Acta Chim. Slov. **1998**, *45*(1), pp. 59-67

(Received 28.1.1998)

ALTERED GENE EXPRESSION IN THE FOETAL RAT TESTIS FOLLOWING THE OESTROGEN EXPOSURE

*Gregor Majdic, #Philippa TK Saunders
*Faculty of veterinary medicine, Gerbiceva 60, 1000 Ljubljana, Slovenia
#MRC - Reproductive Biology Unit, 37 Chalmers street, Edinburgh EH3 9EW, Scotland

Abstract

Several reports in recent years have shown evidence for increasing incidence of male reproductive problems as cryptorchidism, hypospadias and testicular cancer. In addition, several reports have published evidence for decrease in sperm counts in many western countries, which might be as high as 50% in the last 50 years. One hypothesis have linked this problems to increased exposure to oestrogenic chemicals during foetal development. Aim of our studies was to establish whether environmental oestrogens could affect testicular development in rats. Time-mated pregnant rats were treated subcutaneously with either diethylstilbestrol (DES, potent synthetic oestrogen), octylphenol (OP, environmental oestrogen) or oil alone as control on days 11.5 and 15.5 p.c. Animals were sacrificed on day 17.5 p.c. Testes were dissected from their foetuses and either fixed in Bouin's solution, used for RNA extraction or used directly for measuring P450c17 enzyme activity. Lower activity and expression of steroidogenic enzyme P450c17 has been detected in the testes from foetuses whose mothers received either DES or OP. In addition, reduced expression of recently discovered transcription factor Steroidogenic factor-1 (SF-1) has been detected.

Introduction

Sexual differentiation of the male is not just the process of establishing the normal male phenotype but it is also a period when the foundations for fertility in adulthood are laid down. Sexual differentiation and especially development of the male gonads and male phenotype are hormonally regulated and therefore any disturbances of the hormone action in this sensitive period could have far reaching consequences. Recent reports about falling sperm counts and increasing incidence of testicular cancer and other

disorders of development of the male reproductive tract have been hypothetically linked to increased exposure to environmental chemicals which are either oestrogenic or anti-androgenic hormonal mimics. In the present article, the evidence for importance of foetal/neonatal period for functioning of adult reproductive system and some date about the effects of oestrogenic chemicals will be discussed.

Development of the testis and male phenotype

The first morphological event in the development of the testis is appearance of Sertoli cells within the testicular cords (1). Thereafter, these cells control and co-ordinate all the processes relating to testicular development and masculinisation. Sertoli cells secrete antimullerian hormone (AMH), responsible for regression of female reproductive ducts in the male foetus. Via the unknown factors, the Sertoli cells also regulate the regulation and multiplication of early germ cells and also the differentiation and function of Leydig cells, which produce testosterone, hormone responsible for the masculinisation of the whole foetus (1). Simultaneously with their differentiation, Sertoli cells start to proliferate and this continues for the rest of the foetal life and short period unto postnatal life. However, soon after birth, in rats around day 18 p.n. while in humans it is thought to continue at a slow rate until beginning of the puberty, the proliferation of Sertoli cells cease and their numbers remain constant for the rest of the life (2). Therefore the increase of Sertoli cells in the foetal/neonatal period is crucial as the number of Sertoli cells determines the final adult testis size and the capacity for sperm production. This has been clearly shown in studies in animals, when the window of Sertoli cells proliferation has been changed therefore increasing or decreasing their numbers. The regulation of Sertoli cells multiplication is not known yet, however, several hormones are thought to be involved in these processes. This include FSH, which stimulates proliferation of Sertoli cells and thyroid hormones T3 and T4, which seems to be involved in determining the cessation of Sertoli cells proliferation. Experiments in rats have shown that hypothyroidism will prolong the time of Sertoli cells proliferation and therefore result in higher number of Sertoli cells, bigger testis and higher sperm output in adult life. In contrast, hyperthyroidism cause premature cease of Sertoli cells proliferation and finally results in lower testis size and lower sperm production (2, 3, 4).

