

Effects of two DDT metabolites and one synthetic DDE-analogue on testicular steroidogenesis in primary porcine Leydig cells *in vitro* during neonatal development

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Abstract

Although the production and use of the insecticide DDT have been restricted or banned since the 1970s, DDT and its metabolites are persistent in the environment and may still pose a hazard of toxic effects in wildlife and humans. One area, which has been given increasing attention in the past years, is the possible link between exposure to pesticides and disturbance of endocrine functions including reproductive functions. The present study investigated the direct effect of two DDT metabolites and one synthetic DDE-analogue on basal testicular steroidogenesis in primary neonatal porcine Leydig cells *in vitro*. The two DDT metabolites, 3-MeSO₂-DDE and *o,p'*-DDD, as well as the synthetic DDE-analogue, 3,3'-(bis)MeSO₂-DDE, do all have known endocrine effects as they exert toxic effects towards the adrenal cortex in humans and several species. Because of these properties, *o,p'*-DDD is in fact the main drug for adrenocortical carcinoma and Cushing's syndrome.

Neonatal Leydig cells were obtained from castrations, purified by a discontinuous Percoll gradient and the purity of Leydig cells was determined by staining for 3 β -hydroxysteroid dehydrogenase. The primary cultures of purified Leydig cells were then exposed to six different concentrations of each compound up to 20 μ M and solvent control (0.1% DMSO) for 48 hours. The aim was to assess the compounds' effect on cell viability, hormone production (testosterone, estradiol, progesterone and cortisol) and expression of 16 genes involved in testicular steroidogenesis. Only samples exposed to the next highest concentration (10 μ M) were used in the gene expression analysis. The expression in the 10 μ M samples was then compared against the solvent controls.

The results for cell viability showed that only *o,p'*-DDD was cytotoxic at the highest concentration. For hormone production, all three compounds stimulated testosterone secretion with most effect for 3-MeSO₂-DDE and *o,p'*-DDD exposure. 3,3'-(bis)MeSO₂-DDE caused only an increase at the highest concentration. Estradiol production was not much altered in comparison, but a stimulatory effect was seen for 3-MeSO₂-DDE and *o,p'*-DDD, and a decrease was seen with 3,3'-(bis)MeSO₂-DDE. The Leydig cells did not produce progesterone and cortisol. The results for gene expression showed that the three compounds were able to alter the effect of some genes. All genes altered were down-regulated with the exception of one gene and *o,p'*-DDD exerted most effect with down-regulation of eight genes in total. Since 3-MeSO₂-DDE and *o,p'*-DDD caused an increase in hormone secretion at 10 μ M, especially for testosterone, the reduction seen in mRNA levels does not explain the

mechanism behind the stimulatory effect. This might indicate that the compounds interact at the protein level. Taken together, the results suggest that the compounds are able to exert effect on basal testicular steroidogenesis and the possible endocrine effects on the male reproductive tract are thus concerning.

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Abbreviations

ACC	Adrenocortical carcinoma
AKR	Aldo-keto reductase
ANOVA	Analysis of variance
Ca ²⁺	Calcium ion
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CSTEE	Scientific Committee on Toxicity, Ecotoxicity and the Environment
CT	Cycle threshold
CYB5	Cytochrome b5
CYP51	Lanosterol 14 α -demethylase
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP17A1	Cytochrome P450 c17
CYP19	Aromatase
CYP11B1	11 β -hydroxylase
CYP21	21-hydroxylase
Dax-1	Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene-1
DDD	Dichlorodiphenyldichloroethane
<i>o,p'</i> -DDD	2-(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1-dichloroethane
DDE	Dichlorodiphenyldichloroethylene
3-MeSO ₂ -DDE	2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene
3,3'-(bis)MeSO ₂ -DDE	2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA

FCS	Foetal calf serum
FTL	Ferritin light chain
FTH	Ferritin heavy chain
HMGR	3-Hydroxy-3methylglutaryl-CoA reductase
IPCS	The International Programme on Chemical Safety
3 β -HSD	3 β -hydroxysteroid dehydrogenase
17 β -HSD	17 β -hydroxysteroid dehydrogenase
LH	Luteinizing hormone
ng	Nanogram
OECD	The Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
pg	Picogram
RIA	Radioimmunoassay
RNA	Ribonucleid acid
RPM	Revolutions Per Minute
RT	Reverse transcriptase
SF-1	Steroidogenic factor-1
StAR	Steroidogenic acute regulatory protein

1.0 Introduction and background

Today, there is a growing concern about xenobiotic compounds in the environment because of their reproductive and endocrine-disrupting effects for both human and wildlife. The European Commission set up a Working group on Endocrine Disrupters under the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) and the CSTEE working group agreed on the definition of the International Programme on Chemical Safety (IPCS) Steering Group that met at the joint IPCS/Organisation for Economic Co-operation and Development (OECD) Scoping Meeting on Endocrine Disrupters, March 16 to 18, 1998 in Washington, DC;

“An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (Vos et al., 2000).

Endocrine disruption has been identified as a potential global problem (Vos et al., 2000) and there is reported a need for greater awareness about the long-term health consequences associated with exposure to endocrine-disrupting chemicals during early life (Colborn et al., 1993). The pesticides are among one of the groups of chemicals which cause concern with regard to endocrine-disrupting effects (Colborn et al., 1993; Vos et al., 2000). In the past years, effect on the male reproductive system has attracted increasing attention. The reduced male fertility seen in the general population can be caused by many factors, but there exist some indications that pesticides affect sperm quality (Bretveld et al., 2007; Jurewicz et al., 2009).

This study investigated the effect of three compounds on the male reproductive system which all have unknown effect regarding testicular toxicity; two metabolites of the known environmental pollutant and pesticide dichlorodiphenyltrichloroethane (DDT), namely 2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-MeSO₂-DDE) and 2-(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1-dichloroethane (*o,p'*-DDD), and the synthetic compound 2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3'-(bis)MeSO₂-DDE) was also included. Specifically, these three compounds were evaluated for their effect on cell viability, hormone production and gene expression in primary cultures of porcine Leydig cells.

1.1 DDT and its environmental relevance

The organochlorine *p,p'*-DDT was used extensively as an agricultural insecticide in the 1940s-1960s. It was discovered that DDT and its metabolites posed a threat to wildlife and especially birds due to eggshell thinning (Blus et al., 1997; Faber and Hickey, 1973). As other organochlorine compounds, they also possess endocrine disrupting properties with *o,p'*-DDT being the most estrogenic isomer (Fry and Toone, 1981). However, the use of DDT was banned in Europe and many countries in the 1970s, and consequently, the levels have decreased in these areas (Chu et al., 2003; Noren et al., 1996; Weistrand and Noren, 1997). A restricted use of the insecticide is thus allowed in areas where malaria is a considerable problem (Cupul-Uicab et al., 2008; Longnecker, 2005). Despite a restricted use, there is evidence that DDT is transported via the atmosphere (Rapaport et al., 1985; Wang et al., 2010) and DDT can therefore reach remote regions where it never has been used. The previous and the present use of the insecticide are still relevant mainly because it is recognized as a very persistent chemical. The biodegradation half life in soil varies from 2 to 30 years, depending on the conditions (CDC, 2009; Dimond and Owen, 1996).

1.2 Metabolites of DDT

In nature, DDT is degraded to dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) and these stable metabolites are formed by dehydrochlorination or dechlorination of the ethane side chain of DDT (Jonsson et al., 1994). These hydrocarbons have relatively low toxicity, but their lipophilic nature facilitates their accumulation along food chains and the highest levels are therefore found in top-predators (Clarkson, 1995). *p,p'*-DDE, the primary product by degradation, is thus one of the most abundant persistent halogenated hydrocarbons present in human blood and milk worldwide (Smith, 1999). Due to its lipophilic property, animals will tend to excrete DDE by metabolizing it to a more hydrophilic substance; methyl sulphone-containing metabolites of DDE (MeSO₂-DDEs) were first discovered in blubber of Baltic grey seals showing symptoms of adrenocortical hyperplasia in 1976 (Jensen and Jansson, 1976). Hence, MeSO₂-DDEs do also possess hydrophobic properties and can thus be accumulated in the body of mammals and humans (Bergman et al., 1994; Jensen and Jansson, 1976; Lechter et al., 1995). Furthermore, the lipophilic character of both DDE and MeSO₂-DDEs make them capable of transfer from mother to offspring via milk (Azeredo et al., 2008; Jonsson, 1994; Jonsson et al., 1992; Kismul, 2009; Noren et al., 1996). Today, levels of DDE and MeSO₂-containing

metabolites of DDE are therefore still detectable in humans, especially in areas where DDT is still used (Bergonzi et al., 2009; Longnecker, 2005).

1.3 Compounds

The present study aimed to investigate the effect of the two DDT-metabolites 3-MeSO₂-DDE and *o,p'*-DDD on primary neonatal porcine Leydig cells *in vitro*. The effect of another DDE-analogue is also included; 3,3'-(bis)MeSO₂-DDE is made from 3-MeSO₂-DDE by adding an extra methyl-sulphonyl group to the molecule. The three compounds are presented in Figure 1.

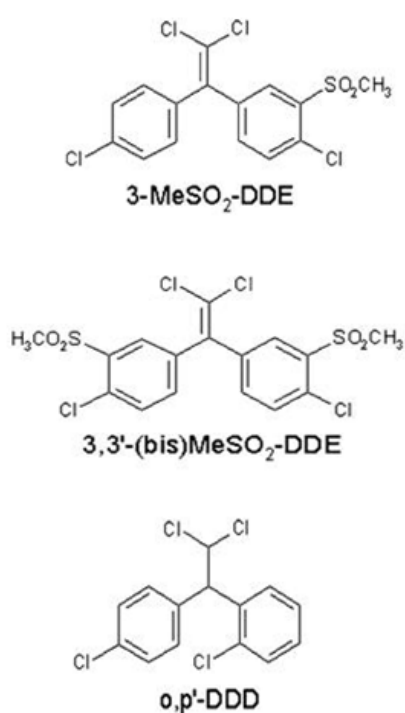


Figure 1. Chemical structures of the compounds used in this study

3-MeSO₂-DDE

In mammals, formation of methyl sulphone metabolites of DDE results from arene-epoxide conjugation with glutathione and subsequent metabolism via the mercapturic acid pathway (Bakke et al., 1982; Brandt et al., 1992; Preston et al., 1984). The toxicological potential of MeSO₂-containing metabolites has been demonstrated by the adrenocorticolytic toxicity induced by 3-MeSO₂-DDE in mice *in vivo/in vitro* after bioactivation by the mitochondrial enzyme 11 β -hydroxylase (CYP11B1) expressed specifically in the adrenal cortex (Jonsson, 1994; Jonsson et al., 1992; Jonsson et al., 1991; Lund et al., 1988; Lund and Lund, 1995). It is metabolized to a reactive and cytotoxic intermediate that binds covalently to the adrenal cortex. Bioactivation of CYP11B1 results in inhibition of the enzyme which gives a decrease in plasma corticosterone levels. Neither 2-MeSO₂-DDE nor *p,p'*-DDE have shown to give similar effect, indicating that the presence and position of the MeSO₂- moiety is crucial (Asp et al., 2009). Besides the toxicity shown for the mouse adrenal cortex, adrenal interrenal cells from chicken (Brandt et al., 1992; Jonsson et al., 1994), human adrenal tissue slices (Lindhe et al., 2002) and the human adrenocortical H295R cell line (Johansson et al., 2002) are also sensitive. However, there is no conclusive evidence that 3-MeSO₂-DDE is toxic in the human adrenal *in vivo*, but the findings indicate that 3-MeSO₂-DDE may be a highly toxic endocrine disrupter in humans and mammals.

3,3'-(bis)MeSO₂-DDE

The synthetic DDE-analogue, 3,3'-(bis)MeSO₂-DDE, also inhibits steroidogenesis and thus decreases corticosterone production in the mouse adrenocortical cell line Y-1 (Asp et al., 2009). As 3-MeSO₂-DDE, the compound produces CYP11B1-dependent cytotoxicity in Y-1 cells, but it is a less potent inhibitor of steroidogenesis and less toxic than 3-MeSO₂-DDE and *o,p'*-DDD (Asp et al., 2009; Lund et al., 1988).

***o,p'*-DDD**

The DDD isomer, *o,p'*-DDD (sold under the name mitotane, lysodren), is a tissue-selective toxicant after being metabolic activated locally in the adrenal cortex and it was shown to exert toxicity to the adrenal cortex in dogs as early in 1949 (Nelson and Woodard, 1949). Toxicity to mink adrenal cortex has also been demonstrated (Jonsson et al., 1993). *o,p'*-DDD is also toxic to humans; it is the main drug for adrenocortical carcinoma (ACC) and Cushing's syndrome (overproduction of glucocorticoids due to a pituitary tumor) due to drug-induced

cell death in the adrenal cortex after a CYP-catalyzed reaction to a reactive acyl chloride which binds covalently to the adrenal cortex (Cai et al., 1995; Martz and Straw, 1980). The compound blocks cortisol synthesis by inhibiting cholesterol side-chain cleavage enzyme (CYP11A1) and CYP11B1 (Martz and Straw, 1980). In contrast to 3-MeSO₂-DDE, mouse adrenal cortex is not sensitive to *o,p'*-DDD (Lund et al., 1988), but the compound is also toxic to adrenal interrenal cells (Brandt et al., 1992; Jonsson et al., 1994). Evaluations of the medical treatment with *o,p'*-DDD show that the compound gives several severe side effects, such as gastrointestinal irritation and central nervous system (CNS) toxicity (Ahlman et al., 2001). There is also a low clinical response with only 35% of patients responding to treatment (Wooten and King, 1993). As ACC is a rare disease, little research has been focused on developing new therapeutic alternatives, but 3-MeSO₂-DDE has been proposed as a possible alternative for the treatment (Lindhe et al., 2002).

1.4 Porcine Leydig cells as a model for testicular steroidogenesis

Testis tissue is organized into two compartments: the tubular compartment and the interstitium. The Leydig cells, discovered by Leydig in 1850, are confined to the interstitial tissue together with macrophages, fibroblasts and blood vessels and the seminiferous tubules are formed by the Sertoli cells (Gnessi et al., 1997). Figure 2 presents an anatomical arrangement of the testis.

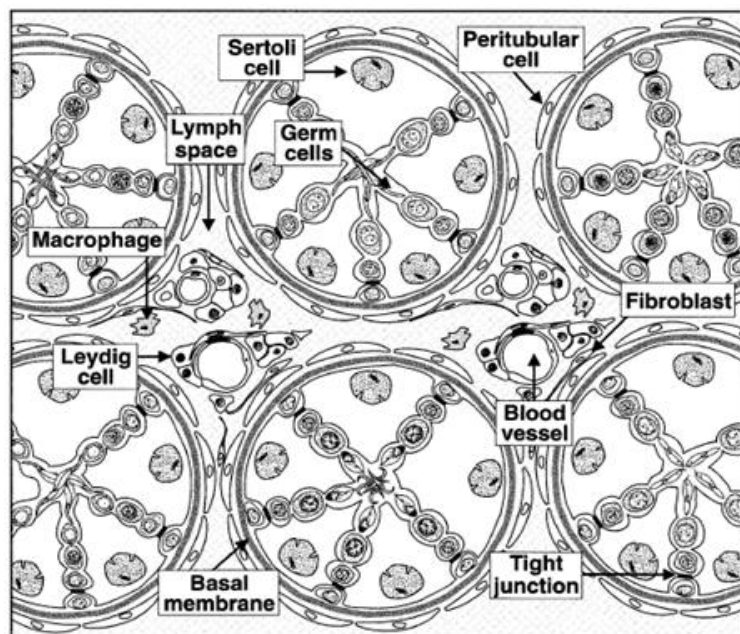


Figure 2. Representation of the anatomical arrangement of an adult testis (rat) (Gnessi et al., 1997).

