Reproductive Toxicology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Reproductive Toxicology

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

A shortened study design for embryo-fetal development studies in the minipig

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ARTICLE INFO

Keywords:
Minipig
Embryo-fetal development study
Mid-term fetal examinations
Pyrimethamine
Skeleton double staining

ABSTRACT

The minipig is accepted from scientific and regulatory perspectives for the safety evaluation of drug candidates on embryo-fetal development. The relative size and the duration of gestation (112–115 days) in the minipig is, however, considered a drawback compared with routine smaller species. We evaluated if study duration and cost could be optimized without impacting scientific validity by performing all terminal procedures around mid-gestation (60 days). At this stage, minipig fetal size is not too dissimilar to full term rabbit and therefore better suited to fetal processing/examination compared with at the end of gestation. Despite encountering higher than anticipated embryo-fetal death, morphological defects clearly associated with a known teratogen, pyrimethamine, were detected. Although the gonads are poorly differentiated macroscopically at mid-term, a histological examination confirmed that external sexing of the fetuses was accurate. Double staining of the bone and cartilage of the mid-term fetal skeleton allowed a more refined examination.

1. Introduction

According to ICH S5(R2) guideline [1], an embryo-fetal development study should be conducted in one rodent and one non-rodent species, with the rat and the rabbit as the default species. However, each species has its own limitations for certain pharmaceutical classes (*e.g.* sex hormones for the rat and antibiotics for the rabbit). Therefore, the guideline states that other non-rodent or rodent species may be appropriate and should be considered on a case by case basis. The non-human primate is essentially reserved for biotechnology-derived products as described in ICH S6(R1) [2] and is of limited use due to length of gestation (5.5 months), small litter size (1–2 offspring/litter) and ethical concerns [3,4]. Conversely, the minipig is a relevant animal model for routine use in regulatory embryo-fetal development studies, at least for small molecules that freely cross the epitheliochorial placenta [5]. Indeed, the minipig has many metabolic similarities with humans (*e.g.* cytochrome P450, hepatic enzymes [5,6,10]). It also has advantageous reproductive physiology compared with the other large animal models such as the dog and non-human primate, including: earlier sexual maturity (3.7–6.5 months), short estrous cycle (21–22 days) and relatively large litter size (5–6 fetuses/litter for primiparous females) [4–9]. Although discussed here as a disadvantage with respect to the small animal species, the duration of gestation in the minipig (approximately 114 days) is, however, shorter than in the non-human primate (approximately 164 days). In the minipig, organogenesis occurs from gestation day (GD) 11 (development of the primitive streak) to GD 35 (closure of the palate) [4,5]. Batch breeding, experimental housing, technical handling, in-life examinations and dosing techniques are well established for the minipig [5,10]. Several miniature swine strains are available such as the Göttingen, Yucatan, Sinclair and Hanford. The Göttingen Minipig (Ellegaard Göttingen Minipigs, Dalmose, Denmark) used in these studies is a genetically defined, purpose-bred and specific pathogen free animal model. Time-mated gilts can also be obtained from Ellegaard [11] so that a lengthy in-house mating phase and the housing of the mating boars can be avoided. In addition, in cases where the minipig has been selected as the non-rodent species for safety assessment, data from previous repeat-dose toxicity studies can limit the extent of preliminary work required to select doses for the pregnant minipig. However, despite these advantages, there remains a tendency to overlook the minipig as a non-rodent species for embryo-fetal development studies, principally for reasons of cost and study duration. Other concerns have been a limited amount of historical control data for fetal observations (partly addressed by Berggren [6] and Ellemann-Laursen et al [7]), malformation clusters with variable background rates (as for other species), large amounts of compound required and extensive housing needs.

The principal aim of these investigations was to evaluate if a reduction in study duration, by performing caesarean section close to

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https://doi.org/10.1016/j.reprotox.2018.06.009
Received 15 March 2018; Received in revised form 8 June 2018; Accepted 18 June 2018

mid-gestation (approximately GD 60), had any detrimental impact on scientific validity of embryo-foetal development studies.

2. Materials and methods

Two successive studies were performed:

The first study (referred to as the "pyrimethamine study" herein) was to evaluate if fetal abnormalities associated with a known teratogen could be detected with fetal examinations performed mid-term (GD 60) [5] compared with current standard examinations around GD 110, *i.e.* close to term. Similar methodology was previously employed successfully for studies in the non-human primate in order to limit study duration [3] before be replaced by ePPND study design.

The second study (referred to as the "refinement study" herein) was performed in order to refine and validate the techniques for fetal examinations at mid-term.

Both studies were reviewed by the Charles River's ethical committee according to the animal health and welfare guidelines [12–14].

2.1. Pyrimethamine study

2.1.1. Test substance

The test substance selected was pyrimethamine due to reported high incidences of fetal malformations without any maternal toxicity [15].

