

MATERIALS AND METHODS

Animals

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

Animal Preparation

All animals were anesthetized with single i.p. dose of pentobarbital (50 mg kg\textsuperscript{-1}, Nembutal\textsuperscript{16}, Abbott Laboratories, North Chicago, USA) and placed in a supine position on an unheated bed. They were kept unanesthetized for histological examination. The tissues surrounding the probe (0.5 × 1.5 cm) with the probe itself were then dissected and the biopsy immersed in 10% formaline for histological examination.

Substances

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

Gastric submucosal microdialysis technique

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

Experimental design

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

Probe performance stability and \textit{in vivo} recovery determination for nitrate

The measurement of NO using microdialysis technique was validated in two consecutive steps employing other two groups (A and B) of pentobarbital-anesthetized rats. First, probe performance stability for nitrate was tested continuously for 7 h (measurements during equilibration period inclusive, group A, n = 5) in one experiment based on an assumption of stable NO production throughout the study period. Microdialysis sampling in gastric submucosa was realized in 30 min intervals at a perfusion rate of 2 μl min\textsuperscript{-1}. As perfusate, normal saline was utilized. Second, \textit{in vivo} recovery of the same probe type was estimated (group B, n = 3) using zero-net flux method originally proposed by Lönnroth et al.\textsuperscript{16}. Four perfusion media of increasing concentrations of sodium nitrate in sterile saline were consecutively applied as follows. After the initial tissue equilibration (1 h) with 10 μmol l\textsuperscript{-1} NaNO\textsubscript{3}, a 30 min sample was collected. The perfusion medium was changed for 15 μmol l\textsuperscript{-1} nitrate and following 30 min equilibration, another sample harvest (30 min) ensued respecting the probe’s lag time (3 min). The experiment
was completed with 50 and finally 120 μmol l⁻¹ nitrate solutions. The results were plotted on a graph and probe recovery was read from the regression equation (slope gradient). Besides microdialysis, these two groups of animals underwent no further experimental treatment. All general steps (anesthesia, surgery, gastric submucosal microdialysis technique including probes but excluding perfusion media, sacrifice and analytical techniques) were equal to the experimental groups of the present study.

**Nutritive blood flow measurement**

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

**Biochemical analyses**

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

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**Fig. 1.** Morphological studies. Panel “a” confirms the position of the microdialysis probe within the submucosal layer of gastric wall (arrow). The catheter is surrounded by noticeable edema and hyperemia with diffuse inflammatory infiltrate of mild degree penetrating at some places into the mucosa. The probe’s tougher inner membrane was slightly damaged during tissue slicing (mu = mucosa, sm = submucosa, mp = muscularis propria). Panel “b” depicts the detail of the probe’s outer membrane permeated by polymorphonuclear leukocytes (arrow). Pictures “c-e” show normal gastric mucosa, i.e. grade 0, and typical lesions of grade I and II – detached cells and necrosis of pits (arrows), respectively. Standard hematoxylin-eosine stain, original magnification “a” 125×, “b” 500×, “c-e” 250×.
ELISA kits (Assay Designs, Ann Arbor, MI, USA) were utilized only. All procedure steps were performed according to the manufacturer’s instructions.

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

Plasma caffeine concentrations were determined using a modified HPLC method by Biederbick et al. The phase was evaporated under nitrogen at 40 °C and the samples were reconstituted in 150 μl of mobile phase, and 30 μl were injected into the HPLC column. Analysis was performed on a 2695 Waters Separations Module equipped with 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). The mobile phase was made up of an aqueous solution of sodiumdihydrogenphosphate (0.5 mmol l⁻¹, pH 5) to acetonitrile (ratio 88:12) and was pumped iso-critically at a flow rate of 0.9 ml min⁻¹. The temperature of the column was set at 40 °C. The quantification of caffeine was performed at 270 nm.

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

**Histological analyses**

Stomach biopsies were taken to evaluate possible effects of caffeine on tissue morphology at microscopic level and for probe position verification. The samples (0.5 × 1.5 cm) were taken from glandular segment surrounding the probe. The tissues were fixed in 10 % neutral buffered formalin and further treated according to standard procedures for hematoxylin–eosin (HE) stain. The sections were evaluated by a blinded professional observer and photographed at 125, 250 and 500-fold magnification. The grading criteria were adopted from Natale et al. and were as follows: grade 0 for normal mucosa, grade I for lysis and segregation of cells on the luminal surface (with intact pit cells), grade II for damage confined to gastric pits with detachment of the surface epithelium and grade III...
which involves injured gastric glands (whole-thickness mucosal necrosis with swelling and possible disconnection of mucosal layers)\(^2\).

