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# A safety study of oral xanthohumol administration and its influence on fertility in Sprague Dawley rats

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Xanthohumol (XN) is a prenylated chalcone, which has been shown to possess a broad range of potential cancer preventive and additional biological activities. In the present study, we have determined the subchronic 4-wk toxicity of XN and monitored its influence on fertility and development of offspring in two fertility studies. Four-week-old female Sprague Dawley (SD) rats were treated with 0.5% XN in the diet or with 1000 mg XN/kg body weight (b.w.) *per* day by gavage for 28 days. No remarkable treatment-related changes in general appearance and b.w. occurred during the study. After autopsy, liver, kidney, lung, heart, stomach, and spleen were examined macroscopically and histopathologically. Relative liver weights of animals in both treatment groups were significantly reduced by 30 – 40% in comparison with the control group, indicating weak hepatotoxicity. Also, mammary glands of treated rats appeared less developed compared to the controls. Consequently, we investigated the influence of XN on rat reproduction. In two fertility studies, XN (100 mg/kg b.w. *per* day), given either for 4 wk prior to or during mating, gestation, and nursing, did not cause any adverse effects on female reproduction and the development of offspring. Noteworthy, treatment of male rats prior to mating significantly ($p = 0.027$) increased the sex ratio of male to female offspring. Overall, lifelong treatment at a daily dose of 100 mg/kg b.w. in a two-generation study did not affect the development of SD rats.

**Keywords:** Fertility study / Hepatotoxicity / Sex ratio / Sprague Dawley rats / Subchronic toxicity / Xanthohumol

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## 1 Introduction

Xanthohumol (XN), a prenylated chalcone and principle prenylflavonoid in hop (*Humulus lupulus* L.), has been reported to exhibit antimutagenic and antioxidative properties and to possess a broad spectrum of cancer chemopreventive activities *in vitro* [1 – 4]. As a first demonstration of its chemopreventive potential, XN at nanomolar concentrations prevented carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture (MMOC) [1]. In addition, XN was found to protect against osteoporosis [5] and arteriosclerosis [6]. Together, these findings open new perspectives of XN application in medical treatment.

XN is commonly ingested by humans in the form of beer and beer mix drinks. Due to the thermal conversion of XN to the isoflavanone isoxanthohumol during the brewing process, the concentration of XN in beer is very low (approximately 0.08 ± 0.03 mg/L in German style lager beers) [1]. Highest levels up to 1.2 mg/L were found in stout and porter beers [7, 8]. Considering the potential future development of XN as a cancer preventive or therapeutic agent, its toxicity as well as its pharmacological properties are gaining increasing interest. So far, little is known about the metabolism, bioavailability, and distribution of XN *in vivo*, particularly after oral administration. In previous studies, the acute toxicity of XN as well as its metabolism was investigated in rats. Female Sprague Dawley (SD) rats were treated with 1000 mg/kg b.w. by gavage. Observing these animals for up to 48 h revealed no adverse effects of XN treatment. Accordingly, a maximal tolerable dose (MTD) of more than 1000 mg XN/kg b.w. *per* day was determined. In that study,

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**Abbreviations: b.w.**, body weight; **f**, female; **F**, filial generation; **m**, male; **P**, parental generation; **SD**, Sprague Dawley; **XN**, xanthohumol

XN was largely recovered in unchanged form: more than 80% of the applied XN was excreted in feces and urine, suggesting that the bioavailability of XN after oral administration is very low [9]. Similarly, Avula *et al.* [10] determined the pharmacokinetics of XN in male Wistar rats. In a first experiment, rats received either oral or intravenous administration of a low dose (20 mg/kg b.w.) of XN. In a second experiment, rats obtained a single oral administration of up to 500 mg XN/kg b.w. Plasma, urine, and feces were collected at varying time points and assayed for their XN content by HPLC. Consistent with our report [9], XN and its metabolites were excreted mainly in feces within 24 h of administration.

Recently, Vanhoecke *et al.* reported the results of a 4-wk safety study of XN in mice. Daily application of about 23 mg/kg b.w. did not lead to any noticeable sign of toxicity in bone marrow, liver, exocrine pancreas, kidneys, muscles, thyroid, ovaries, and surrenal cortex. Furthermore, no differences were noted in the metabolism of proteins, lipids, carbohydrates, and uric acid, as well as in ion concentrations [11].