The proper function of Leydig cells is equally important for normal functioning of reproductive system. Testosterone, main product of the Leydig cells is main factor responsible for masculinisation of the foetus and any interference with its action will have deleterious effects in the male foetus (1). The importance of testosterone is clearly shown in pathological condition called androgen insensitivity syndrome. In this syndrome, the testosterone cannot act due to a mutation in the gene encoding androgen receptor. Subsequently, such foetuses develop female phenotype (or in some cases various degrees of intersex phenotype), despite the presence of the testosterone producing testis in the abdominal cavity (5). Descensus of the testis in the neonatal life into the scrotum is also thought to be regulated by testosterone and cryptorhidism is thought to be a result of improper action of testosterone in the time when descensus should occur (1).

Male reproductive problems

In recent years, several reports have been published about increase in male reproductive problems in western world. These include increased incidence of developmental defects cryptorhidism and hypospadias and increase in incidence of testicular cancer (6). In addition, several reports have shown decline in average sperm counts in western males in last 50 years, which might be as high as 50% (6, 7). While the data about lower sperm counts are still highly debated and will be difficult to prove, the data about increased incidence of cryptorhidism, hypospadias and testicular cancer is most likely to be real. All three conditions are unlikely to be undetected and in several countries, including Slovenia, there exist reliable medial records about their incidence (6).

The cause of these problems is not yet known. Similar problems have been observed in the offspring from mothers, who were treated with potent synthetic oestrogen diethylstilbestrol (DES) during their pregnancies. Namely, in 1950 and 60's, several millions women in Europe and USA were treated with DES as a prevention of miscarriage. Unfortunately, later studies have shown that DES had a very deleterious effects on the development of reproductive system of their offspring. In male offspring of these mothers, very high incidence of cryptorhidism, hypospadias, testicular cancer as well as smaller testis and lower sperm production were detected (8). Similarities between these problems have suggested potential link. Sharpe and Skakkebaek (9) have

reviewed the possible sources of oestrogenic exposure and have suggested that changes in diet, increases in body fat and obesity, together with increases in exposure to environmental pollutants with oestrogenic activity could all contribute to increased exposure to oestrogenic chemicals in present time. The diet of the individuals in many developed countries has radically changed over the last 50 years with an increase in the consumption of milk and dairy products. These may contain oestrogens due to intensive farming with milking occurring during pregnancy. A large increase in use of soya (soya is very rich in phytooestrogens) could also have contributed to an increased exposure to oestrogens in every day life. Furthermore, it is likely that we are all exposed to many oestrogens or chemicals mimicking oestrogen action due to environmental pollution. It has been speculated that usage of contraceptive pills containing synthetic oestrogens, the use of anabolic oestrogens in animal farming and widespread use of chemical agents that exhibit oestrogenic activity by industry may all have contributed to an increasing exposure to oestrogens (9).

Environmental oestrogens

This term is applied to phytoestrogens and man-made chemicals which are released into environment and which have oestrogenic activity either in vivo or in vitro (10). It is known for some time that some PCB isomers and DDT and its metabolites have such activity. However, more worrying, similar action has been shown for several other chemicals including alkylphenols, bisphenol-A and phtalates. The majority of these chemicals have a different chemical structure to endogenous oestrogens and at present it is not therefore possible to assess whether a compound is likely to have oestrogenic activity based on the knowledge of its chemical structure (10). In many instances the oestrogenic activity of these chemicals was discovered accidentally. For example, the observation that oestrogenic substances were released from laboratory plastic ware led to the identification of alkylphenols, in particularly nonylphenol and bisphenol-A as a chemicals with oestrogenic activity (11, 12). In addition, oestrogenic activity has also been shown for phthalate esters, another group of chemicals that are ubiquitously found in the environment (13).

Alkylphenol polyethoxylates were introduced in the 1940s and are widely used in detergents, paints, herbicides, pesticides and many other products (14). Studies by White et al. (15) have shown oestrogenic activity of nonylphenol and octylphenol (OP*) in vitro* and *in vivo* and in addition studies by Sharpe et al. (16) have shown the effects of octylphenol exposure in utero and postnatally on the adult testis size in rats. In this studies, female rats were treated with DES or OP via drinking water during pregnancy and postnatally untill weaning in the doses 100 and 1000μg/L of OP and 10 and 100μg/L of DES, doses probably comparable to human exposure. Their male offspring where sacrificed on day 90 and their testis size and daily sperm production (DSP) assesed. In animals treated either with DES or OP, small but significant reduction in both testis size and daily sperm production were observed therefore showing that exposure to even small doses of these chemicals in fetal and neonatal life could have deleterious effects on developing testis.

