Testicular Function in Biotin-Deficient Adult Rats

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Summary

We have studied testicular function in the biotin-deficient rat biochemically and morphologically. Serum testosterone and luteinizing hormone (LH) levels were decreased significantly in the deficient rats. Administration of biotin or gonadotropins to the deficient rats reversed this decrease in serum testosterone. There was no difference in the serum cholesterol level between the control and biotin-deficient rats. A significant degree of sloughing of seminiferous tubule germinal epithelium was noticed in the biotin-deficient rat testes. Biotin treatment of biotin-deficient rats reversed this condition whereas testosterone treatment was without any effect. The development and maintenance of morphological and functional integrity of the seminiferous tubules appears to require a biotin-mediated step in addition to testosterone.

Key-Words

Biotin Deficiency – Testis – Testosterone

Introduction

Biotin serves as a prosthetic group of the carboxylases four of which occur in animal and human tissues. These are acetyl-CoA carboxylase involved in lipogenesis, pyruvate carboxylase involved in gluconeogenesis, propionyl-CoA carboxylase and beta-methylcrotonyl-CoA carboxylase involved in the metabolism of amino acids (*Wood* and *Barden* 1977). The effects of biotin deficiency in animals are quite severe. Apart from the skin lesions characteristic of this deficiency, various systems such as neuromuscular, immune and reproductive are adversely affected. It is not possible to explain all these changes in the biotin-deficient animal on the basis of the prosthetic group function of biotin. There has been increasing support for the concept of a non-prosthetic group function of biotin (*Dakshinamurti, Chalifour* and *Bhullar* 1985) gained particularly from work with cell culture systems (*Cheng* and *Moskowitz* 1982; *Cohen* and *Gospodarowicz* 1985).

In early work *Shaw* and *Phillips* (1942) reported that the testes of biotin-deficient rats were visually very small in relation to the size of the animal and that the seminiferous tubules were very small and showed signs of degeneration. *Delost* and *Terroine* (1956) also found that the testis size was smaller and weight was less than that of normals. There was evidence of delayed spermatogenesis and decreased number of spermatozoa. They reported that these disturbances in the testes were due to biotin deficiency alone since animals on a restricted but normal diet did not show any of these abnormalities (*Terroine* 1960). The effect of biotin deficiency and biotin supplementation on the production and secretion of testosterone as well as on the morphology of the testis has been investigated in this study. Evidence is presented here to indicate that factors other than testosterone are required for normal spermatogenesis.

Materials and methods

[^{14}C] Leucine was purchased from Amersham, Arlington heights, Il., U. S. A. Human luteinizing hormone (hLH) was a gift from Dr. Salvatore Raiti of NHPP, NIDDK, University of Maryland School of Medicine, U. S. A. HCG, cholesterol oxidase, 4-aminophenazone and amino acids were purchased from Sigma Chemical Co., St. Louis, MO, U. S. A. Anhydrous D (+)-dextrose, Spray-dried egg white, Spector No. 446 salt mixture and choline chloride were purchased from ICN Nutritional Biochemicals, Cleveland, OH, U. S. A.

Animals

Male rats (3 week old, weight 50 ± 5 g) were divided into two groups. Group 1 was fed a biotin-deficient diet (*Dakshinamurti* and *Cheah-Tan* 1968) and Group 2 (control) was pair-fed with biotin-supplemented diet. The animals were used for various experiments after 7 weeks on the diets. To study the effect of biotin, biotin-deficient rats were injected with biotin (20 µg/rat i. p. daily for 6 days). The rats were killed one day after the last injection. To study the effect of hLH and human chorionic gonadotropin (hCG), biotin-deficient rats were injected with hLH (50 IU i. p. daily for 7 days) or hCG (25 IU i. p. daily for 7 days) and the rats were killed one hour after the last injection. In order to study the effect of long-term treatment with testosterone, groups of biotin-deficient rats were implanted with empty or testosterone propionate-filled Silastic (Dow Corning Corp., MI, U. S. A.) capsules (0.2 × 6 cm) for 6 weeks. Rats were killed by de-

Horm. metabol. Res. 21 (1989) 661–665

Received: 11 July 1988 Accepted: 9 March 1989

Table 1 Effects of biotin, human luteinizing hormone (hLH), and human chorionic gonadotropin (hCG) on serum testosterone levels in control and biotin-deficient rats. Values are means ± SEM of 8 separate determinations in each group.

