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Effect of dehydroleucodine on the reproductive tract of male mice

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Summary

The effects of a sesquiterpene lactone, dehydroleucodine, on the reproductive tract were investigated using adult male mice. Dehydroleucodine was dissolved in tap water and administered as drinking water for 30 days. All the parameters were compared with a control group that received only vehicle. Animals were killed by decapitation and the trunk blood, the testes and the epididymes were collected. Plasma concentrations of testosterone and oestradiol, and testicular weight and concentration of spermatids did not change by dehydroleucodine. Nevertheless, in epididymal cauda dehydroleucodine treatment caused a diminution in sperm number, a decrease in the amount of tubular fluid and a reduction in the activity of the hydrolytic enzyme *N*-acetyl-β-D-glucosaminidase. However, the sperm motility was not altered by dehydroleucodine treatment, although sperm binding to zona-free oocytes increased significantly. These results suggest that dehydroleucodine, which has been implicated in the inhibition of aromatase P450, does not affect the plasma concentration of testosterone and oestradiol or testicular activity, whereas altering several epididymal parameters. The epididymis is thus a more sensitive target for dehydroleucodine action.

Introduction

Sesquiterpene lactones are a large and structurally diverse group of plant metabolites (Heinrich *et al.*, 1998). Several biological activities of sesquiterpene lactones have been reported, including anti-tumour (Robles *et al.*, 1995), anti-migraine (Beekman *et al.*, 1997), gastric cytoprotector (Giordano *et al.*, 1992) and aromatase enzyme inhibitor (Blanco *et al.*, 1997) effects.

Aromatases belong to the monooxygenase family of enzymes that metabolise substrates by adding single oxygen atoms to them (Hayaishi, 1962; Mason, 1965). The largest group of monooxygenases is the cytochrome P450 superfamily, where some members of the group participate in the biosynthesis of compounds such as steroid hormones, vitamins and antibiotics (Wang *et al.*, 2009). P450 aromatase is one of them and oxidises only a few substrates that are involved in oestrogen synthesis. This enzyme catalyses the direct production of oestrone from a steroid known as androstenedione (Simpson *et al.*, 1997).

Dehydroleucodine (DhL) is a sesquiterpene lactone of the guaianolide group that has been isolated and purified from the aerial parts of *Artemisia douglasiana Besser*, a medicinal herb used in Argentina (Giordano *et al.*, 1990). It has been shown that DhL inhibits the enzyme P450 aromatase in rat uterus (Blanco *et al.*, 1997, 2001). As P450 aromatase is involved in converting testosterone to oestradiol in testis (Simpson *et al.*, 1997), we evaluated whether the administration of DhL to male mice alters aspects of the reproductive tract. To this end, we analysed the plasma levels of testosterone and oestradiol and the concentration of spermatids in the testis and sperm in the epididymis. In the cauda epididymis, the activity of two hydrolytic enzymes was analysed. In addition, the motility of sperm and the ability to bind to zona-free oocytes was evaluated.

Materials and methods

Animals of experimentation

Adult female golden hamsters were used as source of oocytes.

Two-month-old BALB/c mice fed *ad libitum* were used in the experiments with DhL. DhL at 93% purity was

obtained according to Giordano *et al.* (1990, 1992). A stock solution of 0.2 M DhL in DMSO was stored at 4 °C until use. DhL solution stock was diluted in tap water to 0.4 mM and administered as drinking water to mice (35 mg kg^{-1} body weight per day) over the course of 30 days (treated group). Another set of mice received only the vehicle (DMSO) in the drinking water 30 days (control group).

Blood samples

Mice were killed by decapitation (following the international rules for animal handling of the National Institute of Health) and trunk blood was collected for measurement of serum hormone concentrations.

Oestradiol and testosterone assay

Oestradiol and testosterone concentrations in plasma were measured by radioimmunoassay using commercial kits for total hormones (DSL-4800 and DSL-4100 double antibody radioimmunoassay, respectively, from Diagnostic Systems Laboratories, Webster, Texas, USA).

