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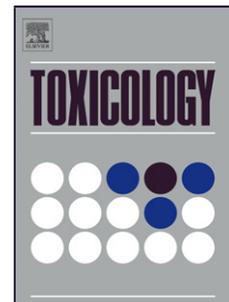


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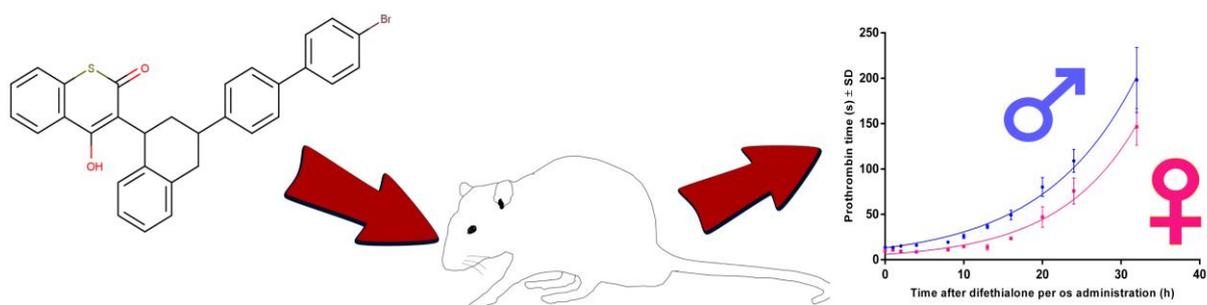
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Graphical Abstract



Abstract

Vitamin K antagonists (VKA) are used either in human medicine to prevent thromboembolic disorders or as rodenticides for pest control management. In rodents, female rats are described to be more tolerant to the action of vitamin K antagonists than males. Nevertheless, the mechanism of this greatest tolerance is still unknown and this study aims to identify the origin of this greatest tolerance after VKA administration. Therefore, difethialone, one of the most powerful VKA was used in this study.

A possible difference in the pharmacokinetics of difethialone between males and females was first investigated. The determination of the pharmacokinetic parameters allowed to exclude a pharmacokinetic origin of the greatest tolerance of females to VKA. Thus, a natural resistance to difethialone of the liver VKOR activity, which is the target of VKA, was thus explored in females. The determination of K_i towards difethialone in liver microsomes allowed to also exclude this hypothesis. Therefore, equipment in vitamin K-dependent clotting factors and properties of vitamin K-dependent clotting factors were explored. Basal activity of clotting factors VII and X were found significantly higher in females of respectively 43% and 21%. Moreover, after VKA administration, half-lives of clotting factors II and X were found significantly longer in females of respectively 27% and 10% and a lag time of 4 hours before the beginning of the decay of factor VII was observed only in females after difethialone administration. The greater tolerance of female rats to VKA is thus due a stronger basal pool of vitamin K-dependent clotting factors VII and X and to a slower decline of vitamin K-dependent clotting factors II, VII and X after VKA administration.

Abbreviations

Gla: carboxylated glutamate

Glu: glutamate

GGCX: gamma-glutamyl carboxylase

VKA: vitamin K antagonists

Keywords: Difethialone; vitamin K antagonist; susceptibility, gender differences; rat.

1 INTRODUCTION

In human medicine, vitamin K antagonists (VKA), derivatives of either 4-hydroxycoumarin or indane-1,3-dione, are widely prescribed in the prevention and the treatment of thromboembolic disorders. Warfarin, a derivative of 4-hydroxycoumarin, is the most used VKA worldwide and is prescribed to 2 million new patients in the USA each year. VKA are also used as rodenticides for pest control management. VKA exert their anticoagulant activity by stopping the recycling of vitamin K, which is the cofactor of oxydo-reduction reactions, including the activation of vitamin-K dependent clotting factors. The activation of vitamin-K dependent clotting factors is performed by the gamma-glutamyl carboxylase (GGCX). In the endoplasmic reticulum, GGCX converts glutamate (Glu) of vitamin K-dependent proteins in carboxylated glutamate (Gla) (Furie et al., 1999). For each conversion, vitamin K hydroquinone has to be converted in vitamin K epoxide. Since the vitamin K dietary intake is not sufficient for the physiological needs (Ferland, 2012), vitamin K epoxide needs to be recycle in vitamin K hydroquinone by a vitamin K 2,3-epoxide reductase (VKOR) activity which involves mainly a vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) protein (Oldenburg et al., 2006). It is this enzyme which is the target of VKA.

Clotting factors II (i.e., Prothrombin), VII, IX and X are vitamin K-dependent proteins involved in the coagulation. These clotting factors need to be gamma-carboxylated to be able to chelate calcium and have a biological activity (Bandyopadhyay, 2008). The uncarboxylated vitamin K-dependent clotting factors circulate in very low level in rat blood (Harauchi et al., 1986). Clotting factor VII is involved in the extrinsic pathway, clotting factor IX in the intrinsic pathway, prothrombin and factor X are involved in the common pathway. Consequently, active vitamin K-dependent clotting factors are necessary for coagulation. By inhibiting VKORC1 protein (Rost et al, 2004; Li et al, 2004), VKA block the gamma-carboxylation of vitamin K-dependent clotting factors, impairing the coagulation function.

VKA are difficult to use because of a narrow therapeutic index and a wide variability of dosage necessary to achieve stable anticoagulation. This variability is partly due to basic

physiological parameters such as age, body weight, but also to co-occurring disorders, drug and food interactions. Sex-differences in the response to VKA could exist. If these differences are difficult to demonstrate in human medicine, such differences have been demonstrated in rats (Gill et al., 1994; Kerins and McNicoll, 1999). Female rats are more tolerant to VKA than males. The underlying mechanism of this resistance is still unknown. Differences of VKOR activity could be associated with the natural resistance of females. Indeed, mutations of VKORC1 enzyme can lead to resistance to VKA (Rost et al, 2004; Watzka et al, 2011; Hodroge et al, 2012; Pelz et al, 2005, 2012). If this VKORC1 mutation-induced mechanism can be excluded in the case of the natural resistance to VKA of females, differences in the expression level of genes encoding putative partner proteins of VKORC1, such as calumenin (Wallin et al, 2001), epoxide hydrolase (Guenthner et al, 1998) or glutathione transferase (Cain et al, 1998), could modify this VKOR activity. Other mechanisms could be suspected to explain the better tolerance of females to VKA, such the existence of metabolic differences between males and females (Huber et al., 1999; Markussen et al., 2007, 2008; Mugford and Kedderis, 1998; Waxman, 1988), or even a natural resistance of the coagulation cascade (Lemini et al., 2007) after VKA administration. The aim of this study is to demonstrate first the greater tolerance of the female rats to VKA compared to males and to characterize this tolerance, and thus to identify the underlying mechanism of this greatest tolerance to VKA.

