The Minipig Research Forum (MRF) – An Introduction to a Unique Network

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Introduction

The Minipig Research Forum (MRF) is a user group focusing on minipigs and its use in biomedical research, both from a practical and scientific perspective. The inaugural meeting was held in December of 2007 in Copenhagen. Since then, annual meetings have been held in various locations throughout Europe, and two meetings have been held in North America.

In many ways the first meeting of the MRF was a natural step to take given the increased interest and extent of use of minipigs in biomedical research. A user group, or forum, seemed the most suitable way to facilitate networking and exchange of knowledge and experience among researchers interested in using this non-rodent species. Meetings are characterised by high quality technical and scientific content and opportunities for networking; the latter being vital to the success of the meetings.

In total more than 400 participants have attended the seven meetings held thus far listening to more than 100 presentations on topics as varied as housing and management; basic behaviour and behavioural assessment; surgery and anaesthesia; advanced sampling techniques; non-traditional dosing techniques; clinical pathology; spontaneous pathology; case studies linking non-clinical and clinical investigations, and much more pertaining to minipigs. The majority of the presentations are available for download via the MRF-website (see Fact Box) offering a true repository of valuable information for the non-clinical experimenter.

The MRF is also present on LinkedIn where a discussion group has been created. It serves as a platform to facilitate discussions between minipigs users as well as an archive where past, present, and future discussions can be accessed (See Fact Box).

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Terms of Reference: Bringing researchers and research model together.

The goal of the MRF is to create a forum where minipig users can interact, discuss and share ideas and knowledge, so that we all may become better at what we do.

Website: www.minipigresearchforum.org
On LinkedIn: http://www.linkedin.com/groups?gid=4219925

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At the European MRF held in Frankfurt in November 2011 the main topics were juvenile and developmental studies and neurobehavioural assessment in minipigs. Abstracts of the presentations are included in this publication and while they do not cover the full content of the presentations they can be seen as appetisers for what was covered in the actual presentations, most of which are available on the MRF website (see Fact Box)

Yours sincerely,
The European MRF Steering Group

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Comparative embryology in the pig, dog, and human

by ***Poul Hyttel***
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Over the recent years, the minipig has become further refined as a human disease model by application of e.g. genetic modifications for mimicking human diseases. Genetic modifications are introduced into the pig by means of cloning by somatic cell nuclear transfer utilizing genetically modified fibroblasts as nuclear donors. In order to use the minipig as a model for processes related to reproduction and embryonic and fetal development, it is of importance to be aware of potential differences in these processes between minipig, human and dog; an alternative model animal. A comparative overview of the sequential steps of initial embryology is presented in the following. Oocyte maturation occurs in the pig in multiple follicles, in dog partly in multiple follicles and partly in oviduct, and in human in a single follicle. In the dog, this particular phenomenon allows for fertilization of the oocyte before it has reached the mature metaphase II stage. Major embryonic genome activation takes place in the pig at the 4-cell stage, in dog at the 8-cell stage, and in human at the 8-cell stage. Blastulation occurs in the pig at Day 5, in dog at Day 8, and in human at Day 5. In the pig and dog the embryonic disc penetrates the trophectoderm, and in the pig a dramatic elongation of the whole conceptus takes place. In human, the embryonic disc does not penetrate the trophectoderm. Gastrulation involves formation of the primitive streak and notochord in all three species establishing the trilayered embryonic disc. Neurulation involves the induction of neural ectoderm by the notochord in all three species. In pig and dog, the amnion is formed by the chorioamniotic folds at Days 15-16 and Day 21, respectively, whereas in human the amnion is formed by cavitation of the epiblast. Placentation in the pig is initiated as a non-invasive attachment at Days 13-14 after which a diffuse, folded, epithelio-chorial, and non-deciduate placenta develops. In dog, placentation is initiated at Days 17-18, it is invasive, and develops into a zonary, lamellar, endothelio-chorial, and deciduate type. In human, placentation is initiated at Days 6-7, it is invasive, and develops into a discoid, villous, hemo-chorial, and deciduate type.

Scientific considerations and practical execution of embryo fetal developmental toxicity studies

by ***Thomas K. Andreassen***
CiToxLAB Scantox A/S

Traditionally rats (and rabbits) are the species of choice for reproductive toxicity studies. However if these species are found unsuitable (e.g. due to metabolism issues) one possible non-rodent alternative is the minipig. In this presentation the use of minipigs in non-clinical reproductive toxicity studies will be introduced. Particular emphasis will be on the use of minipigs in embryofetal developmental toxicity (ED) studies. The possible use of minipigs in fertility and pre-and post natal toxicity studies will briefly be discussed.