Preparation of spermatids, sperm and epididymal content

After 1 month of DhL treatment, mice were sacrificed by decapitation and testes and epididymes were removed and weighed. The number of advanced (steps 17–19) spermatids in the testis was determined by the homogenisation method (Amann & Lambiase, 1969). Each testis was homogenised in a glass-teflon pestle with 0.5 ml PBS and sonicated for 2 min. The suspension was diluted 1 : 10 with PBS and spermatids were counted in a haemocytometer chamber. To obtain the epididymal tubular content (fluid and sperm), the apical tubular end of cauda epididymidis was catheterised with a needle to allow content release by retroperfusion. The tubules were injected with retroperfusion buffer: 20 mM phosphate buffer pH 7.4, 150 mM NaCl, 5 mM, EDTA, 0.5 mM PMSF) using a syringe at low pressure. After collecting tubular content, the tubule interior was dried with air and the epididymis was weighed. The weight of tubular content was estimated as the difference between intact and dried-tubule epididymis (d-te). The tubular content was used for counting sperm and for assaying enzymatic activity. Additionally, enzymatic activity was estimated in d-te and this was taken as the activity in cauda epididymal tissue.

Sperm for both motility and binding tests were obtained from the cauda epididymidis by cutting the tissue in several transverse sections which were layered at the bottom of a 1.7 ml eppendorf tube containing 0.3 ml of nutrient mixture F-10 HAM (Sigma, St. Louis, Missouri, USA) (HAM) supplemented with 25 mM Na bicarbonate, 25 mg ml^{-1} BSA (supplemented HAM) and incubated at 37 °C under 5% CO_2 per air for 1 h. Mobile sperm from the cauda epididymidis swim out into the medium. The number of sperm was counted and the suspension was used for motility and swim-up assays.

The percent of motility was determined from the progressive and nonprogressive movements of sperm.

For the sperm swim-up assay, 5×10^5 sperm from the suspension were layered at the bottom of a 1.7 ml Eppendorf tube containing 1.5 ml supplemented HAM and incubated at 37 °C under 5% CO_2 per air for 30 min. Five fractions of 0.3 ml were collected from the top of the tube and numbered consecutively 1–5. In each fraction, the number of sperm was counted. Sperm from fraction 1 were used in the sperm–oocyte interaction test.

Zona-free hamster oocytes

Oocytes were obtained from hamsters that had been injected intraperitoneally with 30 IU of pregnant mare serum gonadotrophin (PMSG) on the day of oestrus and with 40 IU of human chorionic gonadotrophin (hCG) 48 h later. Eighteen hour after hCG administration, the hamsters were killed by cervical fracture. The fallopian tubes were washed in supplemented HAM (in the following steps, supplemented HAM was used as washing solution), the cumulus and the zona pellucida were dispersed in 1% hyaluronidase, followed by 1% trypsin, under a stereomicroscope. The oocytes were then washed three times and used immediately (De Rosas & Fornes, 1990).

Sperm–oocyte interaction

Drops (0.1 ml) sperm from the swim-up fraction 1 of both control and treated mice were diluted to a concentration of 5×10^5 ml^{-1} in supplemented HAM and were placed under mineral oil in Petri dishes. Thirty oocytes were mixed with 2.5×10^4 sperm and incubated for 30 min at 37 °C in an air chamber with 5% CO_2. The preparation was fixed with 5% glutaraldehyde in PBS and analysed using phase contrast in a Nikon TE 2000-U microscope with a Plan Fluor 20 × lens (Tokyo, Japan).

Enzymatic activity in epididymis

The epididymal tubular content obtained by retroperfusion was centrifuged at 800 ***g*** for 10 min at 4 °C. The supernatant containing the fluid fraction was clarified by centrifugation at 60 000 ***g*** for 30 min and the enzymatic activity was measured.

