CYTOGENETIC, HORMONAL, MILK AND SEMEN STUDIES IN A FERTILE MALE GOAT WITH GYNECOMASTIA

ESTUDIOS CITOGENÉTICO, HORMONAL, DE LECHE Y DE SEMEN EN UN MACHO CABRÍO FERTIL CON GINECOMASTIA

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Abstract

Gynecomastia, the development of mammary glands with secretory activity, was observed in a fertile male goat. Cytogenetic analysis was carried out on GTG-banded chromosomes prepared from peripheral blood lymphocytes cultures. Barr bodies were also sought in PMN neutrophils, in epithelial cells of the mouth and in mammary gland cells. Testosterone (T), growth hormone (GH), prolactin (PRL), estradiol (E2), progesterone (P4), follicle stimulating hormone (FSH), luteinizing hormone (LH) dehydroepiandrosterone (DHEA), dihydroepiandrosterone sulfate (DHEA-SO_4), cortisol (F), 17-α-hydroxyprogesterone (17-OHP4), androstenedione (ΔA4) and pregnenolone (P5) levels were analyzed. Sperm parameters were within normal values defined for bucks. Analysis of milk has revealed no major differences from female goat's milk, except for pH (slight alkaline) and TA (13^{o}T, lower than 20^{o}T). Chromosomes analysis revealed a normal caprine karyotype (60(X,Y)). Barr bodies were not observed in the cell types analyzed. Mean plasma values for hormonal parameters revealed higher levels of GH, P4 and T when compared with a normal male (256 vs 241 ng/mL; 0.176 *vs* 0.128ng/mL; 13.15 *vs* 0.378ng/mL, respectively). The PRL and E2 levels were similar to values determined in a lactating female goat (0.047ng/mL) and to a normal male (58.95 *vs* 49.74 pg/mL), respectively. F and 17-OHP4 had higher levels than both normal buck and female, while P5 level was lowest in the "milking" buck. The ratios calculated for the 3β-hydroxy-Δ5-steroids to 3-oxo-Δ5-steroids were lower in the "milking" buck, when compared to a normal male goat, suggesting a higher activity of the 3-β-hidroxysteroid dehydrogenase enzyme.

Resumen

Ginecomastia, el desarrollo de las glándulas mamarias con actividad secretora, se observó en un macho cabrío fértil. El análisis citogenético se llevó a cabo en los cromosomas obtenidos de cultivos de linfocitos de sangre periférica mediante la técnica de bandas G. También se realizaron búsquedas de Cuerpos de Barr en los neutrófilos PMN, en las células epiteliales de la boca y en las células de la glándula mamaria. La testosterona (T), la hormona del crecimiento (GH), prolactina (PRL), estradiol (E2), progesterona (P4), hormona estimulante del folículo (FSH), luteinizante (LH), hormona dehidroepiandrosterona (DHEA), sulfato de dihidroepiandrosterona (DHEA-SO4) , el cortisol (F), 17 - □-hidroxiprogesterona (17-OHP4), androstenediona (□A4) y se analizaron los niveles de pregnenolona (P5). Los parámetros de esperma se encontraban dentro de los valores normales definidos para el macho caprino. El análisis de la leche no ha revelado diferencias importantes de la leche de cabra, a excepción de pH (ligeramente alcalino) y TA (13oT, inferior 20oT). Análisis de cromosomas reveló un cariotipo caprino normal, (60 (X, Y)). Cuerpos de Barr no se observaron en los tipos de células analizadas. Los valores medios en plasma para los parámetros hormonales revelaron niveles más altos de GH, P4 y T en comparación con un macho normal (256 vs 241 ng / ml; 0,176 vs 0.128ng/mL; 13,15 vs 0.378ng/mL, respectivamente). Los niveles de PRL y E2 fueron similares a los valores determinados en una cabra hembra lactante (0.047ng/mL) y para un varón normal (58,95 frente a 49,74 pg / ml), respectivamente. F y 17-OHP4 tenían niveles más altos que los dos, macho y hembra normal, mientras que el nivel P5 fue más baja

en el macho cabríocon gynecomastia. Las razones calculadas para 3β-hidroxi-Δ5-esteroides/3-oxo-Δ5-esteroides fueron inferiores en el macho estudiado, cuando se compara con un macho normal, lo que sugiere una mayor actividad de la enzima 3 β hidroxysteroid deshidrogenasa.

Introduction

In male goats (*Capra hircus*), the development of mammary glands, with or without galactorrhea, has been observed for years (Jaszcak *et al.*, 2010).

