Reproductive Toxicology 93 (2020) 131–136

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Vitamin K antagonist rodenticides display different teratogenic activity

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ARTICLE INFO

Keywords:
Fetal warfarin syndrome
Vitamin K antagonist
Bromadiolone
Warfarin
Read across

ABSTRACT

Vitamin K antagonists (VKA) are not recommended during pregnancy because warfarin (a first-generation VKA) is associated with a malformation syndrome "the fetal warfarin syndrome" (FWS). VKA are also used for rodent management worldwide. Recently, the Committee for Risk Assessment responsible for the European chemical legislation for advances on the safe use of chemicals had classed 8 anticoagulant used as rodenticides in the reprotoxic category 1A or 1B. This classification emerges from a read-across prediction of toxicity considering the warfarin malformation syndrome. Herein, our study explores the teratogenicity of warfarin at the human therapeutic dose and that of bromadiolone, a second-generation anticoagulant rodenticide. Using a rat model, our study demonstrates that warfarin used at the therapeutic dose is able to induce teratogenicity, while in the same conditions bromadiolone does not induce any teratogenic effect, challenging the classification of all VKA as reprotoxic molecules.

1. Introduction

Vitamin K antagonists (VKA), including in human medicine warfarin, the most used VKA, but also acenocoumarol, phenprocoumon and fluindione, have been the most widely used anticoagulants drugs for the treatment of thromboembolic diseases since the last 70 years. Their use is made difficult by their pharmacological characteristics and side effects (therapeutic window, pharmacological interactions, coagulation monitoring, variable dose-response …) [1]. Pregnancy is a critical period where the toxic risk of anticoagulant drugs is important for women and also for early embryo and fetus. But sometimes, the use of anticoagulant is irreplaceable, especially for women with mechanical heart valve or long-term thromboembolic disease. During pregnancy, VKA could cause maternal outcome such as maternal hemorrhages, systemic thromboembolism or prosthetic valve failure. Warfarin could also leads to fetus outcomes with spontaneous abortion, fetal death (fetotoxicity) or other congenital defects (teratogenicity).

In different cohort studies [2–4], teratogenicity rate (structural congenital anomalies) of warfarin was evaluated at 5.0 % (2,4%–10,5 % depending on the study) for treatment doses between 3 mg/day to 16,5 mg/day. The highest risk of teratogenicity was found between 6–9 weeks of pregnancy with a stronger effect during the 8th week of pregnancy at 5 mg/day of warfarin [5]. The clinical relationship between warfarin dose and teratogenicity rates is unpredictable and must be interpreted cautiously, even if patients who were taking more than 5 mg/day during the 8th week of pregnancy seem to develop more complications [3,6]. The congenital abnormalities found for warfarin are known as "fetal warfarin syndrome" (FWS) or "warfarin embryopathy" and was first described in 1980 by Hall et al. [7]. Characteristics effects of FWS are skeletal malformations such as, nasal hypoplasia (maxilonasal bone hypoplasia) and chondrodysplasia punctata, but also optic atrophy and intellectual disability [8,9]. FWS was initially described for warfarin, but other VKA used in therapeutic (phenprocoumon, acenocoumarol and phenindione) could lead to FWS. Clinical data's were insufficient to compare teratogenicity risk between warfarin and other therapeutic VKA [7,10,5,11].

FWS was investigated by several human clinical studies and experimentally reproduced by studies using rodent models [7,12–15], zebra fish [15] or South African clawed frog (*Xenopus laevis*) [16]. The daily subcutaneous injections of very high toxic dose of warfarin (100 mg/kg) co-injected with 10 mg/kg of vitamin K1 to newborn rat litters for 12 weeks led to the development of FWS [17]. This first teratogenicity study was made using 1500 time the warfarin human therapeutic dose (0.07 mg/kg/day) and correspond more to an accidental use of warfarin. Since today, there is no study about the teratogenic effects of warfarin at human therapeutic dose with mammalian models.