N-Acetyl-β-D-glucosaminidase (β-NAG) and β-galactosidase (β-GAL) activities were assayed fluorometrically by using a 4-methylumbelliferyl substrate according to Barrett & Heath (1977). The enzymatic activity was normalised to the weight of cauda epididymidis, weight of tissue (weight of cauda d-te) and to the weight of tubular content (Belmonte *et al.*, 1998).

Statistical analysis

The values compared were analysed statistically by Student's t-test and the level of significance was set at $P \leq 0.01$.

Results

Oestradiol and testosterone levels

We analysed whether the administration of DhL to mice affected steroid hormone production. Plasma of control mice contained 2.7 pg ml^{-1} testosterone whereas DhL-treated mice had 3.2 pg ml^{-1} (Table 1). The level of oestradiol in plasma was 15.1 ng ml^{-1} in the control and 16.8 ng ml^{-1} in treated animals (Table 1).

Organ weight

The weight of testes and epididymes was not affected by DhL (Table 2). But the epididymal tubular content weight was 10.9 mg in controls and 6.4 in DhL-treated animals (Table 2), indicating a significant reduction with DhL treatment.

Sperm characteristics

In testis, the number of spermatids did not change after treatment (Table 2). However, the number of sperm in cauda epididymidis (for mg of tubular content) was 2.9×10^6 and 1.6×10^6 in controls and treated animals, respectively, a near 50% reduction in the total number of sperm after DhL treatment (Table 2).

The percentage of sperm motility was determined as 69.3 and 71.0 for control and treated animals (Table 1) and the same percentage of sperm from control and treated animals reached each fraction in the swim-up assay (Fig. 1).

Table 1 Concentration of testosterone and oestradiol in plasma

Hormone	Control[a]	Treated
Testosterone (ng ml^{-1})	2.7 ± 0.71	3.2 ± 1.2
Oestradiol (pg ml^{-1})	15.1 ± 2.0	16.8 ± 1.3

[a]Values are expressed as means ± SE of 15 experiments.

Table 2 Weight of testis and epididymis and number of spermatids and sperm

Tissues	Control	Treated
Testis		
Weight (mg)	85.7 ± 1.4	82.2 ± 1.9
Spermatid number mg^{-1} of testis ($\times 10^{-5}$)	1.3 ± 0.13	1.4 ± 0.12
Spermatid number in the testis ($\times 10^{-5}$)	110.2 ± 9.2	115.1 ± 6.3
Epididymis		
Weight (mg)		
Whole	35.6 ± 0.1	35.4 ± 0.7
Cauda	15.4 ± 0.6	16.5 ± 0.8
Cauda tubular cont		
mg mg^{-1} cauda	0.31 ± 0.01	0.18 ± 0.02*
mg per cauda	10.9 ± 0.6	6.4 ± 0.8*
Sperm		
Number in cauda per mg tubular cont. ($\times 10^{-6}$)	2.9 ± 0.6	1.6 ± 0.45*
Number in cauda ($\times 10^{-6}$)	103.2 ± 9.1	56.6 ± 3.7*
Per cent motility	69.3 ± 4.1	71.0 ± 3.2

*$P \leq 0.01$ (compared with controls).
[a]Values are expressed as means ± SE of six experiments.