The etiology of gynecomastia (GM) in mammals seems to be multifactorial. Some authors reported abnormalities in sex chromosomes (Gupta *et al*., 2001) while others found GM associated with normal karyotype (Wang *et al.*, 2009). Other studies suggest that endocrine tumors (testicular, pituitary or adrenocortical) may lead to GM (Barros & Sampaio, 2012, for review) by the increasing of the levels of estradiol/estrogen, steroid precursors, or prolactin.

Endocrine dysfunctions may adversely affect hormonal imbalance between sex steroid hormones. In humans, a key hallmark for GM seems to be the disequilibrium in the ratios between estrogens and androgens. Indeed, male breast tissue contains protein receptors for estrogens and androgens; while estrogens stimulate the proliferation of the mammary ductuls, the androgens antagonize the effects of oestrogens (Pearlman and Carlson, 2006 review).

Familial GM, a rare genetic and endocrine syndrome, was characterized by an overexpression of aromatase (Berkovitz *et al.*, 1985), the enzyme responsible for the conversion of testosterone to estradiol and androstenedione to estrone. The increased aromatization of androgens to estrogens, in extragonadal tissues, results in excessive levels of circulating estrogens. The production of steroid hormones (steroidogenesis) in gonads and adrenal glands is species specific, but little is known in caprine species (Engelbrecht and Swart, 2000). In mammals, cholesterol is the precursor of pregnenolone (P5), the entry into the steroidogenesis which comprises two main pathways, the Δ4 and the Δ5 pathways. In the first, pregnenolone is converted to progesterone and 17α-hydroxyprogesterone (17-OHP4) while in the Δ5 pathway, pregnenolone is converted to 17α-hydroxypregnenolone (17-OHP5) and dehydroepiandrosterone (DHEA). The 17-OHP4 is a potential responsible for the interrelationship of function between the gonads and the adrenal glands. The interplay between the Δ4 and the Δ5 routs involves the 3-β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) enzyme (Conley and Bird, 1997).

A case that describes a deficiency in the enzyme 3-β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) as a cause of postpubertal gynecomastia, in an adult male human with normal gonadal function, was reported by Cavanah and Dons (1993). In humans, there are two 3β-HSD isoenzymes (type I and II) that are expressed by two different genes on chromosome 1p13.1. In Angora goats and sheep, 3-β-hydroxysteroid dehydrogenase differs by five amino acids residues (Goosen *et al.*, 2010) despite the fact that conversion of pregnenolone (P5) by 3βHSD was found to be similar in *Capra aegagrus, Capra hircus and Ovis aries* (Engelbrecht and Swart, 2000).

In this study we described a case of GM associated with galactorrhea in a fertile male goat, which was characterized through the evaluation of the karyotype, hormonal levels, milk and semen parameters.

Material and Methods

A sexually mature fertile male goat (Saanen crossbred) with bilateral mammary gland development was studied. The goat was from a herd at Coimbra region (40º14’35’’N latitude and 8º28’50’’ W). Biometric records (anterior and posterior heights, AH, PH; thoracic perimeter, TP; abdominal perimeter, AP; height on the top of shoulder, HS; rump height, RH; body length, BL; rump width, RW; chest width, CW; thigh circumference, TC; breadth rump, BR) were taken for phenotype evaluation. Clinical examination of reproductive organs was achieved by percutaneous ultrasonography. Cytogenetic analysis was carried out on GTG-banded chromosomes derived from peripheral blood T-lymphocytes cultures according to standard protocols (Smith, 2006) without cell synchronization. Briefly, after blood collection in heparinized tubes (lithium heparin), T-lymphocytes (0.3ml of blood) culture was promoted in Gibco® PB-MAX™ Karyotyping Medium (Invitrogen Corporation, USA) for 71h at 37ºC. The culture was incubated with colcemid solution (10µL/mL) during 1h to arrest metaphases. Cells were then incubated in hypotonic KCl solution (0,075M) and fixed in acetic acid:methanol (1:3, v/v) solution. Cells suspended in fixative were spread on clean glass slides and air-dried. G-banding (Giemsa 10%) was preceded by treatment with trypsin (6mg/mL). Barr bodies were also searched in PMN

neutrophils (Hemacolor® stain), in buccal epithelial cells and mammary gland cells, both by the aceto-orcein method, described by Verma and Babu (1989), and hematoxylin stain.
Semen was examined for volume, colour, mass motility, individual motility, concentration, morphology and viability. Milk analyses comprised fat, protein, lactose and salts content, pH and titratable acidity (TA).
Circulating levels of testosterone (T), growth hormone (GH), prolactin (PRL), estradiol (E2), progesterone (P4), follicle stimulating hormone (FSH) and luteinizing hormone (LH), dehydroepiandrosterone (DHEA), cortisol (F), 17-α-hydroxyprogesterone (17-OHP4), Δ4-androstenedione (Δ4A) and pregnenolone (P5) were determined by a clinical laboratory, and compared with a normal buck and a female.