Warfarin was also used as anticoagulant rodenticide (AR) since the 50's but was rapidly replaced by more potent VKA [18,19] named "super warfarin". Nowadays, the use of VKA is the main method implemented to control rodent populations worldwide. VKA used as AR have all the same mechanism of action and own the same central 4-OH-coumarin core. Nevertheless, their pharmacokinetics properties and

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https://doi.org/10.1016/j.reprotox.2020.02.003
Received 19 November 2019; Received in revised form 17 January 2020; Accepted 5 February 2020
Available online 08 February 2020

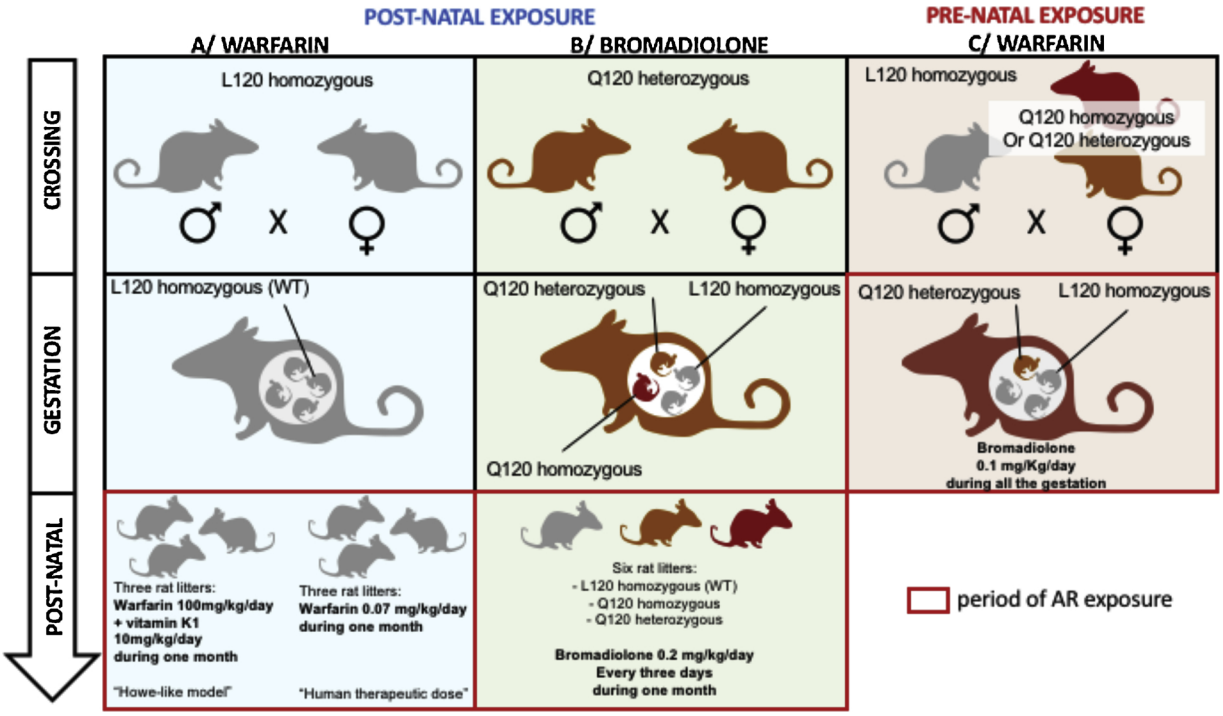

Fig. 1. Diagrammatic representation of post-natal exposure to A/ warfarin and B/ bromadiolone and C/ pre-natal exposure to bromadiolone.

their efficiency are different [20,21] and VKA used as AR are classified according to two generations, the first generation molecules (warfarin, chlorophacinone, coumatetralyl…) requiring repeated ingestions to be lethal but being less tissue-persistent than second generation molecules (difenacoum, brodifacoum, flocoumafen, difethialone and bromadiolone), which are toxic after a unique ingestion.

