University of Pittsburgh

School of Medicine
Department of Cell Biology and Physiology
Center for Research in Reproductive Physiology

W952 Biomedical Science Tower
3500 Terrace Street
Pittsburgh, PA 15261
412-648-8316
Fax: 412-648-8315

Evaluation of the marmoset (*Callithrix jacchus*) as a model for reproductive toxicity

Author: Stefan Schlatt, Ph.D.

e-mail: schlatt@pitt.edu

Date: January 30, 2006

1) Information on the Author

I obtained a PhD from the University Münster, Germany and was awarded a number of postgraduate fellowships including a Welcome Trust Training Fellowship in Reproductive Research and a Heisenberg-Fellowship which is the most prestigeous German Senior Research Fellowship granted by the Deutsche Forschungsgemeinschaft. At present, I am an Assistant Professor at the University of Pittsburgh School of Medicine, PA, USA. In recent years I have been employed and trained at several Centers of Excellence in Reproductive Sciences including the Institute of Reproductive Medicine of the University Münster, Germany; the Institute of Reproduction and Development of Monash University, Clayton, Australia and the Center for Animal Transgenesis and Germ Cell Research of the University of Pennsylvania, Kennett Square, PA, USA. I have explored a wide spectrum of research in reproductive biology including such diverse topics as the photoperiod-dependent regulation of female and male reproductive organs in Djungarian hamsters as well as preclinical research using macaque and marmoset monkeys to analyse the role of inhibin in the testis, the effects of hormones on prepubertal testis development and the development of male contraceptive regimens. I and my colleagues established several milestones for the development of germ cell transplantation as a clinical tool performing the first successful germ cell transplant into a testis of non-human primates and men. Recently, a novel strategy to use testicular xenografting as a tool to generate fertile sperm was described and applied on marmosets, macaques and men. We were also able to describe xenografting as a new tool for exploration of toxic effects during testicular development in rhesus monkeys.

This document has been compiled in response to a request by the American Chemistry Council Phthalate Esters Panel. I have been asked to critique a recent review by Li et al. (2005) and to render my opinion of the general value of the marmoset as a model for human testis development and its use for risk assessments of gonadotoxic exposures in the male. In addition I was asked to express my opinion on the validity and relevance of the Mitsubishi study.

2) Introduction

The marmoset has been intensely used for many years as a non-human primate model to describe the physiology of testicular development and function. In contrast to rodent animal models, this small new world primate shows many similarities with old world primates and man in regard to the general developmental pattern. Like human and old world primates the marmoset enters three periods of development with an active postnatal phase of the hypothalamus-pituitary-gonadal axis, a prepubertal period of quiescence and a re-awakening of the hormonal axis and final differentiation of the male reproductive system during puberty. This pattern is quite different from that in mice and rats which do not show a marked prepubertal period of no or slow gonadal growth and differentiation. Since the marmoset is easy to maintain and to reproduce and – as a primate - appears to be evolutionarily close to man, it has been considered a valuable and clinically relevant animal model to analyze toxic effects on the developing male reproductive system. However, the marmoset also exhibits some interesting and unique features in regard to hormonal regulation of its reproductive functions. A detailed review by Li et al. (2005) that summarizes similarities and differences concludes that interpretation of experimental findings on the testicular effects in marmosets should be made with caution. The present short report revisits the pro- and contra-arguments of using the marmoset as a model for testicular developmental toxicity and concludes that the marmoset presents a useful and valid model to explore several aspects of testicular development. I have also been asked to critically review an unpublished study from the Mitsubishi Chemical Safety Institute using the marmoset as a model to explore the testicular toxicity of DEHP. As discussed below, I conclude that the results of this study should be carefully and critically considered for the evaluation of risks associated with exposure to DEHP.

3) The value of the marmoset as a model for male testis development and evaluation of bias in Li review

3.1 Physiological characteristics of the male marmoset endocrine and reproductive systems

The recent review by Li et al. (2005) is a comprehensive review of the testicular development and function in the marmoset. The authors present the current knowledge on embryonic testicular development and describe the changes occurring during the postnatal period (2-3 weeks) and the pubertal period (6-12 months). They show the striking similarities of organization of the marmoset and human spermatogenic epithelium (both species have a multistage organization of spermatogenic stages per tubular crossection). Although nine stages of the seminiferous epithelial wave have been distinguished originally, many authors have adapted the human staging system for the marmoset indicating quite similar mechanisms of germ cell development and clonal expansion of germ cells in marmosets and man. This organizational similarity has prompted scientists to promote the marmoset as a “suitable model for studies relevant to human testicular function” (Millar et al., 2000).

