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

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Abstract

Although the persistent pesticide DDT has been banned or restricted since the 1970s, DDT and its metabolites may still pose a hazard to wildlife and humans. There is a growing concern regarding endocrine disruptors and their potential role in negative trends in male reproductive health. The aim of this study was to investigate the effects of two DDT metabolites, 3-MeSO$_2$-DDE and *o,p'*-DDD, and the synthetic DDE analogue 3,3'-(bis)MeSO$_2$-DDE on mammalian male reproductive system by evaluate the effect on testicular steroidogenesis in LH-stimulated neonatal porcine Leydig cells *in vitro*. The test compounds are known to disrupt steroidogenesis and cause toxicity in the adrenal cortex of several species, and *o,p'*-DDD is used in the treatment of adrenocortical cancer in humans. 3-MeSO$_2$-DDE has been suggested as an alternative drug and 3,3'-(bis)MeSO$_2$-DDE might reveal any structure-specific effects regarding methylsulfonyl moiety on the steroidogenesis. Testicles were obtained from routine castrations of neonatal piglets and Leydig cells were isolated and purified using a discontinuous Percoll gradient. The primary cultures of Leydig cells were stimulated with LH and exposed to the test compounds for 48 hours in six increasing concentrations (0-20 µM). Cell viability was assessed and the production of testosterone, estradiol, progesterone and cortisol was measured from the cell medium. Gene expression analysis was performed on 16 genes involved in testicular Leydig cell steroidogenesis. The results showed that both 3-MeSO$_2$-DDE and *o,p'*-DDD were cytotoxic at the highest concentration (20 µM), whereas 3,3'-(bis)MeSO$_2$-DDE had no effect on cell viability. All three test compounds decreased LH-stimulated production of testosterone, estradiol and progesterone by the Leydig cells in a concentration-dependent manner. No detectable levels of cortisol were obtained. The gene expression analysis suggested a general suppression in mRNA levels of several genes involved in steroidogenesis induced by all three test compounds. However, the compounds reduced expression of slightly different genes. The decreased expression of some genes involved in steroidogenesis might be associated with the reduced hormone production in exposed cells. This study suggests a steroidogenic disruption in porcine Leydig cells induced by the test compounds. The suppression of Leydig cell steroidogenesis by the two DDT metabolites might be of concern regarding negative effects on male reproduction.

Contents

Abbreviations

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
3βHSD	3β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
ACC	Adrenocortical carcinoma
AKRIC4	aldo-keto reductase family 1, member C4
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CT	Cycle threshold
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP11B1	11β-hydroxylase
CYP17A1	Cytochrome P450 c17
CYP19A1	Aromatase
CYP21	21-hydroxylase
CYP51	lanosterol 14α-demethylase
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
H295R	Human adrenocortical cell line
hCG	Human chorionic gonadotropin
HMGR	3-hydroxy-3-methyl-glutaryl-CoA reductase
LH	Lutenizing hormone
Ml	Milliliter
Ng	Nanogram

o,p'-DDD	1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane
PCR	polymerase chain reaction
Pg	Picogram
POPs	Persistent organic pollutants
Ppb	Parts per billion
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Reverse transcriptase
SF-1	Steroidogenic factor 1
StAR	Steroidogenic acute regulatory protein
TBT	Tributyltin chloride
Y-1	Mouse adrenocortical cell line

1. Introduction

In the present study the DDT metabolites 2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene (3-MeSO$_2$-DDE) and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) and the synthetic DDE analogue 2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethene (3,3'-(bis)MeSO$_2$-DDE) were investigated for possible endocrine effects on testicular Leydig cell steroidogenesis.

1.1 Endocrine disrupting chemicals

The release of persistent organic pollutants (POPs) into the environment has been ongoing for several decades mainly as a consequence of industrial by-products and agricultural productivity. Their persistency to degradation and lipophilic nature make them biomagnifies in food chains and reach high concentrations in adipose tissue of top predators. There is a growing concern regarding several POPs as they might be capable of modulating or disrupting the endocrine system both in wildlife and humans (Colborn et al., 1993; Tyler et al., 1998; Vos et al., 2000). Alteration of endocrine function caused by endocrine disruptors might include several mechanisms, such as interference with synthesis, secretion, action, or elimination of natural hormones in the body (Vos et al., 2000). As hormones play a key role in many vital functions, including sexual development and regulation of fertility, the endocrine disrupting chemicals might have adverse effects on reproduction and male fertility (Eertmans 2003). The endocrine disruptors might act as modulators of sex steroid synthesis by interacting with estrogen and/or androgen receptors to act as agonists or antagonists of hormones or by modulate the activity and/or expression of steroidogenic enzymes (Whitehead 2006).

1.2 DDT

One compound of concern is the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) which belongs to the group of halogenated organic compounds consisting of a backbone of hydrocarbons where hydrogen is substituted with chlorine to varying degrees. The use of DDT started in the 1940s and it revolutionized agricultural production and prevented people from being infected with insect-borne diseases, like malaria and typhus.

DDT is a mixture of several chemicals, but it mostly consist of the isomers *o,p'*-DDT and *p,p'*-DDT (Haller et al., 1945; Thomas et al., 2008). DDT is extremely hydrophobic, chemically very stable and is slowly degraded in nature and is mainly broken down to the persistent metabolites dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) in the environment (Jonsson et al., 1994) which may undergo further biotransformation. The persistency and lipophilic properties make DDT and its metabolites bioaccumulate in body fat and magnify through food chains and accumulate in higher mammals, also in arctic animals (Norstrom and Muir, 1994). DDT and its metabolites have shown weak estrogen and endocrine disruptor effect, causing deleterious effects on reproduction in wildlife (Brandt et al., 1998; Colborn et al., 1993; Guillette et al., 1994; Heath et al., 1969; Sharpe, 1995; Whitehead and Rice, 2006). In addition, the main metabolite *p,p'*-DDE is a potent anti-androgen (Kelce et al., 1995). Even though it has been banned or restricted many places since the 1970s, DDT is still used in large quantities in countries for malaria control (Dua et al., 1996; Longnecker, 2005; van den Berg, 2009). Due to the persistency in the environment and the long-distance atmospheric transportation (Wang et al., 2010) the levels of these persistent organic pollutants are long lasting and may still pose a hazard to wildlife and humans (Crinnion, 2009).

1.3 DDT metabolites and adrenal toxicity

In addition to be potential endocrine disruptors causing reproductive impairment, some DDT metabolites, like 3-$MeSO_2$-DDE and *o,p'*-DDD inhibit steroidogenesis and are toxic to the adrenal cortex of several species (Asp et al., 2009; Brandt et al., 1992; Jonsson et al., 1994). DDT metabolite *o,p'*-DDD is a well known drug in the treatment of adrenocortical carcinoma (ACC) in humans, but due to low efficiency and negative side effects there is a need for treatment alternatives. The mechanisms by which *o,p'*-DDD cause adrenal toxicity is not fully understood, but it might interfere with several steroidogenic enzymes, including cholesterol side-chain cleavage enzyme (CYP11A1) and11ß-hydroxylase (CYP11B1)(Hart and Straw, 1971; Martz and Straw, 1980). The main DDT metabolite *p,p'*-DDE may be transformed to 3-$MeSO_2$-DDE in mammals and 3-$MeSO_2$-DDE undergoes bioactivation by CYP11B1 and cause toxic effects in the adrenal cortex of several species (Brandt et al., 1992; Lindstrom et al., 2008; Lund et al., 1988). Therefore, 3-$MeSO_2$-DDE has been proposed as an alternative drug for treatment of ACC in humans (Lindhe et al., 2002). With major species differences

with regard to adrenocortical toxicity and CYP-dependent covalent binding of both 3-MeSO$_2$-DDE and *o,p'*-DDD (Brandt et al., 1992) further knowledge about these compounds is needed. As the adrenocortical effects of 3-MeSO$_2$-DDE have shown to be structure-specific, synthetic DDE analogue 3,3'-(bis)MeSO$_2$-DDE might reveal any structural requirements for steroidogenic effects depending on the methylsulfonyl moiety. Both adrenal glands and gonads are potential targets for endocrine disruption as adrenal cells and Leydig cells possess steroidogenic activity. Whereas adrenal cortex mainly produces corticosteroids, the hormone producing Leydig cells is involved in androgen and estrogen biosynthesis. The difference is the specific expression of CYP11B1 and 21-hydroxylase (CYP21) involved in the synthesis of cortisol and corticosterone in the adrenal cortex. However, the difference might be limited as testis of mice and humans express these enzymes (Hu et al., 2007; Pezzi et al., 2003) making Leydig cells a good model for studying endocrine effects of the adrenal toxicants.

1.4 Neonatal porcine Leydig cells

The endocrine changes of hormones regulate testicular function during development and the pig testes have a remarkable steroid production (Raeside et al., 2006). The testes are divided into the vascularized interstitial tissue including Leydig cells, macrophages, lymphatic vessels and connective tissue, and the seminoferous tubules which contain Sertoli and germ cells (Gnessi et al., 1997) (Figure 1.1). Leydig cells are found adjacent to the seminoferous tubules and are the steroid hormone producing cells in the interstitial tissue (van Straaten and Wensing, 1978). Leydig cells are stimulated by the pituitary hormone luteinizing hormone (LH) which is the main physiological hormonal stimulator of testosterone biosynthesis by Leydig cells (Colenbrander et al., 1977; Colenbrander and Van Straaten, 1977). The testicular steroidogenesis is dependent on both acute and chronic stimulating effects of gonadotropin LH, including transport and conversion of cholesterol to pregnenolone via the cyclic adenosine monophosphate (cAMP)-pathway and increase of steroidogenic enzyme activity (Lejeune et al., 1998a; Mather et al., 1981). *In vitro* studies confirm the stimulating effect of LH on porcine Leydig cell steroidogenesis (Bernier et al., 1983; Geiger et al., 1999; Lejeune et al., 1998b)
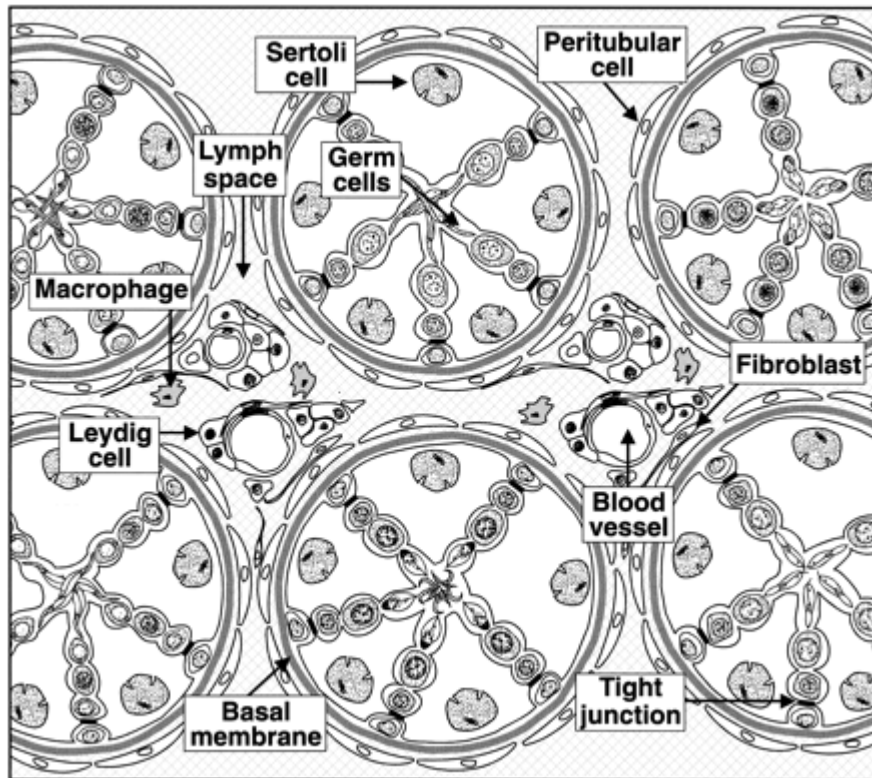
Figure 1.1: Schematic overview over the mammalian testes (rat) and the different compartments, including Leydig cells (from Gnessi et al. 1997)

Pig Leydig cell development occurs in three distinct waves, with the second perinatal wave of development being additional compared to other mammals, only human testes have a similar thriphasic pattern (Prince, 2001; Raeside et al., 2006; van Straaten and Wensing, 1978). The first developmental phase of porcine Leydig cells occurs during fetal life (van Vorstenbosch et al., 1984). The second, perinatal wave of Leydig cell development is between approximately 90 days of gestation to about three weeks after birth when a large volume of well differentiated Leydig cells develops, with active steroid synthesis and secretion in the first weeks after birth (Colenbrander et al., 1978; van Straaten and Wensing, 1978). In the neonatal pig, the majority of the testicular volume, and the percentage of cells, are Leydig cells (van Straaten and Wensing, 1977). During the perinatal phase of development there is a rise in LH and testosterone levels (Colenbrander et al., 1978; Colenbrander et al., 1977). The third wave of development starts just prior to puberty at 13 weeks after birth (van Straaten and Wensing, 1978). Pig testes also produce large amounts of estrogens, and although concentrations of androgens are higher, estrogen concentrations peak during neonatal development in male pigs (Ford, 1983; Raeside et al., 1989). Altogether, neonatal porcine

Leydig cells have an active steroidogenesis with high levels of testosterone and estradiol during the neonatal period associated with highly assessable LH stimuli (Colenbrander and Van Straaten, 1977; Schwarzenberger et al., 1993; Wagner and Claus, 2008). The steroidogenic activity and hormone secretion of neonatal pig Leydig cells is maintained in culture (Mather et al., 1981).

1.5 Aim of Study

DDT and its metabolites DDE and DDD are persistent organic pollutants in the environment and may pose a hazard to wildlife and humans, including their endocrine effect on the male reproductive system. In the present study the effect of the DDT metabolites $3\text{-MeSO}_2\text{-DDE}$ and o,p'-DDD and the synthetic DDE analogue $3,3'\text{-(bis)MeSO}_2\text{-DDE}$ was investigated on the testicular Leydig cell steroidogenesis *in vitro*. Neonatal porcine Leydig cells possess full testicular steroidogenesis which is maintained in culture and stimulated by LH. The compounds investigated in the present study have previously been demonstrated to cause endocrine disrupting effects in the adrenal cortex and are involved in research for improvement of adrenal cancer medicine. In addition to screen for potential endocrine effects on male reproductive function, further knowledge about the compounds steroidogenic effect might contribute to improved therapy of ACC in humans. The main aim was to clarify possible endocrine disrupting effects of $3\text{-MeSO}_2\text{-DDE}$, $3,3'\text{-(bis)MeSO}_2\text{-DDE}$ and o,p'-DDD on mammalian male reproductive system by evaluate the effect on testicular steroidogenesis in LH-stimulated neonatal porcine Leydig cells. In order to evaluate the compounds potential effect on testicular steroidogenesis, neonatal Leydig cells was obtained from pig testicles to

- Quantify the toxicants effect on cell viability
- Quantify the effect on production of testosterone, estradiol, progesterone and cortisol by the Leydig cells
- Quantify the effect on expression of 16 genes coding for enzymes involved in Leydig cell steroidogenesis

Parallel with the study presented here, exposure to the test compounds was performed on unstimulated (without LH) porcine Leydig cells.

1.6 Background

1.6.1 Test compounds

3-MeSO$_2$-DDE

The DDE metabolite 3-MeSO$_2$-DDE (Figure 1.2) was first discovered in the blubber of Baltic grey seals, and has been isolated from several tissues of mammals (Bergman et al., 1994; Jensen and Jansson, 1976; Letcher et al., 1995). DDE is slowly transformed to 3-MeSO$_2$-DDE in mammals by glutathione conjugation (Preston et al., 1984) and further metabolism in the mercapturic acid pathway (Bakke et al., 1982; Brandt et al., 1992). Due to its persistency and lipophilic properties, 3-MeSO$_2$-DDE is found in parts per billion (ppb) amounts in human tissues like liver and adipose tissue, and in breast milk (Chu et al., 2003; Noren and Meironyte, 2000; Weistrand and Noren, 1997). The endocrine disrupting effects of 3-MeSO$_2$-DDE have been reported in adrenal cortex of several species, mice being most sensitive (Lindstrom et al., 2008). 3-MeSO$_2$-DDE undergoes bioactivation to a reactive intermediate by the steroidogenic enzyme CYP11B1 and cause formation of irreversible bound protein adducts, cell death and decreased corticosterone production in the adrenal cortex of mice *in vivo* (Jonsson et al., 1991; Lund et al., 1988; Lund and Lund, 1995). Toxicity and irreversible binding of 3-MeSO$_2$-DDE have also been reported *in vivo* in chicken (Jonsson et al., 1994). 3-MeSO$_2$-DDE cause cytotoxicity and decreased corticosterone production in mouse adrenal cells *in vitro* (Asp et al., 2009). In addition, the *in vitro* bioactivation of 3-MeSO$_2$-DDE in the human adrenal gland suggests that it might be an adrenal toxicant in humans (Jonsson and Lund, 1994). Hence, 3-MeSO$_2$-DDE has been proposed as an alternative drug for treatment of ACC and Cushing's syndrome; an overproduction of glucocorticoids due to a pituitary tumor (Lindhe et al. 2002).