In 2016, the European Chemical Agency had classed the 8 VKA used as AR as reprotoxic based on the data existing for warfarin because these VKA have the same mechanism of action and a common structure according to the read-across procedure. Two (warfarin and brodifacoum) have been classified as reprotoxic with proven toxicity for embryo development (reprotoxic category 1A), and the other (chlorophacinone, coumatetralyl, difenacoum, flocoumafen, bromadiolone and difethialone) with supposed toxicity for embryo development (reprotoxic category 1B (UE) 2016/1179). According to the enforcement decree applicable since March 1, 2018, VKA baits shall now only be supplied for general public with a maximum concentration of 0.003 % w/w, only for indoors and not permanent use. But it is not yet known if all VKA present a proven teratogenicity. This present study compares the teratogenic potentials of warfarin, a first-generation molecule, and bromadiolone, a second-generation molecule, in rats and represents the first approach of warfarin teratogenicity at human therapeutic dose according to a protocol derived from that performed by Howe et al. [17]. To allow the exploration of the potential teratogenicity of bromadiolone, a highly potent anticoagulant rodenticide, laboratory VKA susceptible rats and laboratory VKA resistant rats carrier for the Q120 mutation in the vitamin K epoxide reductase (Vkorc1) gene encoding the target of VKA [22] was used in order to avoid death of the pregnant female, abortion by placental hemorrhage or newborn/embryonic/fetal death by hemorrhage in the absence of the use of vitamin K1.

2. Materials and methods

2.1. Ethical statement

Animal experiment was reviewed and approved by France government under the Directive 2010/63/EU of the protect animals used for scientific purposes. Environment, housing and management of rats were in compliance with rat animal welfare. Experimental procedures on rats were performed according to an experimental protocol following international guidelines and with approval from the ethics committee of the Veterinary School of Lyon.

2.2. Origin and husbandry of animals

OFA Sprague-Dawley rats (each weighing 175 – 200 g) (referred below as L120 rats) were obtained from a commercial breeder (Charles Rivers, L'Arbresle, France) and were acclimated for a minimum of 5 days. Male and female introgressed OFA Sprague-Dawley rats homozygous or heterozygous for Q120 mutation in Vkorc1 (F16-generations) were obtained as described in [22,22] (referred below as Q120 rats or rats homozygous or heterozygous for Q120).

Rats were housed four per cage under a constant photoperiod and ambient temperature. Animals were kept in standard cages, Eurostandard, Type IV® (Tecniplast, Limonest, France) and received standard feed (Scientific Animal Food and Engineering, reference A04) and water ad libitum.

2.3. Genetic characterization of animals

Genetic characterization of animals was performed using allele-specific PCR, as described in [22,23] to detect the Q120 mutation in the Vkorc1 gene.

2.4. Postnatal rat exposure to warfarin or bromadiolone

Postnatal exposure to warfarin was carried out with two different doses, a toxic dose and a therapeutic dose as shown in Fig. 1A. Three OFA Sprague-Dawley rat litters (with males and females newborns) received just after birth daily subcutaneous injection of 100 mg/kg of warfarin and 10 mg/kg of phylloquinone (TVM, Lempdes, France) for one month, as described by Howe et al. [24]. Three other OFA Sprague-Dawley rat litters (with male and female newborns) received just after birth daily subcutaneous injection of 0.07 mg/kg of warfarin

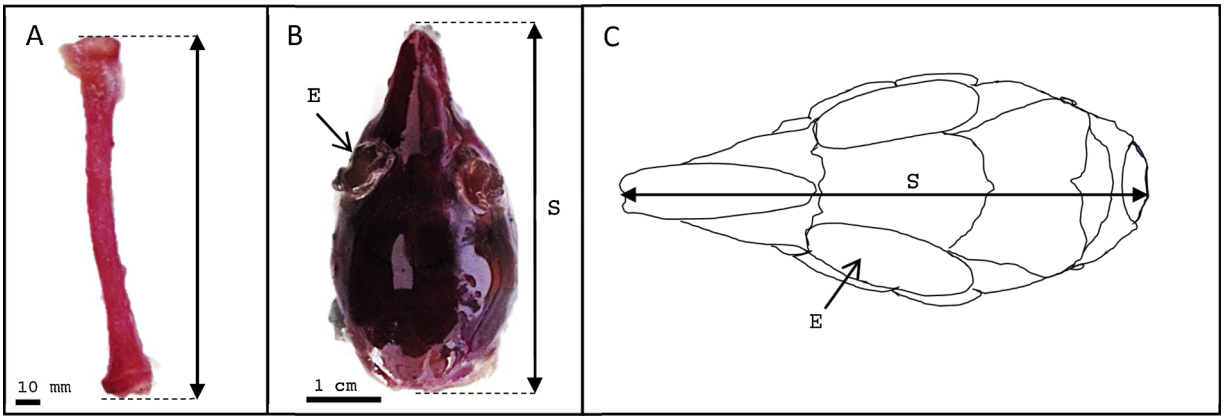

Fig. 2. Post-natal measurement characteristics of radius (A) and skull bone (B and C) of newborns. S = skull and E = eyes. Double arrows indicate length measurement of bones.