The boar testis is recognized by a highly developed interstitial tissue and only the stallion shows a comparable abundance of Leydig cells in the interstitium, suggesting a high capacity for steroid secretion (Fawcett et al., 1973; Raeside et al., 2006). In mammals, Leydig cell development is characterized by two well-defined periods of proliferation and differentiation with the first occurring in the fetal life and the second occurring during prepubertal development (Lejeune et al., 1998a). The boar does also have these two waves, but the pig testicle is unique because of an additional transient wave occurring between 2½ weeks before until 2½ weeks after birth characterized by a large volume of well differentiated cells (Van Straaten and Wensing, 1978). A similar series occurs also in the development of the human and the primate testis (Griswold and Behringer, 2009). In addition, neonatal porcine testes are regarded as a superior system for the study of testicular steroidogenesis: the cell cultures contain a high volume of Leydig cells and they also retain their specific functions in culture for relatively long periods (Mather et al., 1981).

The porcine Leydig cells produce a remarkable list of steroids (Raeside et al., 2006). The testosterone secretion is an essential requirement for spermatogenesis (Ge et al., 2008), for development of the Wolffian duct system during embryonic growth (Ostrer, 2000), the maintenance of accessory sex glands (Thompson et al., 1980) and also for sexual behavior (Cohen-Bendahan et al., 2005). Hence, the male reproductive system is dependent on the secretion of testosterone. In mammals, the synthesis of testosterone proceed either via $\Delta 5$ -metabolites or via $\Delta 4$ -metabolites (Conley and Bird, 1997). $\Delta 5$ -metabolites predominates in the testis of humans and pigs (Ruukonen and Vihko, 1974a; Ruukonen and Vihko, 1974b), thus making pig Leydig cells a good model for studying testicular steroidogenesis.

In addition, the testes do also produce estrogens. Estrogens are essential for normal testicular development where it is involved in regulation of luminal fluid and ion transport (Hess and Carnes, 2004) and the synthesis in boars is much higher than in males of other species (Claus and Hoffmann, 1980; Velle, 1966). Like the adult Leydig cells, neonatal Leydig cells do also produce testosterone and estrogen and a peak in plasma levels occur 2-4 weeks after birth (Colenbrander et al., 1977; Raeside et al., 2006; Schwarzenberger et al., 1993). The luteinizing hormone (LH) is the primary hormone controlling Leydig cell activity and the Leydig cells contain specific LH receptors (Lejeune et al., 1998b; Mather et al., 1982). Binding of the hormone to the receptor causes an increase in cyclic adenosine monophosphate (cAMP) levels followed by secretion of androgens and estrogens.

1.4.1 Testicular steroidogenesis

There are many important aspects regarding testicular steroidogenesis. Gene expression for the cytochrome P450 hydroxylases involved is regulated at the transcriptional level by steroidogenic factor-1 (SF-1; coded by NR5A1) which mediates transcriptional activation in response to cAMP stimulation (Mendelson et al., 2005; Parker et al., 2002; Sandhoff et al., 1998; Sugawara et al., 1996). Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene-1 (Dax-1; coded by NR0B1), another transcription factor whose expression is mostly restricted to steroidogenic tissues (Ikeda et al., 1996), represses SF-1-mediated transactivation of StAR-gene, thus blocking steroidogenesis (Zazopoulos et al., 1997). The presence of ferritin in the cell is also important for steroidogenesis since this protein stores and releases iron in cells and therefore plays a central role in many essential cellular functions (Hentze and Kuhn, 1996). It is composed of two subunits, the ferritin light chain (FTL) and ferritin heavy chain (FTH) (Sammarco et al., 2008).

The production of testosterone starts from cholesterol and involves a number of enzymatically catalyzed steps (Figure 3). There is an extensive documentation confined to the expression and presence of the steroidogenic enzymes in the pig testis (Clark et al., 1996; Conley et al., 1996; Hall, 1991; Inano et al., 1981; Moran et al., 2002; Sasano et al., 1989; Suzuki et al., 1992). Many enzymes are involved in the biosynthesis of cholesterol like lanosterol 14 α -demethylase (CYP51) (Debeljak et al., 2003), but the rate-limiting enzyme is 3-Hydroxy-3methylglutaryl-CoA reductase (HMGR) which catalyzes the conversion of HMG-CoA to mevalonate (Rodwell et al., 1976). The first rate-limiting step from cholesterol to testosterone is the transfer of cholesterol from the outer mitochondria membrane to the inner mitochondrial membrane, a process mediated by the action of steroidogenic acute regulatory protein (StAR) (Stocco, 2001). Further, CYP11A1 converts cholesterol to pregnenolone (Miller, 1995). From here, there are two alternative metabolic routes of pregnenolone utilization to testosterone; either to 17 α -hydroxypregnenolone and dehydroepiandrosterone (DHEA) (Δ 5-pathway) or to 17 α -hydroxyprogesterone and androstenedione (Δ 4-pathway) (Conley and Bird, 1997). One enzyme having two catalytic activities catalyzes the steps in the Δ 4- and Δ 5-pathway; cytochrome P450 c17 (CYP17A1) has both 17 α -hydroxylase and 17,20-lyase activity (Hall, 1991; Nakajin and Hall, 1981). It has been demonstrated that cytochrome b5 (CYB5) is involved with porcine CYP17A1 in the andien- β synthase system (Nakajin et al., 1985). Andien- β synthase activity thus decreases the production of 17 α -

hydroxypregnenolone, but it has been shown that CYP5 also increases the 17,20-lyase activity of CYP17A1 (Katagiri et al., 1982; Nakajin et al., 1985) which will lead to increased DHEA levels. Further in the steroidogenesis, the action of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (Conley and Bird, 1997) is needed to convert DHEA to androstenedione which is then synthesized to testosterone by the action of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (Inano et al., 1981). Estrogens are synthesized from the aromatization of androgens by cytochrome P450 aromatase (CYP19A1) (Conley et al., 1996). In addition, isoforms of 17 β -HSD perform reduction of estrone to estradiol and likewise the reverse reaction (Adamski et al., 1992; Luu-The, 2001). To prevent excess circulation of steroid hormones, active androgens and estrogens are converted to inactive metabolites by the aldo-keto reductases (AKR1C1-AKR1C4), making the steroids substrates for conjugation reactions (Penning et al., 2000).

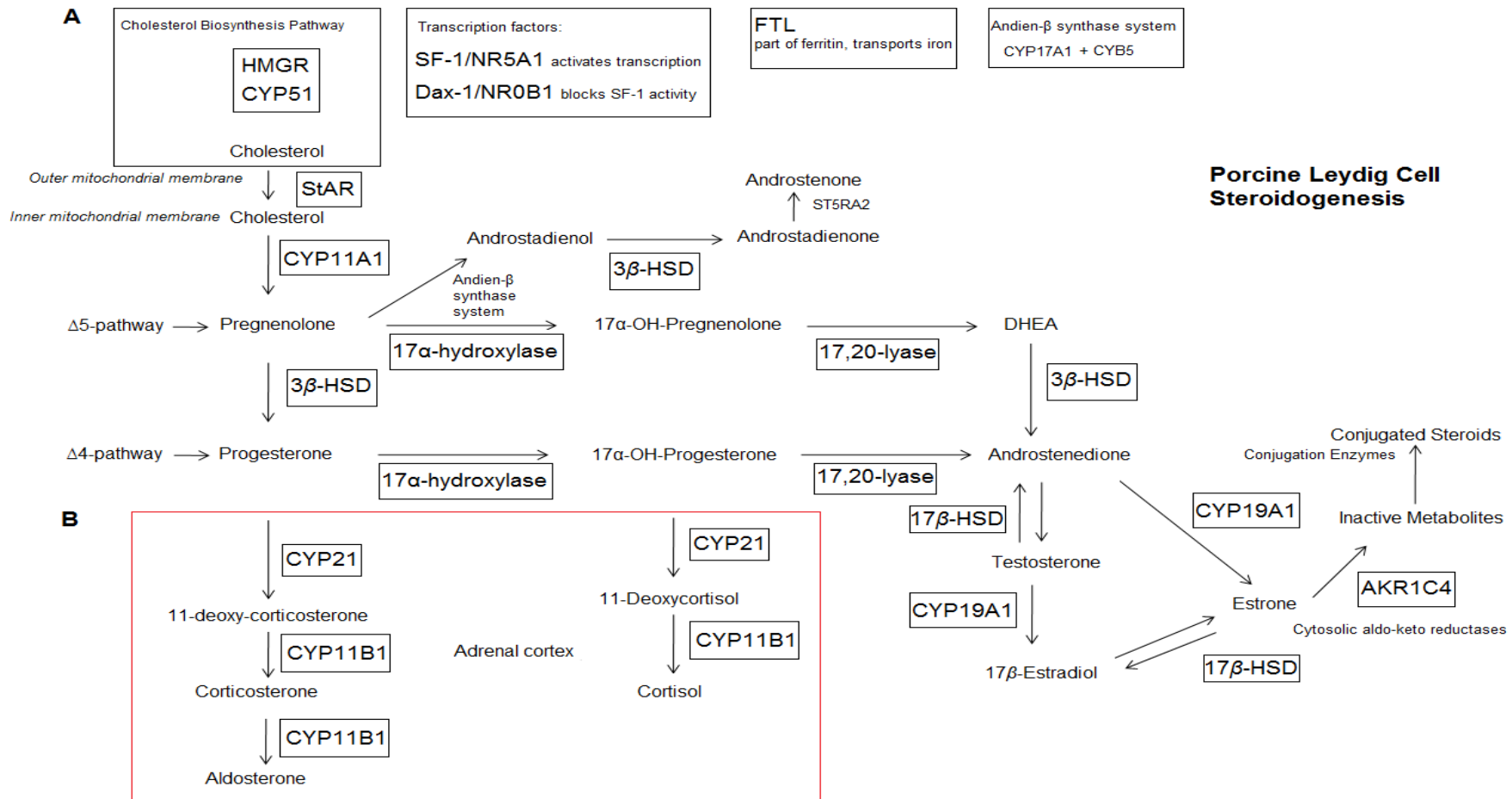


Figure 3. Testicular steroid biosynthesis (A) in porcine Leydig cells. A schematic illustration of the potential pathways from cholesterol to production of testosterone is presented. Part of the adrenocortical steroidogenesis (B) is included to show the potential pathway towards corticosteroids.

1.5 Steroid metabolism in testis tissue and adrenal glands

The three compounds used in this study do all exert toxic properties towards the adrenal cortex. Leydig cells and adrenal cells have tissue-restricted expression of cytochrome-P450 enzymes which accounts for production of distinct steroids in the two tissues (Val et al., 2006). Both are regulated by pituitary hormones and share a common pathway from cholesterol to progesterone (Hu et al., 2007). One of the differences between the cell types is expression of 21-hydroxylase (CYP21) and CYP11B1 in the adrenal cortex which allows secretion of corticosteroids while Leydig cells secrete androgens and estrogens by the presence of CYP17, 17 β -HSD (Hu et al., 2007) and CYP19 (Conley et al., 1996). However, in many higher mammals, some regions in the adrenal cortex like zona fasciculata and zona reticularis also expresses CYP17 and the adrenals and gonads are thus capable of synthesizing similar steroid intermediates (Conley and Bird, 1997).

Expression studies also indicate that Leydig and adrenal cells are derived from a common primordium that divides into separate tissues during embryogenesis (Hatano et al., 1996). Data from real-time polymerase chain reaction (PCR) studies have detected expression of *CYP11B1* in fetal and neonatal testis and *CYP21* in fetal and adult testis of mouse (Hu et al., 2007). Expression of both have been detected in fetal testis of humans (Pezzi et al., 2003). Activity of CYP21 protein has also been detected in fetal and neonatal mouse testis (Hu et al., 2007) and expression of CYP11B1 has been found in adult rat Leydig cells (Wang et al., 2002). One study has performed a similar expression study on porcine testis where expression of *CYP21* was detected (Grindflek et al., 2010). It is discussed, however, if the expression of the two enzymes derives from Leydig cells or from a small population of adrenal-like cells in the interstitium in the developing testis (Hu et al., 2007; Val et al., 2006). Nonetheless, these findings support the link between adrenal cells and Leydig cells.

1.6 Aims of study

Because of the extensive use of the insecticide DDT in the past and the persistency of the mother compound and its metabolites, these halogenated hydrocarbons are still ubiquitous in the environment. Since pesticides have drawn increasing attention towards the reduced male fertility seen in the past years, this study aimed at evaluating the effect of two DDT metabolites (3-MeSO₂-DDE and *o,p'*-DDD) and one synthetic DDE-analogue (3,3'-(bis)MeSO₂-DDE) *in vitro* on neonatal Leydig cells from porcine testicles. The endocrine disrupting effects of the two DDT-metabolites 3-MeSO₂-DDE and *o,p'*-DDD towards humans and animals are well known. Both compounds interact with steroidogenic enzymes which make them toxic to the adrenal cortex and also able to inhibit steroidogenesis, albeit with species differences. Further, *o,p'*-DDD is currently the main drug for adrenocortical carcinoma (ACC) and Cushing's syndrome, but treatment is combined with severe side effects and a low response rate. Due to the known effects 3-MeSO₂-DDE and *o,p'*-DDD pose on adrenal cells and the fact that adrenal and Leydig cells are both steroid producing cells, we wanted to evaluate the effects of these compounds on testicular steroidogenesis *in vitro*. Part of the study was also to contribute to new information about 3-MeSO₂-DDE effect in other areas since this compound has been proposed as an alternative drug for *o,p'*-DDD. The synthetic and structurally related DDE-analogue, 3,3'-(bis)MeSO₂-DDE, was also included in the study to see if an extra methyl sulphonyl group could give a different response. To our knowledge, this is the first time the effect of these three compounds has been evaluated on porcine Leydig cells.

The overall objective in this thesis was to clarify the effect of 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD on the mammalian male reproductive system by evaluating the compounds' effect on testicular steroidogenesis.

To achieve information about the three compounds' effect on neonatal porcine Leydig cells, three experiments were performed in order to;

- Quantify the compounds' effect on cell viability
- Quantify the effects on the production of the following hormones; testosterone, estradiol, progesterone and cortisol.
- Quantify the effects on gene expression by using 16 genes involved in testicular steroidogenesis

2.0 Materials and Methods

For a detailed list about materials, chemicals and preparation of media and solutions see appendix 7.0.

2.1 Chemicals

2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-MeSO₂-DDE) and 2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3'-(bis)MeSO₂-DDE) (purity > 99%) were synthesized by Synthelec AB, Ideon (Lund, Sweden). 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane (*o,p'*-DDD) (purity > 99%) were obtained from Sigma-Aldrich (Stockholm, Sweden).

2.2 Preparation of Cell culture

2.2.1 Collection of Porcine Testicular Tissue

Testis tissue was obtained from male offspring (*Suidae, Sus scrofa*) in approximately 8-12 days old litters from Norwegian Landrace breeding units. Testicles obtained at each collection ranged from 50-90 testicles. The castration was performed by veterinarians from Norwegian School of Veterinary Science (NVH) same morning as the isolation of the Leydig cells took place. Local anaesthesia, 1% Lidocain without adrenalin was given on both sides subcutaneous in the scrotum and in the inguinal string. The skin was washed and then disinfected with 70% ethanol. Standard surgical procedure for castration of male piglets was followed on both sides. Testicles were then immediately placed on ice in a bottle of collection medium (see appendix 7.3 for details on media composition) and transported to NVH. After intervention, 6 mg ketoprofen/kg bodyweight was injected intra muscular as a single treatment. The procedure was always completed within three hours.