Li et al. (2005) point out an unusual uniformity of Sertoli cell morphology throughout the marmoset spermatogenic cycle. In rodents and macaques Sertoli cells show specific morphological features related to specific stages of spermatogenesis. However, it is not clear how stage-specific changes of Sertoli cells can be identified in seminiferous tubules of men and marmosets which show a mixed pattern of spermatogenic stages. Although there are insufficient human data on Sertoli cell morphology to know whether such uniformity occurs in humans, it appears likely that the human and marmoset Sertoli cell show a high degree of similarity in this respect.

Li et al. (2005) quote studies reporting a high efficiency of marmoset spermatogenesis although this finding appears slightly controversial in the literature. They indicate that FSH is the prime regulator of Sertoli cell proliferation which correlates well with the role of FSH in other primates but differs from its less significant role in rodents.

One of the most interesting marmoset specific adaptations is the absence of the LH hormone. In the marmoset and other new world monkeys a CG type hormone is expressed in the pituitary and is responsible for regulation of steroid secretion in the ovary and the testis. This change is accompanied by a deletion of the LH receptor which lost exon 10 and can therefore not bind to LH, but has high affinity for CG. Despite of the exchange of LH by CG the marmoset shows many similarities to man. The function and regulation of FSH and CG and their feedback mechanisms resemble other primates. It is quite interesting to note that the LH receptor deletion in the marmoset mimics the situation in a hypogonadal patient (Gromoll et al., 2000). The availability of the marmoset as a model opened a new research field to explore the molecular mechanisms of hormone specificity of the LH receptor (Gromoll et al., 2003).

Li et al. (2005) propose a generalized steroid hormone resistance in the marmoset. This phenomenon is indeed quite striking and has been proposed for the mineralocorticoid and glucocorticoid hormones. However, whether or not this resistance also exists for gonadal steroids is poorly understood. Although some evidence exists that the response to sex steroids is rather poor, these observations should be balanced with the facts that many aspects of the marmoset in regard to sex steroids are strikingly similar to humans and differ from those in the rodent.

The authors mention that there is little information on marmoset testosterone synthesis and testosterone metabolism. The marmoset, but not the mouse, has sex hormone binding globulin separating the serum testosterone into active free and bound fractions. Due to affinities of these proteins the marmoset appears to have high levels of unbound testosterone in the circulation which is similar to the unusually high levels of glucocorticoids. However, in the absence of solid data on sex steroids it appears poorly justified and premature to transfer the conclusion of high sex steroid resistance from the glucocorticoid and mineralocorticoid system to the sex steroid system.

Some other curiosities exist which – in my opinion- do not impact the use of the marmoset as a model. Intact male marmosets respond with positive feedback to estradiol. Such a response is usually observed in orchidectomized rats or macaques and many homosexual men. The development of the testis relies on the presence of FSH and CG. A delay in testicular development does not have serious consequences, similar to other primates. Our own studies using xenografting as a model system showed the expected outcome that mouse LH can not stimulate the androgen-dependent differentiation of grafted marmoset tissue due to its deleted LH-receptor (Schlatt et al., 2002). Co-grafting with hamster tissue was not sufficient to induce testicular development (Wistuba et al., 2004). Li et al (2005) interpreted our data as indicative of unique factors responsible for marmoset spermatogenesis. However, the more likely explanation is that CG is needed to stimulate organogenesis of the immature testicular fragments and that the testosterone supplied by the hamster graft is insufficient. This explanation is supported by very recent data showing that autografted marmoset testes can develop through puberty (Wistuba et al., 2005), an indication that their hormonal system can drive spermatogenesis in autografted tissue.

In conclusion, the review by Li et al. (2005) presents a useful resource for any scientist interested in the marmoset model. However, the authors' interpretation of the suitability and validity of the model carries an unjustified negative bias.