(corresponding to the human therapeutic dose equal to rat cumulative LD50 for 90 days) for one month.

Postnatal exposure to bromadiolone was carried out with only one dose according to the protocol shown in Fig. 1B. Six rat litters obtained after mates of Q120 heterozygous breeder (given litters with the three genotypes: homozygous for L120, homozygous for Q120 and heterozygous) were given just after birth every three days subcutaneous injection of 0.2 mg/kg of bromadiolone for one month.

After one month four to five treated newborns were randomly chose and euthanized with CO_2. Whole skeleton were stained with Alcian blue/alizarin red double staining as described by Schneider [25]. Measurements of radius and skull were made under binocular magnifying glass with a micrometer as reference. Details of measurements are presenting in Fig. 2. Genotypes of newborns were obtained by genetic characterization.

2.5. Prenatal rat exposure to bromadiolone

Prenatal exposure to bromadiolone was carried out according to the protocol shown in Fig. 1C. Female rat heterozygous or homozygous for Q120 were mated with male L120 rats in order to obtain litters containing L120 fetuses or fetuses heterozygous for Q120.

Date of fertilization was determined by cervical smears by eosine/thiazine staining (Aerospray®, EliTechGroup), presence of spermatozoa corresponding to the first day of the gestation. From the 6th day of gestation (corresponding to the beginning of the organogenesis) to the day before the parturition (day 20), rat females received daily 0.1 mg/kg of bromadiolone by oral administration. At day 20, female rats were euthanized with CO_2. Nine to twelve fetuses randomly chose were removed from placenta and whole skeleton were stained with Alcian blue/alizarin red double staining as described by Schneider [25]. Measurements of radius and nasal bones were made under binocular magnifying glass with a micrometer as reference. Details of measurements are presenting in Fig. 3. Genotypes of fetuses were obtained by genetic characterization.

2.6. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Comparison between two groups was performed using the Multiple t tests with two-stage linear step-up procedure of Benjamini, using GRAPHPAD PRISM 6 software® (GraphPad, San Diego, CA, USA). P-values <0.05 was the accepted level of significance and associated with one star symbol (*); when p-value was lower than 0.01 two stars symbol (**) was used.

3. Results

3.1. Skeletal modification following post-natal exposure to warfarin at toxic and therapeutic doses

Newborn rats were treated with warfarin at 100 mg/kg/day (1500 time the therapeutic human dose) or 0.07 mg/kg/day (therapeutic human dose) and were compared to untreated (corn oil) as shown in Fig. 1A. At the end of the treatment newborn rats did not show evidence of hemorrhage. VKA susceptible L120 rats (Vkorc1-WT) treated daily with *per-os* administration of warfarin at 100 mg/kg/day (corresponding to a cumulative dose of 3000 mg/kg) and subcutaneous injection of vitamin K1 at 10 mg/kg/day for one month just after birth (same treatment as Howe in 1992), presented an average reduction of 15.7 ± 2.3 % of the skull length and 11.3 ± 0.6 % of the radius length statistically significant compared to the untreated group. When warfarin was used at 0.07 mg/kg/day for one month (corresponding to a cumulative dose of 2.0 mg/kg) a significant reduction of about 19.7 ± 1.1 % for skull length was observed, but no effect was noticed on radius length (Fig. 4). Radius bones length was estimated between his two epiphyseal extremities and skull, all along his frontal border. Measurements characteristics are shown in Fig. 2.

3.2. Skeletal modification following post-natal exposure to bromadiolone

To evaluate the teratogenic effect of bromadiolone following chronic post-natal exposure, newborn L120 rats were used as well as newborn homozygous and heterozygous Q120 rats to avoid potential death of all exposed animals by hemorrhage. After exposure of newborns with bromadiolone at 0.2 mg/kg every 3 days for one month (corresponding to a cumulative dose of 2 mg/kg) just after birth, no malformation was noticed compared with untreated regardless Vkorc1 genotypes (Fig. 5).