Animal status	Testosterone nmol/l
Control (Biotin-supplemented)	103.37 ± 8.65
Biotin-deficient	13.63 ± 3.42**
Biotin-deficient, treated with biotin	70.68 ± 9.35
Biotin-deficient, treated with hLH	104.00 ± 11.45
Biotin-deficient, treated with hCG	84.00 ± 11.44

**P < 0.01 compared with all other groups (Duncan's multiple range test).

Table 2 Testicular testosterone level in control and biotin-deficient rats. Values are means ± SEM of 6 separate determinations in each group.

Animal status	Testosterone pmol/g wet wt.
Control (Biotin-supplemented)	157 ± 16
Biotin-deficient	35 ± 11**

**P < 0.01 compared with the control group (Student's unpaired t-test).

Table 3 Serum concentrations of luteinizing hormone (rLH) and follicle stimulating hormone (rFSH) in adult male rats. Values are means ± SEM of 8 separate determinations in each group.

Animal status	rLH ng/ml	rFSH ng/ml
Control (Biotin-supplemented)	0.72 ± 0.09	284 ± 41
Biotin-deficient	0.32 ± 0.02**	244 ± 25

**P < 0.01 compared with the control group (Student's unpaired t-test).

capitation. Blood was collected and serum was used for hormone and cholesterol assays. Testes were removed and frozen immediately on dry ice for hormone and cholesterol assays. Fresh testes were used for in vitro protein synthesis studies. Testes for morphological studies were fixed in buffered formalin and processed for light microscopy.

Assay of Hormones

Testosterone was assayed according to *Yuen, Moon, Mincey* and *Li* (1983) using specific double antibody radioimmunoassay. Rat luteinizing hormone was assayed according to *Monroe, Parlow* and *Midgley* (1968) using specific double antibody radioimmunoassay for rLH utilizing antiserum prepared in rabbit against rLH. Assay was standardized against highly purified rLH. Rat follicle stimulating hormone (rFSH) was assayed according to NPA rFSH radioimmunoassay methodology using materials supplied by NPA.

Assay of Cholesterol

Serum cholesterol was assayed according to *Deacon* and *Dawson* (1979). Total cholesterol assay was based on enzymic hydrolysis through disruption of the lipoproteins using Triton X-100 so that cholesterol and its esters are available for enzymic action. Sodium cholate was added to activate the pancreatic cholesterol esterase. The free cholesterol released from its esters by the action of cholesterol esterase (in addition to the free cholesterol initially present in the sample) was oxidised by atmospheric oxygen in the presence of cholesterol oxidase to hydrogen peroxide and cholest-4-en-3-one. The stoichiometric amount of hydrogen peroxide produced was quantitated by the peroxidase catalyzed reaction with phenol and 4-aminopyrazone to produce a quinoneimine chromogen that absorbs maximally at 500 nm. Testes were homogenized in chloroform-methanol 2:1 and the total lipid was extracted according to *Folch, Lees* and *Sloane-Stanley* (1957). The final extract was dried, resuspended in methoxyethanol and used for cholesterol assay.

In vitro protein synthesis

In vitro protein synthesis was studied in post-mitochondrial supernatant from rat testes prepared according to the procedure of *Lu, Ekstrom, Spicer* and *Richardson* (1978). The in vitro amino acid incorporating system contained the following per ml of the final volume: 2 µmol of ATP, 0.5 µmol GTP, 10 µmol of phosphoenolpyruvate, 50 µmol of $MgCl_2$, 35 µmol of Tris-HCl pH 7.6, 1 µmol of 2-mercaptoethanol, 2 mg of post-mitochondrial supernatant protein preparation from the rat testes, 1 µCi (5 nmol) of L [U-^{14}C]-leucine and 50 nmol of each of the other 19 amino acids were added (*Boeckx* and *Dakshinamurti* 1974). Incubations were performed at 37 °C. The incorporation was stopped by adding equal volume of 10% trichloroacetic acid. The resulting precipitate was washed twice with 5% TCA and the pellet was digested using NCS (Amersham) and radioactivity was determined in a Beckman LS-2800 liquid scintillation spectrometer. Protein was measured according to *Lowry, Rosebrough, Farr* and *Randall* (1951)

Morphological study

Testes were removed and fixed in buffered formalin and subsequently embedded in paraffin. Sections (6 u) were cut and stained with hematoxylin and eosin for light microscopic evaluation. In order to eliminate observer bias, the sections were examined using a coded format without foreknowledge of their source.

