

Protective Effect of Morin against Flutamide-Induced Hepatotoxicity in Male Wistar Rats

Mazhar F. M., Kadry S. M., Hala F. Abd-Ellah, Hend A. Sabry and Saleh A. S.

Department of Zoology, Faculty of women for Arts, Science & Education,
Ain Shams University, Cairo, Egypt

ABSTRACT

Aim of the work: The aim of this work was to investigate the protective role of morin against the hepatotoxic effect of flutamide (FLU) that is widely used drug for treatment of metastatic prostate adenocarcinoma. Administration of FLU to male rats in a dose of 100 mg/ kg b.w. daily for 4 weeks resulted in serious hepatic injury.

Materials and Methods: Male Wistar rats were equally divided into six experimental groups (n = 10): Group I (control group) received appropriate vehicle (carboxy methyl cellulose, CMC) for 8 weeks, Group II (CM group) received CMC for 4 weeks then morin for another 4 weeks, Group III (M group) were treated with morin for 8 weeks, Group IV (CF group) received CMC for 4 weeks then FLU for another 4 weeks, Group V (CMF group) received CMC for 4 weeks then morin for another 4 weeks then received morin associated with FLU for additional 4 weeks and Group VI (MMF group) was pretreated with morin for 4 weeks then treated with morin simultaneously with FLU for additional 4 weeks.

Results: In FLU treated rats, highly significant increases in each of serum ALT, AST, direct and total bilirubin as well as hepatic MDA were observed relative to the control group. Moreover, highly significant decrease in hepatic SOD, GSH and GST activities were observed. On the other hand, administration of morin with FLU resulted in mild and marked reduction in the elevated serum ALT, AST, direct and total bilirubin and hepatic MDA levels induced by FLU intoxication, respectively (regarding co-treatment and pretreatment with FLU).

Conclusion: These data showed protective effect of morin against FLU-induced hepatic damage, especially when administered prior to and concomitantly with FLU.

Keywords: Flutamide; Morin; Hepatotoxicity; Lipid peroxidation and Antioxidant activity.

INTRODUCTION

Flutamide, (FLU) [2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl]-propanamide, is a non-steroidal antiandrogen compound which is devoid of estrogenic, progestational or androgenic activities. This drug is used for treatment of metastatic prostate adenocarcinoma⁽¹⁾. FLU is mainly metabolized in the liver and its metabolites are excreted in the urine⁽²⁾. After oral administration, FLU is well absorbed and its circulating form is almost exclusively the active compound 2-hydroxyflutamide⁽³⁾. In the prostate cancer, tumor cells need testosterone to proliferate. FLU and its active metabolite 2-hydroxyflutamide compete with testosterone to bind to androgen receptors leading to impairment of testosterone signaling and modulation of the testosterone-dependent pathways⁽⁴⁾. Despite its therapeutic benefits, treatment with FLU has been associated with idiosyncratic liver injury and therefore received a black box warning label⁽⁵⁾. Also of interest is the observation that FLU can influence the metabolism of estradiol in particular the 2- and 4-hydroxylation of this essential hormone in patients with prostate

cancer⁽³⁾. FLU also inhibited mitochondrial respiration and adenosinetriphosphate formation⁽⁶⁾. The metabolism of FLU by the cytochrome P-450 system or by other microsomal enzymes results in the formation of reactive metabolites which can lead to lipid peroxidation and consequently to hepatocytic injury. However, the possibility of an immunological mechanism cannot be excluded despite the absence of fever or rash⁽⁷⁾.