Effect of oestrogenic chemicals

In our experiments, time mated female rats were treated on day 11.5 p.c. (just prior to start of gonadal development) and on day 15.5 p.c. (at the start of testosterone production) subcutaneously with 100 or 500μg/Kg of DES in oil, 100 or 600 mg/Kg of OP in oil or oil alone as a control group. Animals were sacrificed on day 17.5 p.c., when the production of testosterone in the foetal testes is at its highest level, and the testes from their foetuses either fixed in Bouins', used for total RNA isolation or used directly for enzyme activity measurements. Administration of either OP or DES resulted in a massive reduction in immunostaining for P450c17 when this was assessed (17) and a parallel reduction in enzyme activity was also found when the testis were removed and homogenised (Fig. 1). The effect of oestrogenic chemicals appear to be at the level of mRNA as in situ hybridisation studies have shown reduced expression of CYP51 gene (17). This decrease could be a consequence of reduced Leydig cell number and/or a decrease in the level of enzyme in the cells. In this instance it appears to be the later, as the immunostaining for 3β-hydroxysteroid dehydrogenase, another Leydig cell steroidogenic enzyme, was comparable in control, OP- and DES-treated animals (17).

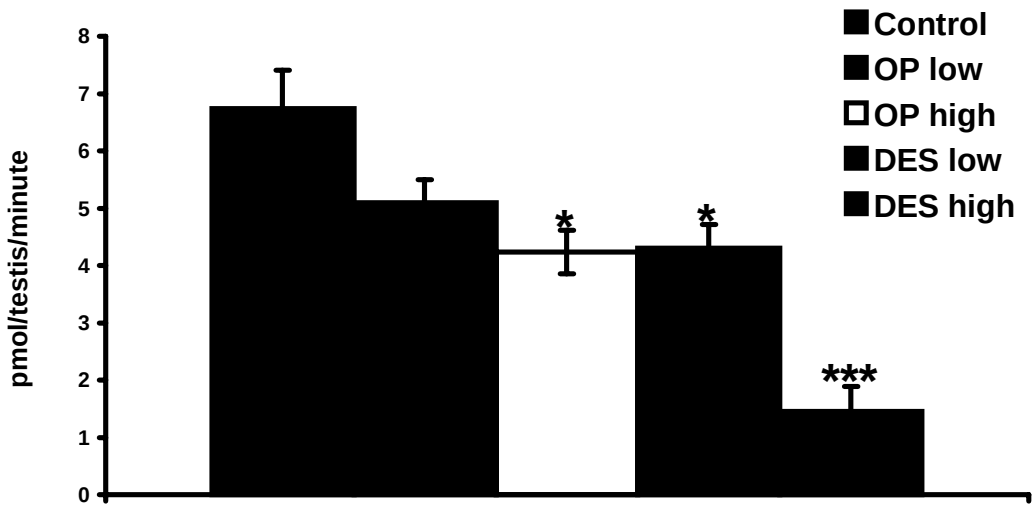

Fig. 1: P450c17 enzyme activity in the foetal testis isolated on day 17.5 p.c. from mothers treated with: Control - oil alone, OP low - 100mg/Kg, OP high - 600mg/Kg, DES low - 100μg/Kg, DES high - 500μg/Kg (* $p<0.05$, *** $p<0.001$).

In additional experiments, the expression of Steroidogenic factor-1 (SF-1) was also assessed. SF-1 is a transcription factor with important function in the development of reproductive tract. Transgenic mice, lacking functional SF-1 gene do not develop either gonads or adrenal glands, have disorganised hypothalamus and unfunctional pituitary gonadotroph cells. The exact function of this factor is not known yet, however, several studies have shown that it regulates expression of several genes with important functions in development of reproductive axis. These include AMH, α-GSU, LH-β subunit, oxytocytine and several cytochromes P450s including P450c17 (18). In our studies, reduced expression of SF-1 was found at both protein and mRNA level (19). Immunostaining showed severely reduced immunoexpression of SF-1 protein and RNAse protection studies have shown similar decrease in gene expression (Fig. 2).