Results

Serum testosterone level in the biotin-deficient rat was reduced drastically to about 13 per cent ($P < 0.01$) compared with the controls (Table 1). Testicular testosterone of the biotin-deficient rat was about 22 per cent of the control level ($P < 0.01$, Table 2). Treatment of biotin-deficient rats with biotin resulted in a significant ($P < 0.05$) increase in serum testosterone. Serum concentration of rLH was significantly decreased ($P < 0.01$) in the biotin-deficient rats compared with the controls, whereas there was no difference in the concentration of rFSH (Table 3). Treatment of biotin-deficient rats with hLH and hCG also led to corresponding increases in serum testosterone levels (Table 1). This response is explained on the basis of the decrease in serum LH in the defi-

Table 4 Serum and testes cholesterol in control and biotin-deficient rats. Values are means ± SEM of 8 separate determinations in each group.

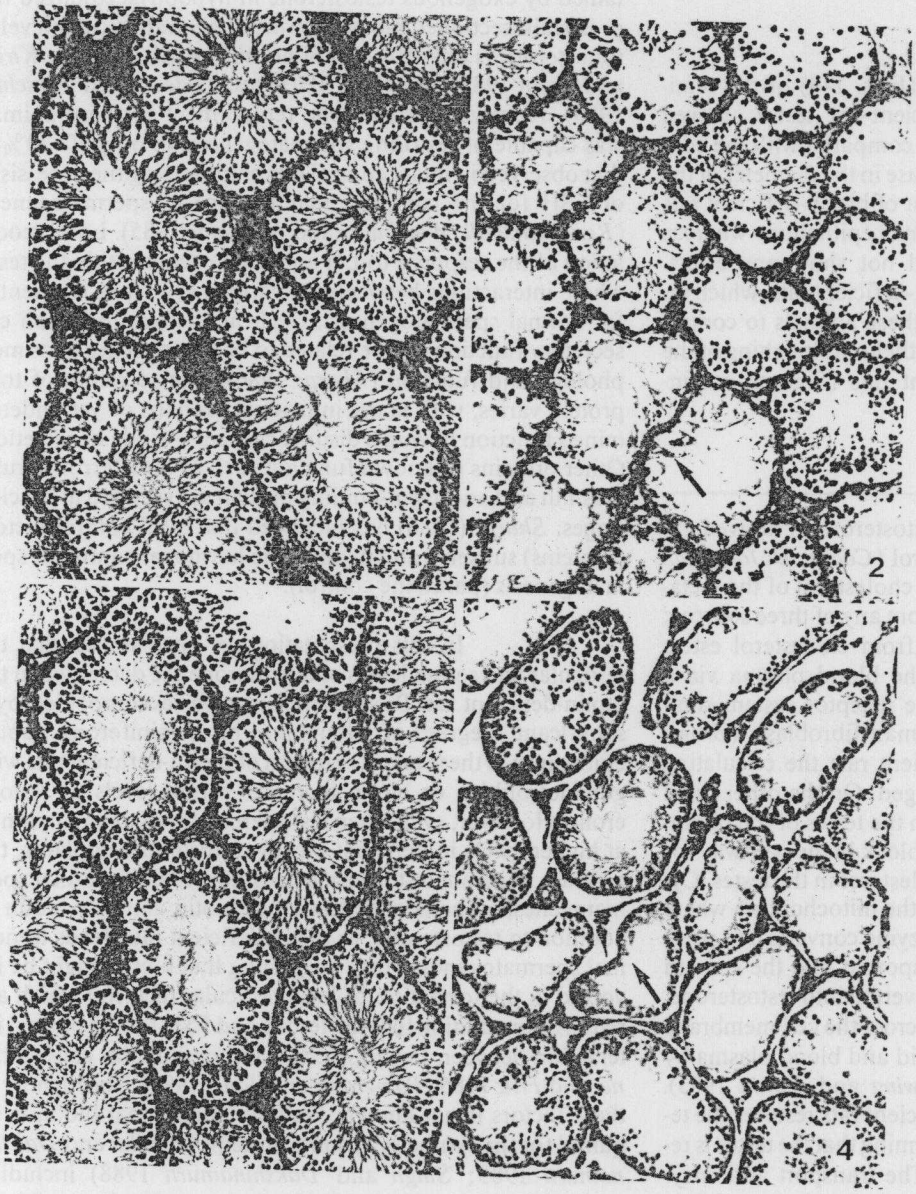
Animal status	Serum cholesterol nmol/l	Testes cholesterol umol/g wet wt.
Control (Biotin-supplemented)	1.36 ± 0.11	923 ± 66
Biotin-deficient	1.39 ± 0.10	1335 ± 87**
Biotin-deficient, treated with biotin	1.73 ± 0.13*	–
Biotin-deficient, treated with testosterone	1.35 ± 0.20	–

*P < 0.05 compared with other groups (Duncan's multiple range test).
**P < 0.01 compared with the control group (Student's unpaired t-test)/

Table 5 Effects of biotin and testosterone on the incorporation of ^{14}C-Leucine into total protein in vitro in adult male rat testes. Values are means ± SEM of 8 determinations in each group.

