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Low dose of carvacrol prevents rat pancreas tissue damage after L-arginine application, while higher doses cause pancreatic tissue impairment

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ABSTRACT

Carvacrol (5-isopropyl-2-methylphenol) is a biologically active monoterpene phenol abundantly present in the essential oils of many Lamiaceae aromatic/ethnomedicinal plants. Herein, we aimed to evaluate the damaging effect of carvacrol to rat pancreatic tissue, but also to assess its possible ameliorative impact on pancreatic damage induced by L-arginine. The toxic and beneficial (in a dose of 10 mg/kg) properties of carvacrol were assessed by measuring serum α-amylase and lipase activities, tissue malondialdehyde (MDA) content, and pathohistological changes in pancreatic tissue. Application of 100/500 mg/kg of carvacrol produced a significant increase in α-amylase activity, followed by inflammatory-cell infiltration and patchy interlobular edema in the pancreas. In the L-arginine-induced pancreatitis model, a dose of 10 mg/kg of carvacrol prevented an increase in α-amylase and lipase activities, and MDA formation, when compared to the animals that received L-arginine only. Animals treated with carvacrol prior to L-arginine administration displayed mild edema and inflammatory infiltration with few necrotic areas. Contrary to that, animals that received only L-arginine showed a massive leukocyte infiltrate with edema and substantial necrotic areas. In our study carvacrol showed significant protective effects and a potential to modulate leukocyte recruitment in pancreatic tissue after L-arginine injection.

1. Introduction

Carvacrol (5-isopropyl-2-methylphenol) is a monoterpenoid, more specifically a *p*-menthane phenol biosynthesized starting from γ-terpinene through *p*-cymene (Baser and Demirci, 2007), and it is highly related (and isomeric) to thymol (2-isopropyl-5-methylphenol). This monoterpene phenol is an abundant constituent of essential oils of many aromatic plants of the Lamiaceae family, herein including many renown genera *Origanum*, *Thymus*, *Thymbra*, *Satureja*, and *Corydothymus* (Ortega-Nieblas et al., 2011; Kirimer et al., 1995). Long before the chemical composition of these essential oils was known, these plants were used in traditional medicines of many nations for the treatment of stomach ache and common cold (Burt, 2004). Carvacrol is likewise used as a flavoring agent in beverages and sweets, and relatively recently as an antibacterial agent for food preservation (Burt, 2004; Ultee et al., 1999). The beneficial effects of traditional medicines have been attributed to the presence of high concentrations of active monoterpenes, such as carvacrol, for which a variety of significant biological properties, including anti-inflammatory (Hajhashemi et al., 2002; Guimarães et al., 2012), antioxidant (Prieto et al., 2007; Kiliç et al., 2016) and antinociceptive (de Cássia da Silveira E Sá et al., 2017), have been described. Apart from these beneficial properties, carvacrol acute oral median lethal dose in rats was proven to be 810 mg/kg (Suntres et al., 2015).

Acute pancreatitis is an inflammatory process affecting the exocrine pancreas tissue accompanied by abdominal pain and elevated serum levels of pancreatic enzymes. It is well described that the first step in the pathogenesis of acute pancreatitis is the activation of enzymes (trypsin, phospholipase A2 and elastase), which cause auto-digestion of the pancreas tissue (Manohar et al., 2017). Under experimental

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conditions, acute pancreatitis could be induced in rats by a non-invasive method - an administration of a large dose of a basic amino acid, L-arginine. Injection of L-arginine causes pancreas damage via different, up to now, not completely understood mechanisms. Besides proteolytic enzymes activation, it has been proposed that oxygen-derived free radicals, mainly generated through the activity of xanthine oxidase (XO), nitric oxide (NO) and inflammatory mediators, play an important role in the acute pancreatitis development at early stages (Su et al., 2006). Recent research demonstrated a major role of oxidative stress as a risk in the development and progression of both acute recurrent and chronic pancreatitis (Robles et al., 2013).