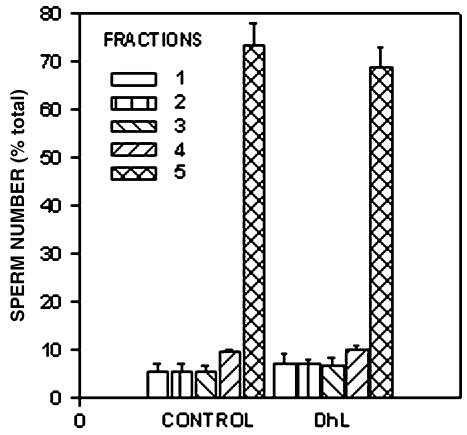

Fig. 1 Sperm swim-up. 5×10^5 sperm from control group (Control) and DhL-treated group (DhL) were layered at the bottom of a 1.7-ml Eppendorf tube containing 1.5 ml supplemented HAM and incubated at 37 °C under 5% CO_2 per air for 30 min. Five fractions (1–5) of 0.3 ml were collected from the top of the tube. The number of sperm in each fraction was expressed as a percentage of total. The data are the average percentage in each fraction ± SEM from six experiments.

To evaluate the capacity of sperm to bind to oocytes, zona-free hamster oocytes were mixed with a suspension of capacitated sperm from fraction 1 of the swim-up assay. After 30 min of incubation 13.3 sperm from control samples and 20.2 sperm from treated samples bound to oocyte, showing a significantly increase with the treatment (Fig. 2).

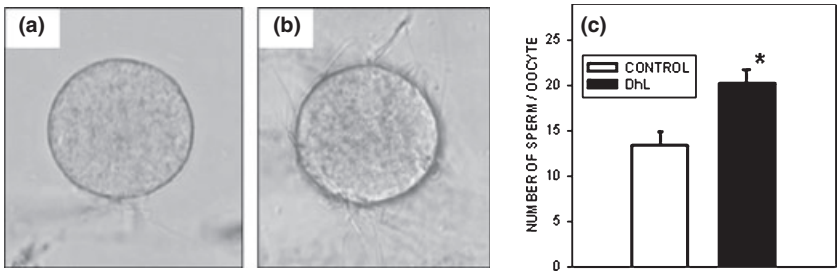

Fig. 2 Mouse sperm binding to zona-free hamster oocytes. Thirty oocytes were incubated with 2.5×10^4 sperm from the control group (a) or treated group (b) for 30 min at 37 °C, and analysed by phase contrast (a,b). The number of sperm that adhered to one oocyte was counted (c). The data represent the average number of sperm bound to oocytes ± SEM from six experiments. $^*P \leq 0.01$.

Hydrolytic enzyme activity in epididymis

Two hydrolytic enzymes were assayed in the epididymis cauda, β-GAL and β-NAG. β-GAL activity did not change after treatment (Table 3). Meanwhile, β-NAG activity in epididymal tissue (assayed in d-te) was 1.7 UI mg^{-1} in both control and treated mice, and in the tubular content was 5.1 and 2.7 UI mg^{-1} for control and treated mice, respectively, indicating a statistically significant reduction in β-NAG activity in treated animals. This low activity of β-NAG persisted when the enzyme activity was assayed in the total tubular content. As the weight of cauda epididymidis tubular content was significantly lower than control (Table 2), nearly 70% of the total β-NAG activity was lost in treated animals and the loss corresponded to the β-NAG activity from tubular content (Table 3).

Discussion

In this study, we show that the administration in the drinking water of 35 mg DhL kg^{-1} body weight per day for 30 days to male mice did not change the level of testosterone or oestradiol in plasma. In addition, the treatment did neither affect the weight of the testis or epididymis, nor the histology of either organ (data not shown). Neither did DhL alter the number of spermatids in the testis.

Table 3 Enzymatic activity in cauda epididymis

Enzyme	Control	Treated
β-GAL		
UI mg^{-1} d-te ($\times 10^{-3}$)	0.6 ± 0.07	0.5 ± 0.06
UI mg^{-1} tubular content ($\times 10^{-3}$)	1.3 ± 0.3	1.6 ± 0.4
UI mg^{-1} (d-te + tubular content $\times 10^{-3}$)	1.9 ± 0.2	2.1 ± 0.3
UI per total tubular content ($\times 10^{-3}$)	20.71 ± 5.2	13.4 ± 3.2
β-NAG		
UI mg^{-1} d-te ($\times 10^{-3}$)	1.7 ± 0.4	1.7 ± 0.7
UI mg^{-1} tubular content ($\times 10^{-3}$)	5.1 ± 1.0	2.7 ± 0.7*
UI mg^{-1} (d-te + tubular content $\times 10^{-3}$)	6.9 ± 0.6	4.4 ± 0.7 *
UI per total tubular content	55.59 ± 3.2	17.3 ± 4.1*

$^*P \leq 0.01$ (compared with controls).