3.3. Skeletal modification following pre-natal exposure to bromadiolone

To evaluate the teratogenic effect of bromadiolone following chronic exposure of embryos/fetuses during the gestation, rat females homozygous or heterozygous for Q120 were used in order to prevent abortion due to placental hemorrhage or death of the pregnant female by hemorrhage in the absence of vitamin K1 administration (Fig. 1C). These females were crossed with male L120 rats in order to obtain fetuses with different genotypes (L120 fetuses and fetuses homozygous and heterozygous for Q120). When these females crossed with male L120 rats were treated with bromadiolone during all the gestational period, offspring were obtained according to Mendel's genetics laws for both genetic crosses (Chi Square Goodness of Fit - data not shown).

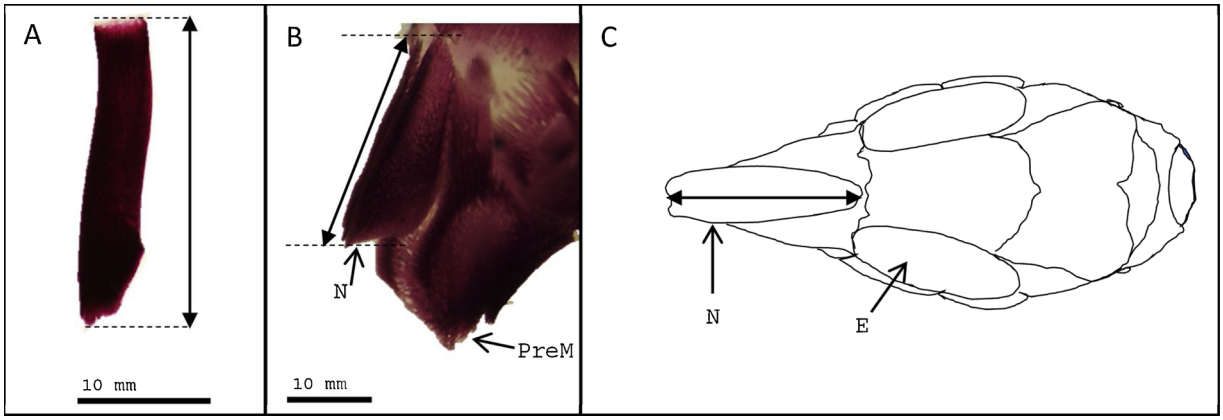

Fig. 3. Measurement characteristics of radius (A) and nasal bone (B and C) of fetuses. N = nasal bone, PreM = premandibular bone and E = eyes. Double arrows indicate length measurement of bones.

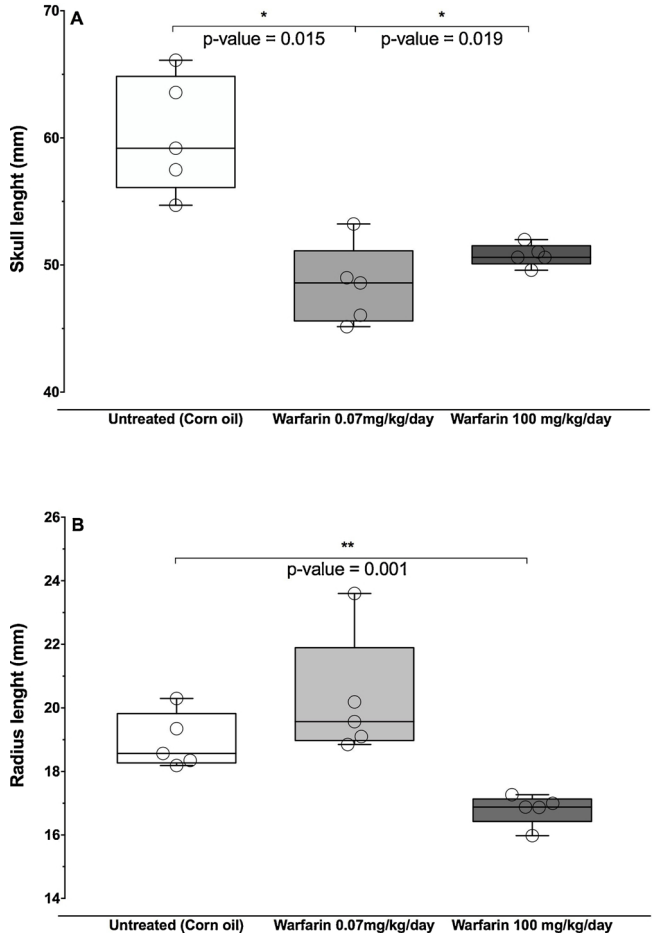

Fig. 4. Skeletal measurement of L120 newborn rats exposed after birth for one month with warfarin every day (0.07 or 100 mg/kg/day), Skull length (A), Radius length (B). * indicates a statistical difference with p-value <0.05 and ** statistical difference with p-value <0.01.