2.2.2 Isolation and Purification of neonatal Porcine Leydig cells

The methods for isolation, subsequent purification and culture of neonatal porcine Leydig cells were adapted from the protocols described by (Bernier et al., 1983; Lejeune et al., 1998b).

All work was performed in a laminar flow hood in a sterile fashion and all glass ware and tools needed were autoclaved beforehand (see appendix 7.2 for details on autoclaved materials). About 12 testicles were collected in a petri dish (VWR, International AS, Oslo,

Norway) containing collection medium. The epididymus and skin of each testicle was peeled off with tweezers and scissors, and then decapsulated by cutting the testicles almost in half with a razor blade (sterile). The parenchyma was scraped off from the skin holding the testicle together and then finely minced with scissors. Collection medium was added and the minced tissue was collected into 50 ml tubes (BD Falcon via VWR). The pellet was left for sedimentation before it was washed several times with collection medium in order to wash out the blood. When a clear supernatant was obtained, about 30 testicles were divided into 50 ml tubes. Further, dissociation medium (refer to appendix 7.3 for details on composition) of 90 ml was put in 500 ml bottle. The bottle was preheated in a waterbath to 34°C (1 bottle per 30 testicles is needed). To these bottles, testis tissue was added and the solution was put on 34°C bath with agitation. During the agitation time the extracellular matrix in the tissue was broken down and cells were freed from the tissue.

After 45 minutes of agitation we harvested collection 1. 50 ml of the supernatant from each bottle was pipetted over a metal filter into a clean beaker. The decanted volume was replaced with new collection medium and put back for further incubation for 45 minutes. The cell suspension was poured into 50 ml tubes and centrifuged for 10 minutes at 1080 Revolutions Per Minute (RPM). The supernatant was discarded, the pellet resuspended and up to 50 ml of collection medium was added. The suspension was left for sedimentation for 5 minutes. Then the supernatant was transferred with a pipette to a new 50 ml tube and left for 15 minutes of sedimentation. After sedimentation the supernatant was transferred to a new tube 50 ml tube and centrifuged for 10 minutes at 1080 RPM. The supernatant was discarded and the pellet resuspended with a little collection medium. Pellet from each 50 ml tube was collected into one 50 ml tube. All tubes were washed with a little media before collecting the pellet together. Collection 1 is now finished and kept in the fridge (4°C).

After the start of the agitation we harvested the solution 3 times in total. We repeated the same procedure each time. Collection 2 is to be harvested after 90 minutes of agitation and collection 3 after 120 minutes. When we harvested collection 3, we poured everything of the testis suspension mix into the metal filter. When finished with collection 3, collections 1, 2 and 3 were pooled together. 5 ml of the pooled cell suspension was then divided on top of the Percoll gradients (Figure 4). The Percoll gradients were made the same morning as the isolation (see appendix 7.3 for details on Percoll gradient composition and layering). The suspension was applied slowly. The break was set on 1 and the gradients were centrifuged for

30 minutes (4°C) at 2140 RPM. After the centrifugation the top layers (21% fraction and 26% fraction) were aspirated with a pipette till about the 12, 5 ml mark of a 50 ml tube. Then we recovered the Leydig cells from the 34% fraction (around 10-7 ml mark).

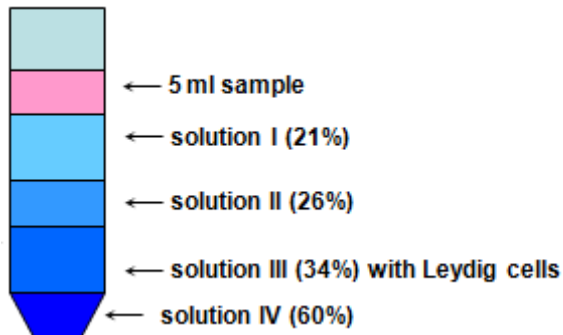


Figure 4. Discontinuous Percoll density gradient. 5 ml of cell suspension is added and after centrifugation the Leydig cells can be obtained from the 34% fraction.

The 34% fraction was transferred to a new 50 ml tube, diluted with collection medium and centrifuged for 20 minutes at 1080 RPM. The supernatant was discarded and resuspended in 20 ml collection medium for about 60 testicles. The cells were filtered through a cell strainer, 70 μ m, white (BD Bioscience via VWR, International AS, Oslo, Norway) into another 50 ml tube and then counted in a Bürker Hematocytometer chamber (Superior, Marienfeld, Germany)

2.2.3 Plating of cells

The cells were plated out in sterile 24-well primaria plates (for hormone readouts and preparation of RNA) (BD Falcon via VWR, International AS, Oslo, Norway) and 96-well MicroWell-plates (for cell viability test) (VWR, International AS, Oslo, Norway). The total number of cells needed per 24-well plate is: $150\,000\text{ cells/cm}^2 \times 2\text{ cm}^2 \times 24 = 7.2 \times 10^6$ cells/plate and per 96-well plate: $150\,000\text{ cells/cm}^2 \times 0.32\text{ cm}^2 \times 96 = 46.08 \times 10^5$ cells/plate. Before plating out the cells, we diluted the cells with complete plating medium (see appendix 7.3 for details on media composition) to 300 000 cells/ml. 1 ml of this suspension was then added to each well in a 24 plate and 100 μ l in a 96 plate. The plates were incubated for 72 hours in a humidified incubator at 34°C and 5% CO₂ to form a monolayer.

2.2.4 3 β -hydroxysteroid dehydrogenase staining for assessment of cell identity

For assessment of cell identity, one round with cytochemical staining for 3 β -HSD was performed on two separate Leydig cell isolations. The Leydig cells turn blue due to reduction of tetrazolium blue dye by the enzyme and this technique is considered specific for Leydig cells in the testis (Huang et al., 2001; Levy et al., 1959). Isolation of Leydig cells were performed as previously described. Cells were plated out in multiwell primary 6-well plates (BD Falcon via VWR, International AS, Oslo, Norway) and left in the incubator for 72 hours at 37°C and 5% CO₂. Medium was removed and cells were washed with phosphate buffered saline (PBS) 0.15 M, pH 7.4. Then 500 μ l trypsin was added for two minutes in order to detach cells from the wells. The trypsin was removed and then 5 ml Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM HEPES, pH 7.4 (DMEM/F12) containing 10% foetal calf serum (FCS) was added to inactivate trypsin. The wells were washed with this medium and then medium with cells was transferred to a tube and centrifuged at 1500 RPM for five minutes. Medium was removed and the cells (300 000 cells/ml) were resuspended and incubated with 2 ml of a solution containing 0.2 mg/ml nitro blue tetrazolium, 0.12 mg/ml 5-androstane-3 β -ol-one and 1 mg/ml NAD⁺ in 0.05 M PBS, pH 7.4 at 37°C in a waterbath for 90 minutes (Huang et al., 2001). Upon development of the blue formazan deposit sites of 3 β -HSD activity, the abundance of Leydig cells was determined with a hemocytometer.

2.3 In vitro exposure of cells

2.3.1 Exposure with 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and o,p-DDD

The test compounds were diluted to 20 mM/ml in dimethyl sulfoxide (DMSO), divided into 50 μ l aliquots and stored at -20°C until use. Before the exposure, the incubation medium was replaced with fresh complete plating medium of 1 ml per well in a 24 plate and 100 μ l per well in a 96 plate. The stock solutions were diluted in DMSO and complete plating medium to yield the final exposure concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 μ M for each compound. The final exposure concentrations were decided due to a study done by (Asp et al., 2009). Control cells were exposed to 0.1% DMSO. Three triplicates for each concentration were used, with three wells left blank with medium only (Figure 5). The cells were incubated at 34°C (5% CO₂) for 48 hours. At the end of the incubation period, the medium from the 24-well plates was collected (for hormone readouts) and stored at -75°C until use. The plates

were then wrapped in parafilm and quickly stored at -75°C to avoid degradation of ribonucleic acid (RNA).

Medium Blank	Medium Blank	Medium Blank	Solvent Control 0.1% DMSO	Solvent Control 0.1% DMSO	Solvent Control 0.1% DMSO
20 μM	10 μM	5 μM	2.5 μM	1.25 μM	0.625 μM
20 μM	10 μM	5 μM	2.5 μM	1.25 μM	0.625 μM
20 μM	10 μM	5 μM	2.5 μM	1.25 μM	0.625 μM

Figure 5: Experimental design of plate lay-out for 3-MeSO₂-DDE, o,p'-DDD and 3,3'-(bis)MeSO₂-DDE.

2.4 Cell viability test with AlamarBlue

After the exposure, assessment of cell viability in the individual wells of the 96-well plates was performed by using AlamarBlue, a redox indicator. In each well the incubation medium was replaced with 100 μl complete plating medium containing 10% AlamarBlue. The plates were let to incubate in 34°C (5% CO_2) in a humidified atmosphere for three hours. Resazurin is the non-fluorescent compound that gives the blue color. Living cells will take this up in the mitochondria and reduce this to resorufin which produces red fluorescence. After incubation 100 μl aliquote from each well was collected into a 96-well transparent well plate (Falcon, Franklin Lakes, NJ). The samples were measured in a Victor³ 1420 Multilabel Counter Spectrophotometer (Perkin Elmer, Shelton, CT, USA) by absorption at 570 and 600 nm wavelength. All 24-well plates were also treated with 10% AlamarBlue for one hour (Due to short incubation time, the readings from 24-well plates were not used)

2.5 Hormone analysis

Frozen medium was thawed and hormones in culture medium were measured by a solid phase radioimmunoassay (RIA) kit. The kit was modified by replacing the standard curve in serum with standards prepared in complete plating medium. The assay under the new conditions was then validated for each hormone and all samples were measured in duplicates. For measurement of estradiol, testosterone and cortisol, Coat-a-CountR kits (Diagnostic Products Corporation, Los Angeles, CA, USA), were used. For estradiol, the standard curve range was 0-4000 pg/ml and the sensitivity of the assay was 9 pg/ml corresponding to 95% binding of the labeled hormone. The interassay variation coefficients (low and high) for estradiol were 6.7% (650.8 pg/ml) and 10.6% (1744.5 pg/ml), respectively. The sensitivity of the

testosterone assay was 0.1 ng/ml, the standard curve ranged from 0-20 ng/ml and the interassay coefficients were 11.7% (2.4 ng/ml) and 14.1% (9.5 ng/ml), respectively. For cortisol, the sensitivity of the assay was 3 ng/ml, the standard ranged from 0-500 ng/ml and the variation coefficients were 9.8% (57.7 ng/ml) and (210.2 ng/ml), respectively. For measurement of progesterone, solid phase radioimmunoassay kit (Spectria, Orion Diagnostica, Espoo, Finland) was used. The sensitivity of the assay was 0.08 ng/ml, standard curve ranged from 0-40 ng/ml and the intervariation coefficients were 5.4% (0.3 ng/ml) and 24.6 (1.5 ng/ml). Hormone levels were measured in Wallac 1470 Wizard gamma-counter (Perkin Elmer, Shelton, CT, USA)

2.6 Gene expression analysis

2.6.1 RNA isolation

The cells were kept in -75°C until isolation of RNA. Total RNA from all 24-well plates was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Crawley, UK) and manufacturer's protocol was followed. The plates were brought in to the lab on ice and 200 µl RLT buffer (lysis buffer) was added to each well. 1000 µl tips were used to scrape off cells and transfer cell lysate to a QIAshredder spin column (Qiagen) in a 2 ml collection tube. Three replicate wells for each sample were pooled into one spin column. (Pooling of three replicate wells was necessary in order to get enough RNA for gene expression analysis). After spinning for 2 minutes at 13000 RPM, 600 µl 70% ethanol was added and mixed well with the homogenized lysate (flow through). Lysate was then transferred to an RNeasy spin column in a 2 ml collection tube, and spun for 15 seconds (1300 RPM). RNA was washed with 350 µl RW1 buffer and centrifuged for 15 seconds (13000 RPM). Prior to further purification, each RNA sample was treated with 80 µl DNase I mixture (10 µl DNase I and 70 µl RDD buffer; Qiagen) to remove deoxyribonucleic acid (DNA) contamination. The DNase I mixture was washed off with 350 µl of RW1 buffer and centrifuged for 15 seconds (13000 RPM). RPE buffer of 500 µl was added with spinning of 15 seconds, this was repeated once more with centrifugation for 2 minutes (13000 RPM). Collection tubes were removed and RNA was eluted with 55 µl RNase-free H₂O (Qiagen) in 1.5 ml tubes. After spinning for 1 minute (13000 RPM), samples were set on ice and 5 µl from each tube was transferred to new tubes which were used for quantitative and qualitative check of the RNA. Samples were stored at -75°C until required.

2.6.2 RNA quantity and quality

The quantity of RNA was determined with a NanoDrop ND-1000-Spectrophotometer (NanoDrop Technologies, Wilmington, DE) where 1 μl of purified RNA sample was added to the instrument. Mean concentrations of purified RNA isolated from neonatal porcine Leydig cells ranged between 94 – 241 ng/ μl for solvent control samples and 52 – 198 ng/ μl for 10 μM samples. The quality was examined with Agilent Bioanalyzer (Agilent Technologies, CA, USA) using the Agilent RNA 6000 Nano LabChip Kit. The bioanalyzer indicated that all RNA samples were of high quality. The samples had all satisfactory ratios of ribosomal RNAs (28S/18S) and RNA Integrity Numbers (RIN) values, with mean of 1.7 and 8.8, respectively. An electropherogram with RNA of high quality from an *o,p'*-DDD exposure is presented in Figure 6.

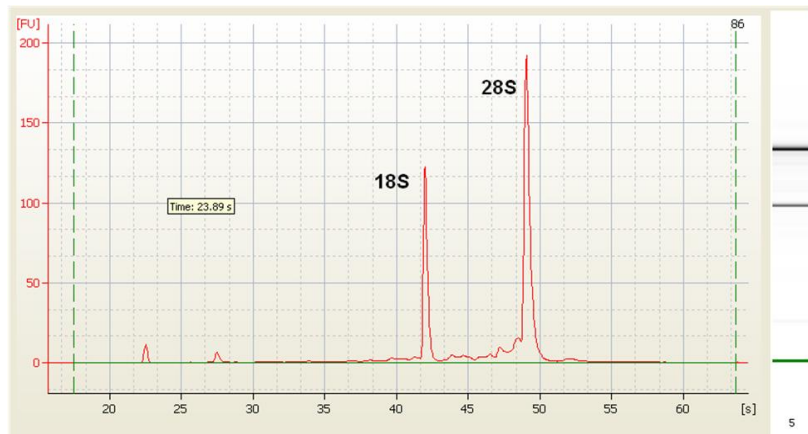


Figure 6. An electropherogram of a RNA sample from Leydig cells exposed to 1.25 μM *o,p'*-DDD by using Agilent 2100 Bioanalyzer. The x axis shows the integrity time (seconds) and y axis represents the fluorescence. Ribosomal RNA peaks of 28S and 18S are indicated in the figure. A gel- image of the RNA products is shown to the right.

2.6.3 Reference genes

In order to have accurate gene expression measurements, it is important to normalize results from the Real-Time Reverse Transcriptase-PCR (Real-Time RT-PCR) experiments to a reference gene that is not affected by the experimental conditions. Six housekeeping genes (*PGK1*, *HPRT*, *S18*, *GAPDH*, *ACTB* and *PPIA*) were analyzed using the geNorm-software (PrimerDesign Ltd, Southampton, UK) in order to predict the most stable reference genes. All genes tested had an M-value of 0.9 or less. The two most stable genes, *ACTB* (cytoskeletal beta actin) and *PPIA* (cyclophilin A), had M-values of 0.27 and were selected as reference genes in this study (Figure 7).