2 MATERIALS AND METHODS

2.1 ANIMALS

OFA-Sprague-Dawley rats were obtained from a commercial breeder (Charle Rivers, l'Arbresles, France) and acclimated for a minimal period of 5 days. The rats were housed four per cage under a constant photoperiod and ambient temperature. In order to characterize the VKOR activity, 8-weeks old OFA-Sprague-Dawley rats (4 males and 4

females) were killed by decapitation. Liver was removed quickly and microsomes were immediately prepared. In order to study the decay kinetics of the vitamin K-dependent blood clotting factors after administration of anticoagulants, 8-weeks old OFA-Sprague-Dawley rats (44 males and 44 females) have been fed with vitamin K3-deficient food (Scientific Animal Food and Engineering, Augy, France), at least 48 hours before the beginning and during the experience. To explore the basal levels of blood parameters, a first group of 8 males and 8 females were killed 48h after the beginning of the vitamin K3-deficient feeding. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at -80°C. The other animals received by force-feeding a *per os* administration of 10 mg/kg of difethialone 48h after the beginning of the vitamin K3-deficient feeding, then a group of 3 males and 3 females was killed 1, 2, 4, 8, 10, 13, 16, 20, 24, 32, 48 and 72 hours after the difethialone administration. Blood was collected in citrate tubes (3.2%; 1:9 v/v) by intracardiac taking and liver was collected and frozen at -80°C.

2.2 MICROSOME PREPARATION

Liver microsomes were prepared by differential centrifugations as described by Moroni (Moroni et al., 1995). Briefly, liver cells were resuspended in 50 mM Phosphate Buffer (pH 7.4) containing 1.15 % (w/v) of KCl. Liver cells were broken by Potter and further submitted to differential centrifugation continuously at 4°C. The 100,000 × g pellet corresponding to the membrane fraction was resuspended by Potter homogenization in HEPES glycerol buffer (50 mM HEPES, 20% glycerol, pH 7.4). Microsomes were frozen at -80°C and used for kinetic analysis. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using serum albumin as a standard.

2.3 VITAMIN K EPOXIDE REDUCTASE ACTIVITY

Microsomal vitamin K epoxide reductase (VKOR) activity was assayed according to a modified protocol previously described by Thijssen, 1987(Thijssen, 1987) and Thijssen and Baars, 1989(Thijssen and Baars, 1989) and Misenheimer and Suttie, 1990(Misenheimer and Suttie, 1990). Briefly, standard reactions were performed in 200 mM Hepes buffer (pH 7.4) containing 150 mM KCl, 1 mM dithiothreitol, 1 mg of total proteins. The reaction was started by the addition of vitamin K > O solution in 1% Triton X-100. After incubation at 37 °C for 30 min, the reaction was stopped by adding 4 mL of iced 1:1 isopropanol/hexane solution. After centrifugation at 5000 × g for 10 min, the hexane layer was removed and dried under nitrogen. The dry residue was immediately dissolved in 0.2 mL of methanol and reaction product was analyzed by liquid chromatography-mass spectrometry.

2.4 VITAMIN K₁ MEASUREMENT

The LC-APCI/MS/MS used was a 6120 Quadrupole LC/MS with an Atmospheric Pressure Chemical Ionisation (APCI) interface and a LCMS Chemstation software from Agilent Technologies (Palo Alto, CA, USA). Chromatographic separation was performed using a XTerra MS C18 column (2.1mm × 50mm, 2.5µm, Waters, Milford, MA, USA) with a mobile phase of methanol, 0.1% acetic acid (96:4) in isocratic conditions. The column temperature was 48°C. The flow rate in the LC column was 0.4mL/min. The injection volume was 20µL. The temperature of the autosampler tray was set to 5°C and the samples were protected from the daylight. Detection was by MS with APCI source in positive mode. Nebulizer pressure was set to 60psi, dry gas temperature to 350°C, dry gas flow to 5 L/min, and vaporizer temperature to 400°C. Capillary voltage was set to 4000V, corona needle to 10µA. Identification criteria for vit K₁ are the retention time (tr= 3.9 min) and the selected ion 451.4. Identification criteria for vit K₁>O are the retention time (tr=2.9 min) and the selected ion 467.0. Linearity and accuracy were tested from 25 to 2000 ng/mL (n=20). The response was

linear throughout the concentration range tested with a coefficient of correlation (r^2) above 0.99. Accuracy was between 80 and 120% of the theoretical concentrations.

2.5 KINETICS

Kinetic parameters (K_m and V_{max}) were obtained from at least two separate experiments after the addition of increasing amounts of vitamin K > O (0.003–0.2 mM) to the standard reaction. The estimation of the kinetic parameters was achieved by the incubation of at least 8 different concentrations of vitamin K > O. Incubations were performed in duplicate. Data were fitted by nonlinear regression to the Michaelis–Menten model using R software.

In order to evaluate the inhibiting effect of warfarin on VKOR activity, inhibition parameters (K_i) were determined after addition of various concentrations of warfarin to the standard reaction. Inhibition parameters were first assessed with 4 different concentrations (0.01, 1, 10 and 30 μ M) and further more precisely determined using concentrations from about 0.05 to $20 \times K_i$. Data were fitted by non-linear regression to the non-competitive inhibition model using Sigmaplot 9.0™ software.