3,3'-(bis)MeSO$_2$-DDE

3,3'-(bis)MeSO$_2$-DDE (Figure 1.2) is a synthetic and structural related compound to 3-MeSO$_2$-DDE, but no toxicity or irreversible binding of 3,3'-(bis)MeSO$_2$-DDE is reported in mice adrenal cortex *in vivo* (Lund et al., 1988). However, the compound decreased corticosterone production and produced CYP11B1-dependent cytotoxicity in mice

adrenocortical cells *in vitro*, although less potent than 3-MeSO$_2$-DDE (Asp et al., 2009). The bioactivation and toxicity of 3-MeSO$_2$-DDE in the adrenal cortex is demonstrated to be structure-specific and dependent on the methylsulfonyl group (Asp et al., 2009; Hermansson et al., 2007; Lund and Lund, 1995). Therefore, 3,3'-(bis)MeSO$_2$-DDE might elucidate any structural requirements of the test compounds effect on Leydig cell steroidogenesis.

o,p'-DDD

The DDD isomer *o,p'*-DDD (Figure 1.2) was first observed to be selectively toxic to the adrenal cortex of dogs, with bioactivation of the side chain to a reactive acyl chloride that induces cell death, decreases glucocorticoid synthesis and produces irreversibly bound protein adducts (Hart et al., 1973; Martz and Straw, 1977; Martz and Straw, 1980; Nelson and Woodard, 1949). The adrenocortical toxic effects of *o,p'*-DDD has also been reported *in vivo* in chicken (Jonsson et al., 1994) and *o,p'*-DDD binds to mouse lung tissue both *in vivo* and *in vitro* (Lund et al., 1989; Lund et al., 1986). *o,p'*-DDD is a well known adrenocorticolytic compound which is used to treat ACC and Cushing's syndrome in humans (Ahlman et al., 2001; Newell-Price et al., 2006) and has been proposed to interact with several steroidogenic enzymes, including CYP11A1 and CYP11B1(Asp et al., 2009; Martz and Straw, 1980). However, the enzyme(s) responsible for bioactivation and the mechanism by which *o,p'*-DDD block glucocorticoid synthesis in the adrenal cortex is still unknown (Ahlman et al., 2001; Cai et al., 1997; Hart et al., 1973; Stigliano et al., 2008). Not all ACC patients respond to *o,p'*-DDD treatment and it also produces gastrointestinal and neurotoxic side effects (Ahlman et al., 2001; Allolio and Fassnacht, 2006). Therefore, treatment alternatives are needed and 3-MeSO$_2$-DDE might be an alternative drug.
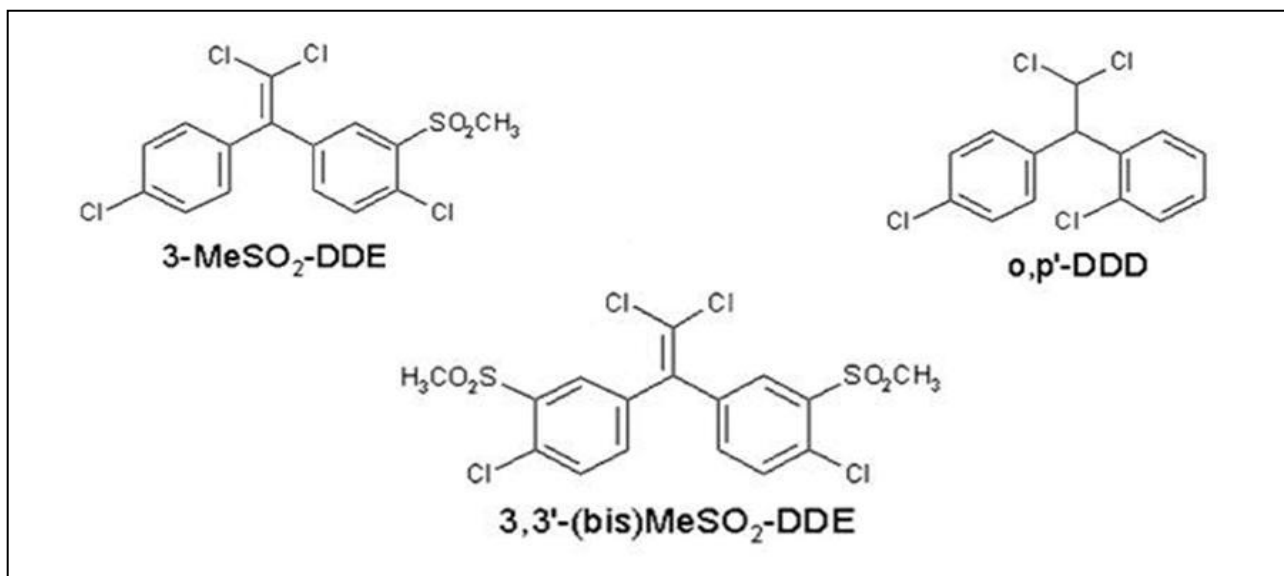
Figure 1.2: Chemical structures of the test compounds used in this study (from Asp et al. 2009).

1.6.2 Steroidogenesis in porcine Leydig cells

The active steroidogenesis and significantly production of steroid hormones by Leydig cells during the neonatal period involves the activity of numerous enzymes in a rather complex pathway (Figure 1.3). Steroidogenic acute regulatory protein (StAR) is involved in transporting cholesterol from the outer to the inner mitochondrial membrane as the first rate-limiting step of steroidogenesis (Christenson and Strauss, 2000; Privalle et al., 1983; Stocco, 2001). Further, CYP11A1 initiate the steroid synthesis by converting cholesterol to pregnenolone (Miller, 1995). The biosynthesis of testosterone and estradiol can then be carried out through two different pathways; Δ4 pathway via progesterone and 17α-hydroxypreogesterone, or by Δ5 pathway via 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) (Conley and Bird, 1997). The Δ5 pathway is predominant in testes of pigs and humans (Ruokonen and Vihko, 1974) which make pig testes a good model for testicular steroidogenesis. 3β-hydroxysteroid dehydrogenase (3β-HSD)(Clark et al., 1996) and cytochrome P450 c17 (CYP17A1)(Sasano et al., 1989) are both involved in the Δ5 pathway, and CYP17A1 catalyzes both 17α-hydroxylase and 17,20 lyase activity (Nakajin et al., 1981) to convert pregnenolone into testosterone and estradiol precursors in neonatal pig testes. Together with cytochrome b5 (CYB5), CYP17A1 make up the andien-β synthase enzyme system which catalyze the metabolism of pregnenolone to androstadienol (Meadus et

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al., 1993; Nakajin et al., 1985). In addition, the interaction between CYP17A1 and CYB5 is important to the 17,20 lyase activity (Hall, 1991). The last step in formation of testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes, which is a large family of enzymes with several forms (Inano et al., 1981). One of them, 17β-HSD4 is involved in inactivation of estradiol to estrone (Adamski et al., 1992; Adamski et al., 1995) whereas 17β-HSD1 catalyze the reverse reaction. The synthesis of estrogens from androgens is catalyzed by the enzyme aromatase (CYP19A1) (Conley et al., 1996). One of the enzymes belonging to the cytosolic aldo-keto reductases (AKR1C4) have been reported to convert active androgens and estrogens into their associated inactive metabolites (Penning et al., 2000).

HMG-CoA reductase (HMGR) and lanosterol 14α-demethylase (CYP51) are important enzymes involved in the cholesterol biosynthesis pathway (Debeljak et al., 2003; Rodwell et al., 1976). Ferritin light polypeptide (FTL) is part of ferritin, an iron storage protein which plays a central role in numerous essential cellular functions (Hentze and Kuhn, 1996). The transcription factor steroidogenic factor 1 (SF-1; coded by NR5A1) is involved in regulation of several steroidogenic genes, including *StAR*, *CYP11A1*, *CYP17A1* and *CYP19A1* (Moe et al., 2007; Sadovsky and Dorn, 2000; Sugawara et al., 1996). The activity of SF-1 might be counteracted by another transcription factor, the dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1; coded by NR0B1) (Ikeda et al., 1996; Yu and Li, 2006).

The steroidogenic output is similar in steroidogenic cells of the adrenal cortex and the testicular Leydig cells. They might share a common origin (Hatano et al., 1996) and adrenal-like cells might migrate into the testes during development (Val et al., 2006). The difference is the specific expression of CYP11B1 and CYP21 involved in the synthesis of cortisol and corticosterone in the adrenal cortex. However, the expression of *CYP11B1* and *CYP21* have been reported in fetal testes of mice (Hu et al., 2007) and humans (Pezzi et al., 2003). In addition, the expression of CYP11B1 is found in rat Leydig cells (Wang et al., 2002).
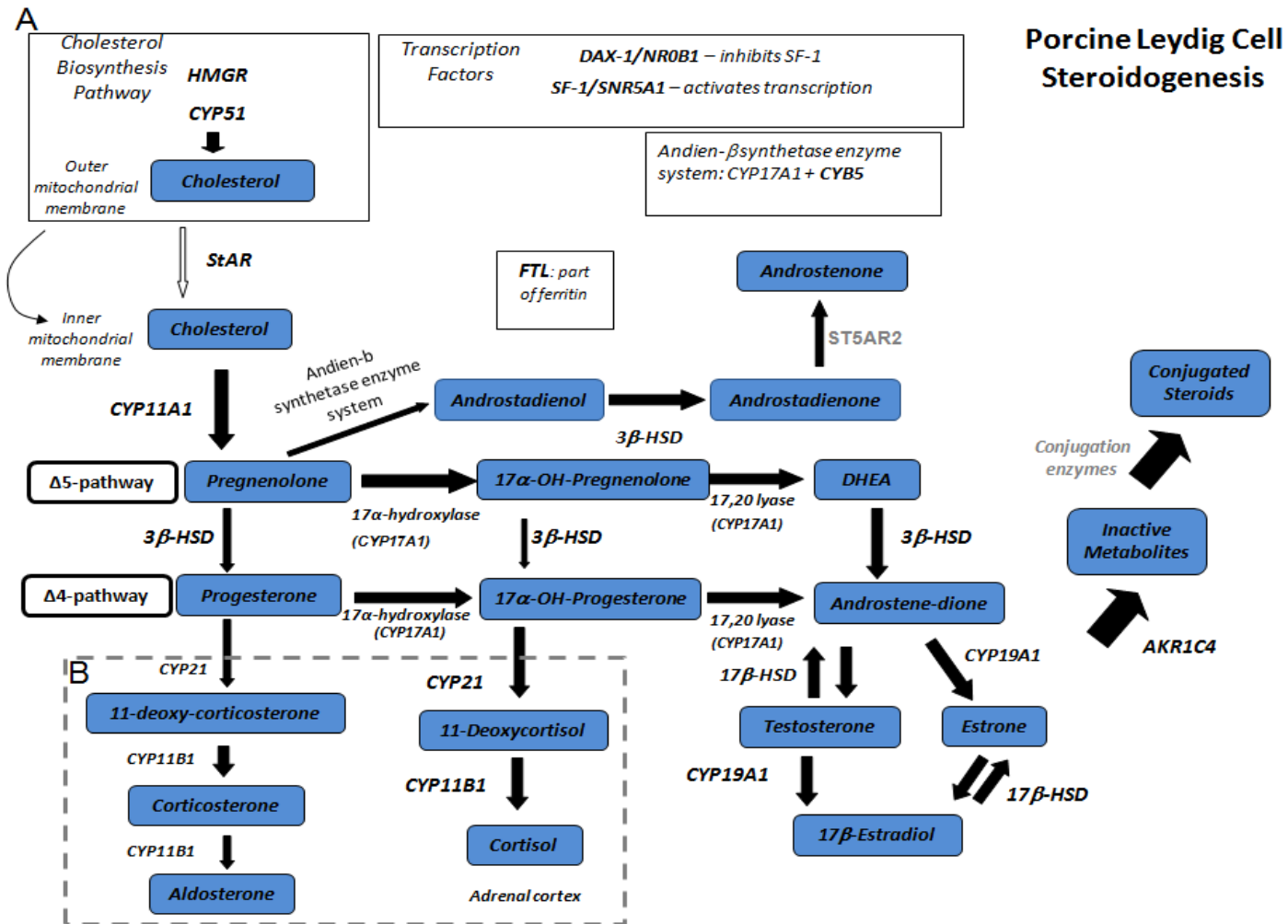
Figure 1.3: Scematic overwiev of some of the enzymes and steroid hormones involved in the steroidogenic pathway of porcine Leydig cells (A). Pathway B presents part of corticosteroid synthesis in adrenal cortex. Refer to text for further details.

2. Materials and Methods

For a detailed list of chemicals, solutions and media see appendix 8.1 and 8.2. For the preparation of discontinuous Percoll gradient see appendix 8.3.

2.1 Chemicals

The compounds 3-MeSO$_2$-DDE (2-(3-methylsulfonyl-4-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene) and 3,3'-(bis)MeSO$_2$-DDE (2,2'-bis(3-methylsulfonyl-4-chlorophenyl)-1,1-dichloroethen) purity >99% were synthesized by Synthelec AB (Ideon, Lund, Sweden). *o,p'*-DDD (1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane) purity >99% was obtained from Sigma-Aldrich, Stockholm, Sweden.

2.2 Isolation and culture of porcine Leydig cells

The isolation and culture of porcine Leydig cells were obtained by the discontinuous Percoll gradient method (Bernier et al., 1983; Lejeune et al., 1998b), using a protocol authored by Dr. Steven Verhaegen, Norwegian School of Veterinary Science. By using differential centrifugation on discontinuous Percoll gradients the isolation of purified populations of Leydig cells is obtained. These primary Leydig cells maintain morphological and functional integrity and provide an *in vitro* model to investigate factors regulating testicular steroidogenesis. Due to time limits and the difficulties in predicting the amount of Leydig cells obtained at the different isolations, cell viability and hormone quantification is assessed on different cells. For cell viability assay two independent Leydig cell isolations/experiments were carried out, whereas three isolations were performed for hormone quantification and gene expression analysis.

2.2.1 Procedure for Leydig cell isolation

Testis from neonatal 8-12 days old Landrace piglets (*Suidae, Sus scrofa*) were obtained from routine castrations at the pig breeder farm Bøhnsdalen in Oslo. The castrations were performed by authorized veterinarians from the Norwegian School of Veterinary Science the same morning as the Leydig cells were isolated by standard surgical procedure for castration

of male piglets. Local anaesthesia was provided by injecting 0.5 ml 1% lidocain without adrenaline subcutaneously on both sides of scrotum and in each spermatic cord. The skin was washed and disinfected with 70% ethanol. After the surgical removal the testicles were immediately put in collection medium (see Appendix 8.2) in bottles and kept on ice (4°C). Immediately after the castration each piglet was administered 30 mg ketoprofen intramuscular for analgesia. Maximum time lapse from first gonad harvest until cell isolation started did not exceed 3 hours. The tissue dissociation for the neonatal boars was performed in cell culture lab in a laminar flow hood in sterile fashion. 500 ml glass flask (1 flask per 30 testicles) was preheated at 34°C. All testes were collected in petri dishes (VWR International AS, Oslo, Norway); about 15 in each dish, in collection medium (see Appendix 8.2). With sterile scissors and pinsets epididymis was removed and in a new petri dish each testis was decapsulated with flat tweezers and scissors and the parenchyma was scraped off. The decapsulated testes were collected in petri dishes with collection medium and the tissue were finely minced with scissors before pipetting into 50 ml Falcon tubes (BD Falcon, New Jersey, USA). The pellet was left to sediment and washed several times with collection medium to wash out blood until a clear supernatant was obtained. Dissociation buffer (see Appendix 8.2) was added to the 500 ml preheated bottles and the tissue suspension above was added and placed in a shaking water bath at 34°C. The extracellular matrix in the tissue was broken down during agitation. Digested tissue was collected after 45, 90 and 120 minutes (collection 1, 2 and 3 respectively). After 45 minutes about half of the solution was decanted through a sterile metal filter into a clean beaker. The decanted volume was replaced with fresh collection medium and incubated further. The cell suspension was pipetted into 50 ml tubes and centrifuged at 1080 revolutions per minute (RPM) for 10 minutes. The supernatant was discarded and pellet resuspended in 50 ml collection medium to sediment for 5 minutes at unit gravity. Supernatant were transferred to new 50 ml tube and then sediment for 15 minutes before transferring to new 50 ml tubes and centrifuge at 1080 RPM for 10 minutes. The final pellet was resuspended in 5 ml collection medium and kept in fridge temporarily at 4°C (=collection 1). After about 90 minutes new half of the solution was decanted in the dissociation buffer and repeated all steps (=collection 2) and after 120 minutes again with the rest of the dissociation buffer solution (=collection 3). Then the three collected supernatants were pooled together and divided over the required number of Percoll gradients (5 ml sample to each gradient).