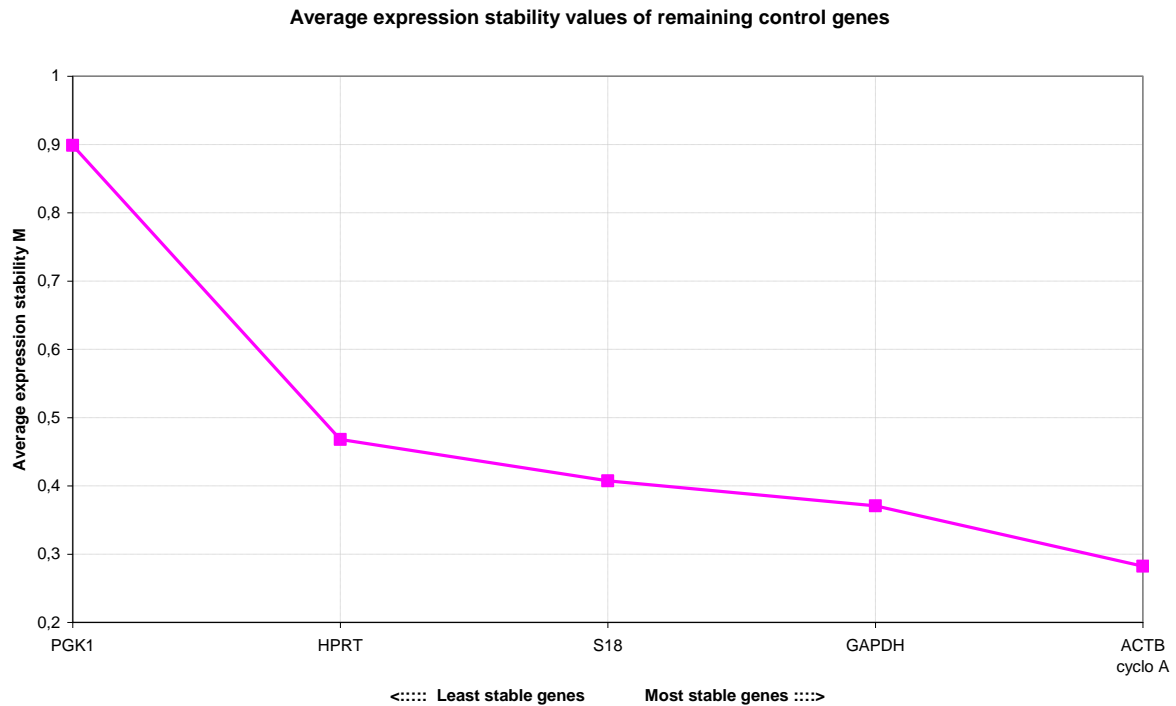


Figure 7. Average expression stability values of six housekeeping genes. The most stable reference genes are centered to the right. *ACTB* and *PPIA* (cyclo A) were most stable and chosen as reference genes.

The expression of *ACTB* and *PPIA* were also reasonable stable, which indicates that exposure with 10 μM of either compounds did not have an effect on expression of the two housekeeping genes (Figure 8).

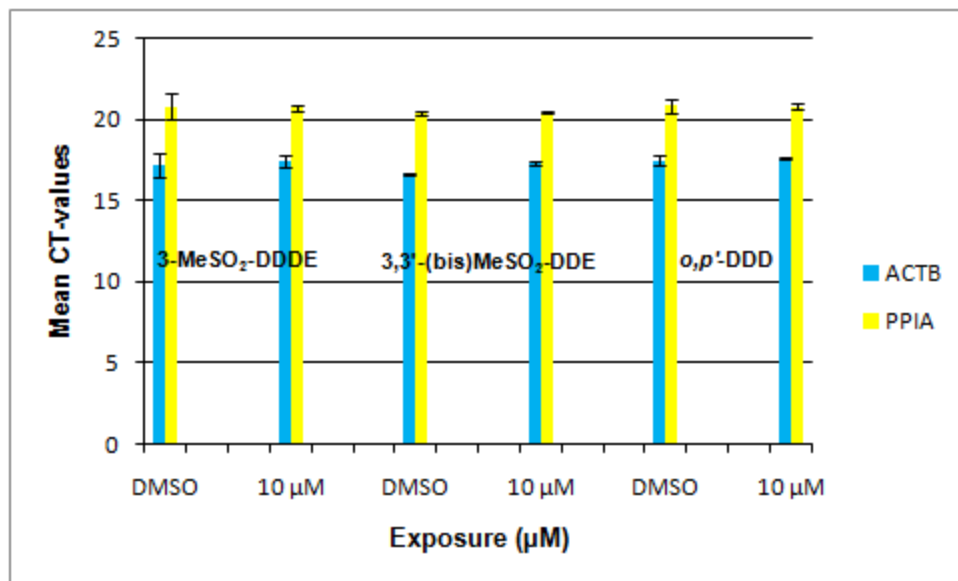


Figure 8. Variation in cycle threshold value (CT-value) for housekeeping genes *ACTB* and *PPIA* in DMSO and 10 μM samples for the three compounds. Mean CT-value \pm SE is shown (n=3).

2.6.4 Primer design

In total, 16 genes involved in testicular steroidogenesis were analyzed. Gene names and primer sequences are listed in Table 1. Primer sequences for *HMGR*, *CYP51*, *StAR*, *CYP11A1*, *CYP17A1*, *HSD3B*, *CYP19A1*, *HSD17B1*, *HSD17B4*, *CYP21*, *CYP11B1*, *CYB5*, *FTL*, *AKR1C4*, *NR5A1*, *NR0B1*, *PGK1* and *S18*-primers were designed using Primer express 1.5 (Applied Biosystems, Foster City, CA, USA) and obtained from Sigma-Aldrich. Specificities of all primers were checked using nucleotide BLAST and primer BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). *HPRT*, *GAPDH*, *ACTB*, and *PPIA* were derived from (Duvigneau et al., 2005). All primer pairs used for the present study were already in house before the experiment started.

TABLE 1**Real-Time RT-PCR primer sequences for genes analyzed**

Gene abbreviation	Gene name	Forward primer, 5'-3'	Reverse primer, 5'-3'
ACTB (reference gene)	Cytoskeletal beta actin	CTCGATCATGAAGTGCACGT	GTGATCTCCTTCTGCATCCTGTC
PPIA (reference gene)	Peptidylprolyl isomerase A (cyclophilin A)	TGCTTTCACAGAATAATTCCAGGATTTA	GACTTGCCACCAGTGCCATTA
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase	CTCGTGGCCAGCACCAATA	GGAAAACGTACCACTGGAGTCAT
CYP51	Cytochrome P450, family 51, subfamily A, polypeptide 1	TATGTGCCATTTGGAGCTGG	CGAAGCATAGTGGACCAAATTG
StAR	Steroidogenic acute regulatory protein	AGAGCTTGTGGAGCGCATG	CATGGGTGATGACTGTGTCTTTTC
CYP11A1	Cytochrome P450 cholesterol side chain cleavage	CACCCCATCTCCGTGACC	GCATAGACGGCCACTTGTACC
CYP17A1	Cytochrome P450 17A1	AGCCAAGACGAACGCAGAA	CCCCAAAGATGTCCGCAAC
HSD3B	Hydroxy-delta-5-steroid dehydrogenase	GGAGGAAGCCAAGCAGAAAA	TTTTAGCGCCTCCTTGTG
CYP19A1	Cytochrome P450 19A1	AAAGCACCCCAGTTGAA	CCACCACTTCGAGTTTTTGCA
HSD17B1	Hydroxysteroid (17 beta) dehydrogenase 1	TCGGGTGCGCATATTGGTGA	GCGCAGTAAACAGCGTTGAA
HSD17B4	Hydroxysteroid (17 beta) dehydrogenase 4	TTGCCATGAGAGTTGTGAGGAA	GTCTTACAAGGGCTCCAAGGG
CYP21	Cytochrome P450, family 21, subfamily A, polypeptide 2	CCATAGAGAACAGGGACCACCT	TAGTCCAGCATGTCCCTCCAC
CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1	GGAGCACTTTGAGGCCTGG	CGCTGTAGTGCCACGGATG
CYB5	Cytochrome b-5	TCAAAGATTGCCAAGCCTTCG	ACAACCAAGTGCTGAGATGGCTG
FTL	Ferritin, light polypeptide	TTCTGGATGAGGAGGTGAAGC	CTTTCGAAGAGGTACTCGCCCA
AKR1C4	Aldo-keto reductase family 1, member C4	AAGTACAAGCCCGTCTGCAAC	TCCTTGGACTTGCAAACTCC
NR5A1	Nuclear receptor subfamily 5, group A, member 1	GCCAGGAGTTCGTCTGCCT	GTTTCGCTTCTCCTGAGCG
NR0B1	Nuclear receptor subfamily 0, group B, member 1	GACCGTGCTCTTAATCCGGA	TCCTGATGTGTTGCTAAGGATC

2.6.5 Real-Time RT-PCR

Real-Time RT-PCR was used in order to investigate if selected genes were up- or down regulated in cells exposed to test compounds of 10 μM compared to solvent control. The assay was already optimized with respect to primer annealing temperatures where a range of temperatures were tested and the lowest CT value was selected. The reaction products were also run beforehand in the lab on an agarose gel to ascertain the presence of a single clear band of the correct size per primer pair.

Initially, the assay was optimized with respect to complementary DNA (cDNA) concentrations and amplification efficiency. A two-fold dilution series was run, where all primers were tested with cDNA concentrations of 5, 2 and 1 ng/ μl . The 1 ng/ μl dilution gave satisfactory CT values (17-30) for all primers and was chosen as the optimal cDNA concentration. A ten-fold dilution series was also performed to evaluate amplification efficiency of each primer pair. Two samples were used; a solvent control sample (DMSO) and a sample representing the three different exposure scenarios pooled together. The cDNA was diluted to concentrations of 30, 3, 0.3, 0.03 and 0.003 ng/ μl . For each primer pair, a standard curve was made. The slope of this curve was used to calculate the efficiency. For all primers the amplification efficiency was between 1.80 and up to 1.99 which means that the amplicon copy number increased 1.80- fold or that 80% of the template was amplified.

First-strand cDNA synthesis by Reverse Transcriptase and quantitative PCR (qPCR) were done using the Superscript III Platinum Two-step qRT-PCR kit with SYBR green (Invitrogen, Carlsbad, CA, USA) and manufacturer's recommendations were followed. The following components were combined to a master-mix enough for 1 reaction: 10 μl 2xRT Reaction Mix, 2 μl RT Enzyme Mix, 2 μl diethylpyrocarbonate (DEPC) and 6 μl RNA sample. This gave an input of RNA of 300, 480 or 600 ng in a total reaction volume of 20 μl . The different amounts of RNA input were due to the variation of the RNA concentrations in the samples obtained from the RNA isolations. All RNA samples were split into technical duplicates prior to cDNA synthesis. For each RNA sample a control with no added reverse transcriptase was included to check for genomic DNA contamination in the qPCR reactions. Negative controls without template and a positive control (with unexposed sample) were included on each plate. The cDNA was synthesized in 96 well PCR plates in a Peltier Thermal Cycler-225 (MJResearch, Waltham, MA, USA) with following configurations:

- 25°C for 10 minutes
- 42°C for 50 minutes
- 85°C for 5 minutes

After the incubation in the PCR machine, the plate was chilled on ice. To remove possible traces of RNA, 1 µl of RNase H was added and the plate was incubated at 37°C for 20 minutes, cDNA was diluted to 1 ng/µl with DEPC water and stored at -20°C until use.

In the real-time RT-PCR reactions, we used half of the amounts recommended in the protocol by the manufacturer. A master-mix containing 12.5 µl Platinum SYBR Green qPCR supermix-UDG, 2 µl DEPC and 5 µl cDNA was prepared. In order to correct for no-amplification related fluorescence, 0.5 µl of Rox dye (diluted 10 times) was also added to the reaction mixture. This master-mix (20 µl) was added to each well in addition to 5 µl diluted primers. The primers had a final working concentration of 200 nM and the resulting cDNA amount was 5 ng (assuming full RT efficiency) in a total reaction volume of 25 µl. Negative controls without RT, positive controls and negative controls without template were also included on each PCR plate. The real-time RT-PCR reactions were run in a DNA Engine Thermal Cycler with Chromo 4 Real-Time detector (MJResearch) and its software Opticon Monitor 3 (Bio-Rad Laboratories, Hercules, CA, USA) with following configurations:

- 50°C for 2 minutes (UDG incubation)
- 95°C for 2 minutes (enzyme activation)

Followed by 40 cycles of

- 95°C for 15 seconds (denaturation)
- 62°C for 30 seconds (annealing)
- 72°C for 30 seconds (elongation)

The absence of primer-dimers, genomic DNA and other DNA contaminations was also monitored during the experiment by including a melting curve from 65 – 90°C, read for 1 second every 0,3°C at the end of each run.

2.7 Statistical analysis

Data were analyzed by JMP 8 software (SAS Institute Inc, Cary, NC, USA) and Microsoft Excel 2007. Two cell viability experiments, three hormone experiments and three qRT-PCR experiments were performed. Where analysis of variance (ANOVA) and multiple comparison tests were used, the three underlying assumptions were required:

- Independent observations
- Normally distribution of data
- Homogeneity of variance

The Shapiro-Wilk's test was used on the observed values for hormone and cell viability data to test for normality. Levene's test was used to evaluate the homogeneity of variances within the observations. In case of non-normality in the dependent variables, a logarithmic or square root transformation was performed to obtain a better fit to the normal distribution. Data were transformed for statistical analysis only.

Due to time limits, cytotoxicity was tested in two independent experiments. The first experiment consisted of three replicates and the second of one replicate for each compound (n=4). Viability measured by fluorescence in the three well triplicates on each plate were combined to one replicate and expressed as percentage of control (=100%). 3,3'-(bis)MeSO₂-DDE data (logarithmic transformed) were normally distributed and 3-MeSO₂-DDE data were close to a normal distribution. The variances for both were homogenous and analyzed with one-way ANOVA. Cell viability data for *o,p'*-DDD failed Levene's test and Kruskal-Wallis test was used as a non-parametric alternative. The medium blank replicates were used as a control against the solvent control replicates to validate the effect on DMSO on viability with a paired two-tailed t-test. Since the solvent controls are not affected by the exposure, the plates within each replicate were combined together as one unit (n=3).

For hormone data, the mean hormone concentrations of nine replicates obtained from three experimental runs were used in the statistical analysis (n=9). A logarithmic transformation was done for estradiol data from the 3-MeSO₂-DDE exposure and a square root transformation was performed for *o,p'*-DDD exposure. The data were normally distributed, except for 3,3'-(bis)MeSO₂-DDE (close to a normal distribution) and the variances were homogenous. For testosterone, 3,3'-(bis)MeSO₂-DDE data were logarithmic transformed to

obtain homogenous variance and a better fit to the normal distribution. These data for estradiol and testosterone were tested with two-way ANOVA and the experimental run was incorporated in the analysis as an independent variable in the regression model together with the exposure groups to explain the response variable. To further include both the exposure groups and the experimental runs in the statistical analysis, the Tukey-HSD test was used to compare the exposure groups against the controls. Linear regression was used to check if the response in hormone production followed a significant trend and the concentration was set as an independent variable.

Testosterone data for 3-MeSO₂-DDE and *o,p'*-DDD failed the Levene's test. The data were prior to statistical analysis expressed as percentage of control in order to remove possible variance in solvent control that could be explained by the experimental run. Kruskal-Wallis test was used for analysis of the data and if significant outcome, each exposure group was then tested against the control with the use of the Bonferroni correction method.