Results

On physical examination, the right-hand mamma was significantly larger than the left, and was pendulous. The testes were normal in texture and size. No other significant abnormalities were observed on physical examination, except for the animal body structure which was above average (data not shown). Results of a complete CBC analysis were within normal limits (Delgado *et al*., 2001) (Table 1).

Table I. Blood panel for the "milking" buck (Buck G) and a normal male (Buck N) (Parámetros sanguíneos en el macho cabrío con ginecomastia (Buck G) y un macho normal (Buck N))

Complete Blood Count (CBC)							
	Buck G	Buck N	Normal Limits[a]		Buck G	Buck N	Normal Limits[a]
Erythrocytes (10^6/ml)	8.69	7.41	7.46–12.30	Leukocytes (10^3/ml)	5.2	8.6	3.55–17.30
Hemoglobin (g/dl)	7.6	11.4	7.42–10.90	Neutrophils (%)	38.9	35.0	9.3–70.7
Hematocrit (%)	20.7	30.0	14.2–26.8	Eosinophils (%)	0.5	2.0	0.54–16
M.C.V. (fl)	23.8	40.5	19.0–23.3	Lymphocytes (%)	59.8	57.0	20.7–78.4
M.C.H. (pg)	8.7	15.4	7.8–11.0	Monocytes (%)	0.7	5.5	0.54–17.2
M.C.H.C. (g/dl)	36.7	38.0	35.9–53.8	Basophils (%)	0.1	0.5	0.10–4.68

[a] – Delgado et al (2001)

One problem in obtaining good G-band preparations was getting a high mitotic index of prophase or early metaphase spreads. For some blood samples the mitotic index was not as high as one might hope. Even so, chromosomes analysis revealed a normal caprine karyotype (60(X,Y)). Likewise, no Barr bodies were observed in the cell types analyzed (Figure 1).

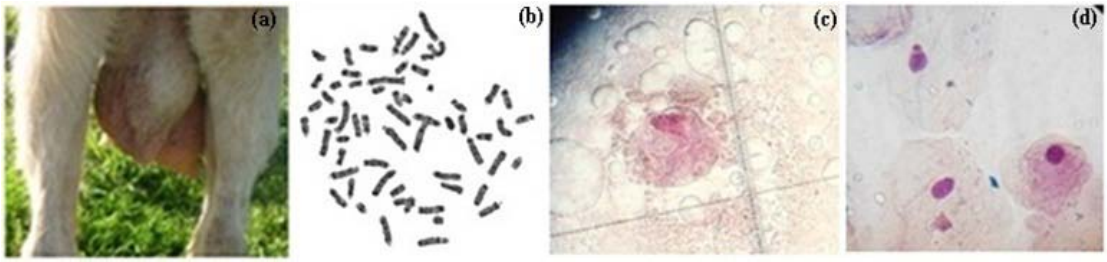

Figure 1. (a) "Milking buck" with developed mammary gland and a normal testis; (b) karyotyping of lymphocytes (1000x); (c) mammary gland biopsy (1000x; aceto-orcein staining); (d) buccal epithelial cells (1000x; aceto-orcein staining). ((a) Macho lactante con una glándula mamaria desarrollada y testículos normales; (b) caracterización del cariotipo en Linfocitos (1000x); (c) biopsia de la glándula mamaria (1000x; tinción con aceto-orcein); (d) celulas epiteliales de la boca (1000x; tincíon con aceto-orcein).

The animal showed normal libido, it was fertile and the female progeny presented no problems of fertility. The quality parameters of semen were between normal values defined for bucks (Table 2).
Analysis of the milk (Table 2) revealed no major differences from milk of goats except for pH (slight alkaline) and TA (13°T, lower than normal values, 20°T).

