

## Antioxidative Effects of Morine in Ischemia-Reperfusion of Kidneys in the Laboratory Rat

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### Abstract

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The purpose of the study was to monitor the antioxidative effect of morine in the conditions of ischemia-reperfusion of laboratory rat kidney tissue. The animals were divided by random selection into two groups (n = 7). The treated group was given morine in peroral doses of 10 mg·kg<sup>-1</sup> in 0.5% solution of methylcellulose (Methocel) E5 once a day, the control group was given only the solution of Methocel E5. After medication was finished on the 20<sup>th</sup> day all animals were subject to kidney tissue ischemia (60 minutes) followed by reperfusion (10 minutes). All animals were subsequently exsanguinated, organs were recovered for histopathological examination and single identification of superoxidismutase, glutathion peroxidase, total antioxidative capacity; and malondialdehyde level in the blood was carried out. We discovered a significant increase ( $p \leq 0.05$ ) of the superoxidismutase catalytic activity in the treated group compared to the group of control ischemia-reperfusion. There was also a highly significant increase ( $p \leq 0.01$ ) of total antioxidative capacity in the treated group compared to the group of control ischemia-reperfusion. The glutathion peroxidase catalytic activity embodied non-significant changes when comparing the treated group and control group. A significant decrease ( $p \leq 0.05$ ) of malondialdehyde level was identified in the treated group compared to the group of control ischemia-reperfusion. The results of biochemical examination show a protective antioxidative effect of morine. The results of histopathological examination support this assumption.

*Antioxidants, superoxidismutase, glutathion peroxidase, malondialdehyde*

Results of intensive research in the last decade have confirmed that pathological increase of free radicals significantly participates in the occurrence and development of several diseases e.g. inflammatory, cardiovascular, degenerative syndromes etc. If the balance between the effect of free radicals and protective mechanisms of the organism is disturbed, it results in the development of a condition known as oxidative stress (Sies 1991). In order to regulate physiological volume of free radicals, the organism has developed antioxidative mechanisms created by natural antioxidants and enzymes e.g. superoxidismutase, redox glutathione system, substances with chelate-bound ions of iron and copper (Rotilio 1994). Remedy of the oxidative damage to the organism is difficult. Much more effective is the way of prevention that consists in minimising the sources of formation of free radicals and strengthening the natural antioxidative mechanism by administering substances that act as antioxidants or so-called free radical extinguishers (Komárek and Strnadová 1992; Nečas et al. 1997; Bartošiková et al. 1998).

The study of biological activity and mechanism of the effect of flavonoids has been the

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subject of interest for a number of years. Flavonoids constitute one of the largest groups of natural phenols. They are usually contained in plants as glycosides and they are also present in fruits and vegetables.

Especially aglycones are pharmacologically effective. Several of them exert hepatoprotective, diuretical, vasodilatation, antibacterial and chemoprotective effects: anti-inflammatory, antidiabetical, antiallergical and other effects were also described

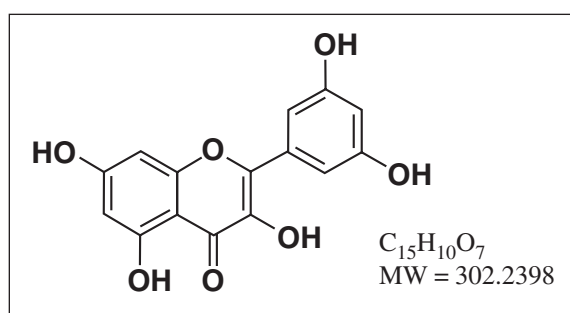


Fig. 1: Chemical structure of morine

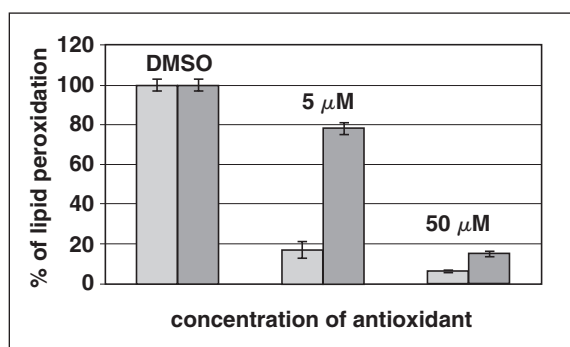


Fig. 2: In vitro inhibition of lipoperoxidation identified in microsomal liver fraction isolated from mice