The Göttingen minipig has been used extensively in toxicological and pharmacokinetic studies in many years. Within reproductive toxicology its use as an alternative non-rodent species in ED studies is increasing, and in comparison to non-human primates the minipig is a good alternative due to the relatively high fecundity (litter sizes >5 in primiparous mothers), low age at sexual maturity (5-6 months), polyestrus cycling (estrus cycle of 21-22 days), gestational length of approximately 113 days, ease of successful mating (pregnancy rate > 90%) and for animal ethical reasons.

In main ED-studies groups of at least 18 mother animals per dose group will be treated from Gestation Day (GD) 11 to GD36 (26 days), which constitutes the period of organogenesis. Caesarean sections are performed on GD110. The uterus and ovary will be examined and an external and visceral examination of the fetuses will be performed at necropsy where after the fetuses will be processed for skeletal and head examination. In preliminary studies caesarean sectioning is performed earlier (between GD21 and GD70) and no visceral, skeletal and head examination will be performed.

CiToxLAB Scantox has until now performed more than 20 ED studies in the minipig, and has thus achieved considerable experience with regard to the conduct and management of ED studies and furthermore has created a large historical database which is important in relation to the scientific evaluation of data obtained.

In the current presentation the practical execution of such studies will be outlined and the challenges and advantages of the model discussed. Further, some historical background ground data on both litter data (uterus, ovary, pre-and post-implantation loss, etc.) and the fetal examinations (external, visceral, skeletal and head) will be presented. In addition, data on hormonal levels (progesterone and estradiol) during the reproductive cycle, synchronization of the estrus cycle, evaluation of cyclicity and mating-success will be presented and discussed.

Alternative approaches for minipig embryo-fetal development studies – mating and early termination

by ***Edward Marsden***
Ricerca Biosciences SAS

The conclusions of the Rethink project on minipigs as models for the toxicity testing of new medicines and chemicals were published in a special issue of the Journal of Pharmacological and Toxicological Methods (*Volume 62, Number 3, 2010*). The articles provide valuable information for those considering selecting the minipig as a non-rodent model for toxicity testing. Reproduction and developmental toxicity is mentioned and the use of the minipig as an alternative non-rodent species is discussed, particularly for embryo-fetal development studies.

However, undertaking embryo-fetal development studies in the minipig is demanding for economic, technical and scientific reasons and the rabbit remains the default non-rodent species. But if the rabbit is not an appropriate model for a given test compound, the minipig is now an established alternative non-rodent species for embryo-fetal development studies.

Working in collaboration with Ellegaard Göttingen Minipigs A/S (EGM), Ricerca Biosciences has been looking at alternative approaches to the current study designs to ensure efficient and cost-effective running of these studies without compromising scientific validity.

We have worked on two principal ideas. The first was to establish a mating protocol to procure the required number of time-mated gilts for a full embryo-fetal development study directly from EGM instead of performing in-house mating. The second was to investigate the feasibility of performing all fetal examinations (external, visceral and skeletal) earlier in gestation instead of close to term.

Feasibility studies have been performed in which EGM synchronised the estrous cycles of selected gilts with the luteinising hormone, Regumate, in order to allow batch mating (up to 18 gilts per week). The animals were delivered during early gestation in order to allow acclimatization for approximately 1 week before the start of dosing. Mating and body weight data were provided by EGM and a transient dip in body weight gain associated with delivery had no impact on the subsequent pregnant status of the gilts or on their litter size.

Early termination is already routine in NHP embryo-fetal development studies at a stage when adequate morphological examinations can be performed (gestation day (GD) 100 with natural birth around GD160). Mid-term caesarean sections are already performed for minipig dose range-finding studies but only external fetal examinations are routinely performed. A feasibility study was therefore undertaken in order to evaluate if fetal abnormalities associated with a known swine teratogen, pyrimethamine (daily gavage at 3.6 mg/kg/day throughout the major period of organogenesis (GD11 to GD35)), could be detected if the fetal examinations were performed earlier (close to GD60) compared with the current standard at term (GD110). With a crown rump length of approximately 10 cm and a mean body weight of approximately 50 g, the GD60 minipig fetus compares closely with a full term (GD29) rabbit fetus. As a consequence, the materials, methods and time required to complete the visceral examinations (by microdissection) and skeletal examinations (after processing for alizarin stained specimens) are also comparable with those for the rabbit. Pyrimethamine-associated fetal abnormalities consistent with those described in the literature, principally limb malformations,

micrognathia and cleft palate, were present amongst the GD60 fetuses.

In conclusion, time-mated females can be obtained directly from EGM in sufficient numbers to perform full embryo-fetal development studies. The females are delivered in time to allow a number of days acclimatization prior to the start of uterine implantation and study dosing on GD11. Fetal examinations performed earlier in gestation are feasible technically, provide numerous practical and time saving advantages with respect to full term caesareans, ultimately reducing study duration and associated costs.