Table II. Mean values obtained for sperm parameters and milk analyses from the "milking" buck (Valores medios obtenidos para los parámetros del esperma y del leche del macho cabrío con ginecomastia)

Sperm parameters		Milk Composition		Microbiology analyses	
Volume (ml)	2,00	Fat (%)	5,55±0,49	*E.coli* (CFU/g)	$<1x10^1$
NTE ($x10^9$)	10,56	Protein (%)	2,95±0,21	*L.monocytog.*(25g)	(-)
Motility (%)	80,00	Lactose(%)	4,25±0,21	Total viable bacteria (CFU/g)	$2,8x10^7$
Vitality (%)	78,00	Salts (%)	0,65±0,07	*C.perfrig.* (CFU/g)	$<1x10^1$
Morphology (%)	91,50	pH	7,2±0,00	*S.coag.*+ (CFU/g)	$<1x10^1$
pH	7,00	TA (ºT)	12,5±0,71		

Mean plasma values for T, GH, PRL, E2, P4, FSH and LH revealed higher levels of GH, P4 and T when compared to a normal male (256 vs 241 ng/mL; 0.176 *vs* 0.128ng/mL; 13.15 *vs* 0.378ng/mL, respectively). The PRL and E2 levels were similar to corresponding values found in a lactating goat (0.047ng/mL) and to a normal buck (58.95 *vs* 49.74 pg/mL), respectively. Furthermore, F and 17-OHP4 had higher levels than a normal buck and a female; in contrast, P5 level was lowest in the "milking" buck (Figure 2).

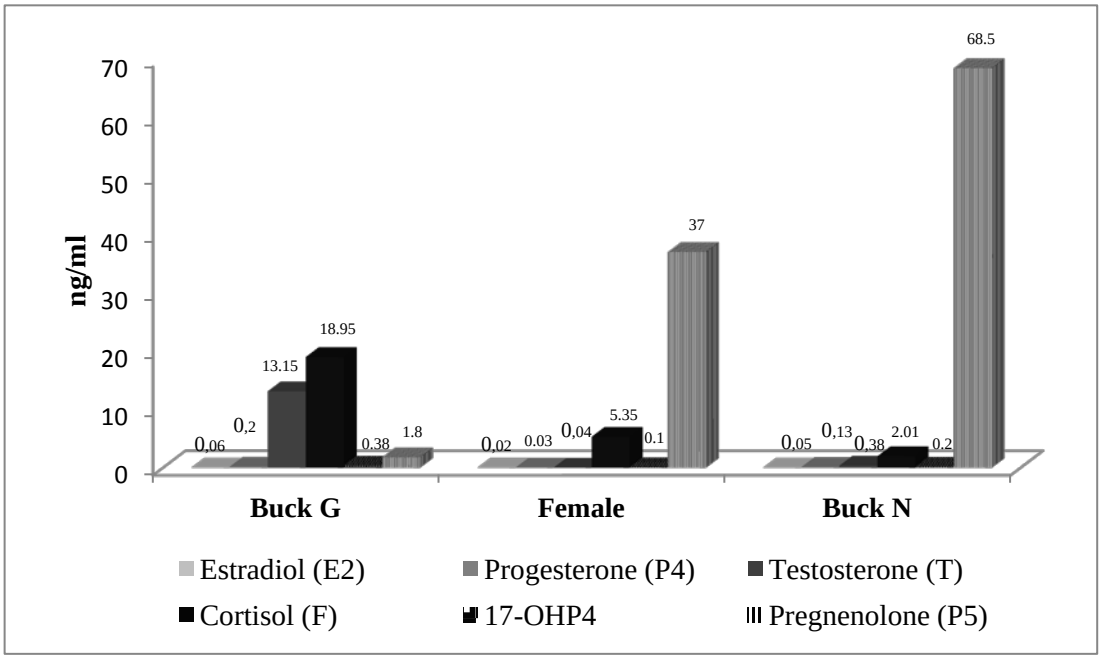

Figure 2. Plasma levels of pregnenolone (P5), 17-α-OH-progesterone (17-OHP4), progesterone (P4), estradiol (E2), testosterone (T) and cortisol (F) in the "milking" buck (Buck G), a female and a normal buck (Buck N) (Los niveles plasmáticos de pregnenolona (P5), 17 - α-OH-progesterona (17-OHP4), progesterona (P4), estradiol (E2), testosterona (T) y el cortisol (F) en el macho cabrío con ginecomastia (Buck G) , una hembra y un macho normal (Buck N))

Product/precursor ratios in serum steroids were determined as an index of the relative activities of steroidogenic enzymes as describe by Ueshiba *et al.* (2002). Δ4A/17-OHP4 ratio, reflecting 17,20-lyase activity, was lowest in the "milking" buck. 17-OHP4/P4 ratio, reflecting 17-hydroxylase activity, was lower than in a female and higher than in a normal buck. The ratios that reflect the 3β-HSD activity, were highest in the "milking" buck for P4/P5 and lower than in a female and higher than in a normal buck for Δ4A/DHEA. E2/T ratio, reflecting aromatase activity, was lowest in the "milking" buck (Table 3).