2.6 DETERMINATION OF DIFETHIALONE CONCENTRATION IN LIVER

Tissue (1g of tissue) was extracted with acetone by using an Ultra Turrax tissue disperser from IKA Labortechnik ® (VWR International, Strasbourg, France). Extract was centrifuged at $3000 \times g$ for 10 min. Supernatant was thus evaporated at 60°C under a gentle nitrogen flow. Dry extract was resuspended in acetonitrile/hexane (v/v 50/50). The hexane layer was eliminated and the rest was dried at 60°C under gentle nitrogen flow. The final dry extract was dissolved in methanol and difenacoum concentration was analysed by HPLC on a reverse phase C-18 column (4.6×150 mm, 5 μ m; Waters, Milford, MA, U.S.A.) at a detection wavelength of 260 nm. The C-18 column was heated 30°C. A gradient elution system was used with a flow rate of 1 mL/min: from 60% acetonitrile/40% water (acidified with 0.2 %

H₃PO₄) to 75 % acetonitrile/25% water at 10 min. The detection limit and the quantification limit were 50 ng/g and 130 ng/g. The specificity of the analytical method was determined by the extraction of three different blank samples of each organs collected from adults rats. The method appeared selective for all the components. The noise level were estimated for each organs and the mean value plus three standard deviations or ten standard deviations were used respectively to calculate the limit of detection (LOD) and the limit of quantification (LOQ). Spiked samples with decreasing concentrations were used to determine those limits chromatographically. The linearity and accuracy were determined on liver and plasma spiked samples. Three series of seven spiked samples were prepared (each one on different day) then extracted and analyzed. The linear regression equations of spiked samples were compared to standards calibration curves ($r^2 > 0,99$).

2.7 QUANTITATIVE REAL TIME-PCR

Total RNAs were extracted from liver first with the Trizol reagent (Invitrogen, Cergy Pontoise, France) then by using SV Total RNA Isolation System (Promega, Charbonnieres, France). The RNA concentration and purity were analyzed using a UV-160A spectrophotometer (Shimadzu, Roucaire, France), measuring spectral absorption at 260 and 280 nm. cDNA templates were synthesized from 100 ng total RNAs in a final volume of 20 μ L containing 500 pmol of oligo(dT)₁₈ and 200 units of Moloney murine leukemia virus Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol.

Quantitative real time-PCR reactions were performed with the MX3000P qPCR Machine (Stratagene, Amsterdam, The Netherlands) using primers for *Ggcx*, *Nqo1*, *Vkorc1*, *Vkorc111*, *Calu* (Calumenin), *F2* (prothrombin), *F7* (factor VII), *F9* (factor IX) and *F10* (factor X). *Gapdh* was used as housekeeping gene. The housekeeping gene was amplified under the same conditions used for the amplification of the target genes. Briefly, in a final volume of 20 μ L, 5 ng of cDNA were added to an optimal amplification reaction mixture containing 5X

HOT BIOAmp Evagreen[®] qPCRMix (Biofidal, Vaux-en-Velin, France) and 200 nM of each primer. Thermal cycling was the following: activation of the HOT BIOAmp[®] DNA Polymerase at 95°C for 5 min and 45 cycles of amplification (95°C for 20s, 60°C for 60s and 72°C for 30s). To determine the specificity of amplification, analysis of product melting was conducted after the 45 cycles of amplification: a melting curve was obtained by increasing the temperature at a rate of 0.01°C per second from 60°C to 95°C. Expression was evaluated in duplicate. The point at which the PCR product is first detected above a fixed threshold, the thermal cycle threshold (C_t), was determined for each sample, and the average C_t of duplicate sample was calculated. To determine the quantity of the target gene-specific transcripts present in different tissues relative to the control, their respective C_t values were normalized by subtracting the C_t value obtained from the *Gapdh* control ($rCt = C_t \text{ target} - C_t \text{ control}$) and the relative concentration was determined with the following formula: 2^{-rCt} .

2.8 DETERMINATION OF CLOTTING FACTOR CONCENTRATIONS

Vitamin K-dependent clotting factors activities (II, VII, IX, X) were measured on citrated plasma using a chromogenic method on Konelab 30i (Thermoscientific) with Hyphen Biomed reagents.

More precisely, factor II was activated by incubation of citrate plasma with Echis Carinatus venom (0.25 mg/mL) in Tris-buffer (0,1mM Tris Hcl, pH 8.3 at 25°C, 0.2% BSA) at 37°C for 10 min. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor VII activity was assayed by incubation of citrate plasma with calcium and tissue factor in excess to allow formation of tenase complexes and addition of a saturating concentration of human factor X activated in factor Xa proportionally to the quantity of tenase complexes formed. Thus, activity of factor Xa was monitored at 405 nm for 2 min after the addition of a specific chromogenic substrate generating para-nitroaniline in the presence of factor Xa.

Factor IX activity was assayed by an initial incubation of thrombin, calcium, phospholipids and Factor XIa with citrate plasma. The addition of a second reagent allowed to add factor VIII:C and factor X in the mixture. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 1 min.

Factor X was activated by the addition of Russel's viper venom. Thus, 50 µg of thrombin chromogenic substrate was added and absorbance was monitored at 405 nm for 2 min.

2.9 STATISTICAL ANALYSIS

Statistical analysis were performed with R v. 3.1.2. Results were expressed as mean values \pm SD or fitted value and confidence interval. Concerning the biochemical part, as the values are in small number, Wilcoxon-Mann-Whitney tests were used to assess the difference between males and females. To compare results between males and females for the basal levels, Student tests were performed as values are normally distributed. For the decrease of each clotting factor after the administration of diféthialone, data were linearized with a logarithmic function. Males and females regression curves were compared on the slope parameters with a Fisher test on the variance. The null hypothesis is that the male and female curves have the same slope.