In the present report, we investigated the subchronic toxicity of XN in female SD rats at daily doses up to 1000 mg/kg b.w. Since XN has been reported to possess antiestrogenic potential *in vitro,* whereas hop is generally associated with estrogenic properties (summarized in Ref. [4]), we also examine its effect on rat fertility.

## 2 Materials and methods

### 2.1 Animal experiments

Four-week-old female SD rats (toxicity studies) and 10-week-old male and female SD rats (mating experiments) were purchased from Charles River Wiga (Sulzfeld, Germany) and were housed in a climate controlled room with a 12-h light/12-h dark cycle. Rats were fed a standard diet (Altromin, Lage, Germany). Animals were given free access to diet and water. All animal experiments were permitted by an ethical committee (Regierungspräsidium Karlsruhe).

### 2.2 Studies on subchronic toxicity of XN

Four-week-old female SD rats were treated with XN for a period of 28 days. Rats were fed either a standard diet enriched with 0.5% XN to achieve a mean dose of approximately 500 mg/kg b.w. *per* day (XN-1), or received XN by gavage at 1000 mg/kg b.w. *per* day in 1% starch suspension (XN-2), in comparison with a vehicle-treated (1% starch suspension) control group ($n = 3$ *per* group). The daily intake of XN in group XN-1 was calculated from the amount of food intake (g) and b.w. (g) assessed daily. After 4 wk of treatment, all animals were sacrificed by heart puncture under ether anesthesia. Blood and organs (liver, kidney, lung, heart, stomach, and spleen) were resected for further investigations. Tissue samples were partly fixed in formalin solution for subsequent histological examination.

### 2.3 Fertility studies

#### 2.3.1 Study 1

Ten-week-old male (m) and female (f) SD rats ($n = 12$ *per* sex) were administered with XN in drinking water (100 mg/kg b.w. *per* day, dissolved in 0.75% EtOH containing 1.5% Tween 20) for 4 wk prior to mating. The rats ($n = 6$ *per* sex and treatment) were divided into four mating groups (m, male; f, female; XN, XN-treated; C, control): group 1, $m_C + f_C$; group 2, $m_{XN} + f_C$; group 3, $m_C + f_{XN}$; group 4, $m_{XN} + f_{XN}$. XN treatment was discontinued during mating, gestation, and nursing. Offspring of all groups was observed and weighed every day for 3 wk.

#### 2.3.2 Study 2 (two-generation study)

In a second experiment, two consecutive generations of female rats were treated with XN. XN (dissolved in 0.75% EtOH containing 1.5% Tween 20) was provided at a daily dose of 100 mg/kg b.w. in the drinking water. Treatment of female rats ($P_{XN}$; $n = 3$) was initiated at 1 wk prior to mating, and continued during mating, pregnancy, as well as nursing of the offspring. Males received XN during mating, since the XN solution was the only source of liquid. Untreated controls ($P_C$; $n = 2$) were carried along. The offspring of treated and control dams was observed for 3 wk. XN treatment was continued in the daughter generation ($F1_{XN}$) of the treated dam group ($P_{XN}$). At the age of 8 wk, XN-treated female rats ($F1_{XN}$; $n = 6$) were mated with male rats, which received XN only during the mating period. The results were compared with an untreated control group ($F1_C$; $n = 3$). Treatments and mating of the different generations are summarized in Table 1.

### 2.4 Determination of plasma testosterone levels

Sodium acetate (0.78 M; 55 µL) and ascorbic acid (0.1 M; 50 µL) solutions were added to plasma samples (500 µL) along with β-glucuronidase (three drops, containing 20 U/mg) in microcentrifuge tubes and incubated overnight (18 h) at 37°C. The reaction was stopped by addition of phosphoric acid (85%, 10 µL). The samples were eluted through primed (methanol, 5.0 mL; distilled water, 5.0 mL) SepPak cartridges, which were washed with acetic acid (2%) in aqueous methanol (5%), and testosterone was eluted with methanol (2.0 mL). The eluates were dried

 