Flavonoids are polyphenolic compounds that can be found in dietary components such as food products, beverages and herbal medicines with different health benefits shown in several studies⁽⁸⁾. Most flavonoids have an antioxidant activity^(9, 10). Morin hydrate, or simply morin (C₁₅H₁₀O₇; 2',3,4',5,7-Pentahydroxyflavone; a light yellowish pigment) is a kind of flavonoid belonging to the group of flavonols, found in almond hulls⁽¹¹⁾, mulberry figs⁽¹²⁾, onion⁽¹³⁾ and guava leaves⁽¹⁴⁾. Over the years, numerous activities have been assigned to this flavone. Morin has been shown to exhibit antitumor⁽¹⁵⁾, antioxidant⁽¹⁶⁾, antidiabetic⁽¹⁷⁾ and anti-

inflammatory⁽¹⁸⁾ activities. Also, morin has been shown to inhibit xanthine oxidase⁽¹⁹⁾, suppress protein kinase C and inhibit the release of inflammatory cytokines, such as interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF) from mast cells⁽²⁰⁾. Morin could also modulate the activities of the metabolic enzymes including cytochrome P450⁽²¹⁾.

The aim of the present study was to elucidate the pathway of FLU- induced hepatic injury through biochemical examinations and to investigate the chemoprotective role of flavonoid morin for relieving FLU-induced hepatotoxicity in male rats and restoring liver functions beyond normal status.

MATERIALS AND METHODS

Drugs and chemicals:

FLU was obtained from Sigma Pharmaceutical Industries, Egypt. Morin was purchased from Sigma-Aldrich Chemicals Co (St. Louis, Missouri, USA). Carboxy methyl cellulose (CMC) was purchased from El- Gomhoryia Chemical Company, Cairo, Egypt. FLU was dissolved in carboxy methyl cellulose (CMC)⁽²²⁾ and was given orally at a dose of 100 mg/kg b.w. / day according to **Sharaf et al.**⁽²³⁾. Morin was dissolved also in CMC⁽²⁴⁾. It was given through gastric intubation at a dose of 50 mg/ kg b.w. / day. This dose was guided by previous studies⁽²⁵⁾.

Animals: Sixty adult male Wistar albino rats weighing 190-200g purchased from animal house of El-Salam Farm, Giza, Egypt, were used in this study. Animals were kept in clean polypropylene cages under standard laboratory conditions (temperature at 25±3°C, relative humidity of 50 ±15% and normal photoperiod of 12h light/dark cycle) with free access to standard dry pellet diet and water *ad libitum*. All the treatments were started after almost 1 week of stabilization from arrival.

Experimental protocol:

The animals were randomly divided into 6 groups of ten rats each as follows:

Group (I) control: Rats received (0.5 ml) of carboxy methyl cellulose (CMC), as vehicle daily through gastric intubation for 8 weeks.

Group (II) (CM): Animals were treated orally with CMC daily for four weeks and then orally administrated morin daily (50 mg/ kg b.w.) for a further four weeks.

Group (III) (M): Animals received morin (50 mg/kg b.w.) orally daily for eight weeks.

Group IV (CF): Rats were orally administrated CMC daily for four weeks and then orally administrated FLU (100 mg/kg b.w.) daily for another four weeks.

Group V (CMF): Animals were orally administrated CMC daily for four weeks then were orally administrated morin accompanied by FLU for extra 4 weeks.

Group VI (MMF): Firstly, rats were orally administrated morin daily for four weeks and secondly received morin in association with FLU daily for additional four weeks.

Absolute and relative liver weights: Liver of control and treated groups were rapidly removed, blotted with a piece of filter paper and weighed for representing the absolute liver weights. In order to obtain a precise measure of the change in organ weights, fresh liver weight was calculated relative to the total body weight, thus:

$$\text{Hepatosomatic index} = \frac{\text{absolute liver weight}}{\text{total body weight}} \times 100$$

Biochemical analysis: The left lateral hepatic lobe was taken, part of it was homogenized and the supernatant was collected and stored at -80°C for MDA, SOD, GSH and GST levels. ALT and AST were measured in plasma by colorimetric method of **Reitman and Frankel**⁽²⁶⁾. Plasma direct and total bilirubin was measured according to the method of **Walter and Gerade**⁽²⁷⁾. Hepatic MDA level was determined by the method of **Yoshioka et al.**⁽²⁸⁾ SOD activity was measured based on the method of **Minami and Yoshikawa**⁽²⁹⁾. Hepatic GSH level was determined by using kits supplied by Biodiagnostic, Egypt according to **Beutler et al.**⁽³⁰⁾. GST activity was assessed by the method of **Habig**⁽³¹⁾.