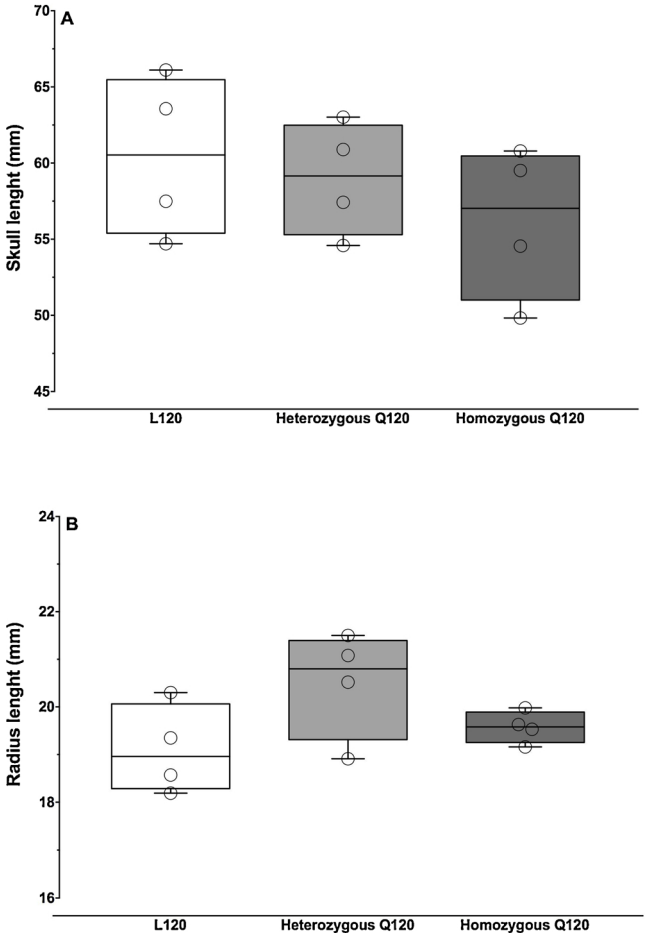

Fig. 5. Skeletal measurement of newborn L120 rats or homozygous or heterozygous newborn rats for Q120 exposed after birth for one month with bromadiolone every 3 days (0.2 mg/kg/3 days). Skull length (A), Radius length (B).

Untreated and bromadiolone treated pregnant rats and their fetuses did not show any evidence of hemorrhage. Fetuses from pregnant rats treated with bromadiolone at 0.1 mg/kg/day during all the organogenesis (corresponding to a cumulative dose of 1.4 mg/kg) did not show nasal or radius bone malformation regardless their VKA resistance phenotypes (Fig. 6). A statistically significant increase of radius bone length was noticed for bromadiolone treated fetuses. Radius bones length was estimated between its two epiphyseal extremities and nasal bones all along its sagittal border. Measurements characteristics are shown in Fig. 3.

4. Discussion

The first aim of our study was to characterize the teratogenicity of warfarin at therapeutic dose because no experimental data is available for such dosage. For that, OFA-Sprague Dawley newborn rats (L120 rats) were exposed every day to therapeutic doses of warfarin from