**Table 1.** Treatment and mating scheme (Fertility study 2)

| | Group $P_{XN}$: 3 female rats | Group $P_C$: 2 female rats |
|---|---|---|
| 1 wk prior to mating | +XN[a)] | – |
| 1 wk mating | +XN[b)] | – |
| 3 wk pregnancy | +XN | – |
| 3 wk nursing | +XN | – |
| | Group $F1_{XN}$: 6 female rats | Group $F1_C$: 3 female rats |
| development until day 60 | +XN | – |
| 1 wk mating | +XN[b)] | – |
| 3 wk pregnancy | +XN | – |
| 3 wk nursing | +XN | – |
| | Group $F2_{XN}$ | Group $F2_C$ |

a) 100 mg/kg b.w. *per* day *via* drinking water.
b) Males received XN (100 mg/kg b.w. *per* day) only during mating (1 wk).

under a stream of nitrogen. The samples were converted to their *O*-TMS ether derivatives by addition of 100 µL MSTFA – $NH_4I$ – ethanethiol (1000 : 2 : 6, v/w/v) and heated at 60°C for 30 min.

Quantitation was achieved by GC-MS in comparison with an external standard curve. Analyses were performed using an HP 5973 mass selective detector coupled to an HP 6890 gas chromatograph. Sample volumes (1 µL) were injected into the gas chromatograph. Separation of analytes was achieved using an HP 5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness). Helium was used as carrier gas with linear velocity of 0.9 mL/s. The oven temperature program: initial temperature 160°C, 160 – 270°C at 4°C/min, 270°C for 20 min. The GC injector temperature was 250°C; the transfer line temperature was held at 280°C. The mass spectrometer parameters for EI mode were as follows: ion source temperature, 230°C; electron energy, 70 eV; filament current, 34.6 µA; electron multiplier voltage, 1200 V.

### 2.5 Statistical analysis

The statistical analysis was carried out using the software package R, version 2.0.1 [12]. The effect of XN treatment on relative organ weights of the subchronic toxicity study was investigated by analysis of variance (ANOVA). Linear models were fitted to test for differences between each of the treatment groups XN-1 and XN-2 and the control group. *p*-Values were adjusted according to the method of Bonferroni-Holm to control the familywise error rate at a level of 5% [13].

The male/female ratio of the offspring in the Fertility study 1 was analyzed by a two-way ANOVA model with factors "treatment male" (control, XN) and "treatment female" (control, XN), including interactions.

## 3 Results

### 3.1 Studies on subchronic toxicity of XN

We performed a subchronic toxicity study with XN in female SD rats (4-wk-old). In a pilot experiment, we attempted to achieve a daily dose of 1000 mg/kg b.w. by feeding a diet enriched with 1.5% XN. However, since the rats refused to eat the XN-enriched diet and lost weight, the experiment had to be terminated prematurely (data not shown). Consequently, in the subsequent experiment described here, the dose of 1000 mg/kg b.w. *per* day was applied by gavage (group XN-2, $n = 3$). In comparison, rats were fed a diet containing 0.5% XN. Measuring the daily consumption of XN by food intake over 4 wk provided an average dose of 645 ± 129 mg/kg b.w. *per* day (group XN-1, $n = 3$). In relation to the b.w. change over time, the dose of XN decreased from 750 ± 165 mg/kg b.w. in the first week of treatment to 537 ± 77 mg/kg b.w. in the fourth week.

In both treatment groups, there was no variation in b.w. and b.w. gain during the feeding period of 4 wk (Fig. 1). Also, the relative size of most organs was not affected by XN

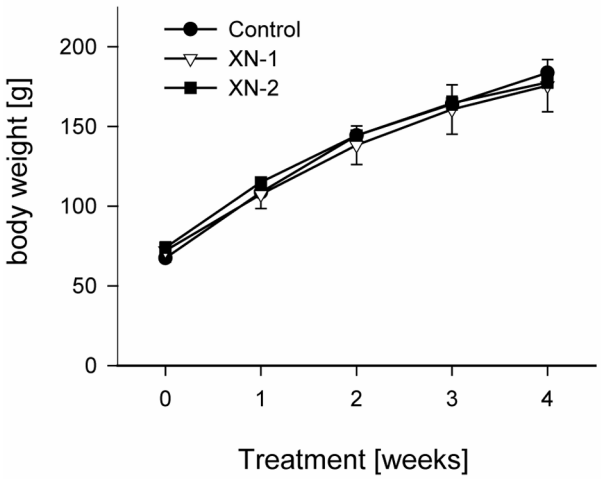


**Figure 1.** Average b.w. ± SEM of female S-D rats ($n = 3$) treated for 4 wk with vehicle (control, ●), 0.5% XN in the diet (XN-1, ▽), or 1000 mg XN/kg b.w. *per* day by gavage (XN-2, ■), respectively.