Congenital abnormalities of the Göttingen minipig

by ***Thomas K. Andreassen***
CiToxLAB Scantox A/S

To obtain more historical background data in the minipig on incidences of congenital abnormalities in the Göttingen minipig population, all still born piglets and piglets dying within 72 hours after birth are being collected in the breeding colony by Ellegaard Göttingen Minipigs A/S. These piglets are being sent to a group of laboratories for evaluation of abnormalities. At CiToxLAB Scantox A/S we have received and evaluated 853 piglets collected during 2010. The group of piglets being collected consisted of still born piglets, piglets euthanized due to visible malformations or due to a general "poor condition" or piglets found dead for known or unknown reasons within the first 72 hours after birth. Due to the diversity in the age/size/history of the piglets the evaluation were restricted to external and visceral examinations, thus no skeletal or head examination was performed.

The most frequently occurring malformations observed in the examined population were cryptorchidism (25.6% of male population), ventricular septum defects (11.5%), polydactyly (9.5%), syndactyly (4.3%), diaphragmatic hernia (4.1%), limb hyperflexion (3.8%), scoliosis (2.5%), cleft palate (2.3%), small/absent gallbladder (2.2 %), limb hyperextension (2.1%), atrium septum defects (1.9%), cleft lip (1.6%) and absent anus (1.1%). In comparison to an earlier study also performed at CiToxLAB Scantox A/S on a similar population of piglets (n=322) collected in 2007 the data indicate an increase in the majority of the most common malformations observed.

These data are valuable tools to be used in combination with study based historical control data when interpreting data obtained in proper embryo fetal toxicity studies. Further, the data can be used as a tool to monitor the "genetic health" of the breeding population over a period of time, and emphasize the importance of keeping a database which is up-to-date.

In addition, the dataset contain information which in combination with breeding information from Ellegaard Göttingen Minipigs A/S gives the unique potential to investigate the possible hereditability of specific malformations.

Genetically modified minipigs – Alzheimer's disease, psoriasis and atherosclerosis

by ***Rikke Westh Thon***
PixieGene A/S

Report of a project funded by the Danish Advanced Technology Foundation

Denmark has established a scientific and commercial stronghold in the fields of health care and agri/food. Interestingly, the position has been achieved through an interaction and flow of know-how between these apparently different fields. The "Pigs and Health" project was based on the very same idea: The natural interaction between human health and agri/food.

Taking advantage of the results of the Sino-Danish Pig Genome Sequencing Project we focused on genetic modification of the porcine genome mainly in the fields of metabolic dysregulation and degenerative diseases. By the use of genetic engineering and novel techniques of reproduction (cloning) we created and produced unique, high value animal models for use in medical research and in the development of new drugs or strategies for human disease prevention.

The purpose of the project was to create disease models to be used in academia as well as in industry. It was primarily based on genetic engineering at the Department of Biomedicine, Aarhus University (AU), cloning technology at Dept. of Animal Science, Faculty of Science and Technology, AU and embryo transfer and caesarean section by Veterinary Reproduction and Obstetrics, KU-LIFE.

The following genetically modified pigs were generated during the project:

The Master Pig: Mini-pigs with suitable sites in their chromosomes for integration of any gene construct designed to elicit a disease process of interest. These Master pigs were produced by Somatic Cell Nuclear Transfer (SCNT) cloning of pig fibroblasts that had received a "reporter gene cassette" with appropriate recombination sites for future exchange of the reporter cassette with the medically relevant gene construct.

The Psoriasis models: Two strains of genetically modified mini pigs carrying α2-integrin and ß1-integrin were developed.

The atherosclerosis models: An ApoE knock-out model has been generated and so has a PCSK9-D374Y mutation based atherosclerosis model. Interestingly, an embryonic germ cell based PCSK9 has also been created. The models are being bred for homozygocity and double mutations and are being phenotyped before commercialization.

The Alzheimer´s model: An APP-sw model of Alzheimer´s disease, born in 2007, has been undergoing phenotyping in the project. A homozygous model has furthermore been bred.

Welfare of the Göttingen Minipig - pigs are curious, social and highly intelligent animals!

by ***Helle Lorentsen***
Ellegaard Göttingen Minipigs A/S

Ellegaard Göttingen Minipigs are purpose-bred miniature pigs exclusively for use in biomedical research.

Regardless of the reason for breeding animals, whether it is for food production, research purposes or simply good company, the animals have some rights, expressed in "The 5 Freedoms". Meeting these rights will result in better animal welfare.

Apart from the general housing and husbandry conditions, which should always be in place, this speech will concentrate on the animals' right to be free from fear and distress and the freedom to express normal behavior.

To meet these needs can prove to be a challenge unless you know what distresses a minipig and what normal behavior of a minipig is. After all, what is actually going on in the mind of a minipig?