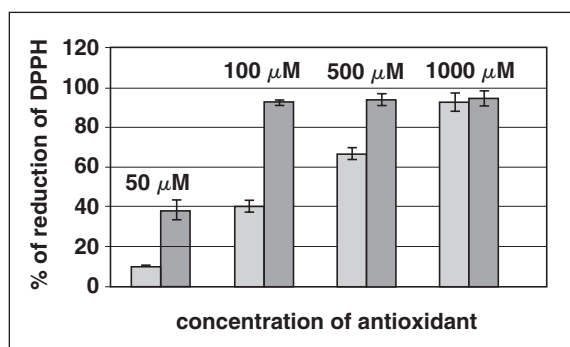


Fig. 3: Determination of antioxidative activity of morine by diphenyl-picryl-hydrazyl (DPPH)  
DMSO = dimethylsulphoxide, BHT = butylhydroxytoluene

(Calomme et al. 1996; Read 1995; Perez et al. 1998; Yamamura et al. 1998). In recent years, closer attention has been paid to examination of their antioxidative activity and the ability to extinguish or trap free radicals (Jovanovic et al. 1994; Rice-Evans et al. 1995; Kubínová and Suchý 1999; Catapano 1997).

Morine (3, 3', 5, 5', 7 - pentahydroxyflavon) is the active substance of *Morus tinctoria* L., from which it was isolated. It belongs to the group of flavonoles: the basic skeleton is substituted by five hydroxyl groups in positions 3, 5, 7, 3', 5' (see Fig. 1).

Testing *in vitro* has proven its chemoprotective activity (Kawabata et al. 1999), anti-mutagenous activity (Choi et al. 1994), antiviral activity (Bunyapraphatsara et al. 2000) and antioxidative activity (Sugihara et al. 1999). In the tests carried out by us *in vitro* the antioxidative activity was confirmed (see Figs 2 and 3). This fact became a stimulus for further research.

The goal of this study was to monitor the antioxidative effect of morine in the conditions of ischemia-reperfusion of laboratory rat kidney tissue. The study itself and its procedure were monitored by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences in Brno. The state of health of all animals was regularly examined several times a day both during the period of the animals' acclimation and during the whole course of the

experiment by the working team whose members are holders of the Certificate on Professional Competence issued by the Central Commission for the Animal Protection pursuant to §17 of the Act on Protection of Animals against Cruelty (No. 246/1992 Coll.) of the Czech National Council.

#### Materials and Methods

Testing the antioxidative effect of morine *in vitro* was carried out by a method of identifying peroxidation of lipids on liver microsomes of the laboratory rat using buthylhydroxytoluen (BHT) as a reference standard (Uchiama and Mihara 1978). The antioxidative effect of morine *in vitro* was also detected by radical 1, 1 – diphenyl – 2 – picryl – hydrazyl (DPPH) (Blais 1958).

The *in vivo* study was carried out on laboratory rats (n = 7) of the Wistar SPF strain (origin - AnLab s.r.o., Brno): all were male, of the same age and comparable physical weight (300 ± 14 g). The animals were placed in a laminary box RIR B-12 by Heto-Holten comp. fed a standard diet (Diet for small laboratory animals SPF M1) and given water *ad libitum*.

After 10 days of acclimation, the animals were divided by random selection into two groups (n = 7). The treated group was given morine in oral doses of 10 mg/kg in 0.5% solution of Methocel E 5 (methylcellulose) once a day. The control group was given only the 0.5% solution of Methocel E5 in the amount of 2 ml also in oral doses once a day.