**Statistics**

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

**RESULTS**

**Histology**

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

**Microdialysis data**

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

**Plasma analytes**

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

**DISCUSSION**

**Effect of caffeine on (sub)mucosal morphology**

Even though macroscopical observations (e.g. a computerized planimetry) are a “golden standard” in the evaluation of gross gastric mucosal injury for their ability to assess the whole organ, they are inconvenient for detection and scoring of minor (visibly hardly discernible) changes. Since, in accordance with literature\(^3\), the latter was also the case in our experiment, gastric mucosal impairment was studied microscopically using standard
histology. However, owing to the exactingness of the suggested method of whole organ histological evaluation\(^2\), we decided for the present time to grade the most prominent cellular/tissue changes found in comparable parts of glandular stomachs (in the vicinity of probe implantation site) of experimental groups. Since this time we did not quantify the lesions, the statistical approach was not feasible. Hence, the present study provides only qualitative data on the effects of microdialysis and caffeine on rat gastric tissue morphology.

**NO measurement using microdialysis**

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

**Effect of caffeine on NO production**

Besides vascular tone regulation, nitric oxide released by Ca\(^{2+}\)-dependent endothelial (e)NOS as well as Ca\(^{2+}\)-independent inducible (i)NOS plays a large number of (patho)physiological roles many of which may be related to mucosal defence and injury\(^{12}\). It is therefore of interest whether caffeine interferes with the release of this reactive pluripotent radical. Bruce *et al.* report of a significant decrease in exhaled NO levels in humans one hour after 100-200 mg caffeine intake\(^{26}\). The latest findings indicate that caffeine (16 mg kg\(^{-1}\) i.v.) may decrease nitric oxide synthase (NOS) expression in rat skeletal muscles\(^{37}\) and attenuate glutamate-induced NO synthesis in murine spinal cord *in vitro*\(^{28}\). Moreover, caffeine negates the protective effect of ischemic preconditioning, i.e. eliminates reactive hyperemia due to the hypoperfusion-induced accumulation of adenosine and enhanced NO production\(^{29}\).

In contrast, endothelium of isolated rat aorta responds to caffeine by promotion of NO synthesis\(^{30}\). In the present study, we failed to detect significant shifts in NO production up to 2.5 h after the application of increasing doses of caffeine. However, eNOS releases NO in nanomolar quantities, changes we may have been unable to detect with the analytical procedure we used while micromolar amounts of NO are produced by activated iNOS as soon as 2 h after application of inducing substance\(^{31}\). Therefore, our results indicate no effect of caffeine on Ca\(^{2+}\)-independent NO production in resting gastric submucosa of anesthetized rats.

**Effect of caffeine on blood flow**

Despite the generally recognized constricting role of caffeine in the brain\(^2\), heart\(^3\), limb\(^4\) or gut\(^5\) vasculature, the literature is inconsistent as far as gastric (sub)mucosal perfusion is concerned. Understandably, the discrepancy with respect to blood flow is reflected in the contrary effects of caffeine on acute induced gastric mucosal injury\(^36,23,12\). Ozturkcan *et al.* showed that a single i.p. injection of 7.5 - 30 mg kg\(^{-1}\) caffeine leads to elevations in rat gastric mucosal blood flow 90 min after drug application\(^39\). Moreover, Koyama *et al.* found that *ex vivo* intraluminal administration of caffeine doses as high as 50–100 mg kg\(^{-1}\) result in a dose-dependent increase in mucosal blood flow lasting up to 90 min\(^12\). Although in the present study some tendencies to decreased nutritive blood flow could be observed 30-90 min after caffeine administration, the results show a lack of statistically significant change in this variable throughout the experiment (Fig. 4b). This would be consistent with other studies showing no direct effect of caffeine on resting blood flow\(^37,39\) and/or induced vascular contractility\(^40\). Along these lines, there is conflict-

<table>
<thead>
<tr>
<th>i. p. caffeine dose (mg kg(^{-1}) b.wt.)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>50</th>
</tr>
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<tbody>
<tr>
<td>plasma caffeine (mg l(^{-1}))</td>
<td>0 ± 0.00</td>
<td>1.43 ± 0.07</td>
<td>11.80 ± 0.42</td>
<td>56.51 ± 1.68</td>
</tr>
<tr>
<td>plasma MDA (μmol l(^{-1}))</td>
<td>0.94 ± 0.05</td>
<td>0.91 ± 0.12</td>
<td>0.84 ± 0.03</td>
<td>0.80 ± 0.15</td>
</tr>
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Table 1. The table summarizes the effect of increasing doses of caffeine on plasma caffeine and malondialdehyde (MDA) levels at the conclusion of experiments. In spite of a negative trend, no statistically significant difference or association between administered caffeine and plasma MDA was found. Data are displayed as means ± SEM of 6 measurements.
The results of the present study indicate that i.p. administration of caffeine in given dosages does not produce acute macroscopic changes to gastric mucosa and is unlikely to alter gastric submucosal nutritive blood flow and nitric oxide production or aggravate systemic oxidative stress level. Additional histomorphometric studies are needed to describe the effect of caffeine on gastric mucosa and further attempts need to be made to explore possible mechanisms whereby caffeine might participate in irritant effects of coffee.

CONCLUSIONS

Effect of caffeine on oxidative stress

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

REFERENCES