Why does it scream? Is it because it hurts, or does it just want to tell you: "Get your hands off me!!"?

Knowing the behavior and nature of the minipig will indeed help both animal and human to an optimal cooperation with a minimum of stress for everybody.

Minipigs are smart creatures, and they will memorize good and bad experiences alike. This feature makes them very trainable for almost any procedure. The philosophy is: the less restraint the less stress - and better welfare.

With the right attitude, technique (positive reinforcement) and treats (!) it is possible to gain the minipig's confidence and train it to do the same things as a dog along the lines of "If you scratch my back, I'll scratch yours!"

Stress is in general an undesirable condition, and total unpredictability can cause stress.

But total predictability can result in boredom. For example are toys left in the pen for days and weeks a poor kind of enrichment as the minipigs often lose interest after minutes/hours. Toys should be changed at least on a daily basis. And bedding-rooting material should be available 24/7.

Why not give individual housed minipigs a little run up and down the corridor? – saying hello to the mates and break the monotony for everyone.

Enrichment of any kind can satisfy the needs of the minipig – but rather than doing the right thing it can be a matter of doing things the right way!

Embryo-foetal development study in the minipig

by ***Ingrid Brück Bøgh[1], Merete Bødker[1], Thomas Andreassen[2]***
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The non-clinical safety evaluation of a peptide with a MW approximately 2000, intended for body weight management, included an embryo-foetal developmental study in the minipig. The objective of this study was to assess potential effects of the test item on pregnancy progress and embryo-foetal development.

During February-April, four groups of a total of 72 primiparous female Göttingen minipigs were dosed subcutaneously once daily from gestation day (GD) 10 to 36, which corresponds to the period of organogenesis in the minipig. A four times loading dose was administered on GD 10 to obtain steady state exposure levels from the start of organogenesis; thereafter daily dosing continued with dose levels of 0, 0.5, 3 and 10 mg/kg/day. Maternal clinical parameters, food consumption, bodyweight and rectal temperature were assessed during the study, and litter data and external, visceral and skeletal development of the foetuses were assessed after euthanasia of dams and offspring on GD 110. In a preliminary study, foetal exposure to the test item had been confirmed 24 hrs after last maternal dose on GD 36.

In all groups dosed with the test item, the maternal rectal temperature was significantly elevated on the day after the first dosing and transient passive behaviour and body weight loss were observed during the first week of treatment. Body weight gain in the test item treated groups during the treatment period corresponded only to approximately 50% of that in the control group, while food consumption was not affected. Transient, reversible hair shedding was observed in all groups, including the control group, however with earlier onset and more pronounced hair shedding occurring in the test item treated dams. No treatment related effect on litter data and foetal malformations was recorded. There was a tendency towards a treatment related effect on foetal weight and size, and the foetal skeletal examination showed slightly enlarged fontanels and a tendency towards reduced ossification of skull bones in the test item treated groups.

The results of this study demonstrate that the test item lowered maternal body weight gain without affecting food consumption, pregnancy maintenance and embryo-foetal growth and development, apart from a slight delay in embryo-foetal development. The latter is considered secondary to the pharmacological effect of the test item on body weight, as it is likely that reduced body weight gain or body weight loss in the dams would affect foetal growth. Hair shedding was considered a physiological response to external stimuli triggering seasonal hair shedding, which was enhanced by the pharmacological effect of the test item.

Histology of the reproductive organs (comparison w. man, dog, NHP)

by ***Noel Downes, Principal Pathologist***
Sequani Limited

The Assessment of many aspects of reproductive toxicity require sexually mature animals as the use of prepubertal or peripubertal animals can result in uninterpretable results or false positives. The time to sexual maturity of many larger laboratory species results in lengths of study that are impractible in a regulatory environment. The mini-pig clearly has a much shorter and clearly defined onset of sexual maturity than either dog or non-human primates.

Detailed microscopic examination has been undertaken of the developing reproductive system of the mini-pig has in order to clarify the reproductive development of the mini-pig

In the testes at 90 days the germinal epithelium looks anatomically complete, sperm production appears to be in full flow and there is sperm in the tubular lumen. The testes has a predominantly adult appearance, The histology suggests reproductive capacity may be present in the male from 3 months and often quoted estimates of 4.5 months for male sexual maturity seem a little high.

Recognition of sexual maturity in female mini-pigs

Mini-pigs are non-menstrual and LH levels are much lower than in humans. As there is no comparable LH surge on ovulation and so there is no good indicator of onset of ovulation. No other parameters in the blood are avaliable to determine sexual maturity. The presence of corpora lutea are often considered as a good indicator of sexual maturity, but in the mini-pig the indications are that the female is reproductively viable prior to this. The ovaries at 90 days have a reasonably adult appearance. There are no corpora lutea but there are indications that ovulation has taken place. It is concluded that the female mini-pig at 90 days may be a suitable reproductive model for the late adolescent or teenager.