treatment (Fig. 2). We found a significant decrease of about 30 – 40% in relative liver weights (normalized to b.w., expressed in %) in comparison to the control (average ± SEM; control: 4.64 ± 0.14%; XN-1: 3.29 ± 0.12%; XN-2: 2.92 ± 0.11%; global test for treatment effects by ANOVA: $p \leq 0.001$; pairwise comparisons: XN-1 *vs.* control, $p \leq 0.001$; XN-2 *vs.* control, $p \leq 0.001$). Histological investigations indicated a loss of glycogen in the livers of XN-treated animals in both treatment groups. Further, a slight increase in proliferation of bile duct cells was suggestive of mild

 

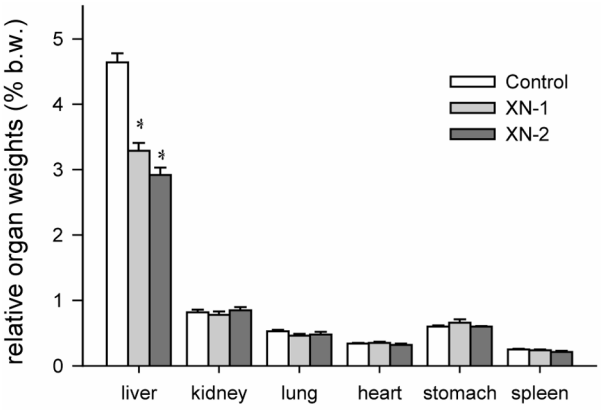


**Figure 2.** Relative organ weights (% of b.w.) of female SD rats (mean + SEM, $n = 3$) after 4 wk of subchronic treatment with XN. Treatment groups: Control (vehicle treatment only); XN-1, 0.5% XN in the diet; XN-2, 1000 mg XN/kg b.w. *per* day by gavage. * Mean ($n = 3$) significantly ($p < 0.001$) different from control (linear model, Wald tests).

hepatoxicity. These findings were confirmed by two independent pathologists (P. Bannasch, F. Amelung, personal communication). No macroscopic or histological signs of toxicity were observed in kidney, lung, heart, stomach, and spleen of either treatment group. In comparison with the control group, the size of the mammary glands of XN-treated rats in both treatment groups was distinctly decreased as observed macroscopically (glands were not weighed).

### 3.2 Fertility studies

The changes in mammary gland size and the potential antiestrogenic activity of XN *in vitro* [1, 4] raised the question whether the compound would influence reproduction *in vivo*. This prompted us to initialize a fertility study in rats.

#### 3.2.1 Fertility study 1

In the first study, male and female SD rats at the age of 10 wk were treated with XN over a period of 4 wk prior to mating. Based on the results of the toxicity study, the daily dose of XN was reduced to 100 mg/kg b.w. administered *via* the drinking water. At this dose, no effects on average b.w. or liver size of the male rats sacrificed directly after mating were observable (average ± SEM; b.w. control: 456.8 ± 16.8 g ($n$ = 11); b.w. XN-treated: 481.2 ± 7.3 g ($n$ = 12); relative liver weight (in % b.w.) control: 3.19 ± 0.065%; XN-treated: 3.30 ± 0.073%).

Treated and control rats were mated crosswise in four groups ($n$ = 6). Litter sizes were in a range of 11 – 17 pups *per* dam (summarized in Table 2), and we did not observe an influence of XN treatment to either male or female rats