In humans, the acute pancreatitis is in more than 10% of cases associated with mortality (Zerem, 2014). There are two major aims in the acute pancreatitis treatment: the first one involves supportive therapy (prompt fluid resuscitation, prevention of electrolyte imbalance, nutritional supplements, analgesics, oxygen supplementation, etc.), and the second one tries to limit pancreatic inflammation and necrosis by interfering with the pathogenesis underlying this disorder (Zerem, 2014). Any new therapeutic option for acute pancreatitis management should strive to be beneficial/active in both of the mentioned aims; thus, the potential drug candidates coming from the pool of bioactive secondary metabolites, carvacrol being a representative of them, might fit in this picture perfectly. Based on the previously established properties of carvacrol, more specifically on its anti-inflammatory (Hajhashemi et al., 2002; Guimarães et al., 2012), antinociceptive (de Cássia da Silveira E Sá et al., 2017) and antioxidant (Prieto et al., 2007) potential, it could be regarded as a potential beneficial agent for acute pancreatitis treatment that would affect disease pathogenesis and act as a supportive (analgetic) agent. A recent study also demonstrated that carvacrol decreased the level of lipid peroxidation and additionally, reduced cell injury in the pancreas of rats with cerulein-induced acute pancreatitis (Kiliç et al., 2016).

The extensive utilization in folk medicine of essential oils containing carvacrol, especially those applications related to stomach ache amelioration, and its numerous biological properties coming from its antioxidant and membrane-interfering nature, led us to first probe its possible toxicity towards rat pancreas and furthermore examine the effect on acute pancreatitis induced by an administration of L-arginine. Carvacrol toxicity and beneficial efficacy, in the mentioned model of experimental pancreatitis, was envisaged to be estimated based on the changes in pancreatic tissue weight, serum levels of α-amylase and lipase, as well as in the tissue MDA levels. Pancreatic tissue micromorphological changes occurring after carvacrol application and during pancreatitis would be studied, as well.

2. Materials and methods

2.1. Drugs and chemicals

All chemicals were purchased from Fisher Chemicals (USA), Aldrich (USA) or Merck (Germany) and were used as received. All drugs/chemicals were prepared freshly and used the same day they were prepared on.

2.2. Animals and housing

Healthy male Wistar rats (200–250 g) were housed at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Niš, Serbia. All animals were maintained under standard husbandry conditions with a temperature of 23 ± 2 °C, relative humidity 55 ± 10% and a 12/12 h-light/dark cycle. Animals were fed with standard, commercial laboratory food pellets and had water available *ad libitum*. The experiments were performed in accordance with the declaration of Helsinki and the European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU) and the experimental protocols were commenced after being approved by the animal ethics committee (decision number: 323-07-00278/2017–052).

2.3. Toxicity studies

Three groups of animals (n = 6) were orally administered (*p.o.*) with 10, 100 and 500 mg/kg of carvacrol dissolved in olive oil. All animals received a volume of 0.4 ml of carvacrol solution by gavage. Animal mortality and gross changes were monitored for the initial 4 h and were finally sacrificed 24 h after the treatment. The animals were weighed, blood and pancreatic tissue were collected/isolated and further analyzed.

2.4. Experimental procedure: the pancreatitis model

Based on the initial toxicity screening and a previously reported study about the *in vivo* effects of carvacrol different doses after oral application (Silva et al., 2012), the dose of 10 mg/kg was chosen for the evaluation of the potential of carvacrol in preventing the onset of acute pancreatitis. Acute pancreatitis was induced by injecting L-arginine in a single dose of 350 mg/100 g of body weight (*i.p.*), chosen according to the review (Hegyi et al., 2004) and our pilot studies, to the three pretreated groups of animals. The three experimental groups of animals (six animals per group) were treated 1 h before an injection of L-arginine was administered. Group I (vehicle treated) received olive oil in a dose of 0.4 ml/kg; group II (carvacrol treated) received 10 mg/kg of carvacrol (0.1 ml per animal); group III (positive control) received allopurinol in a dose of 50 mg/kg. All animals were sacrificed 24 h after the L-arginine injection by an overdose of ketamine (Ketamidor 10%, Richter PharmaAG, Wels, Austria). After sacrification, all animals, and the extracted pancreas tissues were weighed, and the changes in the relative pancreas tissue mass were calculated.

2.5. Biochemical analysis

2.5.1. Serum biochemical analysis

Blood was withdrawn by cardiac puncture, left to clot at room temperature and afterward centrifuged for 20 min at 2000 rpm in order to obtain the serum. Serum α-amylase and lipase activities were determined using an Olympus AU680 Chemistry-Immuno Analyzer (Olympus America Inc., USA).