The mini-pig is clearly a superior model compared to dog and rodent with a reproductive physiology close enough to man to be meaningful. The large litter size and age to sexual maturity are a big advantage over primates.

The mini-pig should be a consideration as a second species for developmental toxicity and under some circumstances may be suitable for specialised fertility assessments.

Appearances of the reproductive organs in peri-pubertal female Göttingen Minipigs

by ***Antonin Torterau[1], Paul Howroyd[2] & Helle Lortensen[3]***
[1]Ecole Nationale Vétérinaire, [2]Ricerca Bioscience, [3]Ellegaard Göttingen Minipigs

Data have not yet been published about the histological features of the female reproductive organs during the peri-pubertal period in the minipig or about the precise age of acquisition of sexual maturity, although it is generally said that females reach sexual maturity at the age of approximately 5 months, by the time the body weight is about 10 to 12kg. In preclinical studies, it is important to know whether the animals used are sexually mature or not, as this can affect responses to the drugs tested, and because histological features of the immature reproductive organs can mimic the effects of compounds under study.

Histological sections of ovaries, uterus, vagina and mammary glands were re-examined from 39 female control minipigs from 10 toxicology studies on pharmaceuticals conducted at Ricerca Bioscience, France. The results were used to assess how the degree of sexual maturity as judged histologically correlates with ovarian and uterine weight, body weight and age.

The developing immune system of Minipigs

by *André H Penninks1, Geertje JD van Mierlo[1], Frieke CF Kuper[2], Cor J Snel[1], Niels-Christian Ganderup[3] and André PM Wolterbeek[2]*

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Changes in the legislative landscape (FDA, EU) in governing approval of new medicinal products has been the main driving force behind the recent focus of the pharmaceutical industries on juvenile toxicity testing. Although the rat is the first species of choice in this kind of research, for logistic and/or scientific reasons the rat may not always be the most appropriate species. For those situations the minipig could serve as a good alternative. To improve the usefulness of the minipig in juvenile (safety) testing, the developmental characteristics of main organ systems of the minipig, including the immune system, still requires additional characterization.

Therefore, the aim of our research was to gain more knowledge about 1) physical and sensory development of the minipig (developmental landmarks), 2) hematological and clinical chemistry parameters during development, 3) some immune parameters during development, and 4) histology of various main organs during development.

Hereto, the physical and sensory development of the piglets of 5 sows was followed starting from birth. At an age of 2, 7, 13, 27 days and 2, 3 and 6 months blood was collected from 4 male and 4 female piglets for hematology (e.g. WBC differentiation, lymphocyte subset analyses), clinical chemistry (e.g. total protein, albumin and albumin/globuline ratio), and immune parameters (lymphoid organ weights and histology). Furthermore, a series of approximately 20 other organs and tissues was collected at these time points to study age-related and developmental histology and background histopathology.

From the results obtained it is evident that at birth the minipig is relatively mature regarding physical and sensory parameters (e.g. eyes open, hair, teeth). From the series of organs/tissues collected some were (almost) fully developed at birth, while others showed distinct postnatal development. The histopathological evaluation of the lymphoid organs showed clear development over time but no obvious differences were noticed in the lymphoid structures when compared with other species, except for the inverted morphology of the lymph nodes, which does not affect lymph node function but only results in different lymphocyte trafficking in the lymph node (1). The nose associated lymphoid tissue (NALT) and the tonsil of the soft palate were already present at birth, but increased in size with age (2).

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Pre- and postnatal expression of P-glycoprotein and CYP3A in the small intestine of the pig: a preliminary investigation

by ***Steven Van Cruchten, Els Van Peer, Chris Van Ginneken***
University of Antwerp

Introduction

Drug transporters and metabolising enzymes are important in the pharmacokinetics of most drugs. Their presence and activity in the small intestine influence oral bioavailability and as such they may determine the clinical efficacy but also the toxicity of orally ingested drugs. Although many of these drugs are also used in (very young) children and differences in oral bioavailability are commonly compared to adults, studies on the ontogeny of drug transporters, such as P-glycoprotein, and metabolising enzymes, such as CYP3A, are scarce. The information is also limited in juvenile animals despite the fact that, from a safety perspective, toxicity studies in one or more species may be required prior to the start of clinical trials in children. The aim of this preliminary investigation was to assess the expression of P-glycoprotein and CYP3A in the small intestine of late gestational pig foetuses and juvenile pigs. The (mini)pig is used in juvenile toxicity studies but is in general also regarded as a good model for human oral absorption of drugs due to its similarities in gastrointestinal morphology and physiology.