3 RESULTS

3.1 MRNA EXPRESSION OF KEY ENZYMES INVOLVED IN THE VITAMIN K CYCLE IN LIVER

The presence of mRNA for *Ggcx*, *Vkorc1* or *Vkorc111* was assessed by real time PCR in liver of 8 weeks-old male and female OFA-sprague Dawley rats. Results are presented in Figure 1. mRNA expression levels were presented relatively to the corresponding male mean mRNA expression level, which was set at 100%. Results are presented in percentage of expression compared to the expression level obtained in male. Expression levels of *Ggcx* and *Vorc111* mRNA were similar between males and females. On the contrary, a significant

difference between males and females was found concerning the expression of *Vkorc1* mRNA coding gene. *Vkorc1* mRNA was found to be 80% more expressed in female liver compared to males (Figure 1).

3.2 VKOR ACTIVITY

To compare the ability to reduce vitamin K epoxide in vitamin K quinone between male and female rats, liver microsomes from 4 males and 4 females OFA-Spradue-Dawley rats were prepared. Then the catalytic properties of the VKOR activity measured in these microsomes were determined. Km and Vmax results are presented in Table 1. A statistic difference was found between males and females only concerning the Vmax value. The specific activity of females was 2.4 fold higher than males.

3.3 BASAL LEVELS IN VITAMIN K-DEPENDENT CLOTTING FACTORS ACTIVITIES

The concentrations of vitamin K-dependent clotting factors (i.e., prothrombin, factor VII, factor IX and factor X) were measured in males and females 48 h after the beginning of the vitamin K3-deficient food. Concentrations were determined relatively for each factors to the corresponding male mean concentration factor (Figure 2A, Table 2) set at 100%. A significant difference was found between males and females for factor VII ($P < 0.001$) and factor X ($P = 0.006$) with a clotting factor concentration in females higher than males of respectively 43 and 21 percent. But considering the factor IX and the prothrombin no significant difference was found.

In addition, a study of the hepatic expression of vitamin K-dependent clotting factor genes: prothrombin, factor VII, factor IX and factor X was performed (Figure 2B). mRNA expression levels were presented relatively to the corresponding male mean mRNA expression level, which was set at 100%. Only Factor VII expression was significantly different between males

and females ($P < 0.001$), females' factor VII expression was 125 percent higher than males' factor VII expression.

3.4 EVOLUTION OF CLOTTING FACTORS AFTER DIFETHIALONE *PER OS* ADMINISTRATION

In order to characterize the decay kinetics of vitamin K-dependent clotting factors, an elevated concentration of difethialone (10 mg/kg) was administered *per os* to 36 male and 36 female rats previously fed with vitamin K3-deficient food for 48h. For each rat prothrombin time was measured and vitamin K-dependent clotting factors activities (prothrombin, factor VII, factor IX and factor X) were measured in citrate plasma. For 3 males, the blood collection was impossible because of their premature death; the first one, ten hours after the difethialone administration and the others seventy-two hours after the administration. Necropsy has showed hemorrhagic lung and abdomen in these rats.

The evolution of prothrombin time after the administration of difethialone for male and female rats is presented in Figure . After the linearization of data (Figure B), a two steps evolution of prothrombin time was pinpointed. A lag time was present in both male and female prothrombin time evolutions. However, this lag time has occurred during 2 first hours in males and 8 first hours in females, lag time slopes were not different from 0 with respectively a p-value of 0.16 and 0.46. Then both prothrombin time evolutions increased with a slope of 0.04 h^{-1} for male rats and 0.05 h^{-1} for female rats. The two increasing slopes were significantly different ($P < 0.001$).

The plasma activities of vitamin K-dependent clotting factors are represented in function of time after difethialone administration in Figure 4. The activity values are represented relatively to the male basal level set at 100%. To assess the evolution of clotting factors, data were linearized with a natural logarithmic function (Figure X2) allowing to determine the slope and the half-life (Table 3). A significant difference was observed between male rats and female rats for the prothrombin and the factor X slope values. Consequently, the half-

lives of these two factors were different between males and females. Concerning female factor VII, only values from 4 hours were including in the linear regression. Indeed, during these 4 first hours, female factor VII activities did not decay. The slope of the female factor VII plateau was not significantly different from 0 (p-value = 0.409).

3.5 EVOLUTION OF THE DIFETHIALONE CONCENTRATION IN LIVER

One, 2, 4, 8, 10, 13, 16, 20, 24, 32, 48 and 72 hours after the *per os* difethialone administration, difethialone concentration in plasma liver was determined. The concentration of difethialone in plasma (data not shown) and liver (Figure 5) increased rapidly after an oral administration in males and females, reached a peak about 8-12 h after the administration, and then decreased slowly an a time-dependent manner. The maximal concentration in plasma and liver was significantly reached earlier in females ($T_{\max}=7.9$ h) than males ($T_{\max} = 11.7$ h). Pharmacokinetic analyses (Table 3) revealed that C_{\max} and $AUC_{0\rightarrow 24h}$ or $AUC_{0\rightarrow 72h}$ were significantly higher in females than males.

4 DISCUSSION

The resistance of female rats to the administration of anticoagulants is a phenomenon well recognized (Gill et al., 1994; Kohn and Pelz, 1999). In practice, discriminating doses of anticoagulants, called ED50 (i.e., effective dose 50), were determined in rats to characterize the susceptibility of rats to anticoagulants. ED50 corresponds to the dose leading to 50% of animals tested with a 5-fold increase of the International Normalized Ratio 24 hours after the administration of anticoagulants. Systematically for all the anticoagulant molecules used as rodenticides, the ED50, determined by the Rodenticide Resistance Action Committee, are higher for female rats than males. For example, as recommended by the technical monograph of 2003, ED50 for difethialone is 0.4 mg.kg^{-1} for male rats; it is 0.5 mg.kg^{-1} for females. Contrary to rats, in humans, the differences in responses after antivitamin K

anticoagulants between men and women are difficult to appreciate. In healthy individuals, available studies are logically always performed with small doses of antivitamin K anticoagulants, while in diseased individuals, used doses and molecules of VKA are often different and moreover, the first evaluations of the coagulation are generally made 3 days after the initiation of the treatment.