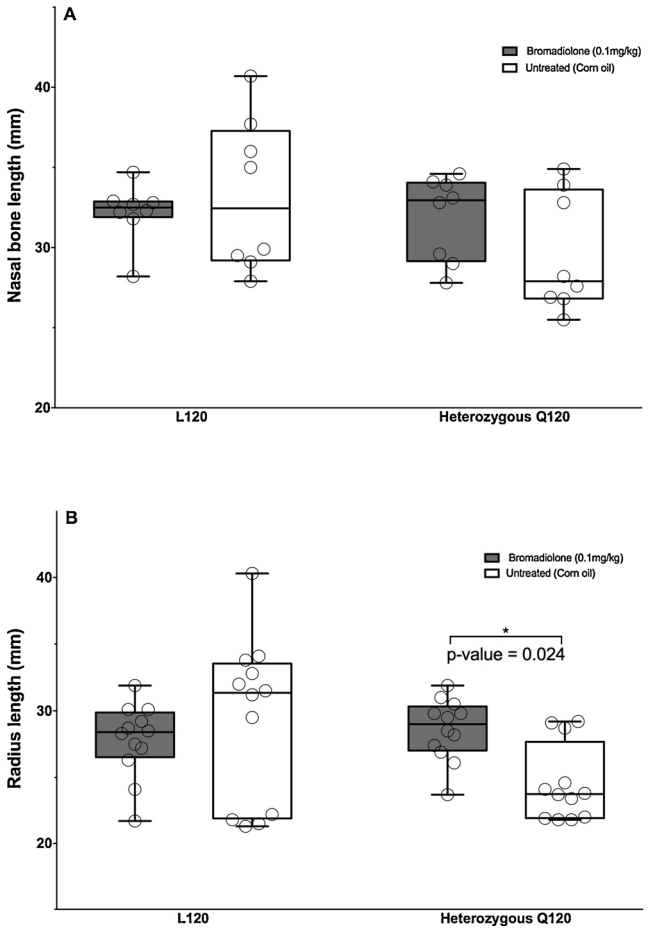

Fig. 6. Skeletal measurement of fetuses of pregnant female rats homozygous or heterozygous for Q120 crossed with L120 rats and exposed throughout gestation with bromadiolone (0.1 mg/kg/day, every day throughout gestation). Nasal bone length (A), radius length (B).

birth to one month. This mammalian model and the period of exposure was decided based on the only mammalian model already described by Howe et al. in 1992 allowing the exploration of the teratogenic effects of warfarin. This period of exposure was chosen because in rats, even if the skeletal development is similar to human, the facial bones and the axial skeleton continue to grow in the days following the birth [26] and the FWS is observed in rats when exposure to warfarin is done at this period. Howe's study evaluated the teratogenic effect of warfarin at doses never used in human to prevent thromboembolic diseases. Indeed, the doses used in his study (100 mg/kg/day) was toxic and 1500-fold higher than the therapeutic dose. To compare effects observed at therapeutic and toxic doses, the model of Howe was performed in parallel of our experimental model. Nevertheless, in Howe's model, because newborn rats received toxic doses, daily administration of vitamin K1 was required to maintain rats alive. In our model, administration of vitamin K1 was not necessary because warfarin was used at lower dose.

Nasal bone length reduction obtained in our study when newborn rats were exposed to warfarin at 100 mg/kg/day co-administrated with vitamin K was similar to that obtained by Howe et al. [17], and allow to validate our exposure method. When warfarin was used at human therapeutic dose (0.07 mg/kg/day, equal to rat LD50 at 90 days for repeated administration), skull length reduction was similar to that obtained at 100 mg/kg/day. On the contrary radius bone formation did not seem to be affected in rats after post-natal exposure to warfarin at human therapeutic dose (0.07 mg/kg/day). This result demonstrates that warfarin at human therapeutic dose is still able to induce skull malformations, but radius formation seems to be preserved at low dose of warfarin. This result is coherent with clinical signs associated to the FWS where skull malformations are dominant [9,11]. Skull development in rats may be more susceptible to warfarin than radius bone development in post-natal period, possibly due to a difference in their development. Indeed, radius ossification begins during the gestation period at the 17th day, while skull ossification could begin for some bones only after birth [26]. Therefore, in post-natal period, warfarin is eventually less susceptible to affect radius bone because ossification is already started contrary to skull.

The second part of our study was to investigate the teratogenicity of other VKA used as rodenticides in order to challenge the European "read-across" approach and the legitimacy of classifying all VKA as reprotoxic. Bromadiolone, a second-generation molecule was chosen in this study because of its different pharmacokinetic and pharmacodynamic properties with warfarin (i.e., very long tissue-persistence, toxic in a single administration) and because of its intensive use in Europe for rodent's management. Because no study was available with this molecule, the study of its potential teratogenic properties was explored in rats as previously done for warfarin. Rats presents a hemochorial placentation as human and placental transfer of VKA are presumably similar between rats and humans. This study was carried out after a prenatal exposure, but also after a post-natal exposure because the FWS was successively reproduced when exposure of rats was done after birth.