After termination of the medication on the 20th day, all animals were subjected to laparotomy in full anaesthesia (2% Rometar 0.5 ml + 1% Narkamon 10 ml, in doses of 0.5 ml solution/100 g of body weight), with preparation and isolation of the left renal artery and placement of a vascular clamp inciting kidney ischemia for 60 min. Afterwards the clamp was loosened, followed by reperfusion of 10 minutes. All the animals were subsequently exsanguinated by blood collection from the left ventricle followed by spectrophotometric single identification of malondialdehyde (MDA) in the serum by the TBARs method (Uchiama and Mihara 1978). After that the identification of superoxididismutase (SOD), glutathion peroxidase (GSHPx), and total antioxidative capacity (AOC) was carried out using the sets of RANDOX, Dublin, Ireland comp. on an automatic analyser COBAS MIRA S. Samples of kidney tissue were recovered for histopathological examination.

The material was fixed in neutral 10% formole and routinely stained by haematoxylin-eosine. Preparations were examined in an optical microscope.

The acquired values of laboratory parameters were processed by Microsoft Excel table processor and statistically evaluated using UNISTAT 5.1 programme and odd T-test where  $p \leq 0.05$  was considered as significant.

#### Results

##### Results of laboratory examinations

The results of laboratory examinations are given in Table 1.

We found a significant increase ( $p \leq 0.05$ ) of the SOD catalytic activity in the treated group as compared with the control group of ischemia-reperfusion.

Table 1  
Values of monitored laboratory parameters expressed as X ± SD

Monitored parameters	Treated group (n=7)	Control group (n=7)
SOD [U/ml]	* 222.21 ± 5.59	200.69 ± 14.83
GSHPx [μkat/l]	1209.00 ± 145.13	1148.14 ± 66.50
AOC [mmol/l]	** 1.14 ± 0.10	0.93 ± 0.08
MDA [mmol/l]	* 23.07 ± 3.04	33.47 ± 9.31

\*  $p \leq 0.05$

\*\*  $p \leq 0.01$

SOD = superoxididismutase

GSHPx = glutathion peroxidase

AOC = total antioxidative capacity

MDA = malondialdehyde

There was also a highly significant increase ( $p \leq 0.01$ ) of AOC in the treated group compared to the control group of ischemia-reperfusion.

The GSHPx catalytic activity showed non-significant changes when comparing the treated group and control group; however, the average GSHPx catalytic activity identified in the treated group was higher than in the control group.

A significant decrease ( $p \leq 0.05$ ) of MDA values was identified in the treated group compared to the group of control ischemia-reperfusion.

#### Results of histopathological examination

Using histopathological preparations, we evaluated the level of damage to the cortical and medullary parts of kidney parenchyma, in particular the condition of all parts of proximal tubules, the content in their lumina, the condition of epithelia, their oedema, loss of the brush border, pyknosis of the cores and loss of their stainability.

The changes were evaluated semiquantitatively in terms of the extent and intensity of damage of several tubules as light, medium and heavy.

#### The treated group

Mild oedema of medullary parenchyma, protein content in proximal tubule lumina approximately in 50-75%, and sporadic hyaline globules were found. Oedema of epithelia of proximal tubules and loss of brush border were present in places. Minimal pyknosis of the cores and loss of stain ability were also detected.

In the interstitium, erythrocytes were found dispersely in 40-50% and polynuclears sporadically (10-20%). Capillaries exhibited non-standard changes: occlusive changes with thrombotizations occurred in 60-70%.

These changes were evaluated as light to medium-heavy kidney parenchyma disability.

#### The control group

Generalised oedema of kidney cortex and protein content was present in almost all proximal tubule lumina, sporadically also in distal tubules. Here and there, apparent hyaline globules in proximal tubule lumina in the size of epithelial cores were detected. Oedema of epithelia, plasma eosinophilia and very frequent loss of brush border were also present. Sporadically apparent pyknosis of the cores and frequent loss of their stain ability were detected, too.

In the interstitium erythrocytes were found dispersely in 80% and polynuclears in 45%. In this group, capillaries also exhibited non-standard changes; however, occlusive changes were quite regularly present in almost 100%.

These changes were evaluated as medium-heavy to heavy kidney parenchyma disability.