**Table 2.** Summary of Fertility study 1

| | Dam | Offspring sex | | Offspring sex ratio | Offspring b.w., g[d] | |
|---|---|---|---|---|---|---|
| | | m (no.) | f (no.) | (m/f) | Day 7 | Day 19 |
| Group 1 $m_c + f_c$ | 1[a] | n.a. | n.a. | n.a. | n.a. | n.a. |
| | 2 | 2 | 12 | 0.17 | 13.6 | 37.0 |
| | 3 | 6 | 8 | 0.75 | 13.4 | 34.9 |
| | 4 | 6 | 8 | 0.75 | 12.6 | 37.0 |
| | 5 | 8 | 9 | 0.89 | 10.9 | 28.8 |
| | 6 | 8 | 7 | 1.14 | 12.3 | 32.0 |
| | Σ | 30 | 44 | 0.74 ± 0.16[c] | 12.5 ± 0.5[c] | 33.9 ± 1.6[c] |
| Group 2 $m_{XN} + f_C$ | 1 | 8 | 4 | 2.00 | 14.7 | 35.8 |
| | 2 | 4 | 11 | 0.36 | 12.3 | 28.8 |
| | 3 | 6 | 6 | 1.00 | 16.0 | 35.0 |
| | 4 | 9 | 6 | 1.50 | 16.0 | 35.4 |
| | 5 | 6 | 8 | 0.75 | 12.9 | 27.2 |
| | 6 | 8 | 5 | 1.60 | 13.7 | 35.2 |
| | Σ | 41 | 40 | 1.20 ± 0.25 | 14.3 ± 0.6 | 32.9 ± 1.6 |
| Group 3 $m_c + f_{XN}$ | 1 | 6 | 9 | 0.67 | 15.0 | 37.2 |
| | 2 | 9 | 7 | 1.29 | 12.9 | 28.9 |
| | 3[b] | n.a. | n.a. | n.a. | n.a. | n.a. |
| | 4 | 4 | 7 | 0.57 | 13.6 | 31.5 |
| | 5 | 6 | 5 | 1.20 | 17.6 | 38.8 |
| | 6 | 6 | 6 | 1.00 | 13.6 | 31.6 |
| | Σ | 31 | 34 | 0.95 ± 0.14 | 14.5 ± 0.8 | 33.6 ± 1.9 |
| Group 4 $m_{XN} + f_{XN}$ | 1 | 11 | 6 | 1.83 | 11.2 | 39.2 |
| | 2 | 5 | 7 | 0.71 | 13.0 | 35.9 |
| | 3 | 8 | 6 | 1.33 | 16.2 | 36.7 |
| | 4 | 9 | 4 | 2.25 | 16.7 | 39.2 |
| | 5 | 6 | 6 | 1.00 | 15.2 | 35.9 |
| | 6 | 10 | 5 | 2.00 | 15.5 | 36.7 |
| | Σ | 49 | 34 | 1.52 ± 0.25 | 14.6 ± 0.9 | 37.3 ± 0.6 |

n.a.: not applicable.
a) Not pregnant.
b) All pups were dead.
c) Average ± SEM.
d) Average b.w. of all pups of one dam.

or both on litter size. We analyzed the sex ratio of the offspring and noticed a slight treatment-dependent shift in the distribution of male and female pups (Table 2). The male/female ratio increased from 0.74 in the control (group 1) to 0.95 when only female rats received XN (group 3). When only males were treated with XN (group 2), the ratio further increased to 1.20, whereas the most male pups were born when both males and females received XN (group 4). Investigation by a two-way ANOVA model regarding the factors treatment male and treatment female, respectively, as well as the interaction of both revealed a significant influence of the factor treatment male on the sex ratio ($p = 0.027$). Neither the factor treatment female nor the interaction of both had a significant effect.

Pups were weighed daily to monitor any influence of XN treatment prior to mating on offspring development. The average weight at birth was in a normal range of 4 – 6 g. All pups developed normally during the first 21 days. Exemplary, offspring b.w. at days 7 and 19 are summarized in Table 2. At day 19, pups from group 4 had the highest average b.w. This might not be surprising since in this group the highest number of male offspring was born.

 

### 3.2.2 Fertility study 2

To investigate the transplacental influence of XN on the development of offspring during pregnancy, we performed a two-generation study (scheme in Table 1). Adult female SD rats (group $P_{XN}$, $n = 3$) were treated with XN (100 mg/kg b.w. *per* day in drinking water) over a period of 1 wk prior to mating as well as during gestation and nursing. Males received XN only during the mating period (1 wk). As summarized in Table 3, litter sizes, within a range of 2 – 15 pups *per* dam, were smaller in both the XN-treated ($P_{XN}$) as well as the control group ($P_C$, $n = 2$) in this second study than in the first study. This might be due to the season, since the first study was conducted in summer, whereas the second study was completed in winter. One dam in the control group delivered only two pups (both male), therefore a comparison of the offspring sex ratio as in Fertility study 1 was not feasible. Offspring was weighed daily to monitor an influence of XN treatment during pregnancy and weaning on offspring development. Smaller litter sizes strongly influenced the average offspring b.w. gain, which was higher than in the first study in both the treated as well as in the control group (Table 3). Overall, all pups developed normally and there was no indication of any adverse effects of XN treatment.