Statistical analysis

All data were statistically analyzed using the statistical package for social science (SPSS)(version 17.0). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) analysis. Differences were considered to be significant at $p < 0.05$. Data were presented as mean ± standard error (S.E.).

RESULTS

Hepatosomatic index: of the control and the experimental groups (CM and M) showed more or less constant levels during the experimental period as presented in Table 1. Treatment of rats

with FLU caused a very highly significant increase ($p < 0.001$) in hepatosomatic index as compared to the control group. However, CMF and MMF groups showed a significant and highly significant decrease in it when compared to CF group, respectively.

Table 1: Hepatosomatic index(g) in control and treated groups.

Groups	Hepatosomatic index (g)
Control	2.56±0.09
CM	2.49±0.02
M	2.52±0.11
CF	6.65±0.42 ^{a***}
CMF	3.81±0.22 ^{b*}
MMF	3.05±0.24 ^{b**}

Data are represented as mean± SE; n= 10

a= control versus CM, M and CF

b= CF versus CMF and MMF

*Significant change at $p < 0.05$

**Highly significant change at $p < 0.01$

***Very Highly significant change at $p < 0.001$

Biochemical results: Serum levels of ALT and AST of control and the experimental groups (CM and M) designated more or less constant levels during the study period as shown in Table 2. FLU exerted a significant ($p < 0.001$) increase in serum ALT and AST levels after 4 weeks of treatment when compared to the control group. Administration of morin for 4 weeks in combination with FLU (CMF group) caused a highly significant decrease in serum ALT and AST levels as compared to CF group. While pretreatment with morin for 4 weeks then accompanied with FLU for another 4 weeks caused a significant ($p < 0.001$) decrease in serum ALT and AST level when compared to CF group.

Table 2: Serum levels of ALT and AST in control and treated groups.

Groups	ALT (U/ ml)	AST (U/ ml)
Control	32.58±2.15	30.69±2.18
CM	30.70±2.66	29.20±1.88
M	29.74±2.29	28.06±1.87
CF	95.60±4.16 ^{a***}	87.55±4.09 ^{a***}
CMF	55.62±3.55 ^{b***}	61.04±2.88 ^{b***}
MMF	40.83±1.84 ^{b***}	48.99±1.54 ^{b***}

Data are represented as mean± SE; n= 10

a= control versus CM, M and CF

b= CF versus CMF and MMF

*Significant change at $p < 0.05$

**Highly significant change at $p < 0.01$

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Serum bilirubin (direct and total) levels demonstrated no significant changes between CM and M groups as compared to the control group. However, a very highly significant increase in direct and total serum levels of bilirubin was evident in CF group when compared to the control ($p < 0.001$). In CMF and MMF, administration with morin to FLU-treated rats induced a significant decline in serum levels of direct and total bilirubin in comparison with CF group as shown in Table 3.

Table 3: Serum levels of direct and total bilirubin in control and treated groups.

Groups	Direct bilirubin (mg/dl)	Total bilirubin (mg/dl)
Control	0.19±0.04	0.31±0.06
CM	0.24±0.03	0.38±0.07
M	0.19±0.05	0.32±0.06
CF	0.80±0.04 ^{a***}	1.56±0.19 ^{a***}
CMF	0.53±0.03 ^{b**}	0.99±0.14 ^{b**}
MMF	0.31±0.04 ^{b***}	0.75±0.14 ^{b***}

Data are represented as mean± SE; n= 10

a= control versus CM, M and CF

b= CF versus CMF and MMF

*Significant change at $p < 0.05$

**Highly significant change at $p < 0.01$

***Very Highly significant change at $p < 0.001$

Data in Table 4 showed no changes in MDA and SOD levels between CM and M groups as compared to the control group. In CF- treated group, a very highly significant increase and decrease in MDA and SOD levels were reported, respectively. Whereas, CMF and MMF groups showed a significant decrease in MDA level and increase in SOD level when compared to CF group, respectively.