In both groups, haemorrhagic changes were predominant in the lower part of the nephron (the area of tubules); the changes in the glomerules were not so regular and marked. The infiltrate was inflammatory, as suggested based on the presence of acute inflammatory changes (see above) when polynuclears prevailed as response to necrobiotic changes. Chronic inflammatory changes in the form of rounded cell infiltrates were almost absent.

Histopathological examination using light microscopy did not make it possible to examine the state of podocytes. This can be carried out only with electron microscope. It was also not possible to determine the type of oedema. On the basis of the described histopathological findings we may assume that it was oedema of a stagnant type. No functional differences in both groups were shown histopathologically.

### Discussion

Morine is an original isolated substance, which so far has been used only sporadically for *in vivo* testing on pathological biomodels. Predominant majority of literature references is

therefore reports *in vitro* studies that frequently compare the effect of the monitored representatives of a flavonoid group between themselves (Sugihara et al. 1999). The substance belongs to polyhydroxylated flavonoids and we assume that under the conditions of *in vivo* studies it could act by means of several mechanisms. Morin could chelate copper and iron which are potential inductors of the reactive oxygen formation. Further it could reduce, owing to its low redox potential, some free radicals, such as hydroxyl and peroxy radicals and superoxide. Morin is capable of regenerating natural antioxidative mechanisms of an organism, for example alphas-tocopherol, ascorbic acid, etc. Morin inhibits cyclooxygenase, lipoxygenase, and glutathione-S-transferase. Thus it could decrease production of reactive forms of oxygen (Raso et al. 2001; Kaneko and Baba 1999; Chen et al. 1996).

Our study recorded a significant difference in the SOD catalytic activity (and partly also GSHPx) in the group treated by morine in comparison with the control group of ischemia-reperfusion. The examined enzymes act intracellularly and their activity is usually linking. Significant higher catalytic activity of SOD identified in the treated group argues for the readiness to eliminate superoxides, clear away hydrogen peroxide and other free radicals causing damage to kidney tissue after reperfusion. We presume that it is a result of previous preventive supply of a substance with proven antioxidative effect *in vitro* to animals of this group. On the basis of the results of histopathological examinations it is not possible to give clear evidence of the disturbance of kidney function in the examined animals. The opinions of individual authors concerning the change of SOD and GSHPx activities as a result of decreasing kidney function vary considerably. Literature sources report both the increased SOD and GSHPx activities (Mimic-Oka et al. 1995) related to the decreasing kidney function and the findings of the decreased or normal SOD and GSHPx activities (Racek et al. 1995) and (Durak et al. 1994), respectively. For a better consideration of the problem it would be desirable to determine the catalase activity in erythrocytes of laboratory animals, which is usually lowered in patients with decreasing kidney function (Durak et al. 1994), and the level of selenium which has antioxidative effects, is part of GSHPx and its deficiency is regularly found in patients with decreasing kidney function (Bonomini and Albertazzi 1995).

The positive effect of supplying antioxidants in conditions connected with ischemia and subsequent reperfusion of kidney tissue is discussed about not only in connection with the improvement of indicators of the antioxidative system values but also in connection with the improvement of currently monitored kidney functions (Lee et al. 1992; Zurovski et al. 1995). The finding of lower catalytic activity of both enzymes in the artificially induced kidney ischemia-reperfusion without previous preventive administration of antioxidants, identified by us in the control group, has been described also by other authors, in some cases even during the ischemic phase (Racek et al. 1997).

A highly significant increase of the AOC values was noted in the treated group compared to the values of the control group. This is a significant difference and it can be assumed that it is again a logical result of the previous supply of a substance with antioxidative effect. One of the reasons of the AOC growth may be attributed to the increased level of the uric acid (Racek et al. 1997). Uric acid should be considered not only as nitrogenous metabolite of purine substances but it also has significant antioxidative effects. The other authors differ in their views on the influence of the decreasing kidney function on AOC changes (Toborek et al. 1992; Jackson et al. 1995).