2.2.2 Purification of Leydig cells

A discontinuous Percoll gradient was used to separate out the Leydig cells from the rest of the cells in the suspension made above. Discontinuous Percoll gradients were prepared as described in Appendix 8.3 the morning of the isolation. 5 ml of the pooled samples was applied carefully and slowly on top of each gradient. The gradients were centrifuged at 30 minutes (4°C) at 2140 RPM. Then we aspirated the top layers with a pipette and with a new one recovered the Percoll 34% layer which should contain the Leydig cells (Figure 2.1). The collected Percoll fraction (5 ml) was diluted with collection medium at least two times and centrifuged at 1080 RPM for 20 minutes. The supernatant was discarded and pellet resuspended in collection medium (20 ml for about 60 testicles) and filtered through a polyester mesh into another 50 ml tube. The cells were counted in a Bürker haematocytometer (Superior, Marienfeld, Germany).
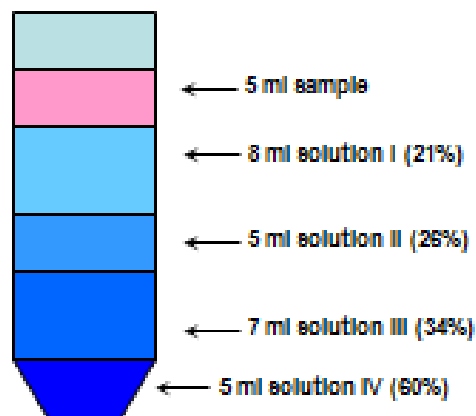
Figure 2.1: The arrows indicate different solutions and final densities in a Percoll gradient. The Percoll 34% fraction contains the purified Leydig cells.

2.2.3 Leydig cell plating

For hormone readouts and RNA extraction the cells were plated out in 24-well cell culture plates (Falcon Primaria, VWR International AS, Oslo, Norway), while plated in 96-well plates (VWR, Norway) for the cell viability test. For the Leydig cell plating we diluted cells at concentration 10^6 cells/ml with complete plating medium (see Appendix 8.2), the cell suspension was adjusted to 300 000 cells/ml. We added 1 ml of this suspension to each well of 24-well cell culture plates and 100 µl were plated out in each well in 96-well plates. Cells

were incubated for 72 hours prior to exposure, to allow the cells to form a monolayer, in a humidified incubator at 34°C and 5% CO_2.

2.4 3β-hydroxysteroid dehydrogenase staining method

The purity of Leydig cells was assessed by histochemical 3β-hydroxysteroid dehydrogenase (3β-HSD) staining were the Leydig cells turn blue by reduction of tetrazolium blue dye (Huang et al., 2001; Levy et al., 1959). The 3β-HSD staining method was performed on two independent Leydig cell isolations to validate the method for isolation and purification of porcine Leydig cells. The purified Leydig cells were seeded out in 6-well plates (Falcon primaria, Becton Dickinson Labware, NJ USA) with complete plating medium and incubated for 72 hours at 37°C and 5% CO_2. The complete plating medium was removed, the cells were washed with PBS 0.15 M pH 7.4 and 500 µl of trypsin was added to each well to let the cells detach. After two minutes trypsin was removed and 5 ml DMEM/F12 including 10% FCS was added to inactivate trypsin. The cells were transferred into a tube and centrifuged at 1500 RPM for five minutes. The supernatant was discarded and pellet resuspended and incubated with 2 ml solution containing 0.2 mg/ml nitro blue tetrazolium, 0.12 mg/ml 5-androstane-3β-ol-one and 1 mg/ml NAD^+ in 0.05 M phosphate buffered saline (PBS), pH 7.4 at 37°C in a waterbath for 90 minutes. Blue formazan deposit sites of 3β-HSD activity developed and we could determine the abundance of Leydig cells by using a haematocytometer.

2.5 In vitro exposure of cells

After 72 hours incubation the Leydig cells were exposed to different concentrations of 3-$MeSO_2$-DDE, 3,3'-(bis)$MeSO_2$-DDE and o,p'-DDD and stimulated with porcine LH. Before the exposure, the medium was refreshed with 1 ml complete plating medium per well in 24-well plates and 100 µl per well in 96-well plates. Stock solutions of the compounds were prepared to 20 mM/ml in DMSO, divided into 50 µl aliquots and stored at -20°C until use. The cells were exposed to the different compounds at concentrations 0, 0.625, 1.25, 2.5, 5, 10 and 20 µM finals solved in DMSO for 48 hours. The exposure concentrations used were decided from a study done by (Asp et al. 2009). Solvent control with 0.1% DMSO final concentration and medium blank with complete plating medium and LH only were included

(Figure 2.2). Immunochemical grade porcine LH was reconstituted in M199 modified Earle's salts, without L-glutamine, sodium bicarbonate and phenol red (Sigma-Aldrich) at 25 ng/ml and stored at -80°C. This working stocks of 25 ng/ml were prepared by further diluting into complete DMEM/F12 and aliquots stored at -20°C. Aliquotes were thawed before use and not refrozen. To each well containing 1 ml of medium, 20 µl LH (25 ng/ml) was added, giving a final concentration of 0.5 ng/ml. For the cell viability assay, 2 µl LH was added to each well in 96-well plates. After 48 hours exposure to the test compounds in a humidified incubator at 34°C and 5% CO_2, the medium in 24-well plates was collected and stored at -80°C for hormone analysis. Plates with cells were stored at -80°C until harvest for RNA extraction. Each exposure was performed in triplicates and carried out as three independent Leydig cell isolations.

In this same study another master student, Irene Sørvik, exposed Leydig cells without LH-stimulation.

Medium +LH	Medium +LH	Medium +LH	0.1% DMSO + LH	0.1% DMSO + LH	0.1% DMSO + LH
Compound 20µM + LH	Compound 10µM + LH	Compound 5µM + LH	Compound 2.5µM + LH	Compound 1.25µM +LH	Compound 0.625µM+LH
Compound 20µM + LH	Compound 10µM + LH	Compound 5µM + LH	Compound 2.5µM + LH	Compound 1.25µM +LH	Compound 0.625µM+LH
Compound 20µM + LH	Compound 10µM + LH	Compound 5µM + LH	Compound 2.5µM + LH	Compound 1.25µM +LH	Compound 0.625µM+LH

Figure 2.2: Setup of exposure to the test compounds in a 24-well plate for hormone analysis and RNA extraction.

2.6 Cell viability assay

Cell viability was estimated using the AlamarBlue Assay (Invitrogen) which incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. The amount of fluorescence produced is proportional to the number of living cells and the more viable the cells are the more capable they are of transforming AlamarBlue fluorescent end products. After 48 hours exposure to the test compounds, the medium was replaced with 100 µl fresh complete plating medium which contained 10% AlamarBlue in each well of the 96-well plates to assess cell viability. The cells were incubated for three hours at 34°C and 5% CO_2. Then a 100 µl sample was collected from each well into a transparent 96-well plate (Falcon, Franklin Lakes, NJ). The absorbance was measured at 570 nm and 600 nm wavelength in a spectrophotometer Victor[3] 1420 Multilabel plate reader (Perkin Elmer, Shelton, CT, USA). In addition, cells in the 24-well plates were treated with AlamarBlue for one hour. This is due to the original plan to incubate the cells in 24-well plates with AlamarBlue prior to hormone quantification, but one hour was not sufficient and 96-well plates was used for the cell viability test. For all cells to be treated equally, all 24-well plates were still treated for one hour with 10% AlamarBlue. Due to time limits, cells used in viability measurements were obtained from two different isolations, with three replicates/plates from the first isolation. Each replicate was performed in triplicates.

2.7 Hormone analysis

Cell medium levels of testosterone, estradiol and cortisol were measured using Coat-A-Count® solid phase radioimmunoassay kits (RIA) (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Samples were measured in duplicate and standards were prepared in complete plating medium. Hormone levels were measured in a Wallac1470 Wizard (Perkin Elmer). The sensitivity of the testosterone assay was 0.1 ng/ml, corresponding to 95% binding of the labeled hormone. The standard curve ranged from 0-20 ng/ml. The interassay variation coefficients were 11.7% (2.4 ng/ml) and 14.1% (9.5 ng/ml) respectively. The sensitivity of the estradiol assay was 9 pg/ml and the standard curve ranged from 0-4000 pg/ml. Interassay variation coefficient were 6.7% (650.8 pg/ml) and 10.6% (1744.5 pg/ml) respectively. The cortisol assay sensitivity was 3 ng/ml and the standard curve range 0-500 ng/ml. The interassay variation coefficients were 9.8% (57.7 ng/ml) and 7.3% (210.2 ng/ml).

Progesterone concentrations were analyzed by a Spectria Progesterone RIA kit (Orion Diagnostica, Espoo, Finland). The standard curve ranged from 0-40 ng/ml and the assay sensitivity was 0.08 ng/ml. Interassay variation was 6.5% (3.87 ng/ml) and 6.1% (12.8 ng/ml) respectively.

2.8 Gene expression analysis

2.8.1 RNA isolation

For the isolation of total RNA we used RNeasy Mini Kit (Qiagen Ltd, Crawley, UK) according to manufacturer's protocol. Briefly, the frozen Leydig cells were lysed by adding 200 µl RLT Lysis Buffer (Qiagen) directly to each well of the cultured plates. Cells were detached with pipette tips (1000 µl). Triplicates from each test concentration were pooled and transferred to a QIA shredder spin column (Qiagen), placed in a 2 ml collection tube, and centrifuged at 13000 RPM for 2 minutes. Samples were treated with 80 µl DNase I mixture (10 µl DNase and 70 µl RDD buffer) (Qiagen) for 10 minutes at room temperature. The samples were eluated in 55 µl RNase Free Water (Qiagen) and stored at -75°C until use. 5 µl of each samples was used for the RNA quality check and quantitative measurements, whereas 50 µl was for the real-time RT-PCR.

2.8.2 RNA quantity and quality

RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) with 1 µl of the eluted sample. The RNA used in the gene expression analysis was isolated from control cells (DMSO) and the cells exposed to the next highest concentration of the test compounds, 10 µM. The mean RNA concentrations isolated and purified ranged between 70.3 – 171.4 ng/µl of RNA for DMSO samples and 72.2 – 148.8 ng/µl of RNA for 10 µM samples. To examine the quality of purified RNA the Agilent 2100 Bioanalyzer (Agilent Technologies Inc, California, USA) was used. Because of the instability of RNA, it is important to check the integrity and degradation of RNA before further analysis. The 2100 bioanalyzer expert software generated the RNA Integrity Number (RIN), ratios of ribosomal RNAs (28S/18S). RNA 6000 Nano LabChip®Kit (Agilent Technologies Inc) was used and the assay guide followed according to manufacturer's instructions.

Results from the bioanalyzer indicated that all samples were of high quality and the mean 28S:18S ribosomal RNA ratio was 1.8 and the mean RIN number was 9.1. The electropherogram showed no degradation or contamination of the RNA samples. An electropherogram with RNA of good quality from Leydig cell sample exposed to *o,p'*-DDD is presented in figure 2.3.
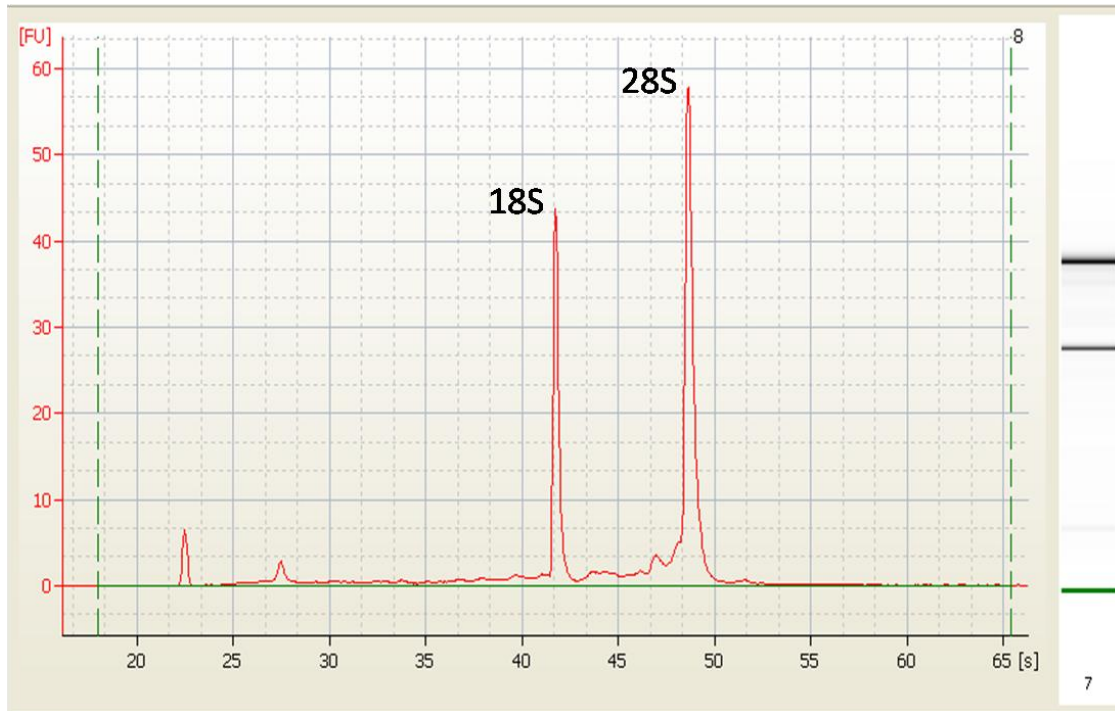
Figure 2.3: An electropherogram of a RNA sample from exposed Leydig cells by using Agilent 2100 Bioanalyzer. The integrity time (seconds) at x axis and the fluorescence (FU; Fluorescence Unit) is represented at y axis. The ribosomal RNA peaks, 18S and 28S, are indicated in the diagram. Gel-image of RNA products is shown to the right.

2.8.3 Reference genes

For the real-time RT-PCR assays 6 housekeeping genes (*ACTB*, *PPIA*, *GAPDH*, *HPRT*, *PGK1*, *S18*) were tested for suitability as reference genes. Using geNorm software analysis (PrimerDesign Ltd, Southampton, UK) the expression stability of these genes was tested. The two most stable genes, *ACTB* (β-actin) and *PPIA* (Cyclophilin A), had M-values at 0.27 and were selected as reference genes in this experiment (Figure 2.4).
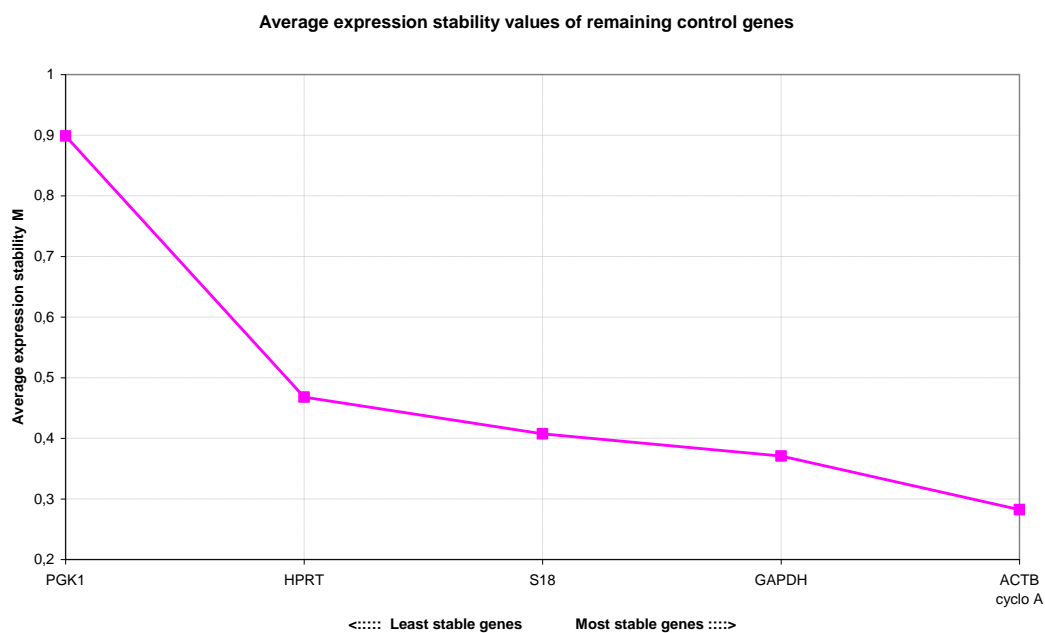
Figure 2.4: Average expression stability values (M-value) of the 6 reference genes tested. *ACTB* and *PPIA* (cyclo A) was selected as reference genes (geNorm).