For analysis of qPCR data, the CT values from the analysis were exported from instrument software to Excel, where the $2^{-\Delta\Delta C_t}$ method was used to create fold changes, and these ratios were log₂ transformed prior to statistical analysis. Expression of the genes was normalized to *ACTB* and *PPIA*. Due to few replicates, it was difficult to detect any alteration on gene expression with Kruskal-Wallis. The log₂ transformed fold change values ($\Delta\Delta C_t * (-1)$) of genes expressed in cells exposed to the test compounds were therefore analyzed by Student t test.

P- values < 0.05 were regarded as statistically significant. In the cases where the Bonferroni correction was used, the *P*- value was reduced six times: $0.05/6 = 0.0083$

3.0 Results

3.1 3 β -HSD staining- purity of Leydig cells

The 3 β -HSD staining was performed to ascertain that cells isolated with a discontinuous Percoll gradient were primary neonatal porcine Leydig cells. Our two preparations with purified cells from testis tissue contained approximately 80% of neonatal porcine Leydig cells.

3.2 Cell viability

Effect on cell viability by 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD was tested with AlamarBlue against the solvent control (0.1% DMSO) after 48 hours incubation. All of the six exposure concentrations were included. Two independent experiments were performed where plates 1-3 belonged to the first experiment and plate 4 to the second experiment.

Since all test compounds were diluted in DMSO, the effect of DMSO on cell viability was also examined (Figure 9). There was no apparent effect that 0.1% DMSO exposure reduced cell viability, but experiment 4 caused a small significant increase (P=0.02).*

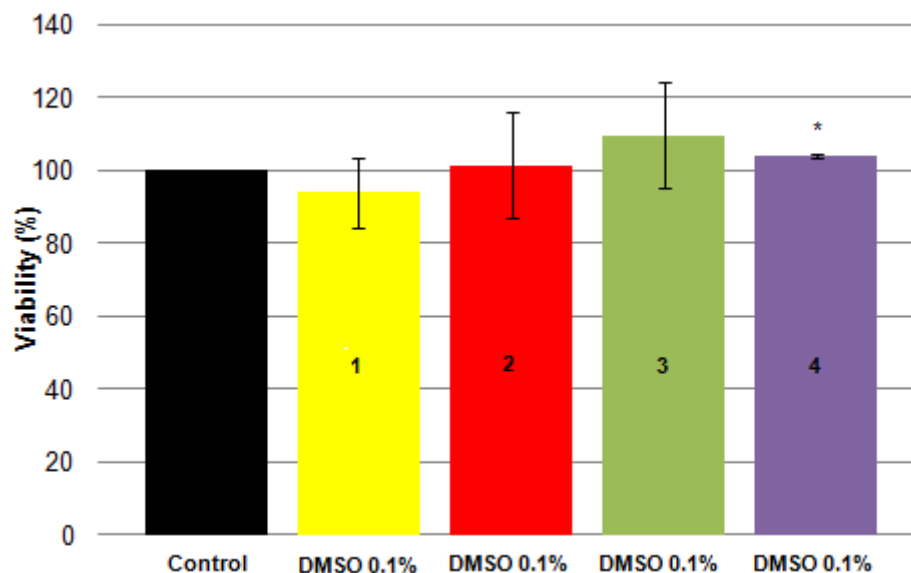


Figure 9: Percent viable Leydig cells exposed to 0.1% DMSO (solvent control) after 48 hours. Viability in controls (medium blank) was set to 100%. The plot shows two independent experiments; the first include replicate 1-3 and the second replicate 4. Mean values with SE are shown for three plates within the replicates (n=3) (paired two-tailed t-test; p<0.05).

In Leydig cells, no significant change in viability was associated with 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE exposure compared to the solvent control (Figure 10A and B, respectively). Both data sets were influenced with some variation between the four plates.

o,p'-DDD (Figure 10C) exposure affected cell viability in Leydig cells. It reduced the viability at the highest concentration (20 μM) to $-1.1 \pm 3.3\%$ (mean \pm SE) from solvent control. There was a significant difference in the dataset (Kruskal-Wallis, $p < 0.05$) which probably could be explained by the reduction at 20 μM. It is therefore likely to assume that only 20 μM exposure with *o,p'*-DDD impair cell viability.

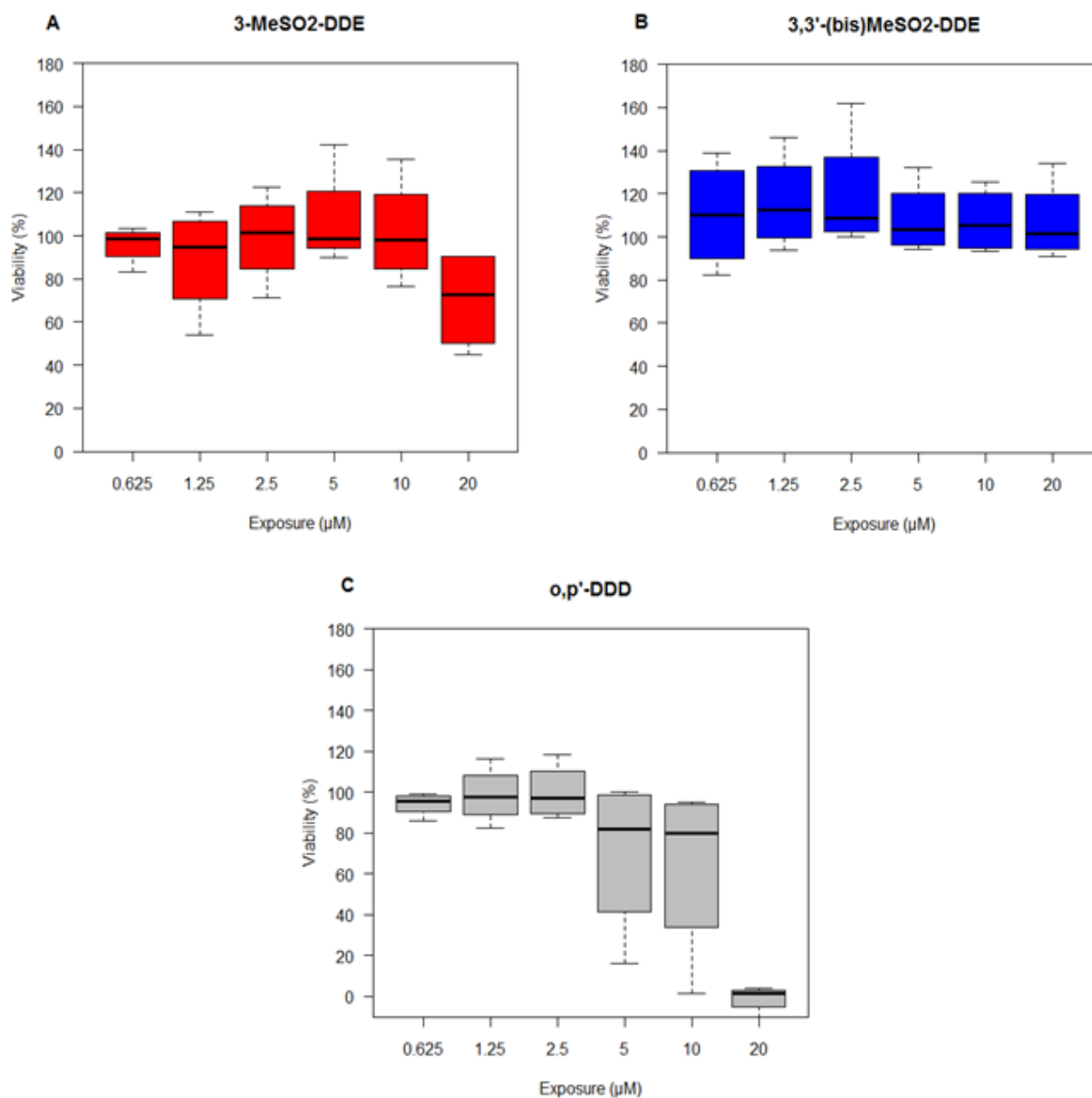


Figure 10. Box plot showing percent viable primary neonatal porcine Leydig cells exposed to A) 3-MeSO₂-DDE (red), B) 3,3-(bis)MeSO₂-DDE (blue) and C) *o,p'*-DDD (grey) after 48 hours incubation. All six exposure concentrations are expressed as percentage of solvent control and compared to solvent control (100%). Results are shown for independent two experiments (four plates) taken together (n=4). Shown are minimum, first quartile, median, third quartile and maximum values.

3.3 Hormone production in primary neonatal porcine Leydig cells exposed to test compounds

Hormone production in neonatal porcine Leydig cells exposed to the three test compounds was measured in three independent experimental runs after 48 hours incubation. Replicates for each exposure concentration on the 24-well plates were treated as biological replicates. The Leydig cells did not produce detectable levels of cortisol and progesterone, so results for these two hormones are not presented. Generally, the compounds caused an increase in hormone production, particularly testosterone.

3.3.2 Testosterone production

In general, the testosterone production increased with increasing concentrations for all three compounds (Figure 11). The testosterone levels in the solvent controls ranged from 0.09-0.6 ng/ml.

3-MeSO₂-DDE exposure increased the testosterone production in a significant concentration-dependent manner (Figure 11A) and all exposure groups were significantly higher than cells exposed to the solvent control (Table 2). 20 μM exerted most effect with almost ten-fold increase from solvent control.

3,3'-(bis)MeSO₂-DDE exposure had less effect on testosterone production compared to 3-MeSO₂-DDE and *o,p'*-DDD (Figure 11B). Only 20 μM was significantly different from solvent control (Table 2) and it gave a three-fold increase in production. There were significant differences between both groups and experimental runs (two-way ANOVA, $p < 0.0001$).

With *o,p'*-DDD, all concentrations gave a significant increase (Table 2) from solvent control in a significant concentration-dependent manner (Figure 11C). 1.25 μM exerted most effect with a 21-fold increase from solvent control while 10 μM and 20 μM leveled it down to almost 19-fold increase and 14-fold in production, respectively.

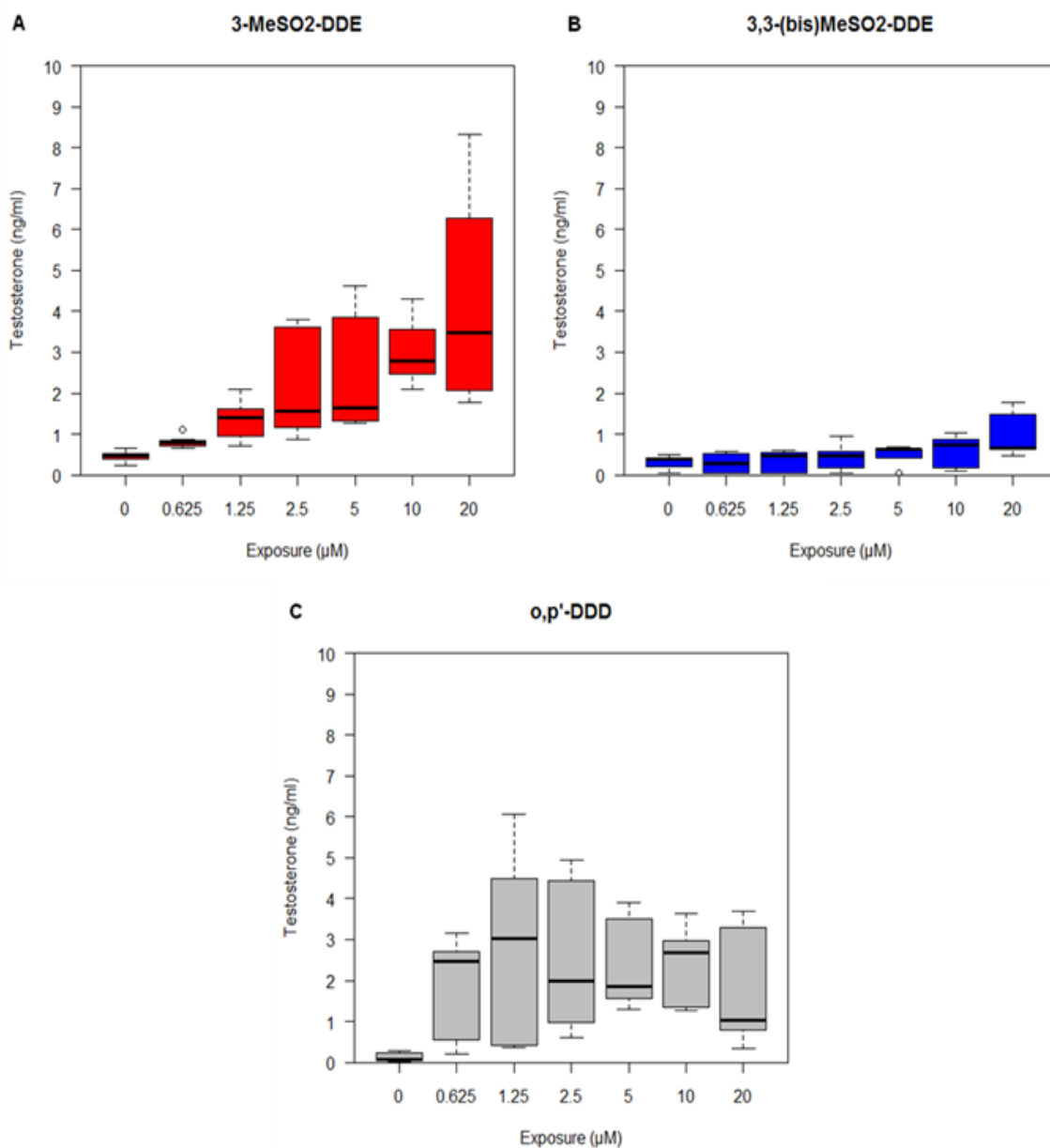


Figure 11. Testosterone production in primary neonatal porcine Leydig cells exposed to A) 3-MeSO₂-DDE (red), B) 3,3'-(bis)MeSO₂-DDE (blue) and C) *o,p'*-DDD (grey) after 48 hours incubation. Testosterone was measured in ng/ml. The plots show minimum, first quartile, median, third quartile and maximum values. Points more than 1.5 times the interquartile range above/below the quartiles are defined as outliers and plotted individually. The data represents three independent experiments, each performed in triplicates (n=9). The statistics are shown in Table 2.

Table 2. Statistical results for testosterone data from Tukey-HSD test after exposure to 3,3'-(bis)MeSO₂-DDE (p<0.05) and for Kruskal-Wallis test with Bonferroni correction after exposure to 3-MeSO₂-DDE and *o,p'*-DDD (p<0.0083). Each exposure group was compared against solvent control (non-significant groups are denoted with n.s).

Compound	0.625 μM	1.25 μM	2.5 μM	5 μM	10 μM	20 μM
3-MeSO ₂ -DDE	p<0.0083	p<0.0083	p<0.0083	p<0.0083	p<0.0083	p<0.0083
3,3'-(bis)MeSO ₂ -DDE	n.s	n.s	n.s	n.s	n.s	p<0.05
<i>o,p'</i> -DDD	p<0.0083	p<0.0083	p<0.0083	p<0.0083	p<0.0083	p<0.0083

3.3.1 Estradiol production

Estradiol production was not affected in the same magnitude as testosterone production (Figure 12). The three solvent controls for the compounds are presented with a large box plot caused by variation between the three isolations (51 – 428 pg/ml). There were significant differences between both exposure groups and experimental runs for all three compounds (two-way ANOVA, $p < 0.0001$).

3-MeSO₂-DDE exposure gave an increase in estradiol production in a significant concentration-dependent manner. 20 μM exerted most effect which resulted in almost two-fold increase compared to cells exposed to solvent control (Figure 12A). All exposure groups except 0.625 μM were significantly different (Table 3).