3.2 Personal judgement of the value of the marmoset as research model for assessment of male reproductive toxicity.

In my opinion there is no doubt that the marmoset is a useful model to explore developmental and toxicological aspects of the testis. Certainly, old world primates appear to be favorable as they maintained a closer physiological relation to humans. However, many similarities in regard to testicular organization, general developmental pattern and hormonal regulation render the marmoset a much more useful model when compared to rodents. The marmoset therefore has its valid place when it comes to the analysis of toxic effects on testicular development. With choice of the correct timepoints this species should be highly useful and informative for exploring the effects on Sertoli cell differentiation, testicular growth and effects of FSH on the testis. The striking similarities to man make it an excellent model for studying effects on germ cell development, the organization of the seminiferous epithelium and changes to the kinetics of spermatogenesis. Due to its unusual gonadotropic and sex steroid regulation it is certainly an interesting biological model for exploring steroid feedback and the functions of the CG-molecule with less relevance for humans in these specific aspects.

4) The value of the Mitsubishi study to determine male reproductive toxicity

4.1 Validity of the results of the Mitsubishi study in respect to pitfalls and shortcomings

This study is a unique effort to explore the effects of DEHP on testicular development in a non-human primate model. In the oral dose toxicity part of the study marmoset monkeys of 90-110 days of age were exposed to three doses of DEHP (100, 500, 2500 mg/kg) and vehicle for 65 weeks. The monkeys received a daily oral dose in corn oil by gavage. The size of the male treatment groups finishing the exposure was 8 (vehicle), 9 (100mg/kg), 10 (500 mg/kg) and 9 (2500 mg/kg). 3 animals in each group were subjected to perfusion allowing electron microscopic analysis of the testes. At the time of sacrifice an impressive number of endpoints were analyzed: 10 hematological parameters, 21 blood chemical parameters, levels of testosterone, estradiol and T3/T4 as endocrine parameters, body weights and organ weights of pituitary, thyroids, liver, pancreas, spleen, kidneys, testes, prostate, seminal vesicles and epididymides. Histological analysis of all organs was performed and additional histochemical (3betaHSD) and ultrastructural analysis was performed in the testes. Testicular homogenates were used to determine counts of spermatids (heads per g testicular tissue) and 6 testicular enzymes were analyzed biochemically. The activities of nine hepatic enzymes were determined in liver homogenates.

The results of the study reveal no statistical changes in clinical parameters and body weights, hematology, blood chemistry, hormone levels and testicular and hepatic enzymes for the male monkey groups. In each DEHP treatment group and in the control group one monkey showed low testis and reproductive organ weights. The authors report that this usually correlated with low body weight and that these three monkeys were therefore considered to be immature. This implies that they consider each other monkey in the study to be fully adult at the time of sacrifice. However, for reasons discussed below, this may not be the case. When summing up the age at the start of the study and the duration of the study, the time point of sacrifice was around 20 months of age which corresponds to the late phase

marmosets go through puberty. It is characteristic for this species that the variability to sexually mature is extremely wide (12-20 months). Therefore, a subgroup of four (or 5, see below) late pubertal monkeys among 36 monkeys is not unusual at this age.

The apparent occurrence of different developmental stages could have been avoided by delaying the time point of sacrifice by several months. While the period of exposure was wisely selected and covered the full period of testis development after weaning, the period of sacrifice means that both adult and late pubertal monkeys were analyzed. The exclusion of some of the treated animals from sperm count analysis may be an indication that poor definition of developmental stages at the time of analysis may have influenced the outcome of the study and might have induced high variability in the data.

I have some questions in regard to the interpretation of testicular histology. Photographs 1 and 2 show the histological patterns of qualitatively and quantitatively normal testes of fully adult marmosets. If these testicular crossections are – as indicated - representative of the control and high dose DEHP groups, these micrographs clearly reveal that the exposure to DEHP had no impact on testis development and spermatogenic induction in marmosets. Photographs 3 and 6 depict a rather unusual pattern of testicular development for a late pubertal monkey and are potentially showing some testicular damage. The degree of vacuolization is quite high and the number of germ cells is rather low for the late stage of pubertal testicular development. It is therefore difficult to determine whether animals 10208 (Fig. 6) and 10204 (Fig. 3) are showing an immature or damaged testicular phenotype. Surprisingly an underdeveloped testis as shown for Mk 10208 has not been reported elsewhere in the study. It might be useful to send all testicular histological samples for a blinded re-analysis to a scientist who has abundant expertise in primate testicular development. Together with the results on testis weight and reproductive organ weights this might enable analysis of subtle effects and confirmation whether the proposed “growing” monkeys are indeed immature or whether their testes are damaged.