The consequences of both these findings are unknown yet. However, reduced expression of P450c17 is certainly expected to result in reduced testosterone production, which could have deleterious effects on masculinisation of the foetus which is occurring at that time. The possible consequences of reduced SF-1 expression could be even more

severe, bearing in mind the importance of SF-1 for development of reproductive system, but further studies will be needed to establish this.

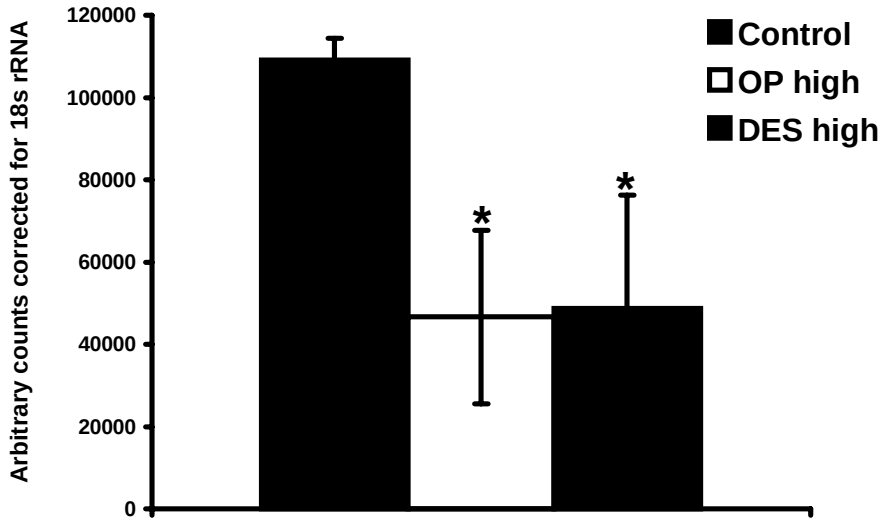

Fig. 2: SF-1 mRNA levels in testes from fetuses of OP and DES treated mothers. Control - oil alone, OP high - 600mg/Kg, DES high - 500μg/Kg (* $p<0.05$).

Conclusion

The available data for man and animals demonstrate clearly that early development of the testis, beginning at the time of sexual differentiation and extending out into early postnatal life, is critical in determining the function of reproductive tract in adult life. This is a very sensitive period and any interference with these processes is likely to have far reaching dire effects. Exposure to exogenous hormones or to chemicals with hormone-like activity thus has the potential to interfere with these processes, an effect which is likely to be irreversible because of the relatively narrow time-window within which these changes occur. The present and other studies (16, 17, 19) demonstrate that indirect exposure of the foetal/neonatal male rat to environmental oestrogenic chemicals administered to the mother, can significantly perturb these early processes by affecting the capacity of Leydig cells to secrete testosterone or reduce the testis size and sperm production (most likely due to reduced Sertoli cell number). It remains to be shown whether these changes actually affect fertility or reproductive function (e.g. behaviour) in

adulthood. The relevance to man of these findings is also unknown and will depend mainly on the level of human exposure to these chemicals. Therefore, it remains the matter of speculation whether these findings are connected with reported increase in male reproductive problems and fallen sperm counts. However, the mechanisms for deleterious action of oestrogenic chemicals on developing foetus exist and further studies will be needed to asses the potential danger for human population.