Female rats of generation $F1_{XN}$ ($n = 6$), which had received XN during their whole lifetime starting with procreation, were again mated and further treated with XN (100 mg/kg b.w. *per* day in drinking water). Offspring numbers and development were compared with a control group ($F1_C$, $n = 3$) as shown in Table 1. Males in group $F1_{XN}$ received XN only during the mating period (1 wk). Lifelong XN treatment did not strongly influence female conception. All except one dam became pregnant without any complications during gestation. Second generation litter sizes were in the range of 8 – 16 pups *per* dam (Table 3). The offspring male/female sex ratio in the XN-treated $F1_{XN}$ group (1.14 ± 0.86) again was slightly increased in comparison with the control group $F1_C$ (0.77 ± 0.26). Average b.w. of pups in the second generation after 7 and 19 days were similar in the XN-treated ($F2_{XN}$) and the control groups ($F2_C$), indicating normal development (Table 3).

**Table 3.** Summary of Fertility study 2

| | Dam | Offspring sex | | Offspring sex ratio | Offspring b.w., g[b] | |
|---|---|---|---|---|---|---|
| | | m (no.) | f (no.) | (m/f) | Day 7 | Day 19 |
| Group $P_C$ | 1 | 4 | 11 | 0.36 | 14.7 | 32.7 |
| | 2 | 2 | 0 | n.a. | 20.0 | 46.5 |
| | Σ | 6 | 11 | n.d. | 17.4 ± 2.6[c] | 39.6 ± 6.9[c] |
| Group $P_{XN}$ | 1 | 6 | 6 | 1.00 | 18.5 | 41 |
| | 2 | 5 | 1 | 5.00 | 20.2 | 49 |
| | 3 | 5 | 3 | 1.67 | 21.8 | 50.1 |
| | Σ | 16 | 10 | 2.56 ± 1.24[c] | 20.2 ± 1.0 | 46.7 ± 2.9 |
| Group $F1_C$ | 1 | 3 | 6 | 0.50 | 17.6 | 47.7 |
| | 2 | 6 | 7 | 0.86 | 15.4 | 41.1 |
| | 3 | 6 | 6 | 1.00 | 20.0 | 51.6 |
| | Σ | 15 | 19 | 0.77 ± 0.15 | 17.7 ± 1.3 | 46.8 ± 3.1 |
| Group $F1_{NX}$ | 1[a] | n.a. | n.a. | n.a. | n.a. | n.a. |
| | 2 | 5 | 10 | 0.50 | 18.4 | [d] |
| | 3 | 6 | 10 | 0.60 | 13.6 | [d] |
| | 4 | 10 | 4 | 2.50 | 18.0 | [d] |
| | 5 | 9 | 6 | 1.50 | 14.6 | [d] |
| | 6 | 3 | 5 | 0.60 | 21.2 | [d] |
| | Σ | 33 | 35 | 1.14 ± 0.39 | 17.2 ± 1.4 | 49.2[d] |

n.a.: not applicable.
n.d.: not determined.
a) Not pregnant.
b) Average b.w. of all pups of one dam.
c) Average ± SEM.
d) Pups were weighed all together without assignment to dams. Total weight was divided by the number of pups. Therefore, the SEM could not be determined.

### 3.3 Determination of plasma testosterone

As a possible explanation for the shift in the sex ratio observed in Fertility study 1, we determined plasma testosterone levels of XN-treated males in comparison with untreated controls by GC-MS analyses. One week after the last application of XN (after mating), levels of free and glucuronized testosterone in the treated group (average ± SEM, 4.6 ± 0.5 nM, $n = 12$) were not significantly different ($p = 0.22$) from the control group (3.6 ± 0.6 nM, $n = 11$).

## 4 Discussion

The aim of the present study was to determine the subchronic 4-wk toxicity of XN and to monitor its influence on fertility and development of offspring in two fertility studies.