3,3'-(bis)MeSO₂-DDE had the opposite effect of 3-MeSO₂-DDE and it decreased the estradiol production in a significant concentration-dependent manner (Figure 12B). 10 μM had most effect and the estradiol production was reduced to circa half of the solvent control. The only concentration which did not cause a significant reduction from solvent control was 0.625 μM (Table 3).

Exposure with *o,p'*-DDD gave a bell-shaped curve response in estradiol production (Figure 12C). All concentrations caused a significant increase in production from solvent control (Table 3). The first concentrations from 0.625 – 5 μM increased the production; 5 μM gave nearly a two-fold increase from solvent control. 10 μM and 20 μM did not give any further increase and the production was reduced to one and a half-fold of solvent control at 20 μM.

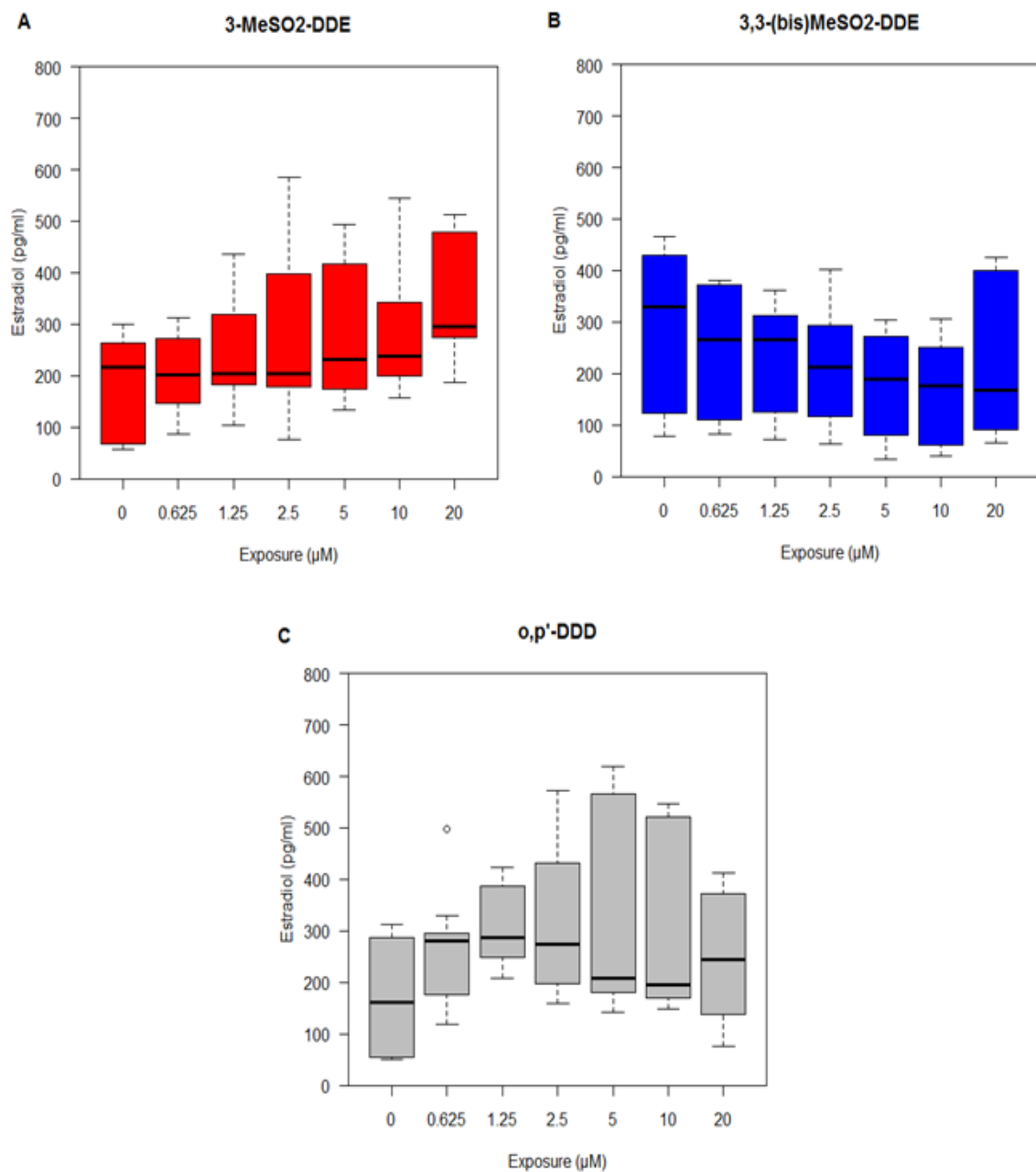


Figure 12. Estradiol production in primary neonatal porcine Leydig cells exposed to A) 3-MeSO₂-DDE (red), B) 3,3'-(bis)MeSO₂-DDE (blue) and C) *o,p'*-DDD (grey) after 48 hours incubation. The estradiol levels were measured in pg/ml. The plots show minimum, first quartile, median, third quartile and maximum. Points more than 1.5 times the interquartile range above the first quartile are defined as outliers and plotted individually. The data represents three independent experiments, each performed in triplicates (n=9). The statistics are shown in Table 3.

Table 3. Statistical results for estradiol data from Tukey-HSD test after exposure to 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD (p<0.05). Each exposure group was compared against solvent control (non-significant groups are denoted with n.s).

Compound	0.625 μM	1.25 μM	2.5 μM	5 μM	10 μM	20 μM
3-MeSO ₂ -DDE	n.s	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
3,3'-(bis)MeSO ₂ -DDE	n.s	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05
<i>o,p'</i> -DDD	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05	p<0.05

3.4 Gene expression results

Gene expression analysis was performed on solvent control samples and 10 μM samples from 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD exposure. The expression of genes was normalized to *ACTB* and *PPIA* and the 10 μM samples were then compared to solvent control. The goal was to investigate if 48 hours of exposure with 10 μM by either compound could up or down regulate genes involved in testicular steroidogenesis. Three independent experimental runs were performed in total.

3.4.4 Effect on gene expression in primary neonatal porcine Leydig cells by test compounds

The test compounds altered the expression of some genes involved in testicular steroidogenesis (Figure 13). Most genes altered were down-regulated while only one gene was up-regulated.

3-MeSO₂-DDE affected the expression of three genes only (Figure 13A). The compound caused a significant reduction in gene expression of *CYP11A1*, *HSD3B* and *CYB5* with most effect on *HSD3B* which was down-regulated three times from solvent control.

CYB11B1, *HSD3B*, *NR5A1* and *FTL* were significantly altered by 10 μM exposure with 3,3'-(bis)MeSO₂-DDE (Figure 13B). Expression of *FTL* was strongest altered and up-regulated three times while the others showed a reduction in expression.

Exposure with *o,p'*-DDD had most effect on expression of genes involved in testicular steroidogenesis (Figure 13C) and eight genes were significantly down-regulated: *CYP11A1*, *CYP17A1*, *CYP19A1*, *CYP21*, *HSD17B4*, *StAR*, *CYB5* and *HMGR*. The most dramatic alteration was seen in the expression of *CYP19A1*, which was down-regulated 12 times.

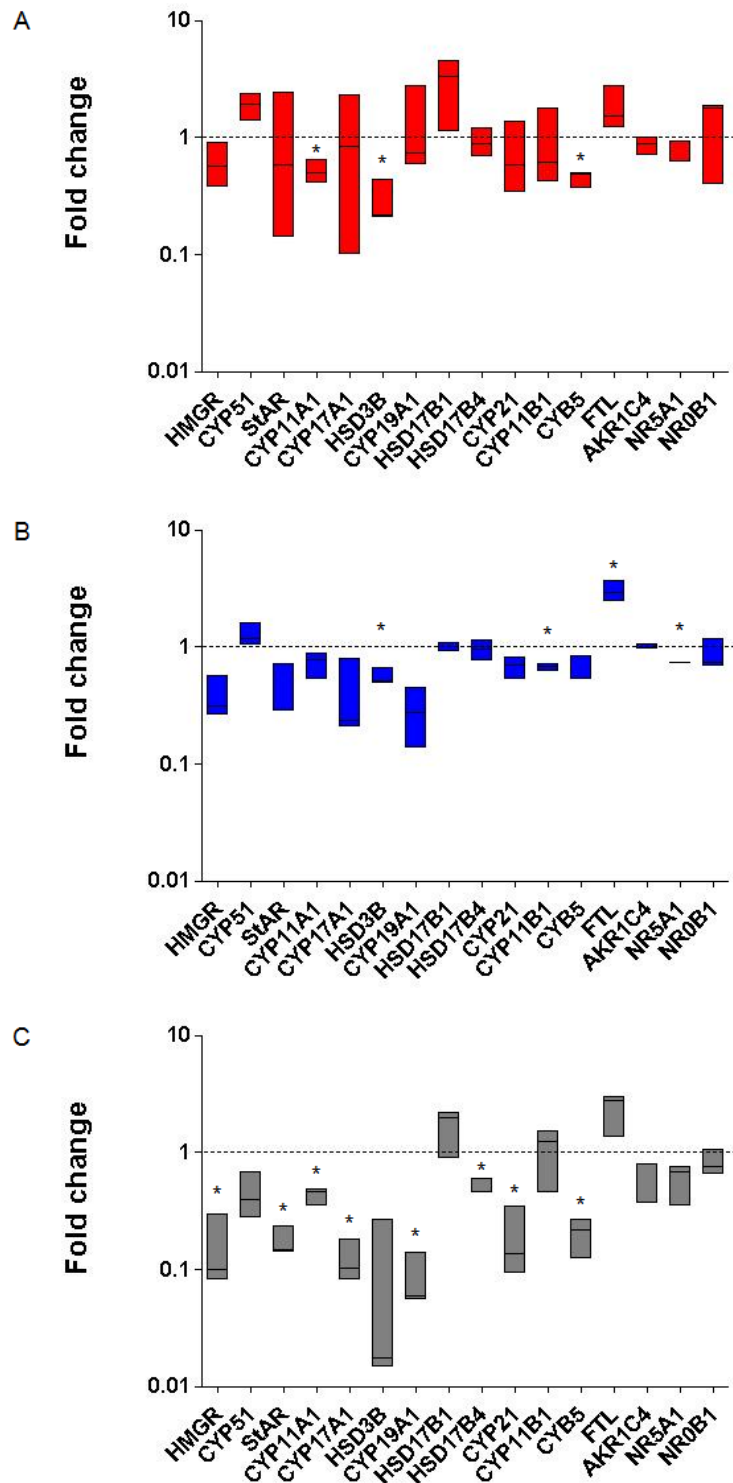


Figure 13. Gene expression of 16 steroidogenic genes in primary neonatal porcine Leydig cells exposed to 10 μ M of A) 3-MeSO₂-DDE (red), B) 3,3'-(bis)MeSO₂-DDE (blue) and C) *o,p'*-DDD (grey) relative to the expression in cells treated with solvent control (set to 1 – dotted line) after 48 hours incubation. The box plots are presented with median (n=3) and the data are shown as fold change ($2^{-\Delta\Delta C_t}$). Fold change values above 1 represent up regulation, while values below 1 represent down regulation. Genes significantly regulated from solvent control are denoted with* (Student's t-test; *P<0.05).

4.0 Discussion

To the best of our knowledge, the three compounds, 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD, used in the present study have not been tested experimentally on primary porcine Leydig cells *in vitro*. This study identified effects by test compounds on basal testicular steroidogenesis in porcine Leydig cells during neonatal development. *o,p'*-DDD was found to be toxic at the highest concentration (20 μM), test compounds were overall able to alter hormone production and they were also able to regulate the expression of some genes involved in testicular steroidogenesis. The causes behind these responses in Leydig cells are not known, but this study provides a contribution to reveal what effects the three compounds may have on Leydig cells and the male reproductive system.

4.1 Purity of Leydig cells

To be certain that the majority of cells isolated indeed were Leydig cells, a cytochemical staining for 3β-HSD was performed. Purity of Leydig cells by our protocol was found to be approximately 80% and this is in agreement with previous reports (Geiger et al., 1999; Lejeune et al., 1998b). Cells not identified as Leydig cells could perhaps represent Leydig cell precursors, fibroblasts, macrophages, sertoli cells, peritubular cells and endothelial cells. There is also a possibility that some of the cells isolated have mixed adrenal and Leydig cells properties, as the presence of a mixed cell type have been confirmed in the interstitium of embryonic and adult mouse testis (Val et al., 2006). Staining was performed once on two separate Leydig cells isolations, so the purity of Leydig cells obtained from each collection is not known. Although the same protocol was followed each isolation some variability is to be expected. One of the most critical steps during the isolation is making up the Percoll gradient. Since many persons were involved in the project, the gradient was not made by the same person each time. This could possibly be one source of variation. Also, depending on how many testes used and the age of the piglets (8-12 days), the percentage obtained from the different collections could vary. This might partly explain why we obtained different amounts of hormone levels and gene expression levels in each experiment. It is well known that isolated primary cultures do vary in their biological responses from batch to batch and to a greater extent than established cell lines.

4.2 Cytotoxicity

Test compounds were diluted in 0.1% DMSO and the effect of DMSO on Leydig cells was therefore evaluated. DMSO gave no significant decrease in cell viability, although one plate seemed to give a small increase. The plates within the first replicate showed some variation from each other, probably due to pipetting errors with a step pipette. No studies have so far evaluated the effect on DMSO on porcine Leydig cells, but it is likely to assume that the concentrations used does not impair cell viability in a considerable manner (Da Violante et al., 2002).

Both 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE did not give any significant reduction in cell viability. In contrast, it is certain that the highest concentration for *o,p'*-DDD was cytotoxic because it gave a very strong reduction in cell viability.

The cytotoxic properties of the test compounds have previously been investigated in other cell lines *in vitro*. These studies have mainly focused on adrenal cells due to 3-MeSO₂-DDE and *o,p'*-DDD known cytotoxic properties on the adrenal gland. 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD have all been tested with same concentrations as used in the current study in Y-1 cells (Asp et al., 2009). MTT cytotoxicity assay was used instead of AlamarBlue and cells were incubated with test compounds for 72 hours. Here, 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE caused a significant concentration-dependent cytotoxicity with 20 μM more potent with 3-MeSO₂-DDE than 3,3'-(bis)MeSO₂-DDE, with reduction to 12 and 66%, respectively. *o,p'*-DDD did not impact cell viability. Effects of 3-MeSO₂-DDE and *o,p'*-DDD on cytotoxicity has also been studied in human adrenocortical cell line H295R (Asp et al., 2010) with same use of concentrations except for 0.625 μM (MTT assay and 72 hours incubation). Here, both compounds decreased cell viability in a concentration-dependent manner with a approximately decrease at 20 μM to 20% with 3-MeSO₂-DDE and 15% with *o,p'*-DDD.

The highest concentration with *o,p'*-DDD exposure on H295R cells is in agreement with current study, but the result for 3-MeSO₂-DDE is not. The results for 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD on Y-1 cells are not in compliance either. This could be due to the fact that adrenal cells and Leydig cells are two different cell types and it could be expected that the compounds will behave differently. On the other hand, it would be likely that the compounds do behave in the same manner, since both Leydig cells and adrenal cells capable of synthesizing similar steroid intermediates (Conley and Bird, 1997). Previous

studies performed with test compounds give a reasonable explanation for the mechanism behind the decrease in cell viability in adrenal cells. As mentioned, it has been shown that 3-MeSO₂-DDE is bioactivated to a reactive intermediate by CYP11B1 in the adrenal cortex of mice *in vivo* and that the reactive intermediate affects the adrenocortical region zona fasciculata where CYP11B1 is expressed (Jonsson et al., 1991; Lund et al., 1988; Lund and Lund, 1995). The reactive intermediate causes formation of irreversible binding to adrenal protein and gives rise to extensive cell death (Lund et al., 1988; Lund and Lund, 1995). Mouse Y-1 cells and H295R cells do also express CYP11B1 (Gazdar et al., 1990; Rice et al., 1989). It is a mitochondrial enzyme catalyzing the final step in the glucocorticoid synthesis with production of cortisol. Although 3-MeSO₂-DDE is toxic in adrenal cortex in mice, the toxicity in the adrenal cortex exhibits considerable species variation (Lindstrom et al., 2008). It binds for instance extensively to hamster and mouse adrenal cortex while guinea pig adrenals were devoid of binding. In rat, only weak binding was observed in the adrenal cortex. 3-MeSO₂-DDE was also reported to be activated and bound by a human adrenal preparation (Jonsson and Lund, 1994) and in zona fasciculata and zona reticularis region in human adrenal tissue slice culture where CYP11B1 is expressed (Lindhe et al., 2002). However, in an unpublished study referred to by Jonsson et al (Jonsson et al., 1991) no binding of 3-MeSO₂-DDE was observed in mouse testicles.