[a]Values are expressed as means ± SE of six experiments.

In the cauda epididymidis, DhL induced a decrease in the activity of β-NAG secreted into the lumen. DhL decreased both the volume of tubular fluid and the number of sperm. Although the number of sperm in cauda epididymidis of the DhL-treated group was significantly lower than in the control group, neither the motility or the swim-up ability of the DhL group sperm was affected. In addition, the capability of treated sperm to bind to zona-free oocyte was higher than in the control, indicating that DhL may induce in the sperm a greater number of affinity sites to oocyte surface, or that DhL may increase the number of capacitated sperm.

P450 aromatase has been found in several cells of the rat testis, Leydig cells, immature Sertoli cells, germ cells and in pre- and post-meiotic germinal cells, including sperm (Tsai-Morris *et al.*, 1985; Janulis *et al.*, 1996, 1998). In adult rats, the administration of an aromatase inhibitor, anastrozole, over 1–2 weeks induced a 50% increase in plasma testosterone concentration (Turner *et al.*, 2000). It was postulated that DhL inhibits the activity of aromatase P450 in rat uterus (Blanco *et al.*, 1997). In any case, we did not find an increase in plasma testosterone concentration, or hypertrophy of Leydig cells after DhL treatment, thus we considered it highly improbable that DhL inhibited P450 aromatase in the Leydig cells, but we do not discard the possibility that DhL is affecting aromatases in other cells.

Aromatase enzymes are also present in epididymal epithelial cells, and aromatase activity is associated with the oestrogen receptors that participate in the secretion of lysosomal enzymes into the duct and in fluid reabsortion (Fisher *et al.*, 1997; Hess *et al.*, 1997)

We observed a decrease in the amount of tubular fluid and in the lumen lysosomal enzyme β-NAG in DhL-treated cauda epididymidis, which could be attributed to an

inhibition in the production of oestrogen by epididymal aromatases.

The transcript for aromatase, aromatase immunoexpression and aromatase activity has been detected in ejaculated sperm (Janulis *et al.*, 1996). Rat epididymal sperm contain active P450 aromatase (Janulis *et al.*, 1998). We found that DhL decreased the number of sperm in the cauda. Perhaps DhL is affecting factors that control the time that sperm inhabit the cauda epididymidis by inhibiting aromatase activity.

However, a greater number of sperm from DhL-treated mice were able to bind to oocytes, possibly because DhL is blocking epididymal aromatases and less oestradiol is interacting with sperm, which decreases their inhibitory activity on sperm capacitation. It was reported that 17-beta oestradiol, by acting rapidly on calcium influx and on protein tyrosine phosphorylation, seems to negatively modulate sperm responsiveness to progesterone, inhibiting both the plateau phase of calcium influx and the acrosome reaction (Luconi *et al.*, 1999; Baldi *et al.*, 2000). Experiments have been carried out in this laboratory to explain the effect of DhL on the epididymis and sperm.

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References

Amann RP, Lambiase JT Jr (1969) The male rabbit. 3. Determination of daily sperm production by means of testicular homogenates. *J Anim Sci* 28:369–374.

Baldi E, Luconi M, Muratori M, Forti G (2000) A novel functional estrogen receptor on human sperm membrane interferes with progesterone effects. *Mol Cell Endocrinol* 161:31–35.