Pyrimethamine (99% purity) was obtained from AK Scientific, Inc. (Union City, NJ, United States). Pyrimethamine was dissolved in 1% carboxymethylcellulose (Prolabo, Fontenay-sous-Bois, France) in water for injection (Laboratoire Lavoisier, Paris, France) at a concentration of 1.8 mg/mL for oral (gavage) administration to mated minipig gilts.

2.1.2. Test system

Thirty-six, 6 to 9-month old Göttingen Minipig gilts (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were mated at the Ellegaard Göttingen Minipigs facility for up to three consecutive days. The first day of mating was regarded as day 0 of gestation (GD 0). The mated females were then delivered to Charles River Lyon Safety Assessment Facility (Charles River, Lyon, France) by day 6 *post-coitum* at the latest. The animals were housed in groups of 3 or 4 of the same dose group in double pens (approximately 2.88 m^2) in a conventional animal unit at the AAALAC-accredited facility with a temperature setting at 20 °C, 12-h light cycle, at least 10 air changes per hour and at least 40% humidity. Animals were fed with pelleted complete commercial diet (SDS SMP MOD(E) SQC) with increasing quantity (600–900 g/animal/day) during the gestation period.

2.1.3. Study design

The animals were divided into two groups of 18 females each; one was administered pyrimethamine at 3.6 mg/kg/day and the other received the vehicle (1% CMC) by gavage. The dosing period complied with regulatory requirements for both groups with exposure throughout the major period of organogenesis (GD 11 to at least GD 35 with one or two extra days for females that mated on up to 3 consecutive days). The groups were further divided into two sub-groups of 9 minipigs each that underwent either mid-term (GDs 59–62) or term (GDs 108–110) caesarean section.

2.1.4. Observations

2.1.4.1. Maternal clinical condition and body weight. The gilts were examined daily and at least once after treatment. They were weighed twice weekly during the period of organogenesis (GDs 11–35) and weekly for the remaining gestation period.

2.1.4.2. Caesarean sections. The females were euthanized by sodium pentobarbitone intravenous injection followed by exsanguination between GDs 59 and 62 or between GDs 108 and 110, and were submitted to a full necropsy examination. Litter parameters were recorded (*i.e.* pregnancy status, gravid uterus weight, number of implantation sites, number and distribution of live and dead fetuses, and early and late resorptions). The number of corpora lutea was arbitrarily considered equal to the number of implantations due to corpora luteal fusion and polyovulation in the minipig. The fetuses were weighed and sexed externally.

2.1.4.3. Fetal examinations. All fetuses (mid-term and term) were euthanized by sodium pentobarbitone intraperitoneal injection and then examined for external (including palate) and fresh visceral (abdominal and thoracic viscera including sectioning of the heart and kidneys) defects. The head of approximately half of the fetuses in each litter was removed and fixed in Harrison's fluid to be examined later by serial sectioning. At GD 110, the visceral examination of the heads was performed after pre-freezing to stiffen the specimens and facilitate sectioning. The eviscerated fetal carcass was processed for skeletal examination. The ossified skeleton was stained with alizarin red s following maceration of the soft tissues in potassium hydroxide solutions (the staining technique was adapted from established methods used for the rat and rabbit). The stained specimens were then cleared and retained in glycerol prior to examination.

2.2. Refinement study

2.2.1. Test system

Five Göttingen Minipig gilts (Ellegaard Göttingen Minipigs A/S, Denmark), of 7 to 13-month-old, were mated at Charles River Lyon Safety Assessment Facility (Charles River, Lyon, France) for up to four consecutive days. The gilts were housed individually or in groups of 2 in the same environmental conditions as the first study.

2.2.2. Study design

The gilts were divided into two groups that underwent either mid-term (3 females) or term (2 females) caesarean section. The gilts were not required to be dosed in this study.

2.2.3. Observations

Maternal clinical condition, body weight and caesarean section were compiled similarly as the first study.

The same maternal terminal procedures, and fetal external and fresh visceral examinations, were performed (mid-term and term) as in the first study. Following visceral examination, the following fetal organs were sampled from both groups (mid-term and term) and preserved in 10% neutral formalin or modified Davidson's fluid: adrenal glands, epididymides, heart, kidneys, liver, lungs, ovaries, testes, thymus, thyroid gland, urinary bladder, uterus and vagina (where applicable). Following hematoxylin and eosin staining, a histopathological examination was performed for all sampled organs from all fetuses.

The eviscerated fetal carcasses of the mid-term fetuses were processed for skeletal examination. The skeleton was stained with alcian blue and alizarin red s (to allow examination of the cartilage and bones, respectively) and then the stain was fixed in ethanol prior to maceration of the soft tissues in potassium hydroxide solutions (staining techniques adapted from methods used for the rat and rabbit [16–20]). The stained specimens were then cleared and retained in glycerol prior to examination.

3. Results

3.1. Pyrimethamine study

3.1.1. Maternal clinical condition and body weight

There were no treatment-related deaths or clinical signs.

There was no adverse pyrimethamine-related effect on mean body weight (including females with no viable fetuses). There was a transient reduction in bodyweight gain for all the gilts (control and treated)