Since the pinpointing of the female rats tolerance to VKA, many assumptions on its origin have been issued. Is the pharmacokinetics of VKA different between males and females? Is the recycling of vitamin K and more precisely the VKORC1 enzyme more resistant to VKA in females than males? Is the half-life of vitamin K-dependent clotting factors longer in females than males? Is the vitamin K storage higher in females than males?

To answer these questions, the greater tolerance of the female rats to VKA was first characterized after administration of 10 mg.kg^{-1} of difethialone by following the time-dependent evolution of the prothrombin time. Difethialone was chosen in this study because it is one of the most powerful rodenticides. No resistance to this molecule has been described until now (Hodroge et al, 2011). An excessive dose of difethialone, corresponding approximately to 20-fold the DL50 (Petterino and Paolo, 2001), was administered to male and female rats. In these conditions, we can assumed that VKORC1 is totally inhibited in males, but also, in females since the inhibition constants of the VKOR activity towards VKA are not different between sexes. Moreover, we can also assume that the complete inhibition of the VKOR activity occurs at the same time, in the liver of male and female rats. Indeed, after *per os* administration, difethialone is totally and rapidly absorbed. The concentration peak in liver occurring sooner in female rats, a faster absorption of difethialone in females can be supposed. Thus, a complete inhibition of VKORC1 in the liver could even occur earlier in females. Moreover, the liver concentration of difethialone was systematically higher in females than males, as described for warfarin (Zhu and Shin, 2005) suggesting a lower metabolism or elimination of difethialone in females than males. Therefore, complete inhibition of VKORC1 is assumed to be at least equivalent between males and females during the first 24 hours following the administration of difethialone.

This complete inhibition of VKORC1 results in a significant increase of prothrombin time 24 hours after the administration of difethialone in males, but also in females. Nevertheless, a lag of at least eight hours in the increase of the prothrombin time was pinpointed in females compared to males (Figure). The lag time was sufficiently important to keep the females' prothrombin times significantly lower than males' for 24 hours, even though the increase of the prothrombin time was significantly higher in females than in males after the lag. Moreover, only males were found dead during the study and the necropsy confirmed the hypocoagulability as the origin of the death. These results argue for a highest tolerance of females compared to rats at least during the first 24 hours and this highest tolerance is not due to a pharmacokinetically- or pharmacodynamically-mediated difference in the inhibition of VKORC1 enzyme.

To go further in the understanding of the origin of females' tolerance, vitamin K-dependent clotting factors activities were measured. Even before the administration of VKA, significant differences were found between males and females. Basal clotting factor VII and X activities were found to be significantly higher in females than males. These results are in agreement with previous results obtained in Wistar rats where factors II and X activities were also observed to be higher in females than males (Kerins and MacNicoll, 1999). In humans, all vitamin K-dependent clotting factors activities were also reported to be higher in women (Favaloro et al., 2005). In OFA-Sprague Dawley rats, these highest activities in females' livers do not seem to be due to higher mRNA syntheses in liver, except eventually for factor VII. Indeed, only, mRNA encoding factor VII were found to be 2.3-fold more expressed in female rats' livers than males, The expression levels of the other mRNA encoding factors II, IX and X were similar between males and females. The highest factor VII and X plasma activities observed in females might be explained by the highest rate of the VKOR activity measured in females livers (i.e., 2.4 fold) compared to males. These highest basal activity of active vitamin K-dependent clotting factors in females are certainly involved in the explaining of the sex-linked clotting time differences previously observed (Lemini et al., 2007). Moreover, this higher initial pool of active vitamin K-dependent clotting factors VII and X

might be a part of the explanation of the highest tolerance of the female rats. Nevertheless, this initial difference is strengthened by longer decay kinetics of vitamin K-dependent clotting factors II, VII and X in females after administration of difethialone. Indeed, half-lives of factors II and X are significantly higher in female rats than males, with half-lives longer of respectively 2.7 and 0.5 hours. For factor VII, no difference of half-lives was observed between males and females. Nevertheless, this absence of difference is due to the calculation method. Since factor VII activities were not modified between the first 4 hours following difethialone administration in females, the calculation of half-life in female was performed in this study considering only the results obtained after the first 4-hours period. Therefore, when the lag time is included in the calculation, loss of 50% of active factor VII activity is reached in 5.8 hours after difethialone administration in females while in males the same loss is reached in only 2.8 hours. Such lag times were previously reported in rats for factors II, VII and X by Kerins and MacNicoll (1999). Nevertheless, lag times were observed in females, but also in males. In our study, the use a vitamin K3-deficient food for a preliminary period of 48 hours before difethialone administration led to an absence of lag times in males for all vitamin K-dependent clotting factors and an absence of lag time in females for factor II and X. The lag time observed in females before the degradation of the factor VII is observed while the VKORC1 enzyme is totally inhibited. The maintained factor VII clotting factor activity might be due to a maintained GGCX activity which is strictly dependent on the bioavailability of vitamin K hydroquinone. This suggests the presence of a storage of vitamin K in female livers only allowing the preservation of the gamma-carboxylation function during the first hours following the VKA-induced inhibition of the vitamin K recycling. The recycling of vitamin K in males should allow to produce just the vitamin K necessary for the physiological needs. In females, this recycling might produce more vitamin K than the physiological. Previous study has already showed that the liver concentration of vitamin K is more important in female rats than in males (Huber et al., 1999). This stock of vitamin K in females seems to be used after the inhibition of the VKOR activity to preferentially maintain the gamma-carboxylation of factor VII, which plays a crucial role in

the initiation of the coagulation. The binding of the gamma-carboxylase to its protein substrates is mediated by a propeptide sequence present in all vitamin K-dependent proteins. Depending of the sequence of this propeptide, its apparent affinities for the carboxylase vary over a 100-fold range. Thus, it was shown that human factor VII and factor X propeptides have higher affinities for the gamma-carboxylase than other propeptides (Chu et al., 1996; Stanley et al., 1999). In rats, no information is available. Our results suggest in rats a higher affinity of the gamma-carboxylase for the propeptide of factor VII compared to those of factor II and X. As the affinity of the gamma-carboxylase for the propeptide is determined by the propeptide sequence, it would be assumed that the gamma-carboxylase affinity is similar between male rats and females. Consequently, the absence of the plateau before the decay kinetics of factor VII and the shortest half-lives of factors II and X in males are certainly due, after the inhibition of the vitamin K cycle, to the absence of the cofactor of the gamma-carboxylase, the vitamin K hydroquinone, while in females the stock of vitamin K allowed to maintain the gamma-carboxylation of factor VII and in a lesser extent factors II and X during few hours after difethialone administration.