Barrett AJ, Heath MF (1977) Lysosomal enzymes. In: A Laboratory Handbook. Dingle JT (ed). Elsevier North-Holland Biomedical Press, Amsterdam, pp 118–120.

Beekman AC, Woerdenbag HJ, Van UW, Pras N, Konings AW, Wikstrom HV, Schmidt TJ (1997) Structure-cytotoxicity relationships of some helenanolide-type sesquiterpene lactones. *J Nat Prod* 60:252–257.

Belmonte S, Maturano M, Bertini MF, Pusiol E, Sartor T, Sosa MA (1998) Changes in the content of rat epididymal fluid induced by prolonged treatment with tamoxifen. *Andrologia* 30:345–350.

Blanco JG, Gil RR, Alvarez CI, Patrito LC, Genti-Raimondi S, Flury A (1997) A novel activity for a group of sesquiterpene lactones: inhibition of aromatase. *FEBS Lett* 409:396–400.

Blanco JG, Gil RR, Bocco JL, Meragelman TL, Genti-Raimondi S, Flury A (2001) Aromatase inhibition by an 11,13-dihydroderivative of a sesquiterpene lactone. *J Pharmacol Exp Ther* 297:1099–1105.

De Rosas JC, Fornes MW (1990) The zona-free hamster oocyte penetration test: a simple procedure using a DNA fluorescent stain to detect the male pronucleus formation. *Microsc Electron Biol Celular* 14:165–171.

Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, Sharpe RM (1997) Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J Endocrinol* 153:485–495.

Giordano OS, Guerreiro E, Pestchanker MJ, Guzman J, Pastor D, Guardia T (1990) The gastric cytoprotective effect of several sesquiterpene lactones. *J Nat Prod* 53:803–809.

Giordano OS, Pestchanker MJ, Guerreiro E, Saad JR, Enriz RD, Rodriguez AM, Jauregui EA, Guzman J, Maria AO, Wendel GH (1992) Structure-activity relationship in the gastric cytoprotective effect of several sesquiterpene lactones. *J Med Chem* 35:2452–2458.

Hayaishi O (1962) Biological oxidations. *Annu Rev Biochem* 31:25–46.

Heinrich M, Robles M, West JE, Ortiz de Montellano BR, Rodriguez E (1998) Ethnopharmacology of Mexican asteraceae (Compositae). *Annu Rev Pharmacol Toxicol* 38:539–565.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB (1997) A role for oestrogens in the male reproductive system. *Nature* 390:509–512.

Janulis L, Hess RA, Bunick D, Nitta H, Janssen S, Asawa Y, Bahr JM (1996) Mouse epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis. *J Androl* 17:111–116.

Janulis L, Bahr JM, Hess RA, Janssen S, Osawa Y, Bunick D (1998) Rat testicular germ cells and epididymal sperm contain active P450 aromatase. *J Androl* 19:65–71.

Luconi M, Muratorim M, Forti G, Baldi E (1999) Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J Clin Endocrinol Metab* 84:1670–1678.

Mason HS (1965) Oxidases. *Annu Rev Biochem* 34:595–634.

Robles M, Aregullin M, West J, Rodriguez E (1995) Recent studies on the zoopharmacognosy, pharmacology and neurotoxicology of sesquiterpene lactones. *Planta Med* 61:199–203.

Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE, Hinshelwood MM, Graham-Lorence S, Sun T, Fisher CR, Qin K, Mendelson CR (1997) Aromatase expression in health and disease. *Recent Prog Horm Res* 52:185–213.

Tsai-Morris CH, Aquilano DR, Dufau ML (1985) Cellular localization of rat testicular aromatase activity during development. *Endocrinology* 116:38–46.

Turner KJ, Morley M, Atanassova N, Swanston ID, Sharpe RM (2000) Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. *J Endocrinol* 164:225–238.

Wang JF, Zhang CC, Chou KC, Wei DQ (2009) Structure of cytochrome p450s and personalized drug. *Curr Med Chem* 16:232–244.