The expression of reference genes, *ACTB* and *PPIA*, were reasonable stable in the different samples which indicates no effect of the test compounds on expression of the housekeeping genes used (Figure 2.5).
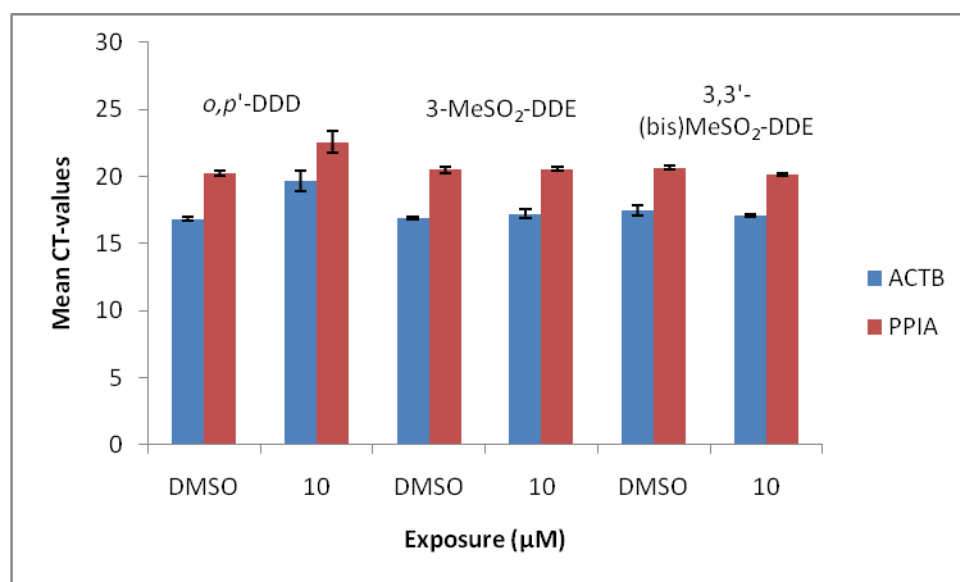
Figure 2.5: Variation in CT-values (mean ± SE) for the reference genes *ACTB* and *PPIA* in the different samples from three experiments.

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2.8.4 Primer design

The genes selected for real-time RT-PCR analysis are involved in the steroidogenesis in Leydig cells and are summarized in Table 2.1. Porcine primers were already in house and had been designed prior to this study. Porcine primers for the reference genes were derived from (Duvigneau et al., 2005) (*ACTB, HPRT, PPIA* and *GAPDH*) or designed using PrimerExpress version 1.5 (Applied Biosystems, Foster City, CA, USA; *PGK1* and *S18*). Primer sequences for the chosen genes; *HMGR, CYP51, StAR, CYP11A1, CYP17A1, HSD3B, CYP19A1, HSD17B1, HSD17B4, CYP21, CYP11B1, CYB5, FTL, AKR1C4, NR5A1* and *NR0B1* were also designed using PrimerExpress version 1.5 (Applied Biosystems). The primers were checked for specificity using nucleotide BLAST and primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2.1. Real-time RT-PCR primer sequences for the analyzed genes. *ACTB* and *PPIA* were used as reference genes.

Gene abbrevation	Gene name	Forward primer 5'- 3'	Reverese primer 5'- 3'
ACTB	Cytoskeletal beta actin	CTCGATCATGAAGTGCGACGT	GTGATCTCCTTCTGCATCCTGTC
PPIA	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	GGAGCACTTTGAGGCCTGG	TTTTCAGCGCCTCCTTGTG
CYP19A1	cytochrome P450 19A1	AAAGCACCCCCAGGTTGAA	CCACCACTTCGAGTTTTTGCA
HSD17B1	hydroxysteroid (17-beta) dehydrogenase 1	TCGGGTCGCATATTGGTGA	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	GGAGGAAGCCAAGCAGAAAA	CGCTGTAGTGCCACGGATG
CYB5	cytochrome b-5	TCAAAGATTGCCAAGCCTTCG	ACAACCAGTGCTGAGATGGCTG
FTL	ferritin light polypeptide	TTCCTGGATGAGGAGGTGAAGC	CTTTCGAAGAGGTACTCGCCCA
AKR1C4	aldo-keto reductase family 1, member C4	AAGTACAAGCCCGTCTGCAAC	TCCTTGGACTTGCAAAACTCC
NR5A1	nuclear receptor subfamily 5, group A, member 1	GCCAGGAGTTCGTCTGCCT	GTTCGCCTTCTCCTGAGCG
NR0B1	nuclear receptor subfamily 0, group B, member 1	GACCGTGCTCTTTAATCCGGA	TCCTGATGTGTTCGCTAAGGATC

2.8.5 Preparation of cDNA

Prior to real-time RT-PCR experiment, the assay had already been optimized with respect to primer annealing temperatures. All products were checked on an agarose-ethidium gel to establish the presence of a single clear band of correct size per primer pair.

cDNA was synthesized by reverse transcriptase (RT) using SuperScript® III Platinum Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen, Carlsbad, CA, USA) according to the manufactures protocol. Approximately 500 ng RNA was used in a final reaction volume of 20 µl, containing 10 µl 2xRT Reaction mix, 2 µl RT Enzyme mix and 2 µl DEPC (Sigma-Aldrich) water in a 96-well PCR plate. For each RNA sample a control with no added reverse transcriptase were included to check for genomic contamination. A negative control without template, in which 6 µl DEPC water was added instead of RNA, and positive control were included. The reactions were set up as technical duplicates. After a quick spin down the reaction were performed in a Peltier Thermal Cycler-225 (MJ, Waltham, MA, USA) with the following program:

- 25°C for 10 minutes

- 42°C for 50 minutes

- 85°C for 5 minutes

Plates were placed on ice; 1 µl of Rnase H (Invitrogen) were added to each well and incubated for 20 minutes at 37°C to remove any traces of RNA. The resulting cDNA was further diluted with DEPC water and stored at -20°C until use.

2.8.6 Real-time RT-PCR

Real-time RT-PCR was performed to check whether the selected genes were up-or down regulated in the exposed cells (10 µM) compared to controls (DMSO). A two-fold dilution serie with three concentrations of cDNA (1, 2 and 5 ng/µl cDNA) were performed to find the best cDNA concentration for the qRT-PCR using Superscript® III Platinum Two-step qRT-PCR kit with SYBR Green (Invitrogen). For the present experiment a concentration of 1 ng/µl cDNA was used. Specific master mix for each gene was prepared with 5 ng cDNA, 200 nM primers, 12.5 µl Platinum SYBR Green supermix-UDG, 0.5 µl ROX dye (10x dilution) and 6

µl DEPC water in each well. The real-time RT-PCR reactions were set up as technical duplicates and included controls without RT. For each primer pair a negative control with no added template and a positive control were included. The q-PCR reactions were carried out using a DNA Engine Thermal Cycler with Chromo 4 Real-Time Detector (MJ Research) and its software Opticon Monitor 3 (Bio-Rad Laboratories, Hercules, CA, USA) with the following configuration:

- 50°C for 2 minutes (UDG incubation)

- 95°C for 2 minutes (enzyme activation)

- 95°C for 15 seconds (denaturation) followed by 62°C for 30 seconds (annealing) and 72°C for 30 seconds (elongation). This step was repeated 40 cycles. Finally the melting curve from 65°C to 90°C read for 1 seconds every 0.3°C.

A 10-fold dilution serie with five concentrations of cDNA was performed to check the amplification efficiency of each primer pair. We used a DMSO control sample and three exposed samples pooled together. The dilution series was 30, 3, 0.3, 0.03 and 0.003 ng/µl cDNA for the two samples. All primers had amplification efficiency between 1.8 and 1.99, which means that over 80% of the template in each PCR cycle was amplified during elongation.

2.9 Statistical analysis

Data were analyzed using Microsoft Excel 2003 and JMP 8 software (SAS Institute Inc 2007, Cary, NC, USA). Due to time limits, cytotoxicity was examined in two independent experiments/cell isolations, with three replicates from one isolation and one replicate from the second isolation (n=4) for each test compound. The effect of solvent control on cell viability was assessed by compare mean viability in cells incubated with 0.1% DMSO to medium blank (100%) with a paired two-tailed t-test (p<0.05). Since medium blank and DMSO are not affected by the exposure of test compounds, all three plates (one for each compound) within each replicate were combined (n=3).

Differences in mean cell viability in exposed cells were compared to DMSO controls (100%) and assessed by one-way ANOVA and Dunnetts test. The mean viability in each sample was

from triplicates of each concentration from four replicates (n=4) with a p-value <0.05 regarded as statistically significant. Cell viability and hormone data were tested for normality by the Shapiro-Wilk test. In case of non-normality in dependent variables a log or square root transformation was performed to make a better fit to the normal distribution. Log transformation of testosterone data and square root transformation of progesterone data from 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE exposed cells gave a satisfactory fit to the normal distribution and were used in the statistical analyses. Further, the variance was assessed for homogeneity by Levenes test. The parametric hormone data from cells exposed to 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE were analyzed by a two-way ANOVA with both exposure concentration and experimental run as independent variables. The differences in mean hormone levels in exposed and control cells were compared by Tukey HSD test to assess which concentrations were different from each other. P-values <0.05 were regarded as statistically significant. The hormone data from *o,p'*-DDD exposed cells was not normally distributed or homogenous in variance and was assessed by non-parametric Kruskal Wallis test. Due to the variation between the isolations, the mean hormone levels were assessed as percent of control (100%) and compared to control group with Bonferroni correction; p<0.0083 (0.05/6 doses) was used as statistically significant. Mean hormone concentrations was measured as technical duplicates, with triplicates of each exposure concentration in three independent experiments (n=9).

Linear regression was used to assess if the response in hormone production followed a significant trend. Hormone concentrations, log or square root transformed concentrations were set as dependent variables and exposure dose entered as independent variable.

From the real-time RT-PCR analysis the mean cycle threshold (CT) from three independent experiments for all tested genes were normalized to the mean of the two reference genes (*ACTB*, *PPIA*) in each sample. The $2^{-\Delta\Delta Ct}$-method was used to create fold changes in gene expression. The log2-transformed fold change values ($\Delta\Delta Ct*(-1)$) were used for statistical analysis by Student t-test with p-values <0.05 regarded as statistically significant.

3. Results

3.1 Purity of Leydig cells (3β-HSD staining)

After the isolation, plating and culturing of Leydig cells, a 3β-HSD staining was performed to assess the amount of Leydig cells in the cell preparation. The cell preparation was found to contain approximately 80% Leydig cells.

3.2 Cytotoxicity of test compounds

The cell viability was investigated by using the AlamarBlue assay. Since all test compounds were diluted in 0.1% DMSO the effect of 0.1% DMSO on Leydig cell viability was examined (Figure 3.1). Cells incubated with complete plating medium and LH only were used as controls. The results showed no significantly decreased cell viability from DMSO exposure compared to medium blank. 0.1% DMSO had no effect on Leydig cell viability.
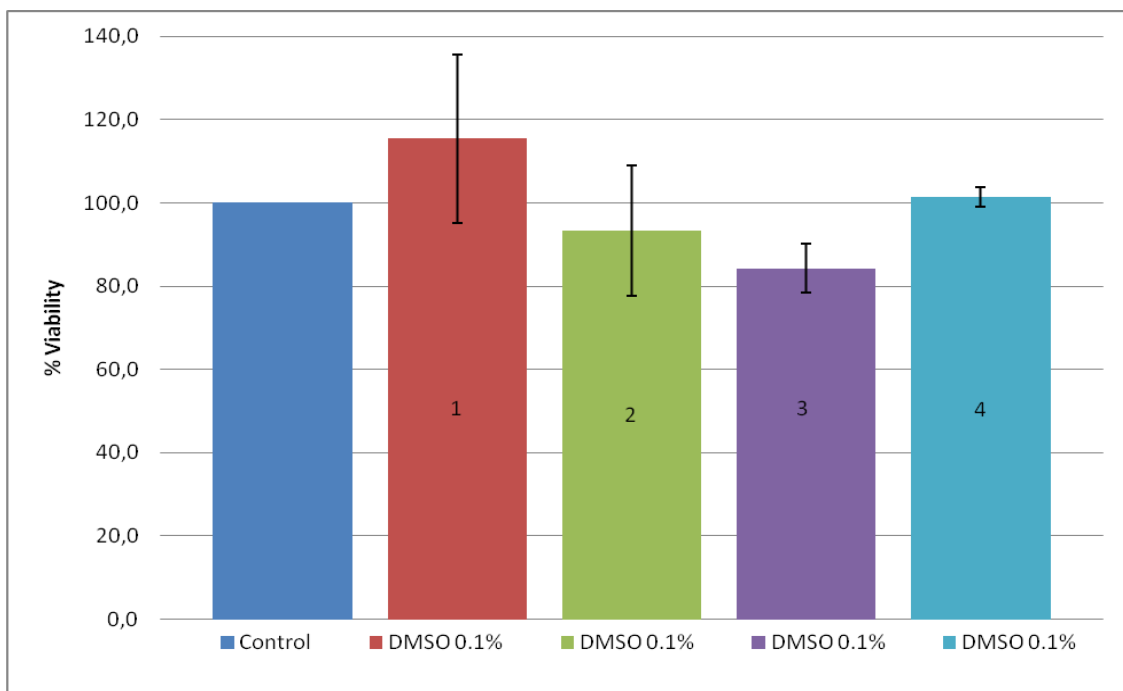
Figure 3.1: Effect of 48 hours 0.1% DMSO incubation on Leydig cell viability as percentage of control (medium blank; 100%). Shown are mean ± SE from three plates (n=3) in four replicates (1-4) from two independent experiments (1-3 within one experiment); two-tailed paired t-test; p<0.05.

Whereas Leydig cells exposed to 3,3'-(bis)MeSO$_2$-DDE had no reduced viability (Figure 3.2 B), cells exposed to both 3-MeSO$_2$-DDE and *o,p'*-DDD had reduced viability compared to control cells (DMSO) (Figure 3.2 A and C). However, only the highest concentration (20 µM) of 3-MeSO$_2$-DDE and *o,p'*-DDD caused significantly reduced viability compared to controls. Cell viability was reduced to 57 ± 8% and 19 ± 14% (mean ± SE) of control levels by 3-MeSO$_2$-DDE and *o,p'*-DDD, respectively. The viability was 91 ± 10% (mean ± SE) in cells exposed to 20 µM 3,3'-(bis)MeSO$_2$-DDE. The exposure concentration (10 µM) used in gene expression analysis did not reduce cell viability compared to control levels, although cells exposed to *o,p'*-DDD had a non-significantly reduction in viability at 65 ± 16% (mean ± SE).
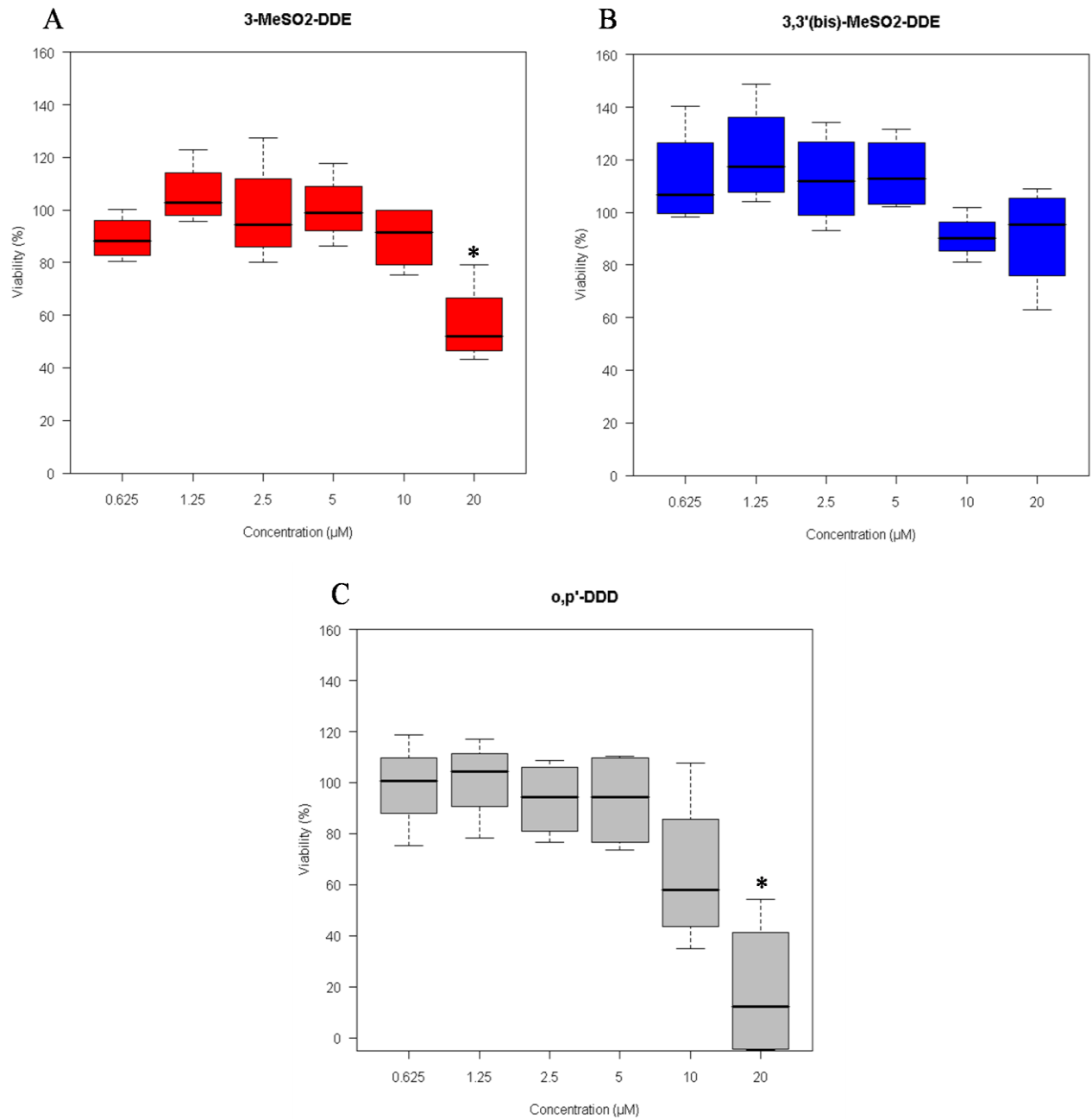
Figure 3.2: Viability of neonatal porcine Leydig cells exposed for 48 hours to A) 3-MeSO$_2$-DDE(red), B) 3,3'-(bis)MeSO$_2$-DDE(blue) and C) *o,p'*-DDD(grey) as percent of solvent control (100%). Shown are median values, maximum and minimum after two independent experiments with four replicates (n=4). *significantly different from control group (Dunnetts test; p<0.05)

3.3 Hormone analysis

Cell medium from LH-stimulated neonatal porcine Leydig cells exposed to 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD was assessed for hormone production by RIA in three independent experiments. The triplicates within each experimental run were treated as biological replicates. The 48 hour exposure of the test compounds to LH-stimulated Leydig cells resulted in a concentration-dependent decrease in production of testosterone, estradiol and progesterone (Figure 3.3, 3.4 and 3.5). Cortisol was found to be secreted in concentrations close to the detection limit of the assay. Large variations were found between the experiments and the solvent control levels varied considerable. There were significantly differences between both exposure concentration and experimental runs for the hormone data exposed to 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE (two-way ANOVA , p<0.0001).