Animal status	In vitro ^{14}C-Leucine incorporation pmoles/mg protein
Control (Biotin-supplemented)	40.05 ± 2.47
Biotin-deficient	32.01 ± 0.98**
Biotin-deficient, treated with biotin	39.71 ± 0.90
Biotin-deficient, treated with testosterone	30.57 ± 1.51**

**P < 0.01 compared with control and biotin-deficient, treated with biotin groups (Duncan's multiple range test).



Figs. 1–4 Testicular histology stained with hematoxylin and eosin. (1) control testis, (2) biotin-deficient, (3) biotin-deficient plus biotin, (4) biotin-deficient plus testosterone. Note well developed seminiferous tubule germinal epithelium from control (1) and biotin-deficient plus biotin treated (3) animals (arrows). In contrast, the testes of biotin-deficient (2) and biotin-deficient plus testosterone treated (4) animals show disruption and thinning of the germinal epithelium (arrows) and decreased seminiferous tubule diameter (arrows). Magnification × 360.

There was no significant difference in the serum concentration of cholesterol, the precursor of testosterone, between the control and deficient rats. Biotin treatment of biotin-deficient rats increased the circulating cholesterol concentration ($P < 0.05$; Table 4). There was however, a significant increase in the testicular content of cholesterol in biotin-deficient rats ($P < 0.01$; Table 4). This is in keeping with our earlier report (*Dakshinamurti* and *Desjardins* 1968) where we found an increase in carcass cholesterol in biotin-deficient rats as compared to the biotin-supplemented rats. We proposed that when the malonyl CoA pathway of acetyl CoA utilization is decreased due to the deficiency of acetyl CoA carboxylase, acetate is preferentially channeled toward acetoacetate and cholesterol.

There was a significant decrease in the protein synthesis in biotin-deficient rat testes compared with the controls ($P < 0.01$; Table 5). Biotin treatment of the deficient rats reversed the decrease in protein synthesis observed in these animals whereas testosterone treatment did not have any effect (Table 5).

Morphological study

Morphological study (Figs. 1–4) of the seminiferous tubules of the biotin-deficient rat testes showed sloughing of the germinal epithelium compared with the controls. Also, there was significant decrease in the diameter of the seminiferous tubules. Biotin treatment of biotin-deficient rats reversed the sloughing of the germinal epithelium whereas long-term testosterone treatment did not show any effect. Also, hLH or hCG treatment of biotin-deficient rats, which increased serum testosterone levels of these animals to control levels, did not reverse the germinal epithelium sloughing of the seminiferous tubules in the deficient rats (Figures not included).

Discussion

In the Leydig cells testosterone is derived exclusively from its precursor, cholesterol (*Cooke, DeJong, van der Molen* and *Rommerts* 1972). This cholesterol of the metabolically active pool can be derived from any of three sources: de novo biosynthesis from acetate, from cholesterol esters stored in lipid droplets, and from the blood plasma via a cholesterol-lipoprotein-cell membrane receptor system similar to that described for cultured human fibroblasts (*Brown* and *Goldstein* 1976). In biotin-deficient rats the circulating cholesterol concentration is not changed. On the other hand there is accumulation of cholesterol in the testes of the biotin-deficient rats. This finding suggests a block in the pathway for the synthesis of testosterone from cholesterol in the testes. Cytosolic cholesterol is transported into the mitochondria where the cholesterol side chain cleavage enzyme converts it to pregnenolone. Pregnenolone is then transported into the smooth endoplasmic reticulum where it is converted into testosterone. Testosterone probably then diffuses across the cell membrane and is trapped in the extracellular fluid and blood plasma by steroid binding macromolecules (*Ewing* and *Zirkin* 1983). HCG or hLH treatment of biotin-deficient rats restores the testosterone level to normal levels confirming that the testes is responsive to the pituitary hormone. The transport and enzymatic machinery is available and intact in the biotin-deficient rat. When stimulated by hLH or biotin, testosterone production and secretion is restored in the deficient rat.