2.5.2. Tissue biochemical analysis

2.5.2.1. Homogenate preparation. Dissected pancreatic tissue was weighed and mixed with a 10-times larger volume of ice-cold distilled water (10% homogenate, 1:10, w/v). The tubes containing the tissues and distilled water were homogenized and centrifuged (10000 rpm, 10 min at 4 °C) afterward. Protein content was determined according to the Lowry's method (Lowry et al., 1951), using bovine serum albumin as the standard for quantification.

2.5.2.2. Pancreatic tissue malondialdehyde (MDA) levels determination. The amounts of MDA were determined following a standard method previously described (Czakó et al., 2000). Briefly, 100 µl of the pancreatic tissue homogenate were boiled at 95 °C with a thiobarbituric acid solution until the color of the chromogen was developed. The intensity of the colored reaction product was measured at 540 nm (Multiscan Ascent (Labsystems, Finland)) and the amount of MDA in each sample was calculated based on a standard curve constructed using 1,1,3,3-tetraethoxypropane as the MDA equivalent. The concentration of MDA in the pancreatic tissues was expressed as nmol per mg of pancreatic tissue proteins.

2.6. Pathohistological analysis

Dissected pancreatic tissue samples were fixed in buffered 10% formalin (w/v) and further processed in order to obtain paraffin molds.

Tissue was cut into 4-5 μm-thick sections (LeicaMicro-Systems, Reuil-Malmaison, France) and stained routinely with hematoxylin and eosin (H&E). Afterward, the stained tissue sections were observed using a light microscope Olympus BX50 (Olympus, Japan) equipped with a digital camera Leica DFC 295 (Leica Microsystems, Germany) under different magnification lenses. The evaluation of the following pathological changes: edema, leukocyte infiltration, necrosis, and hemorrhage, was conducted following the previously described scoring system (Grewal et al., 1994).

2.7. Statistical analysis

Numerical results of the experiments were expressed as the mean ± SD. Statistically significant differences were determined by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons (GraphPad Prism version 5.03, San Diego, CA, USA). Probability values (p) less than or equal to 0.05 were considered to be statistically significant.

3. Results

3.1. Carvacrol increases pancreatic tissue weight

The results of our study show that the treatment with carvacrol elevated the ratio of pancreatic wet weight/body weight (Fig. 1), earlier described as an index of pancreatic edema (Abe et al., 1995, Sandeep and Veeresh, 2012). The relative percentage of pancreatic tissue weight relative to the total rat body weight was found to be significantly increased in animals treated with higher doses of carvacrol (100 and 500 mg/kg), as well as in those animals treated with L-arginine alone or in a combination with carvacrol (Fig. 1). Although, compared to animals that received L-arginine, pancreatic tissue mass was found to be lower in animals treated with allopurinol, an xanthine oxidase (XO) inhibitor, prior to the treatment with L-arginine, such a decrease was not statistically significant (Fig. 1).

3.2. Carvacrol ameliorates α-amylase and lipase activity increase in L-arginine-induced pancreatitis

Table 1 presents the values of serum α-amylase and lipase activities in rats from different experimental groups. Higher doses of carvacrol (100 and 500 mg/kg) produced an increase in serum levels of the two pancreatic enzymes. When compared to the negative control (olive oil group), the levels of serum amylase and lipase activities were significantly higher in the vehicle and L-arginine groups. On the other hand, a pre-treatment with carvacrol (10 mg/kg, *p.o.*) significantly lowered L-arginine-induced elevation of both measured enzymes' activities. Similarly, allopurinol (50 mg/kg; applied *p.o.* as well), produced a reduction in the serum amylase and lipase activities.

Table 1
Serum α-amylase and lipase activities in rats from different experimental groups.