5 CONCLUSION

The tolerance of female rats to vitamin K antagonist administration is due to both the basal level of their vitamin K-dependent clotting factor activities and the half-lives of these clotting factors. Factor II, VII and X activities are higher in females than male rats. Moreover, plasmatic half-lives of prothrombin and factor X are higher in female too. Concerning factor VII in female rats, a lag time before its decay has been observed only in females and seems to be crucial in the tolerance of females to VKA.

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Figure Captions

Figure 1: Hepatic expression levels of genes coding for three enzyme implicated in the vitamin K cycle. GGCX: gene coding for gamma-glutamyl carboxylase; VKR : gene coding for VKORC1; VKL: gene coding for VKORC1L1. * denotes a significant difference between males and females at $\alpha = 0.01$

Figure 2 : Clotting factors activity (A) and hepatic expression (B) were measured after a 48h diet with vitamin K devoid food and before difethialone *per os* administration. Values were compared between males (set at 100%) and females and presented as the mean \pm SD. * denotes a significant between males and females at $\alpha=0.01$. ** denotes a significant between males and females at $\alpha=0.001$.

Figure 3 : Evolution of the prothrombin time after *per os* administration of 10 mg.kg^{-1} of difethialone. Values are presented for each time as mean more or less standard deviation, in second (A) or after linearization with logarithmic function (B). Males' values are presented in black and females' values are presented in grey.

Figure 4 : Evolution of activity of the four vitamin K dependent clotting factors and their linearization after a *per os* administration of difethialone : prothrombin (A); factor VII (B); factor IX (C); factor X (D). The (1) graphs represent the relative mean of activity for each time and their standard deviation; for each factor, the basal concentrations' mean of male rats is set at 100%. The (2) graphs present the same values after linearization by a logarithmic function. Males' values are presented in black and females' values are presented in grey.

Figure 5 : Evolution of difethialone concentration in liver after difethialone *per os* administration. Males' concentrations are presented in black and females' concentrations are presented in grey.