3.3.1 Testosterone production

The reduction in testosterone production by the compounds was most potent compared to reduction in estradiol and progesterone. There was a concentration-dependent decrease in testosterone production in exposed cells compared to controls (DMSO) induced by the 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD (Figure 3.3).

3-MeSO$_2$-DDE reduced testosterone production in the Leydig cells compared to control levels at 38.5 ± 3.9 ng/ml (mean ± SE) (Figure 3.3 A). The testosterone production was significantly reduced at the four highest concentrations compared to unexposed cells (Table 3.1). The testosterone levels were decreased almost six-fold and 12-fold at 10 and 20 µM, respectively.

In Leydig cells exposed to 3,3'-(bis)MeSO$_2$-DDE there was a reduction in testosterone production compared to control levels at 53.5 ± 4.8 ng/ml (mean ± SE) (Figure 3.3 B). The production was significantly reduced at the four highest exposure concentrations (Table 3.1) and was reduced almost seven-fold (10 µM) and 13-fold (20 µM) at the two highest concentrations compared to control.

o,p'-DDD exposure caused reduced testosterone production in Leydig cells compared to control levels at 27.2 ± 6.7 ng/ml (mean ± SE) (Figure 3.3 C). Testosterone levels were significantly decreased at the three highest concentrations compared to unexposed cells (Table 3.1) and were decreased 14- and 23-fold at 10 and 20 µM, respectively.
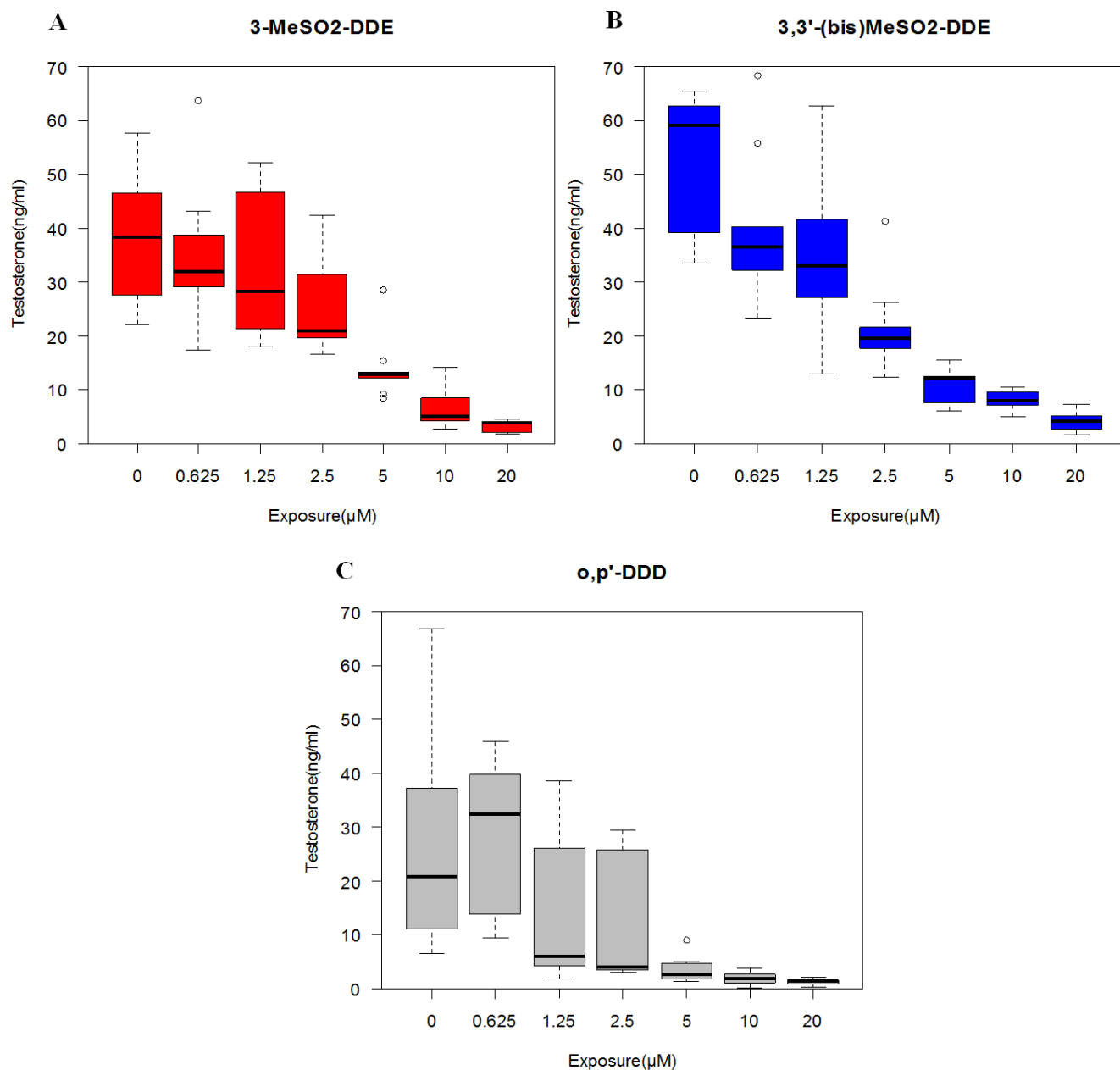
Figure 3.3: Testosterone production in LH-stimulated Leydig cells from neonatal pigs after 48 hour incubation with A) 3-MeSO$_2$-DDE (red), B) 3,3'-(bis)MeSO$_2$-DDE (blue) and C) *o,p*'-DDD (grey). Shown are minimum, first quartile, median, third quartile, and maximum values from three independent experiments performed in triplicates (n=9). Points more than 1.5 times the interquartile range above the third quartile/below the first quartile are defined as outliers and plotted individually(small circle). The statistics is presented in table 3.1.

Table 3.1: Results of the statistical analysis of testosterone data analyzed by Tukey HSD test (p<0.05) (3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE) with both experiment and exposure concentration as significantly variables (two-way ANOVA; p<0.0001). Non-parametric data (*o,p*'-DDD) analyzed by Kruskal-Wallis test with Bonferroni correction (p<0.0083). Each exposure concentration is compared to control group (DMSO), non-significant values; n.s.

Compound	0.625 µM	1.25 µM	2.5 µM	5 µM	10 µM	20 µM
3-MeSO$_2$-DDE	n.s	n.s	p<0.05	p<0.05	p<0.05	p<0.05
3,3'-(bis)MeSO$_2$-DDE	n.s	n.s	p<0.05	p<0.05	p<0.05	p<0.05
o,p'-DDD	n.s	n.s	n.s	P<0.0083	p<0.0083	p<0.0083

3.3.2 Estradiol production

The reduction in estradiol concentrations was less potent than what obtained in testosterone production. Estradiol production in exposed cells was reduced in a concentration-dependent manner by all three test compounds (Figure 3.4).

Leydig cells exposed to 3-MeSO$_2$-DDE had reduced estradiol production compared to control levels at 1205.7 ± 94.7 pg/ml (mean $\pm$ SE) (Figure 3.4 A). The estradiol production was significantly reduced at the two highest exposure concentrations (Table 3.2) with a 1.3-fold and 2.3-fold reduction from control at 10 and 20 µM, respectively.

3,3'-(bis)MeSO$_2$-DDE caused a decrease in estradiol production in Leydig cells compared to unexposed cells 2175.4 ± 137.7 pg/ml (mean $\pm$ SE) (Figure 3.4 B). The production was significantly reduced at concentrations 5, 10 and 20 µM compared to controls (Table 3.2) and was reduced by a 1.3- and two-fold at the two highest concentrations, respectively.

o,p'-DDD exposed cells had reduced estradiol production compared to control levels at 1096.7 ± 210.3 pg/ml (mean $\pm$ SE) (Figure 3.4 C). At exposure concentrations 10 and 20 µM, the estradiol levels were significantly reduced by 2.5- and 3.5-fold compared to controls, respectively (Table 2.3).

Figure 3.4: Estradiol production in LH-stimulated Leydig cells from neonatal pigs after 48 hour incubation with A) 3-MeSO$_2$-DDE (red), B) 3,3'-(bis)MeSO$_2$-DDE (blue) and C) *o,p*'-DDD (grey). Shown are minimum, first quartile, median, third quartile, and maximum values from three independent experiments performed in triplicates (n=9). Points more than 1.5 times the interquartile range above the third quartile is defined as outliers and plotted individually (small circle). The statistics are presented in table 3.2.

Table 3.2: Results of the statistical analysis of estradiol data analyzed by Tukey HSD test (p<0.05) (3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE) with both experiment and exposure concentration as significantly variables (two-way ANOVA; p<0.0001). Non-parametric data (*o,p*'-DDD) analyzed by Kruskal-Wallis test with Bonferroni correction (p<0.0083). Each exposure concentration is compared to control group (DMSO), non-significant values; n.s.

Compound	0.625 µM	1.25 µM	2.5 µM	5 µM	10 µM	20 µM
3-MeSO$_2$-DDE	n.s	n.s	n.s	n.s	p<0.05	p<0.05
3,3'-(bis)MeSO$_2$-DDE	n.s	n.s	n.s	p<0.05	p<0.05	p<0.05
o,p'-DDD	n.s	n.s	n.s	n.s	p<0.0083	p<0.0083

3.3.3 Progesterone production

The progesterone concentrations in the medium of LH-stimulated Leydig cells were very low. Some of the data measured were under the detection limit for the assay, but the changes in mean progesterone levels in relation to exposure concentrations of test compounds showed the same trends as for testosterone and estradiol (Figure 3.5).

Leydig cells exposed to 3-MeSO$_2$-DDE had reduced progesterone production compared to control levels at 1.19 ± 0.15 ng/ml (mean $\pm$ SE) (Figure 3.5 A). The production was significantly reduced at the three highest exposure concentrations (Table 3.3) and was decreased two- and almost three-fold by 10 and 20 µM, respectively.

3,3'-(bis)MeSO$_2$-DDE reduce progesterone production in Leydig cells compared to concentration in solvent control at 0.94 ± 0.09 ng/ml (mean $\pm$ SE) (Figure 3.5 B). At concentrations 2.5, 5, 10 and 20 µM the secretion of progesterone was significantly reduced compared to control levels (Table 3.3). Progesterone was reduced by 3,3'-(bis)MeSO$_2$-DDE about five- and 17-fold at the two highest exposure concentrations.

The progesterone secretion from cells exposed to *o,p*'-DDD was reduced compared to levels in unexposed cells at 1.01 ± 0.25 ng/ml (mean $\pm$ SE) (Figure 3.5 C). The production was significantly reduced in cells exposed to 5, 10 and 20 µM, respectively (Table 3.3) with production reduced by four-fold and nine-fold at the two highest doses, respectively.
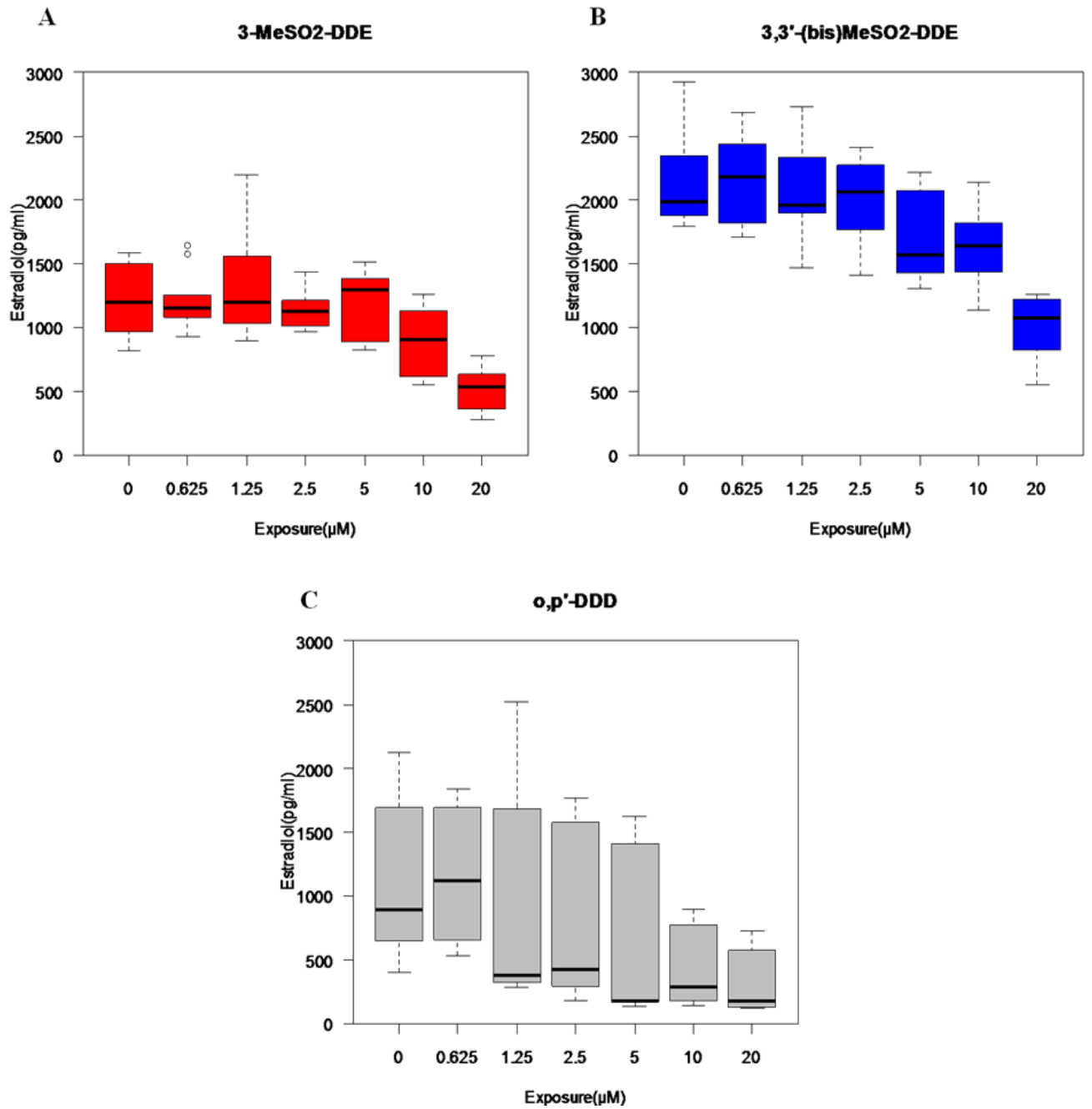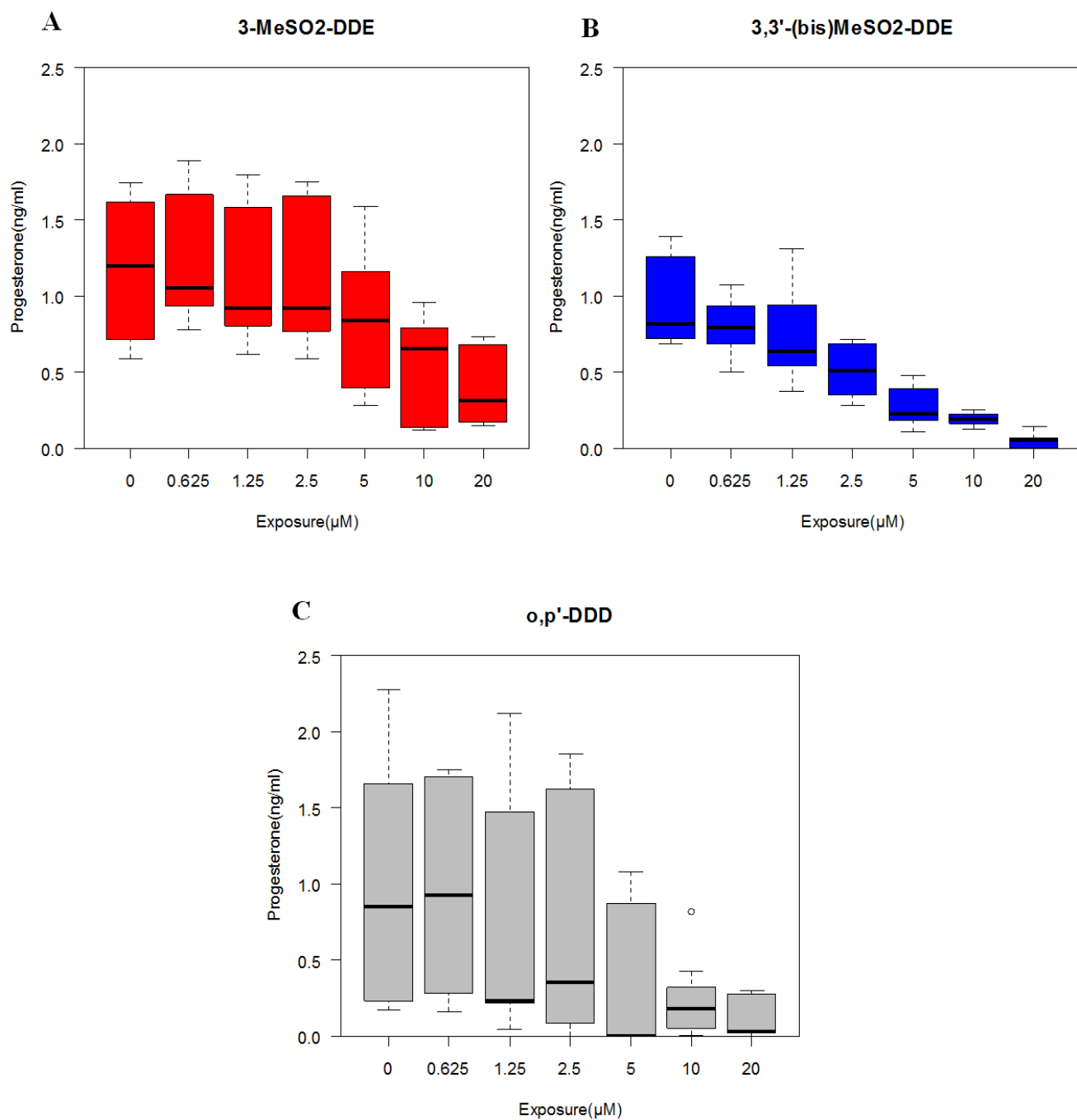
Figure 3.5: Progesterone production in LH-stimulated Leydig cells from neonatal pigs after 48 hour incubation with A) 3-MeSO$_2$-DDE (red), B) 3,3'-(bis)MeSO$_2$-DDE (blue) and C) *o,p'*-DDD (grey). Shown are minimum, first quartile, median, third quartile, and maximum values from three independent experiments performed in triplicates (n=9). Point more than 1.5 times the interquartile range above the third quartile is defined as outlier and plotted individually (small circle). The statistics are presented in table 3.3.