In the 4-wk toxicity study, XN at high daily doses of up to 1000 mg/kg b.w. significantly decreased the relative liver size of treated animals, indicative of mild hepatotoxicity, whereas the relative weights of kidney, lung, heart, stomach, and spleen as well as the average b.w.s were not changed in comparison with the control group. Liver toxicity is an increasing problem associated with the uncontrolled rising consumption of herbal dietary supplements [14]. The liver is primarily responsible for the detoxification of xenobiotics, leading to metabolites that are readily excreted in bile and urine. We and others have demonstrated that XN is efficiently metabolized by human and rat liver microsomes [15 – 17] and recombinant human phase 2 enzymes [18]. Accordingly, the liver was the main target of XN-mediated toxic effects. Since the liver has the ability to regenerate itself, the observed signs of mild hepatotoxicity induced by XN might be reversible after termination of the intervention. Of notice, the concentrations chosen in this toxicity study were considered as maximum tolerable doses to detect potentially harmful effects of the compound. Generally, it is unlikely that animals or humans are naturally

 

exposed to doses of XN in this concentration range. In the fertility studies, we applied a tenfold lower dose (100 mg/kg b.w. *per* day) for 4 wk, and XN treatment did not cause any adverse effects with respect to reproduction. Also, in XN-treated male rats which were sacrificed 1 wk after the last application of XN (after mating), no signs of liver toxicity were observed, and average liver and body weights were not changed in comparison with the control group. This is in line with a recent study published by Vanhoecke *et al.* [11]. XN application for 4 wk to mice at a daily dose of about 23 mg/kg b.w. did not lead to any adverse effects or signs of toxicity. Additional *in vivo* studies of chronic treatment with XN have not been reported until now.

So far, we did not further investigate the cause of the observed reduction in liver weight or the influence of high XN concentrations on liver functions. Based on histopathology, we speculate that the loss in liver weight is caused by a breakdown of glycogen and a significant reduction in hepatic glycogen stores. Further studies have to clarify the underlying mechanisms of hepatotoxicity, which might involve direct cytotoxic processes, oxidative stress, mitochondrial injury, and apoptosis [19]. XN has been shown to inhibit the growth of various cancer cell lines *in vitro* and to induce apoptosis at low μM concentrations [1, 20, 21]. The capacity of hydroxychalcones to cause a breakdown of the mitochondrial membrane potential has been hypothesized as one underlying mechanism of apoptosis induction [21, 22]. Hitherto, it has not conclusively been shown that the antiproliferative potential of XN is selective for tumor cells. At least, XN did not inhibit the proliferation of human primary hepatocytes *in vitro* when treated with XN at a concentration of 10 μM for 24 or 48 h [20].

An additional effect of subchronic treatment with high concentrations of XN was a conspicuous reduction in the size of mammary glands, observed in the toxicity study. XN is known to possess antiestrogenic activity. This was demonstrated *in vitro* in the Ishikawa cell line and by inhibition of aromatase activity [1, 4]. Further, in an *in vivo* rat uterotrophy assay in prepubertal rats, XN treatment reduced unstimulated as well as ethinylestradiol-induced uterine weights by about 30% [23]. In the two fertility studies described here, this potential influence of XN on the development of mammary glands and female reproductive organs did not influence rat reproduction. Overall, XN at a concentration of 100 mg/kg b.w. *per* day did not affect either fertility or mating and nursing abilities of the rats. Also, the development of the offspring of the rats treated with XN was not different from the growth of the control rats' offspring. Similar results were observed in two subsequent experiments when XN was applied either prior to or during mating, pregnancy, and nursing (Tables 2 and 3). Therefore, we conclude that XN does not impair fertility and/or nursing ability of SD rats.

Interestingly, XN treatment of male rats seems to influence the gender distribution of the offspring. We found a significant shift in the sex ratio of male to female offspring influenced by the factor treatment males. Treatment of the females did not affect the sex ratio, neither did the interaction between both treatments. The most likely explanation for a change in the sex ratio is a hormonal mechanism [24, 25]. This consideration might be supported by the fact that XN inhibits aromatase activity and consequently the generation of estrogens from testosterone [4, 23]. This might result in an accumulation of testosterone. To clarify this potential correlation, we measured plasma testosterone levels of males from Fertility study 1. One week after the last application of XN, testosterone levels in the plasma of males treated for 4 wk with XN were not significantly different from controls. These data are consistent with the results of a 4-wk safety study of XN in mice [11]. Another possible reason for the modulation of the sex ratio by the treated males might be an interaction of XN with mechanisms of spermatogenesis, sperm viability, and motility.

In conclusion, the data presented here suggest that 4-wk oral application of XN at doses up to 1000 mg/kg b.w. causes weak hepatotoxicity in female SD rats, but does not influence reproduction and the development of two generations of offspring when given at a daily dose of 100 mg/kg b.w.

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