Group/Serum parameter	α-Amylase (U/L)	Lipase (U/L)
Control (untreated)	2269 ± 216	0.9 ± 0.05
Carvacrol 10 mg/kg	1786 ± 295	1.1 ± 0.1
Carvacrol 100 mg/kg	3057 ± 309**	1.85 ± 0.3
Carvacrol 500 mg/kg	3316 ± 340*	8.8 ± 1.4*
Carvacrol 10 mg/kg + L-arginine	2296 ± 263[a]	6.1 ± 3.3**,[a]
Allopurinol (50 mg/kg) + L-arginine	1944 ± 231[a]	3.4 ± 0.8[a]
Vehicle (10 ml/kg) + L-arginine	4808 ± 257*	10.2 ± 0.9*

Data are given as mean ± SD (n = 6). One Way ANOVA followed by Tuckey's post hoc test, **p < 0.05, *p < 0.001 *vs.* Control (untreated).
[a] p < 0.001 *vs.* Vehicle (10 ml/kg) + L-arginine.

3.3. Carvacrol prevents L-arginine-induced pancreatic MDA increase

Higher doses of carvacrol (100 and 500 mg/kg) were found to cause an increase in pancreatic MDA amounts compared to the control group of animals (Fig. 2). Additionally, in the model of L-arginine-induced pancreatic tissue damage, carvacrol was shown to prevent oxidative lipid damage estimated through the measured MDA levels (Fig. 2), i.e. by decreasing MDA levels compared to the vehicle (10 ml/kg) + L-arginine-treated animals. The positive control, allopurinol, prevented an increase in MDA concentration induced by L-arginine, maintaining them at the same level as found in the untreated animals (Fig. 2).

3.4. In high doses, carvacrol damages pancreatic tissue, but the lower one moderately prevents L-arginine-induced pancreatic damage

Healthy pancreatic tissue obtained from untreated animals appeared uniform, with tightly arranged lobules, and with unaltered acinar cells (Figs. 3A and 4A). When given in the doses of 100 and 500 mg/kg, carvacrol produced pancreatic tissue edema, mainly localized in the interlobar spaces, followed by inflammatory cell infiltration

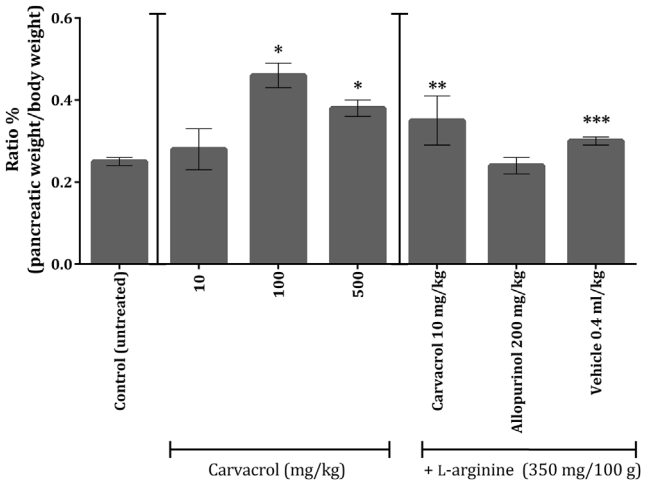

Fig. 1. Pancreatic tissue weight expressed as the ratio (%) to the total rat body weight of animals from different experimental groups. Data are presented as mean ± SD, n = 6; ANOVA, Tuckey's post hoc test *p < 0.001, **p < 0.01 and ***p < 0.05 *vs.* Control group (untreated animals).

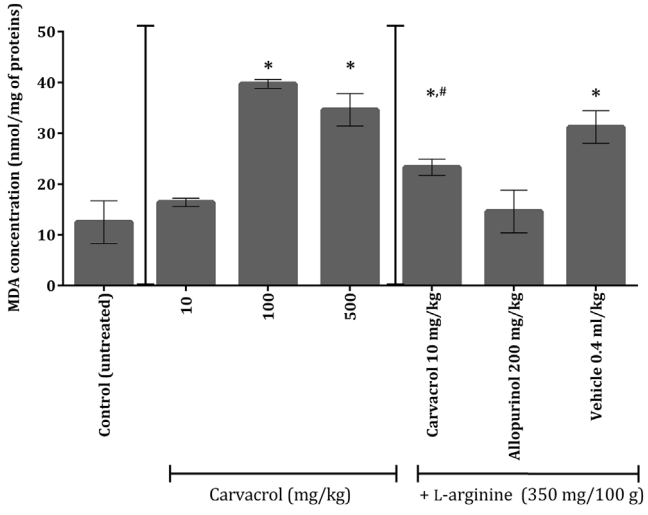

Fig. 2. Pancreatic tissue MDA levels of animals from different experimental groups. Data are presented as mean ± SD, n = 6; ANOVA, Tuckey's post hoc test *p < 0.001 *vs.* Control group (untreated animals); #p < 0.001 *vs.* Vehicle (10 ml/kg) + L-arginine.