Table 4: Levels of MDA and SOD in control and treated groups.

Groups	MDA ($\mu\text{mol}/100\text{mg}$)	SOD (U/ g)
Control	0.56±0.1	0.94±0.07
CM	0.64±0.034	0.94±0.09
M	0.53±0.15	1.03±0.18
CF	2.75±0.23 ^{a***}	0.2±0.05 ^{a***}
CMF	1.26±0.45 ^{b**}	0.55±0.06 ^{b**}
MMF	0.96±0.27 ^{b***}	0.81±0.06 ^{b***}

Data are represented as mean± SE; n= 10

a= control versus CM, M and CF

b= CF versus CMF and MMF

*Significant change at $p < 0.05$

**Highly significant change at $p < 0.01$

***Very Highly significant change at $p < 0.001$

Results showed no significant changes in GSH and GST levels in CM and M groups in comparison to control group. While a very highly significant ($P < 0.001$) decrease was observed in CF treated group when compared to control group. While, rats were treated with morin in both CMF and MMF groups displayed a significant increase in GSH and GST levels as compared to CF group (Table 5).

Table5: The average levels of GSH and GST in control and treated groups

Groups	GSH (mg/ g)	GST (μ mol/ min/ g)
Control	50 \pm 0.43	0.39 \pm 0.05
CM	50.4 \pm 0.59	0.39 \pm 0.04
M	54.9 \pm 1.4	0.42 \pm 0.05
CF	28.4 \pm 1.9 ^{a***}	0.12 \pm 0.03 ^{a**}
CMF	40.1 \pm 0.77 ^{b**}	0.19 \pm 0.02 ^{b*}
MMF	48.2 \pm 1.5 ^{b***}	0.32 \pm 0.03 ^{b**}

Data are represented as mean \pm SE; n= 10

a= control versus CM, M and CF

b= CF versus CMF and MMF

*Significant change at $p < 0.05$

**Highly significant change at $p < 0.01$

***Very Highly significant change at $p < 0.001$

DISCUSSION

FLU is an anti-androgen drug used in the prostate cancer therapy. This drug acts preventing the binding circulating suprarenal androgens to androgenic receptors located in the prostate and prostatic cancer cells. However, FLU treatment is also reported to induce a secondary hepatotoxicity. This calls for the search to avoid such effect on using the drug.

In this study, the results indicated that there is a significant increase in hepatosomatic index in groups treated with FLU (CF group). The present findings were confirmed by **Dourakis et al.** (7) who found that FLU administration in patients with metastatic prostate cancer was associated with the development of hepatic encephalopathy that led to increase in liver weight.

The present results were supported by **Coe et al.** (32) who attributed the increase in hepatosomatic index to hepatocellular hypertrophy and the increase in the activity of cytochrome P450 enzyme after FLU administration to rats. Liver weight gain could be explained to an expansion of the

endoplasmic reticulum caused by enzyme induction (33).

Liver is the primary site of biotransformation of foreign compounds, it is vulnerable to chemicals. Various enzymes are prone to the effects of chemicals and their metabolites. Its primary function is the maintenance of sufficient plasma levels of these metabolites (34). In most cases, these enzymes leak out from the necrotic hepatocytes into blood serum in abnormal amounts. Several of these soluble enzymes have been considered as indicators of liver malfunction and damage (35). In the current work, rats treated with FLU for four weeks exhibited a significant increase in the activities of liver biomarkers (ALT, AST, direct and total bilirubin).