Table 3.3: Results of the statistical analysis of progesterone data analyzed by Tukey HSD test (p<0.05) (3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE) with both experiment and exposure concentration as significantly variables (two-way ANOVA; p<0.0001). Non-parametric data (*o,p*'-DDD) analyzed by Kruskal-Wallis test with Bonferroni correction (p<0.0083). Each exposure concentration is compared to control group (DMSO), non-significant values; n.s.

Compound	0.625 µM	1.25 µM	2.5 µM	5 µM	10 µM	20 µM
3-MeSO$_2$-DDE	n.s	n.s	n.s	p<0.05	p<0.05	p<0.05
3,3'-(bis)MeSO$_2$-DDE	n.s	n.s	p<0.05	p<0.05	p<0.05	p<0.05
o,p'-DDD	n.s	n.s	n.s	p<0.0083	p<0.0083	p<0.0083

3.3.4 Cortisol production

The concentrations of cortisol were close to the detection limit of the assay and are not reported.

3.4 Gene expression analysis

Gene expression analysis was performed on the LH-stimulated Leydig cells with real-time RT-PCR. The gene expression analysis was done after 48 hour exposure to 3-MeSO$_2$-DDE, 3,3'(bis)-MeSO$_2$-DDE and *o,p*'-DDD in three independent experiments. RNA was isolated, cDNA was synthesized and the expression of 16 genes involved in testicular steroidogenesis was measured using real-time RT-PCR. Relative quantification was done with solvent control (DMSO) as calibrator and exposure dose (10 µM) of the three compounds as test sample. The efficiency test of primers gave a satisfactory result of all primers tested. There was no effect of the test compounds on expression of the housekeeping genes used. Most of the genes examined were down-regulated in cells exposed to the test compounds compared to solvent control (Figure 3.6; p<0.05).

In cells exposed to 3-MeSO$_2$-DDE, the eight genes *HMGR, StAR, CYP17A1, HSD3B, CYP11B1, CYP21, CYB5* and *NR5A1* were significantly down-regulated compared to controls and fold change values are shown in Figure 3.6 A. The genes were slightly down-regulated between 1.6-3.4 times compared to control.

Cells exposed to 3,3'-(bis)MeSO$_2$-DDE had significantly down-regulation of the following seven genes; *HMGR, CYP11A1, HSD3B, CYP11B1,CYB5, AKR1C4* and *NR0B1* (Figure 3.6 B). *FTL* was significantly up-regulated compared to controls. The genes were down-regulated

between 1.6-3.7 times, whereas *FTL* was up-regulated almost two times compared to controls. 3,3'-(bis)MeSO$_2$-DDE especially induced down-regulation of *CYP11B1*, almost 12 times compared to control.

o,p'-DDD induced down-regulation most strongly of the test compounds. Eight genes involved in testicular steroidogenesis were down-regulated in cells following *o,p'*-DDD exposure compared to controls; *CYP51, CYP17A1, HSD3B, CYP19A1, HSD17B4, CYB5, AKR1C4* and *NR5A1* (Figure 3.6 C). Both *CYP17A1* and *CYP19A1* were down-regulated almost 19 times compared to control, while *HSD3B* levels decreased more than 60-fold. The other genes were down-regulated between 1.7-5.6 times compared to control. *HMGR* and *CYP21* were not significantly down-regulated due to only two replicates.

The exposure to 3-MeSO$_2$-DDE, 3,3'(bis)-MeSO$_2$-DDE and *o,p'*-DDD to porcine Leydig cells induced a general down-regulation of several genes involved in the testicular steroidogenesis, however, the compounds significantly altered slightly different genes. All three test compounds induced down-regulation of *HSD3B* and *CYB5*. A large variation in gene expression results of some of the genes was found between experiments.
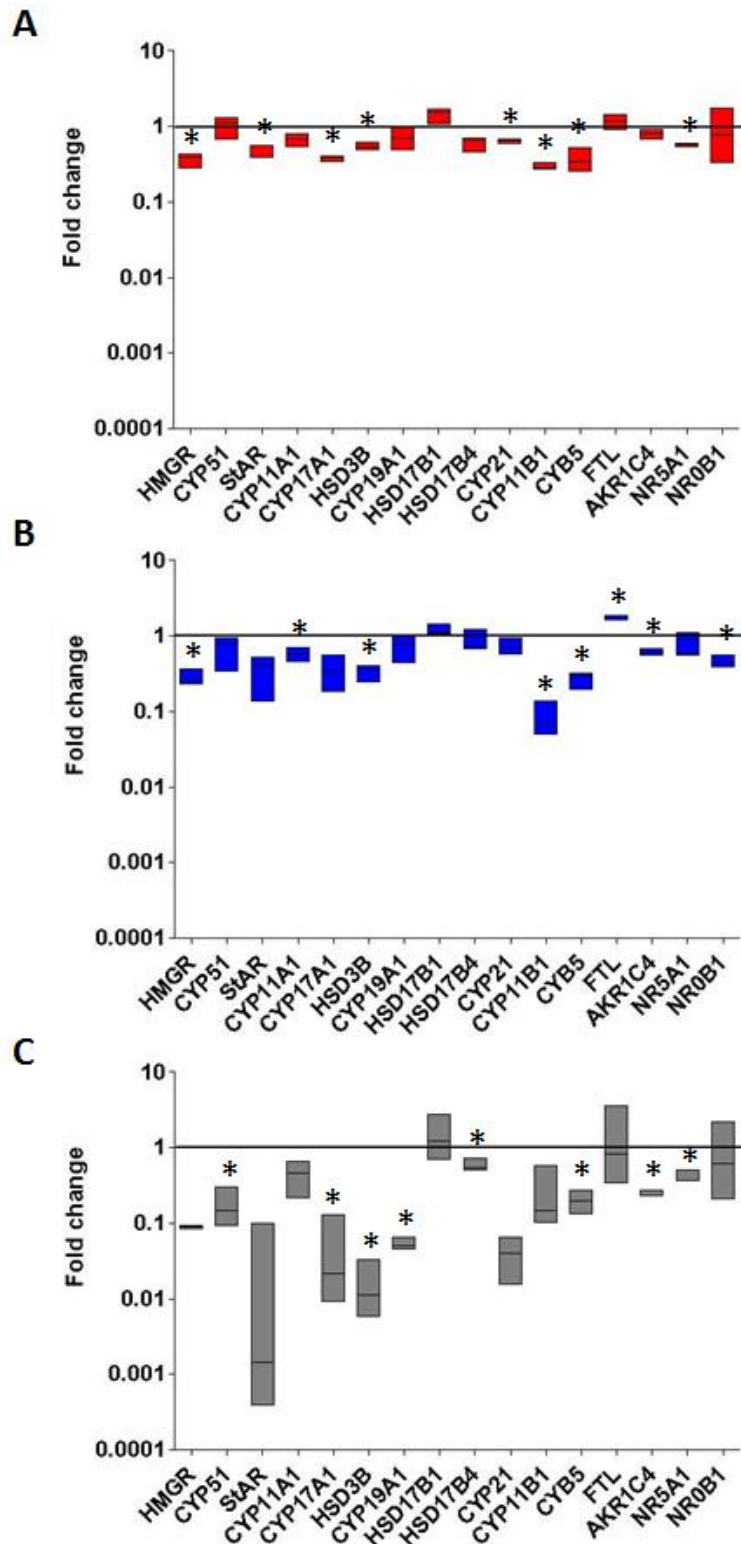
Figure 3.6: Gene expression of 16 steroidogenic genes in LH-stimulated Leydig cells after 48 hours incubation with 10 µM of A) 3-MeSO$_2$-DDE (red), B) 3,3'-(bis)MeSO$_2$-DDE (blue) and C) *o,p*'-DDD (grey) relative to DMSO control (line at 1). Shown are maximum, minimum and median fold change values from three independent experiments (n=3, exept n=2 for *CYP21* and *HMGR* in panel C) on a logarithmic scale. Values above 1 indicate up-regulation, while values below 1 represent down-regulation compared to control. * statistically different from control; Student t-test; p<0.05.

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4. Discussion

To the best of our knowledge this is the first study investigating the steroidogenic effects of the three compounds 3-MeSO$_2$-DDE, 3,3'(bis)-MeSO$_2$-DDE, and *o,p'*-DDD in LH-stimulated neonatal porcine Leydig cells. All three compounds caused suppressive effects on neonatal Leydig cell steroidogenesis with concentration-dependent decreased hormone production and a general reduction in gene expression. The highest concentration (20 μM) of 3-MeSO$_2$-DDE and *o,p'*-DDD was cytotoxic to the Leydig cells.

The suppressive effects of DDT metabolites 3-MeSO$_2$-DDE and *o,p'*-DDD on Leydig cell steroidogenesis in the present study might support the growing concern regarding persistent organic pollutants and their endocrine disruptive effect and potential role in negative trends in male reproductive health. The main DDT metabolite DDE which is widespread in the environment is converted to 3-MeSO$_2$-DDE in mammals, and both 3-MeSO$_2$-DDE and to less extent DDD metabolites of DDT are present in human tissues (Chu et al., 2003). However, *o,p'*-DDD is less common in the environment than the isomer *p,p'*-DDD (Falandysz et al., 1999; Pazou et al., 2006; Ssebugere et al., 2010) thus, 3-MeSO$_2$-DDE might be of most concern considering exposure to wildlife and humans. Another aspect to the present study was to contribute to further knowledge about steroidogenic effects of 3-MeSO$_2$-DDE since it is suggested as alternative drug to *o,p'*-DDD in the treatment of ACC in humans.

4.1 Purity of Leydig cells

The purity of Leydig cells was assessed by histochemical 3β-hydroxysteroid dehydrogenase staining and the preparation in this study was found to contain approximately 80% Leydig cells. A purity of 80% is considered acceptable and in compliance with several studies (Brun et al., 1991; Geiger et al., 1999; Lejeune et al., 1998b; Li, 1991), although the content of Leydig cells might exceed 90% (Fombonne et al., 2003; Nakajima et al., 2003). Cells that were not identified as Leydig cells may represent Leydig cell precursors, Sertoli cells, macrophages, fibroblasts, endothelial cells and red blood cells. There might also be a possibility of a mixed cell type of adrenal-like and Leydig cells during development in the testis (Val et al., 2006). The determination of Leydig cell purity was not performed at the cell solutions used in the experiments for cell viability, hormone quantification and gene expression analysis, but the same Percoll gradient method were used every time. Some

variation in Leydig cell content might have been caused by variable quality of Percoll gradient layers and difficulties when sucking up the layer which contains the Leydig cells.

4.2 Cytotoxicity of test compounds

The possible cytotoxic effects of the compounds were assessed by the AlamarBlue assay. The effect of solvent control (0.1% DMSO) on the purified porcine Leydig cells was examined since the test compounds were diluted in 0.1% DMSO. The 0.1% DMSO did not have any effect on Leydig cell viability compared to control cells in the present study. DMSO is a well known solvent for chemicals and it was expected that 0.1% DMSO did not alter the cell viability (Da Violante et al., 2002). The variation observed between the replicates could be due to inexact pipetting, differences in duration of AlamarBlue incubation in each plate and variations in primary cell culture between the two cell isolations.

Both 3-MeSO$_2$-DDE and o,p'-DDD caused significantly decreased cell viability at the highest concentration (20 μM) compared to control cells, whereas 3,3'-(bis)MeSO$_2$-DDE had no effect on Leydig cell viability. o,p'-DDD seemed to be the most potent compound, with two of the experiments causing zero viability at 20 μM and a non-significantly reduction at 10 μM. Due to variation in primary cultures, several experimental runs should be included to evaluate the compounds effect on cell viability. Nevertheless, in all replicates there was decreased cell viability at the highest concentration of 3-MeSO$_2$-DDE and o,p'-DDD.

Similar to the present study, both 3-MeSO$_2$-DDE and o,p'-DDD decreased viability in human adrenocortical carcinoma cell line (H295R) in a concentration-dependent manner, although 3-MeSO$_2$-DDE was the most potent drug (Asp et al., 2010). Further, 3-MeSO$_2$-DDE caused concentration-dependent cytotoxicity in forskolin-stimulated mice adrenocortical cell line (Y-1) at concentrations equal to what is used in the present study, whereas o,p'-DDD only caused a slight, but non-significantly decrease in cell viability (Asp et al., 2009). This is in contrast to the present study were o,p'-DDD seems to induce cytotoxicity more potent than 3-MeSO$_2$-DDE. The differences in potency between the compounds might be due to species differences (Asp et al., 2010; Brandt et al., 1992) and different types of cells. The cytotoxicity of 3-MeSO$_2$-DDE is CYP11B1-dependent in mice (Asp et al., 2009), but as porcine Leydig cells are not reported to have CYP11B1 enzyme activity, the cytotoxicity in the present study

might involve other mechanisms. In addition, the cytotoxicity following 3-MeSO$_2$-DDE exposure in H295R cells was not counteracted by the CYP11B1-inhibitor etomidate, indicating that other steroidogenic enzymes might be involved in the toxic effect (Asp et al., 2010). The drug-induced cell death of *o,p'*-DDD in the adrenal cortex occurs after CYP-catalyzed bioactivation and might involve several steroidogenic enzymes, including CYP11B1 and CYP11A1 (Hart and Straw, 1971; Hart et al., 1971). The exact enzyme(s) responsible for the bioactivation is not known (Ahlman et al., 2001; Stigliano et al., 2008). Therefore, both 3-MeSO$_2$-DDE and *o,p'*-DDD might interfere with several steroidogenic enzymes that can activate the compounds to reactive intermediates and hence cause cytotoxicity in the porcine Leydig cells, as suggested in adrenal cells. However, this is only one possible mechanism.