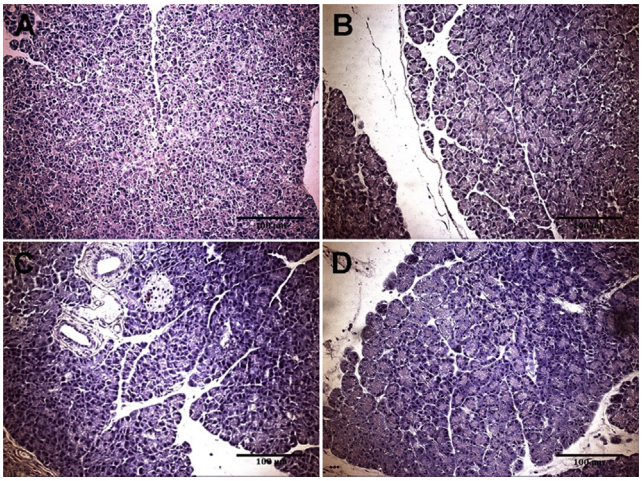

Fig. 3. Histological appearance of the pancreatic tissue from healthy rats (A) and from those treated with carvacrol at the doses of 10 mg/kg (B), 100 mg/kg (C) and 500 mg/kg (D).

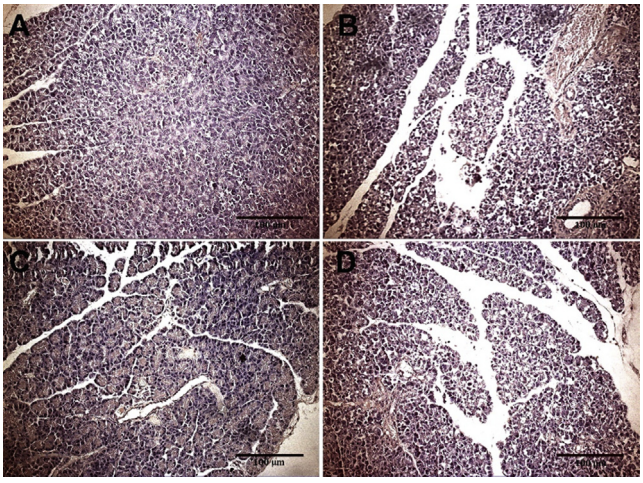

Fig. 4. Histological appearance of pancreatic tissue from healthy rats (A) and those with acute pancreatitis (B–D). Significant tissue edema and inflammatory cell infiltration were seen in the animals treated with the vehicle (B) and carvacrol at a dose of 10 mg/kg (C); almost healthy pancreatic tissue was seen in the animals that received allopurinol (D) following the tissue damage induction.

(Table 2, Fig. 3). A high dose of L-arginine led to significant tissue edema, affecting even spaces between acinus cells, and acinar cell necrosis, with a massive inflammatory cell infiltration, probably as a response to cell death (Fig. 4B and C, Table 2). Acinar cells in these rats displayed vacuolar degeneration and, occasionally, disintegration (Fig. 4B). Similar pathological changes were displayed by animals treated with carvacrol and L-arginine, and animals treated with L-arginine only; however, their intensity was less pronounced (Fig. 4C, Table 2). Application of allopurinol prior to L-arginine almost completely prevented acinar cell necrosis, while interlobar edema and mild inflammatory cell infiltration was still present (Fig. 4D, Table 2).