There was a significant loss of monkeys during the study. About 15% of the experimental animals died during the exposure period and were replaced. Marmosets are highly sensitive and show high mortality rates when exposed to poor hygiene conditions or poor diet. The main reason for this high death rate (also known as wasting syndrome) is intestinal infection. Marmosets have a short gut transit time, easily and frequently develop diarrhea, and eventually develop colitis leading to a rapid loss of body weight and death. The animal husbandry conditions in the study which exposed isolated single monkeys to a solid chow without supplementation of additional fruit or additional protein components were suboptimal to maintain the animals in good health. These monkeys live in social family groups and isolation might have been an additional stressor throughout the experiment. In addition, the effect of daily exposure to approximately 0.2-0.5 ml of corn oil must have had additional negative effects on their digestive tract. The amendments and deviations from the study protocol as outlined in the report (see 4.4.3: addition of milk powder to the diet and 5.4: discontinuation of oral exposure in 6.6% of applications) show that the animal care staff and veterinarians experienced serious problems in respect to the health and the nutritional status of the monkeys.

Given these factors, it is possible that the lack of statistical significance in this study might be due to high inter-animal variations in many of the analyzed parameters. Reasons for the high variation could be the different developmental status of the animals at the time of sacrifice, poor health of some of the monkeys, and/or a high individual variability known for marmosets.

4.2 Personal judgement of the value of the Mitsubishi study for risk assessment

Despite of the above described shortcomings this study presents strong evidence that DEHP had **no major** effect on testicular development even after very long and intense DEHP exposure. The study analyzed many different parameters of which hardly any showed a significant change. Unfortunately, the poor design of the study and suboptimal animal husbandry render this study not fully conclusive.

The oral dose toxicity study has generated a unique and wide set of data in a primate species which – as outlined above – has been used and should be considered in the future as a valuable model for the study of testicular development. Although the marmoset has an unusual androgen-CG feedback loop, the most striking effect of DEHP in other animal models is related to direct effects on Sertoli cells during testicular growth. In regard to this parameter the marmoset should be considered an excellent animal model. The analysis of the histological testicular parameters in the Mitsubishi study are therefore valid and impressively confirm a minor – if any – negative effect of DEHP exposure on Sertoli cell development and induction of spermatogenesis.

In my opinion the Mitsubishi study can therefore not be fully neglected and should be carefully and critically considered for evaluating the risk of gonadotoxic effects in humans after exposure to DEHP.

Yours sincerely,

(Stefan Schlatt, Ph.D.)

(5) References

Gromoll J, Eiholzer U, Nieschlag E, Simoni M. Male hypogonadism caused by homozygous deletion of exon 10 of the luteinizing hormone (LH) receptor: differential action of human chorionic gonadotropin and LH. J Clin Endocrinol Metab. 2000; 85:2281-6.

Gromoll J, Wistuba J, Terwort N, Godmann M, Muller T, Simoni M. A new subclass of the luteinizing hormone/chorionic gonadotropin receptor lacking exon 10 messenger RNA in the New World monkey (Platyrrhini) lineage. Biol Reprod. 2003; 69:75-80.

Li LH, Donald JM, Golub MS. Review on testicular development, structure, function, and regulation in common marmoset. Birth Defects Res B Dev Reprod Toxicol. 2005 74:450-69.

Millar MR, Sharpe RM, Weinbauer GF, Fraser HM, Saunders PT. Marmoset spermatogenesis: organizational similarities to the human. Int J Androl. 2000 23:266-77.

Schlatt S, Kim S, Gosden R. Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and grafting. Reproduction 2002; 124: 323-329.

Wistuba J, Mundry M, Luetjens CM, Schlatt S. Cografting of hamster (Phodopus sungorus) and marmoset (Callithrix jacchus) testicular tissues into nude mice does not overcome blockade of early spermatogenic differentiation in primate grafts. Biol Reprod. 2004; 71:2087-91.

Wistuba J, Luetjens CM, Wesselmann R, Nieschlag E, Simoni M, Schlatt S. Meiosis in Autologous Ectopic Transplants of Immature Testicular Tissue Grafted to Callithrix jacchus. Biol Reprod. 2005 Dec 21; [Epub ahead of print]