Methods

Pig foetuses (n = 5; 90-115 days of gestation) and piglets of Day 0 (n = 5), 3 (n = 6), 10 (n = 4) and 28 days of age (n = 3) were obtained from Large White x Landrace pregnant sows at the slaughterhouse and after natural delivery at a local farm, respectively. The piglets were anaesthetised by an intraperitoneal injection of sodium pentobarbital, followed by exsanguination. The small intestine (middle part of the jejunum) was dissected, rinsed, formalin-fixed and embedded in paraffin. A mouse anti-human monoclonal antibody (Ab C219, Abcam, ab3364, Cambridge) was used for immunohistochemical evaluation of P-glycoprotein, whereas rabbit anti-human polyclonal antibodies were used for CYP3A4 (ab1254, Millipore, Billerica) and CYP3A7 (ab10323, Millipore, Billerica).

Results

The at term piglets (day 0 of age) and the piglets at 3, 10 and 28 days of age showed a clear staining of P-glycoprotein at the apical surface of the intestinal villi. There was no staining visible in the crypts. P-glycoprotein was absent in the small intestine of the pig foetuses. These results are similar to man, in which expression of P-glycoprotein in the small intestine occurs at birth and is also absent prenatally. Regarding CYP3A, both isoforms were present in the cytoplasma of the villous enterocytes of all age groups. This seems in contrast to human data, where CYP3A7 is known to be the foetal and neonatal isoform and CYP3A4 the adult isoform. However, cross-reactivity of the antibodies to both isoforms can be the reason for this apparent discrepancy.

Conclusion

These preliminary results suggest that the ontogeny of P-glycoprotein in small intestine is similar in pigs compared to humans. Regarding the ontogeny of CYP3A, RT-PCR will be used to unravel the different isoforms in pig small intestine.

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Neurobehavioral assessment of minipigs – an overview

by ***Peter Clausing***
Consultant

Watching a (mini)pig in the pen – mostly sleeping unless food is coming - seems not very suggestive for its use in neurobehavioral studies. Therefore, it is not surprising that only a few laboratories have used minipigs in this type of investigations so far. The few that did, however, did this with remarkable success. With the emergence of the minipig as a preferable non-rodent species in non-clinical studies it seems reasonable to have a closer look at their use in the area of neuroscience and behavior.

The lab of N.M. Lind (which does no longer exist) has to be given credit for the most comprehensive series of studies published between 2002 and 2007 using Göttingen minipigs. These studies ranged from spontaneous object recognition to response-to-novelty and open-field behavior, acoustic startle and various learning tasks. Learning was also assessed by Ferguson et al. (2009). Interestingly, they previously used identical operant tasks for rats, rhesus monkeys and humans, facilitating cross-species comparisons. Further open-field testing was performed in (mini) pigs after s.c. amphetamine dosing (van der Stay et al. 2009) and prenatal cocaine exposure (Laferriere et al. 1995). The determination of odor detection threshold as a means to use olfaction for operant-conditioning (Søndergaard et al. 2010) is another interesting feature. The minipig appears to be a very suitable model for Parkinsonism (e.g. Nielsen et al. 2009) and has been proposed as a model for the study of antipsychotics (van der Stay et al. 2009). Behavioral assessments have been supplemented by PET scanning, neurochemical and histological methods in particular with regard to the dopaminergic system (Mikkelsen et al. 1999; Lind et al. 2005; Nielsen et al. 2009).

In summary, the minipig emerges as a valuable neuropharmacological model and appears to be very suitable for advanced non-clinical safety studies.

Scientific considerations and practical execution of a minipig juvenile study

by ***Nanna Grand, DVM***
CiToxLAB Scantox A/S

Most pre-clinical safety evaluation of pharmaceuticals has been carried out using adolescent or adult animals. Safety data based on preclinical juvenile studies or clinical trials in children are thus generally limited for pharmaceuticals used in the pediatric population. Pharmaceuticals are often prescribed based on professional experience as well as calculated, based on the bodyweight of the child.

As children may react differently from adults to administered pharmaceuticals due to developing organ systems, the need for juvenile animal studies in the pre-clinical evaluation of potential novel pharmaceuticals has been emphasized and is today a regulatory requirement for new pharmaceuticals with intended use in children or adolescents.

The minipig is already a widely used non-rodent species in the pre-clinical toxicity testing of potential novel pharmaceutical and shows many advantages when considered as a juvenile animal model. Certain issues due to the husbandry and size of the juvenile animals have to be recognized before carrying out a juvenile study. Handling, dosing and obtaining study relevant data often need to be approached differently from study designs used for adult animals.

Furthermore, data obtained in the juvenile study may differ from adult animal data and knowledge of normal data for the juvenile animal population is of crucial importance when evaluating obtained results.

Scientific considerations and practical aspect when designing a juvenile minipig study as well as normal data relevant for evaluation of results will be presented.