4. Discussion

The proposed mechanisms by which L-arginine causes acute pancreatitis involve reactive oxygen species (ROS) and inflammatory mediators/cytokines (Su et al., 2006). This study also revealed that NO can contribute to the oxidative-mediated tissue injury suggesting its participation in the processes of L-arginine-induced pancreatic damage and disease development. In L-arginine-induced pancreatitis, the elevation of pancreatic wet weight/body weight ratio clearly demonstrated a pronounced edema formation at 24 h following pancreatitis induction (Takács et al., 2002), which is in accordance with the results from our experiments (Fig. 1). A previous study suggested that endogenous NO plays an important role in the development of pancreatic edema in L-arginine-treated rats, probably by increasing the vascular/microcapillary permeability and protein extravasation. In our study, the treatment with carvacrol, following that of L-arginine, was unable to prevent an increase in pancreatic edema formation estimated both through wet pancreas tissue mass and pathohistological examination (Fig. 1, Table 2). It is interesting to mention that carvacrol alone, in higher doses, caused pancreatic edema estimated using the same method (Fig. 1, Table 2), which was evident as a patchy and/or diffuse interlobular septum distension on microscopic tissue sections. In several studies, carvacrol was proven to possess significant anti-edematous activity in various murine models (Silva et al., 2012; Lima Mda et al., 2013; Zhong et al., 2013), sometimes even in doses that were here proven to cause pancreatic tissue edema (Table 2). On the other hand, allopurinol, an XO inhibitor, prevented an increase in tissue edema, which is only logical since XO is known to be the source of oxygen-derived free radicals which further participate in the well-known pathophysiological mechanisms of pancreatic tissue edema formation. However, the evidence of mild tissue edema, at the level of interlobular spaces, was still visible in the positive control group (Fig. 4D, Table 2).

Inflammatory cell infiltration in rat pancreatic tissue after administration of L-arginine was a clearly visible finding (Fig. 4, Table 2). The lower dose of carvacrol was able to prevent an increase in inflammatory cell infiltration to a similar extent as allopurinol (Table 2). Beneficial properties of carvacrol were discovered in different animal models of inflammatory diseases as well. Namely, earlier studies revealed that carvacrol significantly reduced proinflammatory cytokine level (TNF-α) in pleural lavage and suppressed leukocytes migration in the model of carrageenan-induced pleurisy (Guimarães et al., 2012). The anti-inflammatory effect of carvacrol was also recently evaluated in the acetic acid-induced gastric lesion model, where the oral treatment with this monoterpene improved the healing process of gastric ulcers (Silva et al., 2012). Additionally, in *in vitro* conditions, carvacrol non-selectively inhibited prostaglandin production by COX-1 and COX-2 and it is believed that the anti-inflammatory action of carvacrol is mainly related to its inhibitory effect on prostaglandin production (Landa et al., 2009). Results from the cerulein-induced acute pancreatitis study demonstrated that the intraperitoneal pretreatment with another

Table 2
Pathohistological scoring of pancreatic tissue from the experimental group animals.

Group/Parameter	Edema	Inflammation	Necrosis	Hemorrhage
Control (untreated)	0	0	0	0
Carvacrol 10 mg/kg	0	0	0	0
Carvacrol 100 mg/kg	1.0 ± 0.8	0.5 ± 0.5	0	0
Carvacrol 500 mg/kg	1.25 ± 0.5	1.0 ± 0.4	0.5 ± 0.6	0
Vehicle (10 ml/kg) + L-arginine	4.0 ± 0.3	3.0 ± 0.7	1.6 ± 0.5	0.6 ± 0.1
Carvacrol 10 mg/kg + L-arginine	3.6 ± 0.5	1.2 ± 0.6	1.7 ± 0.5	0.3 ± 0.2
Allopurinol (50 mg/kg) + L-arginine	1.0 ± 0.0	0.9 ± 0.2	0	0

monoterpene, α-pinene, inhibited cytokine production in isolated cerulein-treated pancreatic acinar cells (Bae et al., 2012).

After an *i.p.* administration of L-arginine, rats should develop acute pancreatitis characterized by a rise in serum amylase and lipase levels (Melo et al., 2010). In accordance with the previous report, in our study, the induced pancreatitis with L-arginine led to a marked increase in serum amylase and lipase levels 24 h after the commencement of the experiment. Namely, serum levels of both enzymes are important diagnostic markers for acute pancreatitis, both clinical and experimental, and are usually elevated within 4–8 h after the initial L-arginine injection, with a peak in activity after 24 h (Melo et al., 2010). A recent study showed that carvacrol can inhibit amylase activity in an *in vitro* assay, with an IC_{50} value of 152.3 μg/ml (Govindaraju and Arulselvi, 2018). Although one could argue that a dose of 500 mg/kg given to animals could have possibly reached IC_{50} in the serum, the suggested inhibiting activity was not apparent/observed. This could potentially be explained by a much more pronounced cell-damaging effect of carvacrol than its potential to inhibit amylase activity.