Exposure to 3,3'-(bis)MeSO$_2$-DDE did not reduce the cell viability in the neonatal porcine Leydig cells. A previous study demonstrated cytotoxicity following 3,3'-(bis)MeSO$_2$-DDE exposure in mice adrenal cells, although less potent than 3-MeSO$_2$-DDE (Asp et al., 2009). Since the methylsulfonyl moiety seems to be important for CYP11B1-dependent toxic effects of 3-MeSO$_2$-DDE in adrenal cells (Hermansson et al., 2007; Lund et al., 1988) the extra methylsulfonyl group might then explain why 3,3'-(bis)MeSO$_2$-DDE is nontoxic in the present study. If interference with enzymes may cause cytotoxicity, it might be that structure-specific bioactivation of the compounds by other enzymes than CYP11B1 could explain the difference in cytotoxic effects of the compounds reported here.

In summary, *o,p'*-DDD and to a less extent 3-MeSO$_2$-DDE caused cytotoxicity at 20 µM, whereas 3,3'-(bis)MeSO$_2$-DDE exposure had no effect on Leydig cell viability. The different effect on cell viability by the compounds might be structure-dependent.

4.3 Effect of test compounds on LH-stimulated Leydig cell steroidogenesis

The present study examined the steroidogenic effects of two DDT metabolites and one synthetic DDE analogue in primary cultures of LH-stimulated neonatal porcine Leydig cells. The three test compounds 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE, and o,p'-DDD caused a reduction in testosterone, estradiol and progesterone secretion by the Leydig cells in a concentration-dependent manner. In addition, the test compounds induced down-regulation of several genes involved in Leydig cell steroidogenesis. The decreased hormone production was observed at concentrations lower than the cytotoxic concentration (20 μM) of 3-MeSO$_2$-DDE and o,p'-DDD, indicating that the decrease in hormone production was not due to cytotoxicity. In addition, 3,3'-(bis)MeSO$_2$-DDE exposure reduced hormone production in Leydig cells without affecting cell viability. However, since the cytotoxicity assay was performed on cells other than those used for hormone quantification, and obtained from only two independent experiments, reduced hormone production due to less viable cells cannot be excluded. The large variations in hormone and gene expression results between experiments may reflect that primary cultures of Leydig cells were used in the present study. Primary cultures are different than established cell lines in reflecting the individuals the cells are obtained from and hence there will be some variation. Sources of variation could include variation in Percoll gradient quality, Leydig cell content and differences in number and age (8-12 days) of male piglets from the litters between the cell isolations.

4.3.1 Effect on hormone production in LH-stimulated Leydig cells

The cells were stimulated with the physiological hormone stimulator LH which maintain the steroidogenic activity and increase the hormone secretion in primary porcine Leydig cells (Bernier et al., 1983; Lejeune et al., 1998b; Mather et al., 1981).

The LH stimulated cells were then exposed to 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and o,p'-DDD, which led to a similar pattern of reduced testosterone, estradiol and progesterone production for all three compounds. A reduction in hormone production by these compounds has previously been demonstrated in adrenal cells and linked to interference with steroidogenic enzymes at the protein level (Asp et al., 2009). The compounds might have acted in a similar manner in the Leydig cells from the present study and caused the observed

decrease in hormone production. Reduced activity of steroidogenic enzymes is indeed related to decreased hormone production in Leydig cells (Akingbemi et al., 2000; Ohno and Nakajima, 2005). For instance, both testosterone and progesterone are reduced in porcine Leydig cells by the specific CYP-inhibitors ketoconazole and aminoglutethimide by inhibition of CYP11A1 (Brun et al., 1991). However, the possible interaction between enzyme activity and the compounds was not investigated in the present study.

After exposure to 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD, testosterone production seemed to be more reduced than estradiol and progesterone production. Testosterone concentrations was still measured in levels elevated compared to estradiol and progesterone, and since testosterone is the main steroid hormone synthesized by Leydig cells, it may be most affected by suppression of Leydig cell steroidogenesis. The low amounts of progesterone might reflect its role as an intermediary compound in the biosynthesis of steroids in the testes (Raeside et al., 2006; Ruokonen and Vihko, 1974). In addition, the Δ5 pathway (Figure 1.3) of androgen and estrogen synthesis predominates in pigs, this pathway converts pregnenolone to testosterone via 17α-hydroxypregnenolone and not via progesterone (Ruokonen and Vihko, 1974). However, since some progesterone levels were obtained, the steroid synthesis through Δ4 pathway to progesterone might also occur (Conley and Bird, 1997). As progesterone was reduced in a similar way as testosterone and estradiol, it might suggest that the test compounds affect the steroidogenesis cascade upstream.

In the present study, cortisol levels were not detected by the assay, either from unexposed or exposed cells, indicating no production of cortisol by the Leydig cells. Cortisol participate in regulating sperm output in mature boars (Wagner Claus 2004) and corticosteroids might inhibit testosterone production by cultured porcine Leydig cells (Bernier et al., 1984). The activity of CYP11B1, the enzyme synthesizing cortisol, is not previously reported in porcine Leydig cells, although found in rat Leydig cells (Wang et al., 2002).

3-MeSO$_2$-DDE exposure caused a reduction in testosterone, estradiol and progesterone secretion, with only the highest concentration being cytotoxic. 3-MeSO$_2$-DDE has previously been demonstrated to inhibit corticosteroid production in adrenal cells due to interaction with the steroidogenic enzyme CYP11B1 at the protein level (Asp et al., 2009; Johansson et al., 1998; Johansson et al., 2002; Lindhe et al., 2002). Since the production of testosterone, estradiol and progesterone does not involve CYP11B1 (Figure 1.3), the reduction in hormone

production following 3-MeSO$_2$-DDE exposure in the present study is likely to involve other steroidogenic enzyme(s) or mechanisms not previously described to be affected by 3-MeSO$_2$-DDE.

Reduction of testosterone, estradiol and progesterone production was induced by 3,3'-(bis)MeSO$_2$-DDE exposure. In adrenal cells, the reduction in corticosteroid production by 3,3'-(bis)MeSO$_2$-DDE is less potent compared to 3-MeSO$_2$-DDE and o,p'-DDD (Asp et al., 2009). The bioactivation of 3-MeSO$_2$-DDE by CYP11B1 is reported to be structure-specific (Hermansson et al., 2007; Lund et al., 1988), hence the extra methylsulfonyl group of 3,3'-(bis)MeSO$_2$-DDE could affect the affinity to other steroidogenic enzymes and cause different steroidogenic effects by the compounds. However, reduction in hormone production did not seemed to be less potent by 3,3'-(bis)MeSO$_2$-DDE exposure, at least for testosterone and estradiol.

All three hormones decreased following o,p'-DDD exposure. The reduced steroid hormone secretions at the highest concentration could be caused by reduced cell viability. o,p'-DDD has previously been reported to reduce testosterone and progesterone synthesis in H295R cells, although the mechanism at which o,p'-DDD inhibit adrenal steroidogenesis is still unknown (Stigliano et al., 2008). Several steroidogenic enzymes might be involved in the reduction of hormone production by o,p'-DDD, and it is previously reported to inhibit CYP11A1 (Hart et al., 1973; Hart et al., 1971) which could inhibit steroid hormone production in Leydig cells.

In summary, all three compounds decreased LH-stimulated hormone production in cultured porcine Leydig cells in a concentration-dependent manner. The reduction in progesterone as well as testosterone and estradiol might indicate that the compounds affect the steroidogenesis upstream.

4.3.2 Effect on gene expression in LH-stimulated Leydig cells

Expression of 16 genes involved in Leydig cell steroidogenesis were investigated after exposure to 10 μM 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and o,p'-DDD. The overall trend in gene expression suggests that all three compounds induce down-regulation of several genes relative to expression in control cells, suggesting that the compounds interfere at mRNA level. The suppressive trend in expression of some genes might be an explanation to the observed reduced hormone levels following exposure to the compounds at the same concentration.

The results indicate that the three compounds reduce mRNA levels of slightly different steroidogenic genes. *o,p'*-DDD seems to reduce the gene expression levels more potently compared to 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE. Although not significantly cytotoxic at 10 μM, the reduced cell viability by *o,p'*-DDD at this concentration cannot be excluded.

The three compounds each significantly altered expression of eight genes, all down-regulated except for one. 3-MeSO$_2$-DDE exposure significantly induced down-regulation of *HMGR, StAR, CYP17A1, HSD3B, CYP11B1, CYP21, CYB5* and *NR5A1*, whereas 3,3'-(bis)MeSO$_2$-DDE exposure significantly reduced expression of *HMGR, CYP11A1, HSD3B, CYP11B1,CYB5, AKR1C4* and *NR0B1*. In addition, 3,3'-(bis)MeSO$_2$-DDE caused an significant up-regulation of *FTL*. *o,p'*-DDD exposure significantly induced *CYP51, CYP17A1, HSD3B, CYP19A1, HSD17B4, CYB5, AKR1C4* and *NR5A1*(Figure 3.6). The overall trend suggests the compounds induce a suppression of gene expression along the steroidogenic pathway.

Expression of genes involved in steroidogenesis

The genes *CYP51* and *HMGR* encode enzymes involved in cholesterol biosynthesis (Debeljak et al., 2003; Rodwell et al., 1976) and steroidogenesis is critically dependent on cholesterol as a substrate. The inhibition of CYP51 has been reported to reduce serum testosterone levels in humans (Miettinen, 1988). Several of the genes down-regulated in the present study are encoding enzymes involved in Leydig cell steroidogenesis (Figure 1.3) and reduced activity of these enzymes might decrease hormone production by Leydig cells (Brun et al., 1991; Nakajima et al., 2005; Ohno and Nakajima, 2005; Shi et al., 2010; Walsh and Stocco, 2000). The StAR protein mediates the first important step in transfer of cholesterol from the outer to the inner mitochondrial membrane (Stocco, 2001) hence reduced activity of StAR might inhibit steroidogenesis. The reduction of *StAR* mRNA might account for decrease in StAR protein and inhibition of progesterone synthesis in mouse Leydig cells exposed to lindane (Walsh and Stocco, 2000). The next rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by CYP11A1 and inhibition of CYP11A1 in porcine Leydig cells has been demonstrated to suppress progesterone and testosterone synthesis (Brun et al., 1991). Further, several enzymes are involved in the Δ4 and Δ5 pathway for the synthesis of androgens and estrogens, including CYP17A1 and 3βHSD (Conley and Bird, 1997). Decrease in *CYP17A1* expression has previously been linked to reduced testosterone secretion in

neonatal porcine Leydig cells (Nakajima et al., 2005). CYB5 interacts with CYP17A1 and are important for the 17,20 lyase activity in the androgen synthesis (Nakajin et al., 1985). The CYB5 protein is widely involved in biological processes, being a component of electron transfer chains in several pathways, (Bonfils et al., 1981; Grindflek et al., 2010; Schenkman and Jansson, 2003). Thus, a decrease in expression of these genes might explain the reduction in steroid hormone production observed in this study. Even though the compounds might reduce expression of slightly different genes, all three compounds reduce mRNA levels of genes that might be associated with reduced hormone production, for example the expression of *HSD3B* and *CYB5* were significantly reduced by all three compounds.

The reduced estradiol production following *o,p*'-DDD exposure might have been caused by the decreased expression of *CYP19A1*, which codes for the enzyme aromatase that converts testosterone to estradiol (Conley et al., 1996). Another enzyme involved in estradiol production encoded by *HSD17B4* and is capable of converting estradiol to estrone (Adamski et al., 1995). Reduction in mRNA levels of this gene could be associated with reduced formation of estrone and can therefore not explain the reduction in estradiol levels measured in the present study. The level of estrone production was not measured and the relative amounts of estrone vs estradiol cannot be discussed. However, there were no significantly alterations in expression levels of *HSD17B1*, the enzyme converting estrone to estradiol.

The last step in testosterone synthesis is the convertion of androstenedione to testosterone catalyzed by another type of hydroxysteroid dehydrogenase is HSD17B3 (Andersson et al., 1996; Luu-The, 2001). However, expression of *HSD17B3* was not included in the present study. Another possible mechanism by which measured steroid levels would be low is through the action of AKR1C4. AKR1C4 is involved in converting androgens and estrogens to their inactive metabolites which might reduce the levels of circulating steroid hormones and the steroids might be substrates for conjugation reactions (Penning et al., 2000). However, if the activity of AKRIC4 was responsible for reduction in hormone levels, an increase in *AKR1C4* expression would be expected rather than the reduction observed in the present study.

The only gene significantly up-regulated in the study was *FTL*, following 3,3'-(bis)MeSO$_2$-DDE exposure. *FTL* encodes the ferritin light polypeptide which is part of ferritin, an important protein for many cellular functions. Increased mRNA levels of *FTL* could possibly

increase ferritin which in turn could increase the iron storage capacity of the cell (Hentze and Kuhn, 1996). This might be one possible mechanism for the sustained cell viability seen in 3,3'-(bis)MeSO$_2$-DDE exposed cells.

Expression of transcription factors

Gene expression levels of two transcription factors involved in steroidogenesis was investigated, *NR5A1* and *NR0B1*. The steroidogenic transcription factor NR5A1 (SF-1) is an important transcriptional activator which increase mRNA levels and induce transcription of *CYP11A1, HSD3B, CYP19A1, CYP17A1, CYP21* and *StAR* (Sadovsky and Dorn, 2000; Sugawara et al., 1996). Hence, reduced mRNA levels of *NR5A1* could result in down-regulation of these genes, and the subsequent reduction in hormone secretion observed in the present study. Both 3-MeSO$_2$-DDE and *o,p'*-DDD did reduce mRNA levels of *NR5A1* which might have affected the other genes; *HSD3B, CYP17A1*(both), *StAR, CYP21*(3-MeSO$_2$-DDE) and *CYP19A1*(*o,p'*-DDD). The other transcription factor investigated, NR0B1 (DAX-1), has previously been shown to inhibit expression of *StAR, HSD3B* and *CYP17A1* (Lalli et al., 1998), which are genes with suppressed expression in the present study. After 3,3'-(bis)MeSO$_2$-DDE exposure, the expression of *NR0B1* was down-regulated, hence the observed down-regulation of *HSD3B* expression cannot be explained by the activity of this transcription factor.

Expression of *CYP21* and *CYP11B1*

Gene expression levels of the steroidogenic enzymes CYP11B1 and CYP21 were investigated as the compounds are adrenal toxicants. Neither CYP11B1 nor CYP21 are involved in androgen and estrogen synthesis, but synthesize cortisol (Figure 1.3). Nevertheless, the expression of both *CYP11B1* and *CYP21* observed in this study support the suggestion that adrenal and Leydig cells might originate from a common precursor (Hatano et al. 1996, Val 2006). The expression of *CYP11B1* and *CYP21* are previously reported in fetal testis of mice (Hu et al., 2007) and humans (Pezzi et al., 2003), and might be expressed by a subpopulation of fetal Leydig cells or adrenal-like cells in the testes (Hu et al., 2007; Val et al., 2006). The obtained Leydig cell purity of 80% does not exclude that other cells than Leydig cells might express *CYP11B1* and *CYP21*, as proposed in mice. The mRNA levels of *CYP11B1* and *CYP21* were reduced after 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE exposure, but as no detectable cortisol was measured in the medium either from control cells or exposed cells,

CYP11B1 and CYP21 enzyme activity might not be present. Since corticosteroids might inhibit Leydig cell function and suppress testosterone secretion, inhibition of *CYP11B1* translation may prevent high levels of corticosteroid production by the testes, and hence prevent decreased testosterone secretion (Hu et al., 2007). Nonetheless, expression of *CYP11B1* and *CYP21* is found in the porcine testes in the present study in accordance to previous findings in other species (Hu et al., 2007; Pezzi et al., 2003; Wang et al., 2002).

Effect of compounds on LH-induced cAMP-pathway

The general suppression of mRNA levels as well as hormone production by the compounds might be linked to interference with LH function. The Leydig cells were stimulated with LH which increases the mRNA level of several enzymes in the testicular steroidogenesis (Clark et al., 1996; Clark et al., 1994; Debeljak et al., 2003; Sugawara et al., 1996) and boost cholesterol transfer to the inner mitochondrial membrane via the cAMP - protein signaling pathway (Dufau, 1998). Since binding of LH to its receptor will increase cAMP levels and steroidogenic activity in the Leydig cells, reduced cAMP-synthesis might therefore decrease steroid hormone secretion and expression of steroidogenic enzymes (Laurich et al., 2002; Li, 1991; Penhoat et al., 1988; Ronco et al., 2001). Testosterone production and *CYP17A1* mRNA levels induced by LH/hCG-stimulation was reduced after exposure to tributyltin chloride (TBT) in neonatal porcine Leydig cells by reduced intracellular cAMP levels (Nakajima et al., 2005). The mother compound DDE might inhibit the generation of cAMP (Whitehead and Rice, 2006). Reduced cAMP levels might therefore be one possible mechanism for decreased steroidogenic activity causing suppression in hormone production and gene expression.