An overview of the maturation and development of metabolism in minipigs – and humans

by ***Lars Dalgaard, PhD***
LD ADME Consult

A literature overview of the ADME related parameters of relevance for drug development for use in paediatrics is presented and will include:

- age dependant pharmacokinetics, absorption, distribution, clearance and half-life
- age dependant metabolism with emphasis on cytochrome P450
- Species similarities and differences between humans and pigs with regards to enzymes involved in the metabolism of drugs, endocrine steroids, pheromones, cholesterol and bile acids

The Göttingen minipigs are often used as alternative to dogs or non-human primates in toxicological studies. In a recent review it is concluded that the minipig could be the prime large animal model (Puccinelli, et al., 2011), because of the resemblance with human enzymes involved in drug metabolism. Unfortunately, the knowledge about the development of drug metabolism in juvenile minipigs is sparse. Recently, two CYP enzymes have been studied in livers from foetal and neonatal minipigs (Hermann and Skaanild, 2011) that shows promising results with regard to the resemblance with the development in humans. In other studies enzymes that are involved in the production of bile acids in the liver were shown to be up-regulated in weaned piglets (Lewis, et al., 2000;Lundell and Wikvall, 2003;Lundell and Wikvall, 2008). Another age dependant development of metabolism is causing the boar taint (Andresen, 2006) that is due to the presence of compounds like16-androstenes (mainly 5-alpha-androstenone, a pheromone) and skatole. In conclusion, it seems that the drug metabolising enzymes studied have developed within the first weeks after birth, while enzymes involved in cholesterol metabolism changes gradually with age and steroid hormones with sexual maturation, like in humans.

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Training and anticholinergic treatment (Biperiden) of pigs in the cognitive holeboard: a comparison study between Göttingen Minipigs and conventional pigs

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There is a rising interest in the use of pigs in biomedical brain research studies. However, the popularity is increasing faster than the development of necessary and valid behavioural tasks. We designed a spatial cognitive holeboard task for pigs. With this task we are able to look at several learning and memory parameters in intact animals of various sizes and ages. Previous experiments have shown that this apparatus yields replicable and robust results and that subtle group differences can be made visible.

The holeboard apparatus (constructed by *Ossendrijver Installatiebedrijf, Achterveld, the Netherlands*) is a square arena (530 x 530 cm) in which sixteen food bowls are evenly distributed. These food bowls can be accessed by an animal when it lifts a ball located on top of the bowl with its snout. The ball is held in place by a framework; when an animal retracts its head, the ball falls back in place. Each animal is assigned its own fixed configuration of rewarded bowls: out of sixteen bowls, four are rewarded with food.

Behaviour (latencies, strategies and 'hole' visits) in the holeboard is registered automatically. Every bowl is equipped with a magnet-sensor (*Turck Industrial Automation, Zwolle, the Netherlands*) and if the ball (equipped with a magnet) is lifted, this increase in distance between the magnet and sensor is registered. The signal is sent via an interface to a laptop and customized software (*Blinq Systems, Delft, the Netherlands*) records all signals and automatically stops a trial after an animal has found all rewards.

A group of female Göttingen minipigs (*Ellegaard Göttingen minipigs a/s,* n=8) and conventional pigs (Duroc X Yorkshire and Duroc X Danish landrace, n=8) were, after a proper habituation period, trained in the cognitive holeboard (two successive trials, twice daily during 13 weekdays) on their specific individual configuration of rewarded bowls. Training started when the animals were 12 weeks old. Learning curves of both groups were compared. We found conventional pigs as well as minipigs well able to acquire this task. After intensive training, both lines reached nearly errorless asymptotic performance levels. Differences between lines were found to be limited. Only reference memory performance tended to be a little lower in the Göttingen minipigs.

Furthermore, a validation study was performed. To acquire a dose-response curve of Biperiden (an acetylcholine antagonist) after oral administration, all animals were treated with Biperiden six times (+ 1 control test) spread over a 4 week period. During pre- and post-testing days animals were trained once a day (two successive trials), as during a test day. The doses were 0.05, 0.15, 1.5, 5, 15 and 20 mg/kg body weight. Averaged over all doses and sessions, the pig lines did not differ for the working and reference memory performance. A marginal Dose by Sessions interaction indicated that reference memory impairment induced by Biperiden was dose dependent.

The automated cognitive holeboard seems to be a suitable task for mini- as well as conventional pigs. These different pig lines both are found to be able and willing to perform in this task and the line differences are limited. This makes –depending on the research question– both lines suitable for use in this task and comparisons possible. Oral Biperiden administration seems to be safe in pigs and minipigs. Our data cautiously indicates memory consolidation to be partially affected by Biperiden. However, we suggest that it should be evaluated further to show if Biperiden is really able to affect the learning process and/or memory consolidation in the pig.