Table III. Ratios obtained for hormonal data of male goats with (Buck G) and without gynecomastia (Buck N) and a normal female (Ratios obtenidos para los datos hormonales de machos cabríos con (Buck G) y sin ginecomastia (Buck N) y una hembra normal)

Ratios	Buck G	Buck N	Female
E2: T	4.48×10^{-2}	1.31	5.56
P4:P5	0.10	0.002	0.0008
17OHP4: P4	2.11	1.54	3.33
Δ4A:17OHP4	7.89×10^{5}	15.00×10^{5}	30.00×10^{5}
Δ4A:DHEA	2.31×10^{5}	1.76×10^{5}	4.29×10^{5}

Discussion

GM represents a benign proliferation of the breast glandular tissue (Johnson & Murad, 2009) that is stimulated by the presence of androgens, estrogens, progesterone and prolactin receptors in the receptive cells. This provides support for the hypothesis that GM is steroid-dependent (Carlson, 2011). Braunstein (1999) hypothesized that the imbalance between estrogen actions relative to androgen action at the breast tissue level appears to be the main etiology of GM.

Ormandy *et al.* (1997) demonstrated that in breast cancer cells, progesterone and sex steroid receptors increase with PRL; in contrast, androgen receptors decrease. Their results indicate that sex steroid and PRL receptors are coexpressed and cross-regulated. However, despite prolactin receptors having been verified in man with GM, most men with GM do not have elevated serum prolactin levels (Barros and Sampaio, 2012). Recently, Wang *et al.* (2009), conjecture that a single nucleotide polymorphism (SNP) in the prolactin gene could be related with gynecomastia in bucks. In our study, the "milking" buck presented PRL levels comparable to a lactating goat. A genetic approach in order to determine the SNP status in the prolactin gene of the "milking" buck is planned.

Some authors report that serum concentration of estrogen in patients having tumors of the Sertoli cells are usually increased, which may lead to GM (Pearlman and Carlson, 2006; Bing and Bai, 2012). In our case study, ultrasonographic examination of reproductive organs allowed us to reject breast malignancy.

In face of our results, common causes of GM were excluded. In order to establish the cause of the origin of GM associated with galactorrhea, serum levels of GH, PRL, P5, P4, DHEA, D4A, T, E2, F, FSH and LH were determined and compared with the levels obtained from normal males and females of the control group; T and E2 showed increased values in the buck goat studied. The result for T and PRL levels obtained in our work is in accordance with that found by Pilo et al. (2011), in two bucks with GM and galactorrhea. However, those authors found no apparent changes in estrogen levels and they could not confirm the hormonal origin of the GM associated with galactorrhea.

Conley and Bird (1997) stated that steroid production is regulated largely by the relative levels and tissue–specific array of steroidogenic enzymes expressed at cellular level. Gonodal and adrenal steroidogenesis in mammals are mechanistically controlled by the Δ4/Δ5 metabolism. During adrenal steroidogenesis the enzymes 3*β*-HSD, P450c17, and P450c21 play a pivotal role in channeling metabolites: if a 3*β*-HSD deficiency is encountered, the *Δ*5 pathway will be favored and adrenal androgen production will be high. In the case of *Δ*4 pathway, the relative activities of P450c21 and P450c17 will determine the distribution of the common substrate, P4, between mineralocorticoid and glucocorticoid precursors (Hiwatashi and Ichikawa,1981; Kater and Biglieri, 1994; Conley and Bird, 1997).

As the 17-OHP/P4 ratio, reflecting 17-hydroxylase activity, was higher in the "milking" buck, when compared with a normal male, the increase in this enzyme may produce the increased F observed in serum. Gynecomastia has been also associated with aromatase activity (Braunstein, 1999) however, our data suggest that higher levels of 17-OHP4, P4, T and F occurring in buck G, together with similar E2 levels that occur in both buck G and buck N, are associated with a higher activity of 3β-HSD.

Conclusion

The ratios calculated for the hormones from the steroidogenesis pathways, suggest an overexpression of the 3-β-hidroxysteroid dehydrogenase (3β-HSD) enzyme in the male goat with gynecomastia and galactorrhea.

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