It should be noted that in H295R cells the specific inhibitor of CYP11B1, etomidate, did not affect either cytotoxicity or protein binding of 3-MeSO₂-DDE (Asp et al., 2010). This inhibitor did decrease both irreversible binding and cytotoxicity of 3-MeSO₂-DDE in Y-1 cells (Asp et al., 2009; Hermansson et al., 2007). Since 3-MeSO₂-DDE is bound to adrenocortical regions where human CYP11B1 is expressed (Lindhe et al., 2002), the question was raised whether it is the parent molecule that binds proteins in human cells or if bioactivation is performed by other enzyme(s) in addition to CYP11B1. There are no studies which have confirmed the presence of CYP11B1 enzyme in Leydig cells. It is therefore not likely to assume that 3-MeSO₂-DDE could reduce cell viability in porcine Leydig cells via this enzyme. However, if bioactivation is performed by other enzyme(s) in addition to CYP11B1 in adrenal human cells, it should not be ruled out if unknown enzymes could bioactivate 3-MeSO₂-DDE and cause reduction in cell viability in pig Leydig cells as well. 3-MeSO₂-DDE had a non-significant reduction at 20 μM to 70%, but with only two independent experiments performed, we need more replicates to clarify if this compound can impair cell viability in Leydig cells.

In contrast, 3,3'-(bis)MeSO₂-DDE did not show the same capacity as 3-MeSO₂-DDE to accumulate in the adrenal cortex in mice (Lund et al., 1988). It might be that this compound has less affinity for CYP11B1 and the extra methyl-sulfonyl group could possibly give 3,3'-(bis)MeSO₂-DDE the ability to be less cytotoxic than 3-MeSO₂-DDE, for example by steric hindrance of enzyme binding. This could perhaps be one explanation for why 3,3'-(bis)MeSO₂-DDE did not show any capacity to reduce cell viability in Leydig cells and was less potent than 3-MeSO₂-DDE in mouse Y-1 cells.

o,p'-DDD is known to be toxic in the adrenal cortex of bovine, dogs, minks, chickens and humans through a CYP-catalyzed reaction to a reactive acyl chloride which binds covalently to primarily mitochondrial proteins (Brandt et al., 1992; Cai et al., 1995; Hart et al., 1973; Martz and Straw, 1980). As mentioned previously it is therefore the main drug for Cushing's syndrome and ACC. It blocks cortisol synthesis by inhibiting CYP11A1 and CYP11B1 (Martz and Straw, 1980). Mice do not respond with toxicity in adrenal cortex after a single dose (Lund et al., 1988; Lund et al., 1986). Surprisingly, *o,p'*-DDD was demonstrated to be bioactivated and bound in the Y-1 cell line (Hermansson et al., 2007), but as mentioned, cell viability is not affected (Asp et al., 2009). It did also become activated and covalently bound in the mouse lung (Lund et al., 1986). *o,p'*-DDD seems therefore to be activated in different tissues within same species and toxicity within the adrenal gland seems also to be species dependent. The mechanism behind toxicity in porcine Leydig cells is not known, but it could be possible that enzymes may convert it to a reactive metabolite since this is shown for enzymes in the adrenal gland. It should be noted that conclusive evidence for which or what enzymes responsible for the bioactivation in adrenal cells is lacking (Ahlman et al., 2001; Hart et al., 1973).

4.3 Effect on basal testicular steroidogenesis

4.3.1 Hormone production

The present study evaluated effects of the compounds on basal estradiol and testosterone production in primary neonatal porcine Leydig cells. We found that the test compounds showed a capacity to alter hormone production, particularly testosterone.

The change in hormone production in Leydig cells could be caused by several mechanisms. However, since the compounds are shown to interfere at the protein level in adrenal cells, it is likely to assume that they would behave in the same manner in Leydig cell as well. Due to the fact that the three compounds showed an overall ability to stimulate basal steroidogenesis, it

is a possibility that the activity of the enzymes involved in testicular steroidogenesis was not inhibited, but stimulated. A reduction in enzyme activity would most likely give a decrease in testosterone and estradiol secretion as this is confirmed in other exposure studies on Leydig cells (Akingbemi et al., 2000; Murugesan et al., 2008; Ohno et al., 2005). Thus, stimulation in activity of one or more enzymes could be true for 3-MeSO₂-DDE since this compound caused an increase in production for both hormones, particularly for testosterone. 3,3'-(bis)MeSO₂-DDE exposure had least effect on hormone secretion; the increase seen for testosterone was much less potent than with 3-MeSO₂-DDE and this difference in response is probably because of the extra methyl sulphonyl group. For estradiol, however, the effect was opposite and a decrease was seen. *o,p'*-DDD exposure did as 3-MeSO₂-DDE cause a stimulatory effect on testosterone secretion. It also stimulated estradiol secretion, but to a lesser extent and the response seemed to fit a bell-shaped curve more than a straight line. For both hormones, low concentrations of *o,p'*-DDD seemed to stimulate hormone secretion, but the production did not increase further with higher concentrations which could suggest that the stimulatory effect ceased off. The least increase in hormone production was seen at 20 µM, but since 20 µM was toxic for the cells it is difficult to interpret the cells ability to produce hormones at this concentration.

The test compounds' effect towards adrenal cells is well known. The adrenal gland secrete mainly corticosteroids and as expected, the compounds give mostly a decrease in basal glucocorticoid secretion in Y-1 cells and H295R cells which is caused by their known interactions with CYP11B1 and CYP11A1 (Asp et al., 2009; Asp et al., 2010). The three compounds had different ability to reduce hormone secretion, but 3,3'-(bis)MeSO₂-DDE was less potent than 3-MeSO₂-DDE which is in agreement with current study. Since CYP11B1 is not involved in synthesis of testosterone and estradiol or is not to our knowledge present in porcine Leydig cells, the mechanism behind the decrease in glucocorticoid secretion in adrenal cells is consequently not related to an increase or decrease in hormone secretion in the Leydig cells. No detectable levels were found of cortisol either, which also confirms that this protein is not strongly expressed in neonatal porcine Leydig cells.

The activity of enzymes involved in testicular steroidogenesis are all important for the synthesis of androgens and estrogens and if the activity of one of these enzymes become affected due to exposure this will disturb the steps further down in the steroidogenic pathway (Figure 3). The first crucial step involves StAR which transports cholesterol from the outer to inner mitochondrial membrane (Stocco, 2001). This step is dependent of the presence of

cholesterol and also the presence and activity of the StAR protein. Hence, a blocking of StAR activity would thus prevent further conversion of cholesterol while stimulation in activity would give more substrate for the next rate-limiting enzyme CYP11A1 which converts cholesterol to pregnenolone (Miller, 1995). Due to the fact that *o,p'*-DDD inhibits CYP11A1 in adrenal cells, it is possible that it could inhibit CYP11A1 in porcine Leydig cells as well. This would prevent the conversion of cholesterol and thus suppress testosterone secretion. An interaction with CYP11A1 could perhaps explain why the stimulatory effect of *o,p'*-DDD ceased off at the highest concentrations.

Porcine Leydig cells utilize pregnenolone mostly via the $\Delta 5$ -pathway to 17α -hydroxypregnenolone and DHEA (Ruukonen and Vihko, 1974b), but fluxes through $\Delta 4$ -pathway to progesterone and then to 17α -hydroxyprogesterone and androstenedione may also occur (Conley and Bird, 1997; Nakajin et al., 1981). Since no detectable levels of progesterone were found in this study, this suggests that the testosterone production occurred via the $\Delta 5$ -pathway. It is the presence of CYP17 which directs the synthesis towards androgens via the $\Delta 4$ - and $\Delta 5$ -pathways (Fluck et al., 2003) and the activity of this enzyme is thus crucial for the secretion of sex steroids. The $17,20$ -lyase activity of CYP17 is also dependent on CYB5 which has been shown to increase this activity (Katagiri et al., 1982). CYB5 is also involved in the andien-beta synthase system together with porcine CYP17A1 which stimulates synthesis of androstenone from pregnenolone and progesterone (Figure 3), a pheromonal hormone responsible for boar taint (Nakajin et al., 1985).

Another important enzyme, 3β -HSD, is involved in both the production of androstenone (Moe et al., 2007) and in testosterone synthesis where it converts DHEA to androstenedione (Conley and Bird, 1997). Androstenedione is the substrate for testosterone and one enzyme that might explain the increase seen in testosterone secretion for all the compounds, though only at $20 \mu\text{M}$ for $3,3'$ -(bis)MeSO₂-DDE, is 17β -HSD which converts androstenedione to testosterone (Inano et al., 1981). The 17β -HSDs consist of a large family; it is 17β -HSD type 3 (in testis only) that catalyzes the conversion from androstenedione to testosterone, type 1 catalyzes the reduction of estrone to estradiol while type 4 catalyzes the oxidation of estradiol to estrone (Luu-The, 2001; Whitehead and Rice, 2006). The activity of 17β -HSD type 3 is important and if inhibited this would seriously impact testosterone production (Ohno et al., 2005). CYP19 catalyzes the synthesis of estrogens to androgens (Conley et al., 1996); it produces both estrone and estradiol from androstenedione and testosterone, respectively (Figure 3). Thus, the activity of both 17β -HSD type 1 and CYP19 determines the amounts

estradiol produced by the testis. The enzymes which convert active androgens and estrogens to inactive metabolites, AKR1C1-AKR1C4 (Penning et al., 2000), could also explain the response in hormone production since reduced activity of these enzymes would lead to an excess of circulating hormones.

4.3.2 Gene expression

In samples exposed to 10 μ M of either compound, the expression of 16 genes was evaluated to determine if the test compounds could exert effect at the transcriptional level as well. Unfortunately, this study did not evaluate the effect by the compounds on *HSD17B3* since primer pairs were not in house before the experiment was started. The expression of *CYP21* and *CYP11B1*, whose corresponding enzymes are adrenal cortex specific, was evaluated and we found that they were expressed in neonatal pig Leydig cells. This is in consistency with previous results where *CYP11B1* was expressed in fetal and neonatal testis and *CYP21* in fetal and adult testis of mouse (Hu et al., 2007). Both have also been found to be expressed in fetal testis of humans (Pezzi et al., 2003). The expression of *CYP21* and *CYP11B1* in neonatal pig Leydig cells is further evidence for a link between adrenal and Leydig cells, but the question remains if the expression is caused by a mixed cell type of adrenal and Leydig cells or by a subpopulation of Leydig cells (Hu et al., 2007; Val et al., 2006). However, alteration in expression of these genes will not be discussed because the presence of the enzymes would not affect the synthesis of androgens and estrogens, nonetheless.

Both 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE were less potent than *o,p'*-DDD; 3-MeSO₂-DDE down-regulated three genes (*CYP11A1*, *HSD3B* and *CYB5*) while 3,3'-(bis)MeSO₂-DDE caused down-regulation of three (*CYP11B1*, *HSD3B*, *NR5A1*) and up-regulation of gene (*FTL*) (Figure 13). *o,p'*-DDD caused significant down regulation of eight genes (*CYP11A1*, *CYP17A1*, *CYP19A1*, *CYP21*, *HSD17B4*, *StAR*, *CYB5*, *HMGR*). This suggests that the compounds also interfere at mRNA level. Since the altered genes were mostly down-regulated with the exception of one gene, the compounds did probably somehow reduce the transcription rates or cause a decrease in mRNA stability.

Due to the response seen in hormone secretion there is a possibility that the compounds could interfere with the regulation of LH concentrations and receptor binding since this is the primary hormone regulating Leydig cell activity (Lejeune et al., 1998b; Mather et al., 1982). However, if this was the case then this interference probably did not have a stimulatory effect due to decreased mRNA levels. Binding of LH to its receptor activates synthesis of cAMP

and levels of cAMP are involved in maintaining and stimulate the expression of enzymes involved in testicular steroidogenesis (Clark et al., 1996; Mason et al., 1984; Nakajima et al., 2005). It has for instance been shown in neonatal pig Leydig cells that *CYP17A1* mRNA level induced by LH stimulation decreases with tributyltin chloride (TBT) exposure (Nakajima et al., 2005).

Since 3-MeSO₂-DDE only had an impact on three genes, it is reasonable to assume that this is not caused with interference with LH. At 10 μM, 3-MeSO₂-DDE showed a stimulatory effect on testosterone secretion. It is therefore interesting that the mRNA levels of *CYP11A1*, *HSD3B* and *CYB5* were decreased since the presence of the corresponding enzymes is important for the synthesis of testosterone. A possible decrease in mRNA levels would probably lead to less translation into protein, reduced protein levels and reduced conversion of steroids to androgens and estrogens. The gene expression results for 3-MeSO₂-DDE did therefore not explain the increase seen in hormone secretion at 10 μM.

3,3'-(bis)MeSO₂-DDE significantly affected the expression of four genes which might also suggest that the compound did not interfere with LH. 3,3'-(bis)MeSO₂-DDE did not exert stimulatory effect on testosterone secretion at 10 μM, but it caused a decrease for estradiol. The fact that it did cause reduction in mRNA levels for *CYB11B1*, *HSD3B* and *NR5A1* seemed not to exert an impact on the production of testosterone. SF-1 (coded by *NR5A1*), one of the transcription factors investigated, is involved in mediating transcriptional activation in response to cAMP which will lead to an increase in mRNA levels, normally of *CYP11A1*, *HSD3B*, *CYP17*, *CYP19*, *CYP21* and *StAR* (Mendelson et al., 2005; Parker et al., 2002; Sadvovsky and Dorn, 2000; Sandhoff et al., 1998; Sugawara et al., 1996). However, the reduced mRNA levels for *NR5A1* caused by 3,3'-(bis)MeSO₂-DDE did not seem to affect mRNA levels for the other genes, except for *HSD3B*. *CYP19A1* had though a non-significant reduction (Figure 13) which could perhaps explain the decrease seen in estradiol production, but more experimental runs would be needed to associate reduced estradiol levels with reduced mRNA levels for *CYP19A1*. 3,3'-(bis)MeSO₂-DDE did also significantly up-regulate *FTL*. Increased mRNA levels of *FTL* could suggest that sustainable amounts of ferritins were made, thus increasing the iron storage capacity of the cell (Hentze and Kuhn, 1996). If the iron storage capacity was increased, it might be one explanation for why the cell viability was sustained with 3,3'-(bis)MeSO₂-DDE.

o,p'-DDD had most impact at mRNA level due the strongly reduction in mRNA levels seen for many of genes at 10 μ M (Figure 13). An interference with the regulation of LH is perhaps more likely for this compound since many of the cytochrome P450 steroid hydroxylases and *StAR* mRNA were down-regulated (Clark et al., 1996; Sugawara et al., 1996). Since *CYP11A1*, *CYP17A1*, *CYP19A1*, *CYP21*, *HSD17B4*, *StAR*, *CYB5* and *HMGR* were significantly affected and because of the importance of the corresponding enzymes in testicular steroidogenesis, the reduced mRNA levels would suggest low levels of testosterone and estradiol secretion. However, neither of the hormones had decreased production compared to solvent control at 10 μ M, so the reduced mRNA levels did not seem to affect the response seen in hormone secretion. Dax-1 (coded by *NR0B1*) is known to repress SF-1-mediated transactivation of *StAR* (Zazopoulos et al., 1997), but since *NR0B1* mRNA levels is unaffected by *o,p'*-DDD exposure we cannot link reduced levels of *StAR* mRNA to this transcription factor. *o,p'*-DDD did affect mRNA levels of *HMGR*. *HMGR* is the rate-limiting enzyme in cholesterol synthesis (Rodwell et al., 1976) and the activity of the corresponding enzyme thus determines the cholesterol levels in the cell. This enzyme is therefore often targeted to treat hypercholesterolemia (Pak et al., 2008) and since *o,p'*-DDD reduced *HMGR* mRNA levels it should be further looked in to if this down-regulation could affect the amounts of the cholesterol produced by the cell.