In summary, both hormone quantification and gene expression analysis indicated a suppressed effect on Leydig cell steroidogenesis induced by the test compounds. The reduction in mRNA levels of some genes might be associated with reduced hormone production, although the compounds suppressed expression of slightly different genes. Gene expression was reduced slightly more potent by *o,p'*-DDD than 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE.

4.4 LH-stimulated versus basal steroidogenesis

As part of the same project and carried out parallel with the present study, exposure of 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD was investigated on basal steroidogenesis by another master student, Irene Sørvik. The findings suggest different response in basal steroidogenesis compared to what obtained in LH-stimulated Leydig cells in this study. First, the basal steroid hormone concentrations and mRNA levels were lower compared to LH-stimulated levels, confirming the stimulatory effect of LH. The study (unpublished) report a slight stimulatory trend of basal steroidogenesis, with induced concentrations of testosterone, and to a less extent estradiol, after exposure to the test compounds (Sørvik, 2010) which is opposite of the suppression observed in the present study. A similar response is reported in cultured rat Leydig cells, with inhibition of LH-mediated testosterone production, but stimulation of basal steroidogenesis (Midzak et al., 2007). They suggested that inhibition of the mitochondrial electron transport chain might block LH-stimulated testosterone production through suppression of several steps of the steroidogenic pathway, but increase the basal steroidogenesis through a calcium-mediated mechanism. A similar explanation could be involved in the present study. The steroidogenic stimulation by LH involves increased cholesterol transfer and mitochondria play a central role as transfer of cholesterol to the inner mitochondrial membrane is the rate-limiting step in the synthesis of steroid hormones. Disruption of mitochondrial function might therefore have a suppressive effect on the Leydig cell steroidogenesis, also reported in mouse Leydig cells (Allen et al., 2006). The inhibition of the mitochondrial electron transport chain caused reduction in testosterone production in the LH-stimulated rat Leydig cells and was associated with reduced ability of the cells to produce cAMP and with reduced steroidogenic enzyme activities. Another effect of electron transport chain inhibition is increased intracellular calcium levels (Duchen et al., 1990; Midzak et al., 2007). Increase in calcium concentrations has been reported in parallel with increased testosterone synthesis and the calcium-dependent increase of testosterone might be independent on cAMP (Sullivan and Cooke, 1986). Inhibition of electron transport chain might therefore be involved in both suppression of LH-stimulated and increase of basal Leydig cell steroidogenesis. The suppressed effect of 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD on LH-stimulated porcine Leydig cell steroidogenesis might involve disruption of mitochondrial function and reduction in cAMP levels, as one possible mechanism.

4.5 Reasons for concern?

The present study report that $3\text{-MeSO}_2\text{-DDE}$, $3,3'\text{-(bis)MeSO}_2\text{-DDE}$, and $o,p'\text{-DDD}$ suppressed the steroidogenesis in neonatal porcine Leydig cells indicating a disruptive effect on testicular steroidogenesis. Reduction in testosterone production might be of concern as testosterone is important for male sex development and testis function (Gnessi et al., 1997). There is an increased concern regarding endocrine disruptors and their effect on male fertility (Eertmans et al. 2003) and the mother compounds DDT and DDE are already known endocrine disruptors that might impair reproduction in wildlife (Kelce et al., 1995; Sharpe, 1995; Sohoni and Sumpter, 1998; Whitehead and Rice, 2006). The suppressive effects and endocrine disruption on Leydig cell steroidogenesis by the DDT metabolites $3\text{-MeSO}_2\text{-DDE}$ and $o,p'\text{-DDD}$ might be of concern considering negative impact on mammalian reproduction. The compounds reduced hormone production with increasing concentrations and caused cytotoxicity at the highest concentration. When revealing endocrine disrupting effects of compounds using cell systems, it is important to keep in mind whether the *in vitro* effects and the doses used are relevant and applicable to *in vivo* exposure. As DDT metabolites are persistent and lipophilic, the test compounds are highly fat soluble and thus might concentrate to a higher degree in adipose tissue, breast milk (Kanja et al., 1992) and reproductive tissues such as testes (Szymczynski and Waliszewski, 1983). Both $3\text{-MeSO}_2\text{-DDE}$ and $o,p'\text{-DDD}$ derives from DDT metabolites that are widespread and persistent in the environment. $3\text{-MeSO}_2\text{-DDE}$ and $o,p'\text{-DDD}$ accumulates in fat tissues of minipigs, but $3\text{-MeSO}_2\text{-DDE}$ is more slowly eliminated (Hermansson et al., 2008). $3\text{-MeSO}_2\text{-DDE}$ is present in tissues of mammals (Bergman et al., 1994; Letcher et al., 1995), humans and in breast milk (Chu et al., 2003; Noren et al., 1996; Noren and Meironyte, 2000). In areas where DDT is still in use or banned at a later date, the levels of DDE in human breast milk are higher (Azeredo et al., 2008). The suppressive effects on Leydig cell steroidogenesis during neonatal development might be of concern considering exposure of $3\text{-MeSO}_2\text{-DDE}$ through breast milk to suckling newborns, as reported in mice (Jonsson et al., 1992) and pigs (Kismul, 2009). Therefore, human risk assessment of $3\text{-MeSO}_2\text{-DDE}$ is suggested to be focused on breast-fed infants. This might be considered as $3\text{-MeSO}_2\text{-DDE}$ is suggested as a potential drug for ACC, exposure during development might cause disruption of Leydig cell steroidogenesis. $o,p'\text{-}$ DDD is also present in the environment, although in less concentrations than the isomer $p,p'\text{-}$ DDD (Falandysz et al., 1999; Ssebugere et al., 2010). In addition to the known toxic effects of $o,p'\text{-DDD}$ in adrenal cortex it might also disrupt Leydig cell steroidogenesis as reported in the

present study. Human malignant Leydig cell tumor has been reported to respond well to *o,p'*-DDD treatment (van der Hem et al., 1992) and *o,p'*-DDD is reported to have estrogen activity characteristics (Tyler et al., 1998). 3,3'-(bis)MeSO$_2$-DDE is a synthetic DDE analogue and hence is not of concern regarding exposure from the environmental, but it has been investigated as a part of the improvement of ACC therapy in humans. Toxicants capable of affecting Leydig cell function may cause spermatogenic damage resulting from androgen deficiency and as Leydig cells play a crucial role in testicular functions they might be target of testicular toxicity *in vivo* (Steinberger and Klinefelter, 1993). Even though experimental concentrations at which endocrine effects are observed are usually above those being measured in the body, the lack of information about mechanisms and metabolism into potentially more active compounds cannot be ignored (Whitehead and Rice, 2006). Although not possible to directly extrapolate to *in vivo* effects, *in vitro* studies might give an indication of possible endocrine disrupting effects and the mechanisms causing them. Porcine Leydig cells in culture is a good model for evaluating steroidogenic effects of compounds (Brun et al., 1991) and the *in vitro* results obtained in the present study indicate an endocrine disrupting effect on Leydig cell steroidogenesis.

5. Conclusions

To the best of our knowledge the present study is the first to investigate possible endocrine disrupting effects of 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD on LH-stimulated neonatal porcine Leydig cells. The results suggest a suppressive effect of the test compounds on LH-stimulated Leydig cell steroidogenesis, but with large variation in studied endpoints between experiments.

Both 3-MeSO$_2$-DDE and *o,p'*-DDD reduced Leydig cell viability with cytotoxic effect at the highest concentration (20 µM). *o,p'*-DDD was the most potent compound, whereas 3,3'-(bis)MeSO$_2$-DDE did not have any effect on cell viability in the present study. This might indicate that the cytotoxic effects are structure-dependent and that the extra methylsulfonyl group makes 3,3'-(bis)MeSO$_2$-DDE less toxic.

3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD suppressed testosterone, estradiol and progesterone synthesis in LH-stimulated neonatal porcine Leydig cells in a concentration-dependent manner. No detectable levels of cortisol were obtained. The results suggest an inhibitory effect of all three compounds on Leydig cell testicular steroid hormone production. The reduction in progesterone secretion as well as testosterone and estradiol might indicate that the compounds inhibit Leydig cell steroidogenesis cascade upstream.

Overall, the expression of genes involved in Leydig cell steroidogenesis was decreased by the three test compounds, although one gene was up-regulated following 3,3'-(bis)MeSO$_2$-DDE exposure. The compounds reduced expression of slightly different genes, and the results might indicate a more potent reduction following *o,p'*-DDD exposure compared to 3-MeSO$_2$-DDE and 3,3'-(bis)MeSO$_2$-DDE. The decreased expression of some genes in the exposed Leydig cells might be associated with the reduced synthesis of steroid hormones observed at the same concentration.

The potential endocrine disruption of 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD on Leydig cell steroidogenesis might be of concern as Leydig cells play a crucial role for male reproductive function and development by the secretion of testosterone and estradiol.

6. Future perspectives

The variation between the experiments is a consequence of working with primary cell cultures, and further replicates should be included for more conclusive results. An additional gene important for testosterone synthesis, *HSD17B3*, could be included to the gene expression assay.

To investigate the mechanisms behind the suppression of Leydig cell steroidogenesis induced by 3-MeSO$_2$-DDE, 3,3'-(bis)MeSO$_2$-DDE and *o,p'*-DDD, further studies are needed. Previous studies in adrenal cells suggest steroidogenic disruption by the compounds caused by interaction with steroidogenic enzymes at the protein level. Therefore, the inhibitory effect on Leydig cell hormone production by the compounds might involve interaction at the protein level and possible alterations in enzyme activity could be interesting to investigate.

The suppression of Leydig cell steroidogenesis might involve inhibition of LH-stimulation following exposure to the compounds, therefore intracellular cAMP levels could be measured in exposed and control cells. Inhibition of the mitochondrial electron transport chain might suppress Leydig cell steroidogenesis, hence the compounds effect on mitochondrial function could be investigated.

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8. Appendix

8.1 Chemicals

Chemical	Producer
3-MeSO$_2$-DDE	Synthelec AB, Ideon, Lund, Sweden
3,3'-(bis)MeSO$_2$-DDE	Synthelec AB, Ideon, Lund, Sweden
o,p'-DDD	Sigma-Aldrich, Stockholm, Sweden
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich AS, Oslo, Norway
LH (tuenre.pLH.ig,)	Tucker Endocrine Research Institute, Atlanta Georgia, USA
ITS + Premix	BD Biosciences via VWR, International AS, Oslo, Norway
NuSerum	BD Biosciences via VWR, International AS, Oslo, Norway
Ham's F-12 and Dulbecco's Modified Eagles Medium supplemented with 1.2 mg/ml sodium bicarbonate and 15 mM Hepes, pH 7.4 (500 ml) (DMEM/F12)	Gibco, Invitrogen, Carlsbad, CA, USA
Ham's F-10 Nutrient mixture	Biological Industries, Israel
Collagenase-dispase (500 mg)	Vibrio alginolyticus/Bacillus polyxema, Roche Neuss, Germany
Penicillin/Streptomycin/Neomycin (PSN)	Invitrogen, Carlsbad, CA, USA
Percoll (500 ml)	Sigma-Aldrich AS, Oslo, Norway
AlamarBlue	Invitrogen, Carlsbad, CA, USA
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich AS, Oslo, Norway
M199 modified Earle's salts	Sigma-Aldrich AS, Oslo, Norway
Fetal calf serum (FCS)	Fisher Scientific, Pittsburgh, PA, USA
Lidocain	Haukeland Hospital Pharmacy, Bergen, Norway
Ketoprofen	Merial SAS, Lyon, France
Trypsin EDTA	Gibco Invitrogen, Carlsbad, CA, USA
Nitro blue tetrozolium	Sigma-Aldrich AS, Oslo, Norway
5-androstane-3β-ol-one	Sigma-Aldrich AS, Oslo, Norway
NAD$^+$	Sigma-Aldrich AS, Oslo, Norway

8.2 Preparation of Solutions and Media

Collagenase/dispase stock

For the collagenase/dispase stock 500 mg collagenase (Roche, Neuss, Germany) were dissolved in 10 ml of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM/F12). This produced a 100x collagenase stock solution (50 mg/ml), which were divided into 1 ml aliquots and stored at -20°C.

Collection medium

The collection medium was made from DMEM/F12 (500 ml) including 10 ml antibiotics penicillin, streptomycin and neomycin (PSN).

Dissociation medium

The dissociation medium was prepared on the day of use by mixing 75 ml DMEM/F12 supplemented with PSN (10 ml PSN to 500 ml DMEM/F12) in a sterile flask. 2 ml aliquots of the collagenase/dispase (100x) stock were thawed and diluted with 8 ml of the DMEM/F12 medium and 5 ml Fetal Calf Serum (FCS) were added. We filtered through a sterile 0.2 μm filter (VWR International AS, Oslo, Norway) and added to the remaining DMEM/F12. This is enough to digest about 30 testicles in a 500 ml flask. The dissociation medium was kept at 4°C until use and put at 34°C water bath before the start of the testicle decapsulation.

Complete plating medium

The complete plating medium was prepared by taking 500 ml DMEM/F12 and adding 10 ml of PSN, 5 ml ITS + Premix and 12.5 ml NuSerum and stored at 4°C.
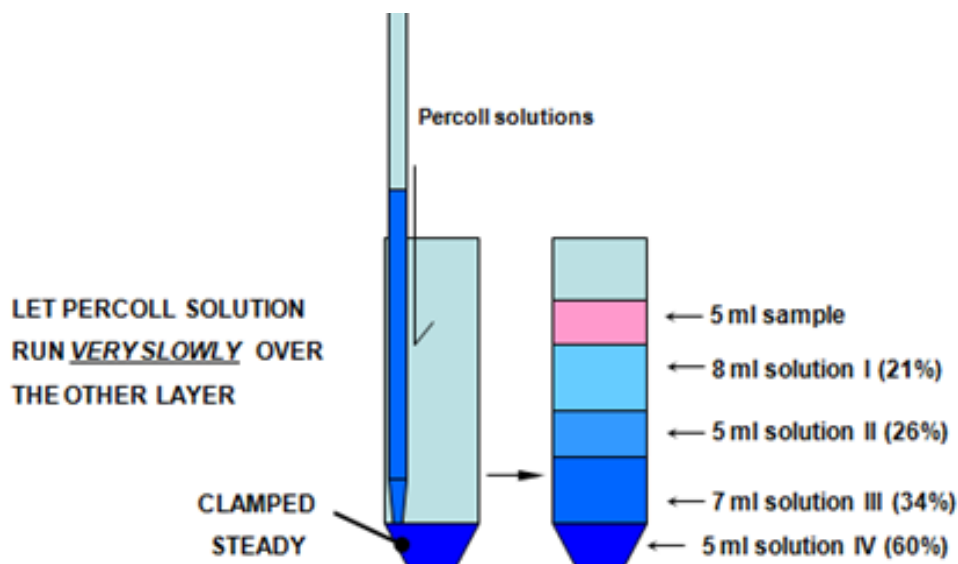
Percoll solutions

For preparation of the Percoll solutions with the required densities (for 12 gradients) we mixed 13 ml 10x Ham's F10 nutrient mixture with 117 ml of undiluted Percoll which creates a Percoll 90% isosmotical solution (130 ml). 220 ml of DMEM/F12 and four labelled 50 ml sterile Falcon tubes is ready. To each tube the required amount of Percoll 90% and DMEM/F12 was added for the four individual Percoll dilutions (Table 8.1). The Percoll dilutions were stored at 4°C until use and are sufficient to construct 12 gradients.

Table 8.1 Construction of Percoll solutions with the required amounts of Percoll 90% and DMEM/F12 (12 gradients).

Solution (nr)	Final density (%)	Percoll 90% (ml)	DMEM/F12 (ml)
I	21%	24.5	82.1
II	26%	18.1	45.8
III	34%	40.5	66.1
IV	60%	42.7	21.3
		Total = 125.8	Total = 215.3

8.3 Construction of Percoll discontinuous gradient

A steady application of the solutions yields the most reproducible gradients. For each gradient we prepared a 50 ml tube and added 5 ml of the most dense Percoll solution IV (60%) to the bottom of the tubes (Figure 8.1). 7 ml of solution III (34%) in a 10 ml pipette is placed at the meniscus of the previous layer to form a liquid bridge and then we very slowly empty the pipette. Then a clean layer is formed on top of the previous one. This is repeated carefully with 5 ml of solution II (26%) and finally with 8 ml of solution I (21%). It is important that enough space is left at the top of the tube, which to pipette the 5 ml sample of the dissociated testicular cells.

Figure 8.1: Making the Percoll discontinuous gradient. Each of the solutions (I-IV) was carefully added to a 50 ml tube to make the gradients.