In view of the present data, it could assume that FLU administration to rats caused highly significant increase in ALT and AST activities in serum. This increase may be a consequent of increased lipid peroxidation and membrane damage. Lipid peroxidation is caused by the dangerous effect of free radicals on target tissues. Also, the elevation in serum bilirubin can be explained by increased lipid peroxidation as well as glutathione depletion (36). Furthermore, the elevation of transaminases and bilirubin levels were related to FLU induced toxicity to hepatocytes by the cytochrome P450-mediated formation of metabolites as reported by **Matsuzaki et al.** (2). They also attributed the increase in bilirubin level to FLU administration that led to retention of bile salts by inhibition of bile salt export pump (BSEP)-mediated taurocholic transport (37, 38). BSEP is the transport protein primary responsible for biliary excretion of unconjugated bile salts which are more hepatotoxic than amino acids (taurine & glycine) conjugated metabolites (39). In the other way, the level of bilirubin in serum was related to the function of hepatic cells. A high concentration of bilirubin in serum is an indication for erythrocytes degeneration rate caused due to liver injury when treated with hepatotoxin (40).

Lipid peroxidation is an auto-catalytic, free-radical-mediated destructive process, whereby polyunsaturated fatty acids in cell membranes undergo degeneration to form lipid hydroperoxides. These latter compounds then

decompose to form a wide variety of products, including low molecular mass hydrocarbons, hydroxyl aldehydes, fatty acids, ketones, alkenals and alkanals, in particular malondialdehyde (MDA) ⁽⁴¹⁾. In this context, several studies reported that FLU is a well-known inhibitor of complex I and the electron transfer in mitochondria leading to perturbation of cellular respiration⁽⁴⁾. Also, this inhibition of complex I led to generation of increased levels of superoxide radicals released into the matrix through the reduction of molecular oxygen by NADPH/ubiquinone oxidoreductase ⁽⁴²⁾. Alternatively, FLU has been shown to produce reactive metabolites via bio activation by CYP1A2, 3A4, and 2C19 ⁽⁴³⁾. Specifically, aromatic hydroxylation leads to the formation of a reactive quinone amine. In addition, FLU has a potentially reactive aromatic nitro group ⁽⁴³⁾ that could undergo redox cycling ⁽⁴⁴⁾. The nitro group can be also reduced to amine, followed by oxidation to diamine, which would be prone to subsequent nucleophilic attack at the substituted positions of the ring ⁽⁴³⁾.

In the present study, it was found that the level of MDA was significantly elevated by FLU administration in (CF) group. This is in agreement with **Catala** ⁽⁴⁵⁾ who mentioned that MDA content was evaluated as an endpoint, indicative of the extent of lipid peroxidation, since any increase in MDA level reflects the enhancement of lipid peroxidation. This increase was attributed to metabolism of FLU by the cytochrome P-450 enzymes to the reactive electrophilic 2-hydroxy-flutamide which induced activation of Kupffer cell phagocytes. Activated Kupffer cells released the superoxide anion radical, reactive oxygen species and chemotactic cytokines ⁽⁴³⁾.

In the current work, there is a decrease in SOD level in rats of CF treated group and this was attributed to generated ROS by FLU administration was pointed out that under aerobic conditions, oxygen readily accepts the one-electron reduction product of nitroaromatics which is a substructure of FLU or its intermediates to generate superoxide anion and subsequent ROS and RNS that led to a decrease in SOD level ⁽⁴⁶⁾.

GSH is involved in the maintenance of the normal cell structure and function, probably through its redox and detoxification reactions. GSH detoxifies toxic substances so, protects

cellular thiol groups ⁽⁴⁷⁾. GSH can effectively scavenge free radicals and other oxygen species through non-enzymatic and enzymatic process by conjugation with GST ⁽⁴⁸⁾. GST catalyzes the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics, leading to elimination or conversion of xenobiotic-GSH conjugates thus, preventing their interaction with cellular proteins and nucleic acids ⁽⁴⁹⁾.