Povzetek

Več člankov je v zadnjih letih objavilo ugotovitve, da pri ljudeh narašča pojavnost problemov na moških spolnih organih kot so kriptorhizem, hipospadije in rak na modih, objavljenih pa je bilo tudi več poročil, ki kažejo na to da se je povprečno število semenčic v ejakulatu pri moškem v zadnjih petdesetih letih zmanjšalo kar za polovico. Eden od možnih vzrokov za povečanje pojavnosti teh problemov je povečana izpostavljenosti snovem z estrogenim delovanjem. Namen naših raziskav je bil ugotoviti možen vpliv teh kemikalij na razvoj moda pri podgani. Breje podgane so prejele podkožno Dietilstilboestrol (DES), onesnaževalec okolja Oktilfenol ali samo olje (kontrolna skupina) na dan 11.5 in 15.5 brejosti. Podgane so bile žrtvovane na dan 17.5 ter iz njihovih zarodkov izolirana moda, ki so bila fiksirana v Bouinovi raztopini, uporabljena za izoliranje RNK ali pa uporabljena neposredno za merjenje encimske aktivnosti. V modih podgan, katerih matere so bile tretirane z omenjenima estrogenima snovema je bila ugotovljena zmanjšana aktivnost encima P450c17 ter tudi zmanjšana izraženost gena, ki kodira ta encim, vendar pa to ni bilo na račun manjšega števila Lejdigovih celic saj imunohistokemično barvanje z uporabo protiteles proti encimu 3β-hidroksisteroidna dehidrogenaza (3β-HSD) ni pokazalo očitnih razlik v številu teh celic med tretiranimi in kontrolnimi živalmi. Poleg zmanjšane izraženosti gena CYP17 pa je bila ugotovljena tudi zmanjšana izraženost gena SF-1, ki kodira jedrni hormonski receptor Steroidogeni faktor-1.

References

[1] F.W. George and J.D. Wilson, The Physiology of Reproduction, Raven Press, New York, 1994, pp 3-28.
[2] R.M. Sharpe, The Physiology of Reproduction, Raven Press, New York, 1994, pp 1363-1434.
[3] R.A. Hess, P.S. Cooke, D. Bunick and J.D. Kirby, *Endocrinology*, **1993,** 132, 2607-2613.
[4] L.H. Van Haster, F.H. de Jong, R. Docter and D.G. de Rooij, *Endocrinology*, **1993**, 133, 755-760.
[5] C. Quigley, A. De Bellis, K. Marschke, M. El-Awady, E. Wilson and F. French, *Endo. Rev.*, **1995**, 16, 271-321.
[6] A. Giwercman, *Hum. Rep.*, **1995**, 10, 158-164.
[7] E. Carlsen, A. Giwercman, N. Keiding and N.E. Skakkebaek, *B. Med. J.*, **1992**, 305, 609-613.
[8] R.J. Stillman, *Am. J. Obst. Gynae.*, **1982**, 142, 905-921.
[9] R.M. Sharpe and N.E. Skakkebaek, *Lancet*, **1993**, 341, 125-126.
[10] R.M. Sharpe, *Curr. Opinion in Urol.*, **1994**, 4, 295-304.
[11] A.V. Krishnan, P. Stathis, S.F. Permuth, L Tokes and D Feldman, *Endocrinology*, **1993**, 132, 2279-2286 .
[12] A.M. Soto, H. Justicia, J.W. Wray and C. Sonnenschein, *Environ. Health Perspec.*, **1991**, 92, 167-173.
[13] S. Jobling, T. Reynolds, R. White, M.G. Parker and J.P. Sumpter, *Environ. Health Perspec.*, **1995**, 103, 582-586.
[14] G.C. Naylor, J.P. Mierure, J. Weeks, F.J. Castaldi and R.R. Romano, *J. Am. Oil Chemists Soc.*, **1992**, 69, 695-703.
[15] R. White, S. Jobling, S.A. Hoare, J.P. Sumpter and M.G. Parker, *Endocrinology*, **1994**, 135, 175-182.

[16] R.M. Sharpe, J. Fisher, M.R. Millar, S. Jobling and J.S. Sumpter, *Environ. Health Perspec.*, **1995**, 103, 1136-1143.
[17] G. Majdic, R.M. Sharpe, P.J. O'Shaughnessy and P.T.K. Saunders, *Endocrinology*, **1996**, 137, 1063-1070.
[18] K.L. Parker, BP Schimmer, *Endo. rev.*, **1997**, 18, 361-377.
[19] G. Majdic, R.M. Sharpe and P.T.K. Saunders, *Mol. Cell. Endocrinol.*; **1997**, 127, 91-98.