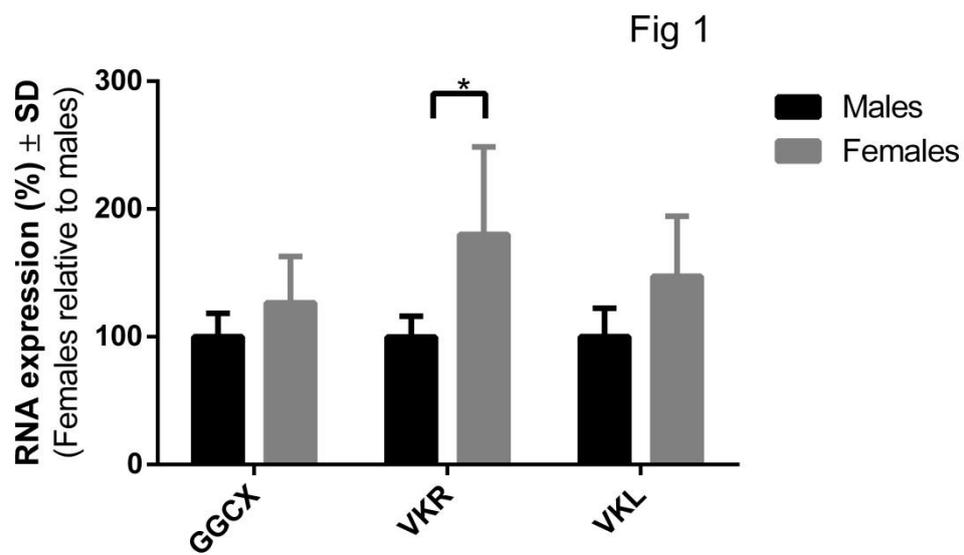


Fig 2

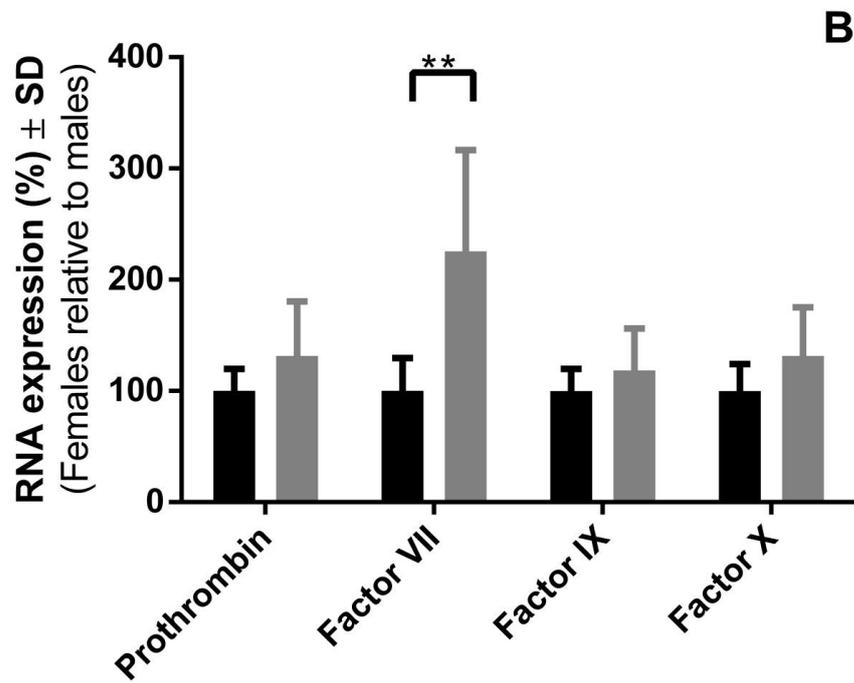
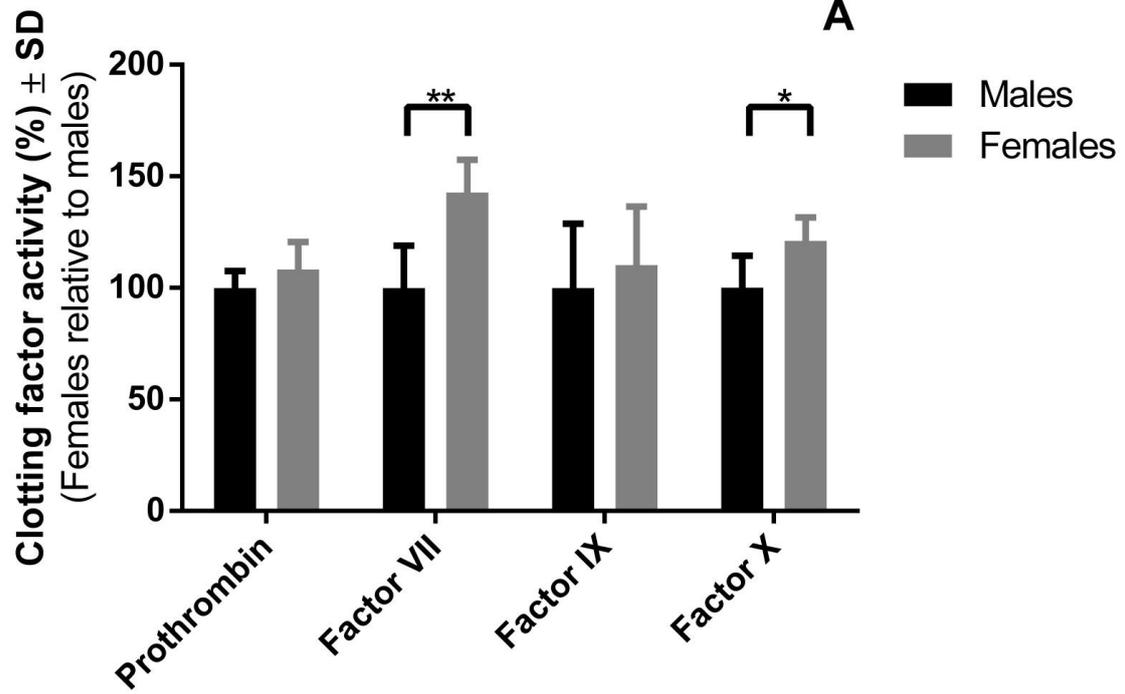