It is previously described that after *i.p.* application of L-arginine, detected changes in serum amylase and lipase levels follow the endoplasmic reticulum disorganization, metabolic/protein synthesis alterations and progressive morphological damage of pancreatic acinar cells (Kishino and Kawamura, 1984; Tashiro et al., 2001). It is possible that the detected changes in enzyme activities are the consequence of progressive morphological damage of the pancreatic acinar cells seen on tissue sections from the animals treated with L-arginine (Fig. 3, Table 2). Tashiro et al. (2001) described that in pancreatic acinar cells, the actin network is involved in the changes of cell mechanical properties, especially in the process of zymogen granules exocytosis and their fusion with the cell membrane. In *in vitro* models of osteoclast formation, carvacrol prevented the formation of the actin ring (Deepak et al., 2016), suggesting that it might affect cell cytoskeleton organization within acinar cells as well.

One of the pathophysiological mechanisms connected to the acute pancreatic damage involves ROS which act as initiators of chain reactions and are known to cause damage of cell structure molecules (e.g. lipids and proteins). A recent study described that carvacrol efficiently scavenges free radicals including peroxyl radicals, superoxide radicals, H_2O_2 and NO (Aristatile et al., 2015), and this was attributed to the presence of a phenolic hydroxyl group (OH) in its molecule. The weak acid character of carvacrol facilitates the reaction with free radicals.

In the present study, we also determined the amount of MDA in pancreatic tissue, which is considered to reflect cell and/or organelle membrane ROS-mediated damage. It is suggested that these morphological changes, which result in enzyme leakage, are a direct result of focal cytoplasm degradation and damage of cell organelle membranes (Andrzejewska and Jurkowska, 1999). A previous study showed that carvacrol decreases the extent of lipid peroxidation and, additionally, reduced pancreatic tissue cell-injury in a model of cerulein-induced acute pancreatitis in rats (Kiliç et al., 2016). Having in mind these morphological alterations, one could expect a significant increase in MDA content after the application of higher doses of L-arginine. The results related to the amounts of MDA in pancreatic tissue of animals treated with allopurinol and L-arginine are in accordance with previous findings that suggest significant beneficial properties of this XO inhibitor (Czakó et al., 1998; Czakó et al., 2000).

It is worth mentioning that carvacrol, when administered in doses equal to or higher than 100 mg/kg, also produced a significant increase in MDA concentrations compared to the healthy animals. Although the effectiveness of carvacrol was previously evaluated on numerous occasions (Baser, 2008; Sharifi-Rad et al., 2018), this is the first *in vivo* study showing that at these relatively high doses carvacrol induces oxidative lipid damage demonstrated by elevated MDA levels. On one previous occasion, carvacrol was found to cause cell membrane damage (estimated through MDA levels) when applied in higher concentrations; however, in lower ones, it prevented H_2O_2-induced cell damage (Ozkan and Erdogan, 2012). From this study, investigators concluded that under cell culture conditions carvacrol acts as a prooxidant when cells are exposed to higher concentrations, while when they are exposed to lower ones and some oxidizing agent it acts as an antioxidant. These results might be translated to our findings where lower doses of carvacrol did not increase pancreatic tissue MDA content and even prevented an increase in MDA levels induced by L-arginine, while higher doses (100 and 500 mg/kg) significantly increased MDA level in healthy animals.

5. Conclusions

Taking into account all of the above, in a very complex *in vivo* network, carvacrol probably affects the function of multiple cellular mediators involved in inflammatory, oxidative, and other signaling pathways. However, its potential is very limited since it barely prevented edema formation during an early phase of pancreatitis development, but at the same time, it reduced values of serum biochemical parameters (serum amylase and lipase activities) and prevented an increase in MDA formation. The changes observed on the microscopic level in animals with pancreatitis and those treated with carvacrol were of a slightly lesser extent than those seen in the negative control group animals, *i.e.* those with only pancreatic damage.

Conflicts of interest declaration

The authors declare no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Transparency document

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