Compared to warfarin, bromadiolone is a highly effective anticoagulant molecule that can cause very low-dose anticoagulant effects. The ED50, corresponding to the effective dose able to induce 24 h after administration an increase in prothrombin time by a factor of 5 is only 0.5 mg/kg, the LD50 being 1.125 mg/kg in one administration. This strong anticoagulant activity is a difficulty to study the reprotoxic potential of this molecule. The exploration by Howe et al. [17] of the teratogenicity of warfarin daily administered at a toxic dose was enabled by daily co-administration of vitamin K1 that could have minimized, masked or modified observed effects. To study the reprotoxic potential of bromadiolone, we chose to use anticoagulant-resistant animals due to a mutation of the VKORC1 enzyme that is the target of VKA [27,28]. The Q120 mutation in the homozygous state allows the animal to receive doses increased by a factor of 8 without presenting hemorrhagic signs [22], in the heterozygous state, the doses can be increased by a factor of 4. To expose the fetuses daily to bromadiolone, we crossed homozygous or heterozygous females for Q120 with susceptible L120 males. The Q120 females being resistant to bromadiolone, they were able to receive high doses of bromadiolone throughout their gestation (corresponding to a cumulative dose of 1.4 mg/kg) without presenting any hemorrhagic disorder allowing them to complete their gestation. Since these females were crossed with susceptible L120 males, the reproductive effects of bromadiolone were observed in susceptible L120 fetuses as well as in fetuses heterozygous for Q120. The genotyping of the litters resulting from these crosses allowed us to estimate the impact of exposure on embryonic mortality, in particular on that of the sensitive fetuses. The genotypic proportions obtained were similar to the expected proportions (Chi Square Goodness of Fit - data not shown), demonstrating the non-mortality of susceptible fetuses during gestation despite exposure to bromadiolone. This non-deviation of genotypic proportions is surprising and reveals an absence of toxicity of such concentration of bromadiolone for fetuses even for sensitive L120 fetuses suggesting a limited placental transfer of bromadiolone. Moreover, no teratogenic effect was detected following fetal exposure, either in susceptible L120 fetuses or heterozygous for Q120 fetuses. To evaluate the effects of post-natal exposure to bromadiolone, we exposed L120 newborns rats or newborn rats homozygous or heterozygous for Q120 with bromadiolone. Knowing that bromadiolone is a highly persistent molecule, we administered to newborns that molecule every 3 days, that is to say, 8 administrations over a month to reduce the handling of newborns. Since the cumulative dose must be less than the

LD50, we administered 0.2 mg/kg 8 times. At such a dose, no significant decrease in the length of bone structures was observed. The methodology being validated by the tests carried out with warfarin, the absence of modifications of the bone structures could be due to the dose chosen to carry out this study. Since no hemorrhage has been observed in sensitive newborns treated, the dose tested could be increased (knowing that such exposure seems extraordinarily unlikely by using baits containing bromadiolone in bait boxes). However, it is important to note that the bromadiolone concentration administered in this study is equal to the cumulative 3-day dose of warfarin causing effects on skull length (0.07 mg/kg/day or 0.21 mg/kg every 3 days). Our results demonstrate that bromadiolone and warfarin anticoagulant rodenticides in our experimental conditions do not show similar embryotoxic potential. The observed difference could have a pharmacokinetic origin with hepatic uptake or plasma protein binding different between both VKA and therefore a different tissue distribution especially at the level of the conjugation cartilages which are constitutionally poorly irrigated tissues. Further works needs to be performed to explain the difference observed and to confirm the classification of all VKA as reprotoxic molecule. This classification has led the European Authorities to define a limit concentration in baits not to be exceeded for sale to the general public (Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012). This will lead to a decrease in concentrations of active substances in baits and may have a negative impact on the selection of resistant rodent strains. Numerous mutations of Vkorc1 leading to resistance have been described in rats and mice in Europe [20,23,29] and a decrease in active substance concentrations in baits could promote the dispersion of these resistance alleles and make it even more difficult to manage rodents.

Declaration of Competing Interest

The authors declare no conflict of interest.

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