The reduction seen in mRNA levels did not explain the response in hormone levels at 10 μ M for the three compounds. This leads to the suggestion that the compounds' effect is caused by interaction at the protein level. However, it has been discovered that mRNA expression patterns are not necessarily consistent with protein expression patterns. The differential expression of mRNA and protein from neonatal and prepubertal pig testes has been evaluated for *CYP11A1*, *CYP17*, *CYP19*, 3 β -HSD and 17 β -HSD 4 (Choi et al., 2009) and except in the cases of *CYP19*, changes of protein abundance during early neonatal development were not consistent with the patterns of mRNA expression. For prepubertal testis the mRNA expression pattern consisted with protein expression patterns. Hence, these results suggested the existence of posttranscriptional regulatory mechanisms on the expression of steroidogenic enzymes in the pig testis during early neonatal development which results in the increases of plasma and testicular steroid hormone concentrations during early neonatal development (Choi et al., 2009). If posttranscriptional regulatory mechanisms do occur in neonatal pig Leydig cells, it should be considered when effect by compounds is to be evaluated on testicular steroidogenesis.

4.4 Basal versus LH stimulated steroidogenesis

This study evaluated the effect of the 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD on basal steroidogenesis. However, the effect on LH-mediated testosterone and estradiol synthesis was also evaluated in the same study by another fellow masterstudent (Tanum, 2010). The results from this study confirmed the stimulating effect of LH as the hormone levels in the solvent controls were much higher compared to unstimulated cells. LH did also stimulate the cells to produce progesterone, but to a lesser extent than testosterone and estradiol.

In LH-stimulated cells, all three compounds managed to reduce progesterone, testosterone and estradiol production from solvent control with the highest concentrations, and mRNA levels for most of the genes were also reduced at 10 μM. In fact, this suggests that all three compounds inhibit LH stimulated hormone secretion, but it is interesting that the overall effect on basal hormone synthesis was opposite. Another study with similar results can thus give a suggestion for the mechanism behind the stimulation on basal hormone secretion: myxothiazol exposed to rat Leydig cells was found to inhibit LH-mediated testosterone synthesis, but a stimulating effect was observed for basal steroidogenesis (Midzak et al., 2007). These results indicated that inhibition of the mitochondrial electron transport chain blocked LH-stimulated testosterone production through suppression of a number of steps in the steroidogenic pathway and that basal steroidogenesis was stimulated through a calcium-mediated mechanism. Other known electron transport chain inhibitors did also stimulate basal testosterone production in the same study. The stimulatory effect was linked to the fact that inhibition of the electron transport chain has been shown to increase intracellular calcium levels in a number of cell types (Duchen et al., 1990; Midzak et al., 2007). Further, Ca²⁺ concentrations have been demonstrated to increase in parallel with testosterone production and Ca²⁺-dependent stimulation of testosterone is also cAMP independent (Sullivan and Cooke, 1986).

4.5 Possible reasons for concern?

Due to the extensive use of DDT in the past and also because of the restricted use today, levels of DDE and 3-MeSO₂-DDE are present in milk and tissues of both humans and mammals. Consequently, there is a chance that these metabolites are transferred from mother to offspring via milk. A recent study showed an expressed 3-MeSO₂-DDE excretion via milk in lactating minipig sows and concentrations in milk were 30-40 times the concentration of

the maternal plasma (Kismul, 2009). These sows were given a single dose of 3-MeSO₂-DDE (15 mg/kg body weight) two days *post partum* which caused the piglet plasma concentrations of 3-MeSO₂-DDE to be 3 times higher than in the sows. Because of these findings, it was postulated that milk secretion is the only efficient elimination pathway of 3-MeSO₂-DDE and suckling offspring is therefore at risk for being highly exposed.

Both 3-MeSO₂-DDE and *o,p'*-DDD accumulate in fat tissues, but elimination of 3-MeSO₂-DDE is slow compared to *o,p'*-DDD although plasma concentration of 3-MeSO₂-DDE reaches higher levels than *o,p'*-DDD (Hermansson et al., 2008). These findings confirm that there are no other efficient elimination pathways for 3-MeSO₂-DDE besides milk. In addition, *o,p'*-DDD is much less common than *p,p'*-DDD in soil (Pazou et al., 2006; Ssebugere et al., 2010) and it is also not biomagnified in a considerable manner (Falandysz et al., 1999). 3-MeSO₂-DDE is found in human tissues (Chu et al., 2003) and breast milk (Noren et al., 1996; Smith, 1999) and it is isolated from large marine mammals (Jensen and Jansson, 1976; Lechter et al., 1995). Of the two DDT metabolites, 3-MeSO₂-DDE is the one which should be given increasing focus with regard to risk assessments. Leydig cells play a central role in male reproductive function with the secretion of testosterone and estradiol and the new knowledge of 3-MeSO₂-DDE effect on Leydig cells is especially concerning due to the fact that disturbances during early stages of life can affect further development and male fertility. Furthermore, since 3-MeSO₂-DDE is proposed as an alternative drug for *o,p'*-DDD for treatment of ACC and Cushing's syndrome, the compound's effect on the Leydig cells should be considered when it is to be evaluated for treatment.

The effect 3,3'-(bis)MeSO₂-DDE posed on the Leydig cells is not environmentally relevant, but we confirmed that the extra methyl sulphone group gave a different response in the Leydig cells compared to 3-MeSO₂-DDE. This may be of importance for the search of a new drug candidate for ACC and Cushing's syndrome. Although there is a possibility that the current drug for ACC and Cushing's syndrome may be replaced in the future, we have discovered that *o,p'*-DDD not only possesses toxic properties towards the adrenal cortex, but also towards Leydig cells.

5.0 Conclusions and future work

The described study was carried out to determine the effect of 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD on testicular steroidogenesis in neonatal porcine Leydig cells.

The compounds exerted different effects on cell viability. 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE did not impair cell viability while *o,p'*-DDD was cytotoxic at the highest concentration. All three compounds increased testosterone production, but 3,3'-(bis)MeSO₂-DDE caused only effect at the highest concentration. Hence, it appears that the extra methyl sulphonyl group of 3,3'-(bis)MeSO₂-DDE makes the compound to a less potent stimulator of testosterone production than 3-MeSO₂-DDE in porcine Leydig cells. Estradiol production was also altered for all three compounds, but not in the same magnitude as testosterone. Both 3-MeSO₂-DDE and *o,p'*-DDD seemed to have a stimulatory effect, while 3,3'-(bis)MeSO₂-DDE caused a decrease. The Leydig cells did not produce progesterone and cortisol. The compounds exerted effect on gene expression and the genes altered were all down-regulated with the exception of one with 3,3'-(bis)MeSO₂-DDE. *o,p'*-DDD caused most effect with down-regulation of eight genes, all important for testicular steroidogenesis. 3-MeSO₂-DDE and 3,3'-(bis)MeSO₂-DDE caused reduction in expression of three genes each. However, with the use of two replicates only for cell viability where three of the four plates are from the same batch of cells, the evidence for the compounds' effect on cell viability is not fully conclusive. The dataset for the hormone results, especially estradiol, and the gene expression results were influenced with large spread between the three experimental runs. More experimental runs would therefore have given the results even more strength, especially for the cell viability results.

Due to the results obtained, further studies should be conducted. With regard to cell viability, it should be examined if *o,p'*-DDD could exert cytotoxicity in same manner as it does in adrenal cells at 20 µM. More replicates for 3-MeSO₂-DDE could also clarify if this compound could be cytotoxic at 20 µM as well. As for hormone and gene expression results, the reduction in mRNA levels cannot be linked to the response seen in hormone secretion at 10 µM. Because of the compounds' overall ability to stimulate hormone secretion, stimulation in enzyme activity should be investigated. It would for instance be interesting to see whether the three compounds were capable of stimulating basal steroidogenesis through increased levels of Ca²⁺ by inhibition of the mitochondrial electron transport chain. If the compounds do

interfere at the protein level, protein expression should also be examined. The mRNA product for the protein involved in the making of testosterone (17β -HSD 3) was unfortunately not included in this study. The expression of this gene should therefore be included in the future. It is also important to evaluate effect on cAMP levels because the amount of cAMP in the cell determines the amounts of hormones produced. In addition, due to the interference with the LH-stimulation it would be interesting to include mRNA product for the LH receptor and to investigate if the compounds are able to interact with the LH receptor.

Taken together, the results suggest that 3-MeSO₂-DDE, 3,3'-(bis)MeSO₂-DDE and *o,p'*-DDD are capable of disturbing the male reproductive system by affecting basal testicular steroidogenesis in neonatal porcine Leydig cells, with 3,3'-(bis)MeSO₂-DDE being the least potent.

6.0 References

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7. Appendix

7.1 Chemicals and solutions

Chemical	Product information
3-MeSO ₂ -DDE	Synthelec AB, Ideon, Lund, Sweden
3,3'-(bis)MeSO ₂ -DDE	Synthelec AB, Ideon, Lund, Sweden
<i>o,p'</i> -DDD	Sigma-Aldrich, Stockholm, Sweden
Dimethyl sulfoxide	Sigma-Aldrich, Oslo, Norway
Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) 1:1 supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM HEPES, pH 7.4 (500 ml)	Gibco Invitrogen, Carlsbad, CA, USA
F-10 Nutrient Mixture (Ham's) (10X)	Biological Industries, Kibbutz Beit Haemek, Israel
Percoll (500 ml)	Sigma-Aldrich, Oslo, Norway
NuSerum	BD Bioscience via VWR, International AS, Oslo, Norway
ITS + Premix	BD Bioscience via VWR, International AS, Oslo, Norway
Foetal calf serum	Fisher Scientific, Pittsburgh, PA, USA
Collagenase-dispase (500 mg)	Vibrio algionolyticus/Bacillus polyxema, Roche Neuss, Germany
Pencillin/streptomycin/neomycin	Invitrogen, Carlsbad, CA, USA
AlamarBlue	Invitrogen, Carlsbad, CA, USA
Diethylpyrocarbonate	Sigma-Aldrich, Oslo, Norway AS
Lidocain	Haukeland Hospital Pharmacy, Bergen, Norway
Ketoprofen	Merial SAS, Lyon, France
Trypsin EDTA	Gibco Invitrogen, Carlsbad, CA, USA
Nitro blue tetrazolium	Sigma-Aldrich, Oslo, Norway AS
5-androstane-3 β -ol-one	Sigma-Aldrich, Oslo, Norway AS
NAD ⁺	Sigma-Aldrich, Oslo, Norway AS

7.2 Materials

Autoclaved materials needed for Leydig cell isolation

6 tea or steel filter
4 pinsettes
3 bottles (500 ml)
3 glass containers
2 big scissors
3 small scissors

7.3 Solutions and media

Solutions and media used in isolation and culture of Leydig cells

Collagenase/Dispase Stock

500 mg collagenase was dissolved in 10 ml of DMEM/F12. The collagenase stock solution (50 mg/ml) was divided into 1 ml aliquots in nuc cryo tubes and stored at -20°C.

Collection Medium

The medium was made from DMEM/F12 (500 ml) with 10 ml of added penicillin/streptomycin/neomycin (PSN). 5 bottles were made. 2 bottles (about 300 ml of media) were needed for the collection of testicles and were put in a box for the castration team.

Dissociation Medium

75 ml DMEM/F12 and PSN (10 ml PSN to 500 ml DMEM/F12) was put in a sterile bottle. 2 ml of the Collagenase stock was diluted with 8 ml of the DMEM/F12 and 5 ml of FCS was added. This solution was filtered through a 0.2 µm sterilized filter and added to the remaining DMEM/F12. The amount made is enough to digest about 30 testicles in a 500 ml bottle. The dissociation medium was kept at 4°C until use and before starting up the testicle decapsulation it was heated up to 34°C.

Complete Plating Medium

To 500 ml of DMEM/F12, 10 ml of PSN, 12.5 ml of NuSerum, and 5 ml ITS + Premix was added. The medium was stored at 4°C.

Percoll solutions

Percoll 90% isosmotic solution was made by mixing 13 ml F-10 Nutrient Mixture (Ham's) with 117 ml of undiluted Percoll. Four 75 cm² bottles (T75) (BD Falcon, New Jersey, USA) were marked from I-IV and with final density concentrations (see Table 4). To each tube the required amount of Percoll 90% and the required amount of DMEM/F12 was added. The Percoll dilutions were stored at 4°C before the layering of the Percoll solutions into a discontinuous gradient.

Table 4. Required amounts of Percoll 90% and DMEM/F12 for 12 gradients

Final Density (%)	Percoll 90% (ml)	DMEM/F12 (ml)
21%	24.5	82.1
26%	18.1	45.8
34%	40.5	66.1
60%	42.7	21.3
	= 125.8	= 215.3

Percoll gradient

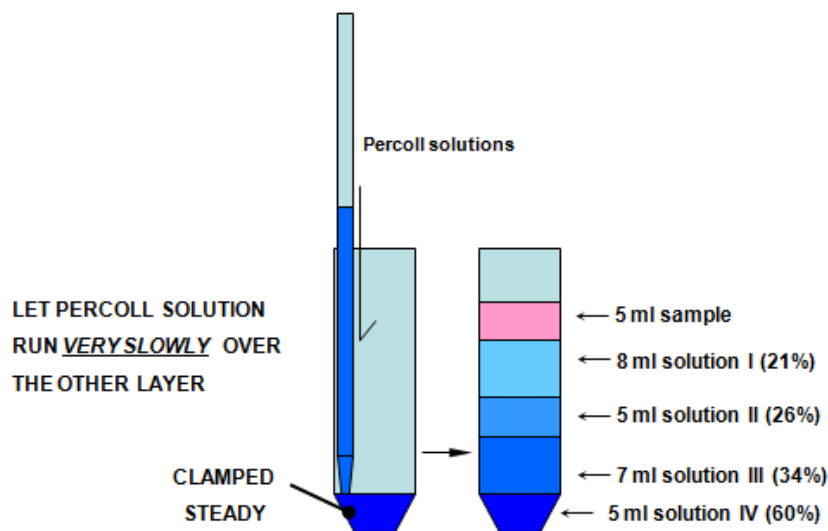


Figure 14. Layering of the Percoll solutions into a discontinuous gradient

The gradients were prepared on the day of isolation. One 50 ml Falcon tube is needed per gradient (about 8 testicles for one gradient). In the first step 5 ml of solution IV was added to each tube. Then 7 ml of solution III, 5 ml of solution II and 8 ml of solution I was added.

Solutions III-I were overlaid carefully on top on the previous layer by placing the pipette point at the meniscus. This created a liquid bridge. We emptied the pipette slowly through this liquid bridge and a clean layer formed on top of the previous one.