The results of statistic comparison of MDA values in both groups show significant changes of this toxic by-product of lipoperoxidation in the treated group compared to the control group. When comparing results of the studies performed a number of authors agree with the elevated MDA concentrations in plasma or erythrocytes (Kuroda et al. 1985;

Racek et al. 1995) in patients with decreasing kidney function, it may, however, be caused not only by its increased formation from lipid peroxides but also by its decreased renal elimination (Racek et al. 1997). Furthermore, MDA may modify proteins and lead to changes similar to those observed during their glycation (Racek et al. 1997; Roselaar et al. 1995).

Free radicals play the main role in the pathogenesis of kidney damage upon reperfusion after previous ischemia. During the ischemic period there is only anaerobic glycolysis in the tissue and its ATP production is insufficient. There is not enough energy to keep the diaphragm processes and  $\text{Na}^+$  entering the cells. During reperfusion, these ions are replaced by ions of  $\text{Ca}^{2+}$ , which cause the inability of mitochondria to produce ATP and activate endocellular proteases and phospholipases. Apart from degradation of cell diaphragms, also arachidonic acid is released and free radicals and other cytotoxic products emerge in its metabolism. Another source of free radicals is represented by activated neutrophil granulocytes, mitochondrial oxidation chain during very low partial oxygen pressure and the metabolism of catecholamines released in stress situation. Hypoxia leads also to the change of xanthine dehydrogenase into xanthine oxidase, which provides the emergence of two superoxide radicals during biosynthesis of uric acid. The volume of released radicals leads to damage of the organ (Racek et al. 1997).

Flavonoids belong to the substances that are given increased attention due to their antioxidative activity and ability to extinguish or trap free radicals. According to some authors, the antioxidative effect of this group is probably the essence of the so-called vitamin effect of these substances (Torel et al. 1986). Literature sources state that a human with conventional kind of nourishment daily consumes around 1 kg of these substances (Bisset et al. 1991). Lately flavonoids have also become a part of vitamin mixtures and food supplements, therefore the amount consumed by the organism can also considerably exceed the usual values.

The laboratory results of our study show a distinct antioxidative effect of the tested morine flavonoid in the conditions of incited ischemia-reperfusion of kidney tissue in an experiment. The results of histopathological examination support this assumption. This pilot study could become an impulse for further preclinical analyses with further verification of the effect of this substance in clinical practise.

#### **Antioxidační efekt morinu u ischemie-reperfuzie ledvinné tkáně laboratorního potkana**

Cílem studie bylo sledovat antioxidační efekt morinu v podmínkách ischemie-reperfuzie ledvinné tkáně u laboratorního potkana. Zvířata byla metodou náhodného výběru rozdělena do dvou stejných skupin ( $n = 7$ ). Skupině léčené byl podáván morin v dávce 10 mg/kg v 0,5% roztoku Methocelu E 5 perorálně 1x denně, skupině kontrolní byl podáván pouze roztok Methocelu E 5. Po ukončení medikace 20. den byla u všech zvířat v celkové anestezii provedena ischemie ledvinné tkáně (60 min) s následnou reperfuzí (10 min). Poté byla zvířata utracena vykrvením, byly odebrány orgány pro histopatologické vyšetření a v krvi jednorázově stanoveny superoxiddismutáza, glutathionperoxidáza a celková antioxidační kapacita a hladina malondialdehydu. Byl zjištěn statisticky významný vzestup katalytické aktivity superoxiddismutázy ( $p \leq 0.05$ ) a statisticky vysoce významný vzestup celkové antioxidační kapacity ( $p \leq 0.01$ ) u léčené skupiny ve srovnání se skupinou kontrolní ischemie-reperfuzie. Katalytická aktivita glutathionperoxidázy vykazala nesignifikantní změny. Dále byl zjištěn statisticky významný pokles hladiny malondialdehydu ( $p \leq 0.05$ ) u léčené skupiny ve srovnání se skupinou kontrolní ischemie-reperfuzie. Výsledky biochemického vyšetření ukazují na protektivní antioxidační efekt morinu. Histopatologické nálezy tuto domněnku podporují.

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