Fig 3

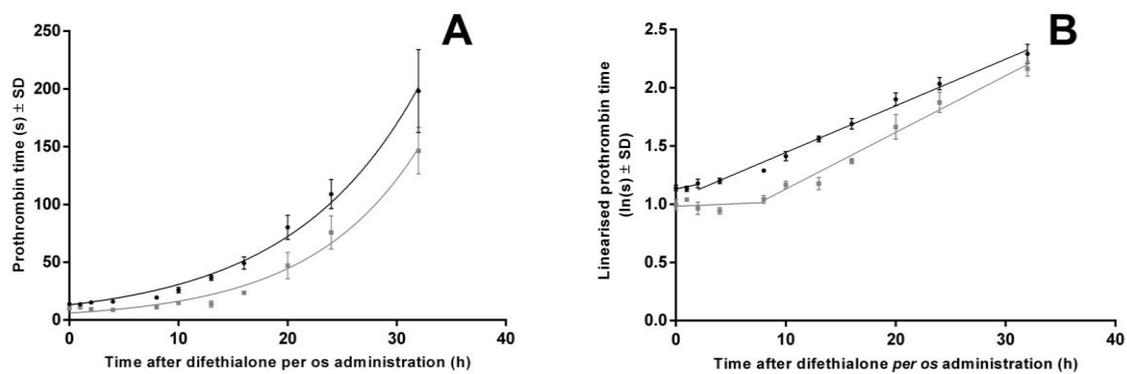


Fig 4

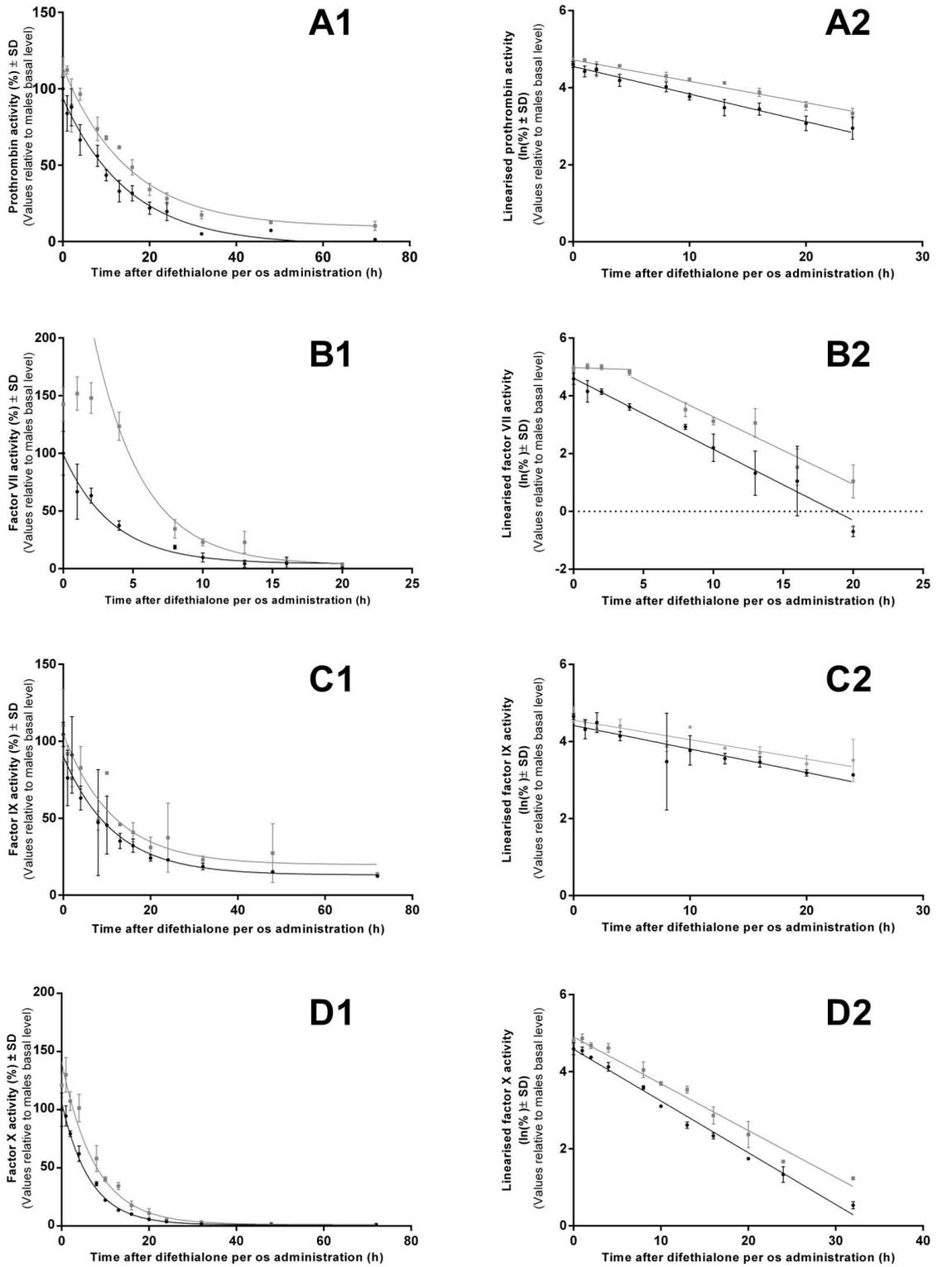
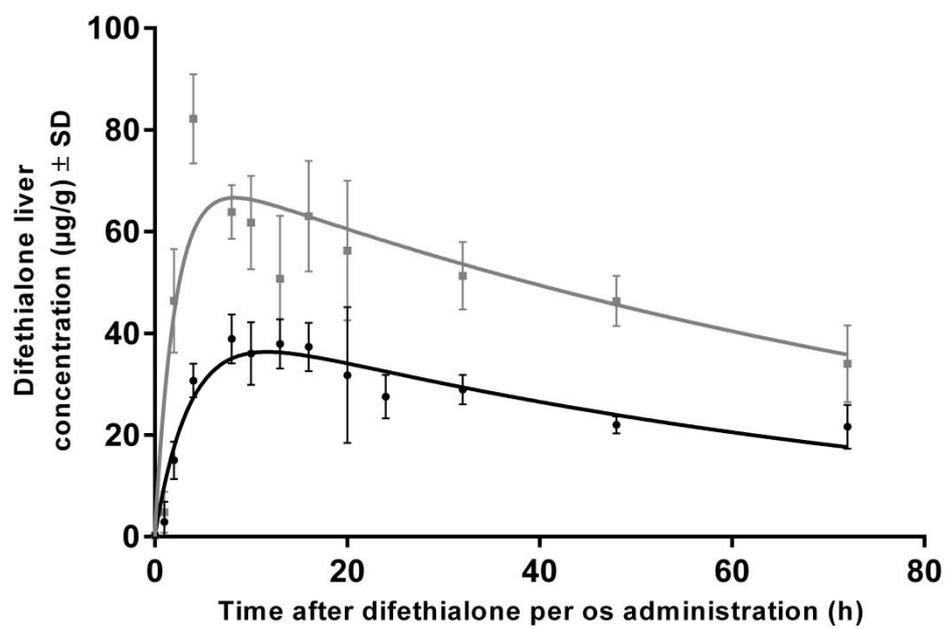


Fig 5



Tables

Table 1 : Kinetic parameters of VKOR activity measured in liver of male and female rats

Gender	Km (μM)	Vmax ($\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	Ki (μM)
Males	48.9 [32.9-76.8]	72.9 [46.7-98.3]	0.26 [0.21-0.36]
Females	110.2 [75.87-173.6]	178.2 [129.6-280.6]	0.31 [0.13-0.45]
P-value	0.057	0.029	0.657

Table 2 : Values of parameters from linear regressions performed on linearized data of each clotting factor activity evolution after *per os* administration of difethialone. Values are presented with 95% confidence interval.

Clotting factors	Gender	Slope (h^{-1}) absolute values	p-values	Half-live time (h)	R ²
Factor II	Male	0.071 [0.065-0.078]	<0.001	9.7 [8.9-10.6]	0.9373
	Female	0.056 [0.051-0.061]		12.4 [11.3-13.6]	0.9421
Factor VII	Male	0.25 [0.22-0.27]	0.596	2.8 [2.6-3.1]	0.9375
	Female	0.23 [0.19-0.28]		3.0 [2.5-3.7]	0.8862
Factor IX	Male	0.061 [0.041-0.081]	0.362	11.4 [8.6-16.7]	0.6110
	Female	0.050 [0.038-0.063]		13.8 [11.0-18.4]	0.7327
Factor X	Male	0.13 [0.13-0.14]	0.0035	5.2 [5.0-5.4]	0.9872
	Female	0.12 [0.11-0.13]		5.7 [5.4-6.1]	0.9741

Table 3 : Hepatic pharmacokinetic summary of difethialone treatment in male and

	$AUC_{0 \rightarrow 24h}$ ($\mu\text{g/g}\cdot\text{h}$)	$AUC_{0 \rightarrow 72h}$ ($\mu\text{g/g}\cdot\text{h}$)	C_{max} ($\mu\text{g/g}$)	T_{max} (h)
Male	$753 \pm 123^*$	1921 ± 291	36.3 ± 6.0	11.7 ± 0.06
Female	$1376 \pm 209^*$	3578 ± 560	64.5 ± 9.7	7.9 ± 0.3
p-value	<0.05	<0.05	<0.05	<0.001