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An investigation into behaviour, learning and memory assessments in the juvenile Göttingen minipig treated with haloperidol, d-amphetamine, or scopolamine

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Over the last decade, the requirement for safety evaluation of medicinal products in the paediatric population has intensified, thereby accelerating the need for non-clinical safety investigations. The value of the minipig as a non-rodent model for non-clinical juvenile toxicity testing is becoming increasingly appreciated. In order to comply with both FDA and EU guidance documents released in 2006 and 2008, respectively (1, 2), there is a need to establish methods for developmental neurotoxicity assessments in the juvenile minipig to monitor key central nervous system (CNS) functions, reflex ontogeny, sensorimotor function, locomotor activity, reactivity, learning and memory. In the absence of any published data in the juvenile minipig, this study employed the use of two distinct tests, an adjusted holeboard test (3) to assess cognitive performance and a ten-minute open field test (4) to assess behavioural response which had previously been used in a preliminary study at Sequani (5).

Twenty-one naïve juvenile Göttingen minipigs were used to investigate techniques for behaviour, learning and memory assessments in relation to treatment with Haloperidol, *d*-amphetamine, and Scopolamine. The dose levels were selected following a review of the literature (2, 6, and 7). These substances were selected to impair normal responses in order to assess the selectivity and sensitivity of each technique for potential use on regulatory safety evaluation studies.

An adjusted holeboard test (4 of 16 holes baited) was used to assess cognitive performance after administration of Scopolamine. Results showed that the time taken to complete the test was increased for animals dosed with Scopolamine when compared with the concurrent controls; however, there were no discernible differences between the Scopolamine treated animals and concurrent controls for the number of re-visits to baited holes (Working Memory) and to unbaited holes (Reference Memory).

A 10 minute open field test was used to assess behavioural response of each animal in a testing arena after administration of either Haloperidol or *d*-amphetamine. Haloperidol and *d*-amphetamine produced marked changes in motor behaviour and decreased explorative behaviour, consistent with responses documented in the literature. A clear distinction was determinable between the behavioural profiles of these compounds and the concurrent controls.

In conclusion, this investigation indicated that the design of the 10 minute open field test was capable of detecting behavioural changes in juvenile Göttingen minipigs treated with haloperidol or *d*-amphetamine, however, further refinement or investigations are required to assess the utility of the adjusted holeboard test for learning and memory assessments, the functional observation battery may be further enhanced by including a visual discrimination task in addition to the aforementioned neuro-behavioural assessments.

Bottle raising minipigs for juvenile studies

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A pilot study was undertaken to investigate hand-rearing of neonatal Göttingen minipigs by bottle feeding with a sow milk substitute, as one of the objectives of a research and development project at Huntingdon Life Sciences, in preparation for further work to evaluate infant formula components. Piglets were farrowed normally and after 24 hours were removed from the sow and bottle feeding commenced. Initially the piglets were fed every 2 hours and the interval between feeds was then progressively increased to 6-hourly at 16 days post partum. From 18 days port partum, hand feeding was discontinued and ad libitum feeding via a multi-teat feeder introduced until the animals were finally weaned at 4 weeks of age.

Clinical health was monitored and growth and development was assessed by bodyweight gain, crown/rump measurements and tibial length, in comparison with naturally suckled, conventionally cross-fostered piglets. All hand-reared piglets remained in good health and growth performance compared favourably with naturally suckled piglets overall. It was concluded that hand-rearing Göttingen minipigs from 24 hours post partum using the selected bottle-feeding regime was successful and practicable, and can be used where appropriate in neonatal juvenile toxicity studies or other neonatal investigations in minipigs.

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The Pediatric Investigation Plan (PIP), regulatory experiences and considerations when using the mini pig as a non-rodent species

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In 2007 the European Paediatric Regulation came into force and a new scientific expert committee of the European Medicines Agency (EMA), the Paediatric Committee (PDCO) was established. The PDCO is responsible for assessment of the content of paediatric investigator plans (PIP) and the adoption of scientific opinions (which are binding on applicants). A PIP is intended to outline the plan for development of a medicinal product in the pediatric population, defining the design and timing of the quality, nonclinical, and clinical measures needed to support the marketing authorization application for pediatric use. The EMA Juvenile Tox guidance document can help the applicant determine the need for non clinical Juvenile Studies and outlines the general 'considerations' when designing a Juvenile Toxicity Study, one of these being species selection.

The mini pig is becoming more frequently used as an accepted alternative non-rodent species in nonclinical safety testing and can provide a robust model to support development of a pediatric medicine.

The presentation will provide a number of examples of PIPs where the mini pig has been chosen as the species for the Juvenile Toxicity study, and will discuss the rationale for species choice, any regulatory feedback on the PIP and where available, study results.

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