In the current study, there is a decrease in GSH and GST levels in rats treated with FLU for 4 weeks. This is in accordance with **Shet et al.** ⁽³⁾ who reported that the administration of FLU decreased the glutathione (GSH)/ glutathione disulfide (GSSG) ratio and total protein thiols. This was associated with an early increase in phosphorylase activity (Ca²⁺-dependent enzyme), decrease in cytoskeleton-associated protein thiols and the release of lactate dehydrogenase with subsequent loss of cell viability.

A novel GSH conjugate of FLU was reported in human liver microsomes, suggesting that the P450-mediated oxidation of FLU via a nitrogen-centered free radical could be one of several bio activation pathways of FLU that led to increment of oxidative stress and hence depletion of cellular glutathione as suggested by **Sharaf et al.** ⁽²³⁾.

In the present investigation, morin administration with FLU (CMF and MMF groups) could minimize changes in the body weight and absolute and relative liver weights.

Also, there is a decrease in ALT, AST and bilirubin sera levels in CMF and MMF groups where the last group showed more recovery than CMF group. Furthermore, morin administration decreased cyclophosphamide (CPX) induced toxicity and maintained ALT and AST in their normal levels ⁽⁵⁰⁾. Similar observations were reported by **Magdalan et al.** ⁽⁵¹⁾ and **Subash and Subramanian** ⁽⁵²⁾ who found that treatment with morin prevented hepatotoxicity caused by toxic substances, improved the status of antioxidants and decreased liver markers enzymes. So, use of morin as protective agent was better than its use as a therapeutic in this study.

The present findings are in agreement with those of previous studies that reported the decreased activities of these enzymes may be

due to the prevention of intracellular enzyme leakage resulting from cell membrane stability or cellular regeneration⁽²⁵⁾. They showed that morin had hepatoprotective and antifibrogenic effects against dimethylnitrosamine (DMN) induced hepatic injury and inhibited the elevation of ALT, AST and total bilirubin levels. It also reduced the expression of collagen type I, transforming growth factor beta (1) and alpha-smooth muscle actin thus preventing hepatic fibrosis induced by DMN⁽⁵³⁾.

In the current study, CMF and MMF groups showed moderate and marked decrease in MDA level, respectively through scavenging free radicals. So, morin protects hepatocytes from oxidative damage induced by FLU due to its antioxidative effect⁽¹⁶⁾. As a flavonoid, it has an antioxidant effect and decreased the adverse effects of ROS that caused oxidative damage to macromolecules such as lipids, DNA and proteins of hepatocytes. So, it caused a decrease in ALT, AST, bilirubin and MDA and an increase in SOD, GST and GSH⁽¹⁶⁾.

Current results are in agreement with earlier studies pointing that morin has beneficial effects on the liver damage by enhancing antioxidant enzyme activity and help to scavenge superoxide and hydroxyl radicals. So it decrease MDA and SOD levels^(14, 24). They mentioned that flavonoid derivative of morin fully reversed the CPX-induced changes, especially decreasing MDA level in the liver⁽⁵⁴⁾. Similarly, it was reported that morin flavonoids is potent free radical scavenger and antioxidant due to its phenolic ring and hydroxyl substituents which can function as effective antioxidant by virtue of its ability to quench free radicals and maintain SOD level to normal level^(55, 56).

CONCLUSION

From the present data, it could be concluded that administration of the anticancer drug, FLU to adult male rats was found to induce biochemical alterations which were relatively improved by the administration of flavonoid morin especially in case of prior to administration with FLU. The ameliorative effect of morin may be resulted from protecting hepatocytes from free radical and maintaining protein metabolism and synthesis. So, there is no doubt that morin is consider as powerful

hepatoprotective substance that can protect the hepatocytes through different mechanisms.

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