The biotin-deficient rat has decreased serum LH as well. The decrease in circulating LH in the biotin-deficient rat indicates that pituitary synthesis and/or release of this hormone is affected in deficiency. The testes does respond to exogenous LH by increasing testosterone production. However, this increase in testosterone produced by LH treatment or by administration of exogenous testosterone to the biotin-deficient rat does not restore the testes to its normal biochemical function and morphological appearance. Administration of biotin is capable of doing this.

In mammals spermatogenesis is primarily dependent upon testosterone (*Sharpe* 1987). It is produced by the Leydig cells and acts upon the sertoli and peritubular cells of the seminiferous tubules and via processes which are virtually unknown, drives spermatogenesis (*Sharpe* 1986). There has been much discussion about the need for testosterone to maintain spermatogenesis. Spermatogenesis can be maintained by exogenous testosterone in hypophysectomized rats or in stalk-sectioned monkeys when the intratesticular level of testosterone is only 10–20% of normal (*Buhl, Cornette, Kirton* and *Yuan* 1982; *Marshall, Wickings, Lüdecke* and *Nieschlag* 1983). However, testosterone replacement in these animals was capable of restoring spermatogenesis to only 60–70% of that observed in intact control rats emphasizing the necessisity of FSH for the complete maintenance of spermatogenesis (*Rea, Marshall, Weinbauer* and *Nieschlag* 1985). Local modulation of the response to LH and FSH occurs within the testis where interactions between the various cell types is essential for normal spermatogenesis. Cyclical changes in Sertoli cell secretory function can be related to known changes in its morphology and function (*Sharpe* 1986). The secretion of total protein varies, with many individual proteins of yet undetermined function having a distinct cyclical episode of secretion. Other proteins of known function such as transferrin, ceruloplasmin and androgen-binding protein are secreted at specific stages. *Sharpe* (1986) has indicated that local control factors (proteins) subserve and mediate the hormonal control of spermatogenesis (paracrine control).

In this presentation we have shown that the testicular and serum levels of testosterone are decreased in the biotin-deficient rat. Biotin deficiency was accompanied by a significant degree of sloughing of seminiferous tubule epithelium in these rats. Treatment of biotin-deficient rats with gonadotrophins or biotin increases serum levels of testosterone. However, even when testosterone levels are maintained at higher levels in deficient rats by testosterone implants, the increase in serum testosterone does not result in normal spermatogenesis. The administration of biotin alone or biotin in addition to testosterone, to biotin deficient rats, leads to normal spermatogenesis, thus suggesting that biotin might be involved in the formation of local testicular factor(s) which are required in addition to testosterone and FSH for the normal interaction between Leydig, Sertoli and peritubular cells (*Skinner* and *Fritz* 1985; *Pomerantz* and *Jansz* 1987). The identity of these factors is not known. We have shown that biotin is required for the synthesis of some proteins (*Bhullar* and *Dakshinamurti* 1985; *Singh* and *Dakshinamurti* 1988) including testicular proteins. The biotin requirement of various cell lines

in culture has been related to the role of biotin in the synthesis of some growth factor (*Cheng* and *Moskowitz* 1982; *Dakshinamurti, Chalifour* and *Bhullar* 1985; *Cohen* and *Gospodarowicz* 1985; *Collins, Morell* and *Stockert* 1987). It is possible that a similar event mediated normally by biotin is necessary for the action of testosterone in the complex process of spermatogenesis. This effect of biotin seems to be related to its role in increasing testosterone production and secretion, and also in regulating the synthesis of certain proteins which might include the local (paracrine) factors necessary for normal spermatogenesis.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada.

References

Bhullar, R. P., K. Dakshinamurti: The effect of biotin on cellular functions in HeLa cells. J. Cell. Physiol. 123: 425–430 (1985)

Boeckx, R. L., K. Dakshinamurti: Biotin-mediated protein biosynthesis. Biochem. J. 140: 549–556 (1974)

Brown, M. S., J. L. Goldstein: Receptor-mediated control of cholesterol metabolism. Science 191: 150–154 (1976)

Buhl, A. E., J. C. Cornette, K. T. Kirton, Y. D. Yuan: Hypophysectomized male rats treated with polydimethylsiloxane capsules containing testosterone: Effects on spermatogenesis, fertility and reproductive tract concentrations of androgens. Biol. Reprod. 27: 183–188 (1982)

Cheng, D. K., M. Moskowitz: Growth stimulation of Rous-Sarcoma virus-transformed BHK cells by biotin and serum lipids. J. Cell. Physiol. 113: 487–493 (1982)

Cohen, D. C., D. Gospodarowicz: Biotin and choline replace the growth requirement of Madin-Darby canine kidney cells for high density lipoproteins. J. Cell. Physiol. 124: 96–106 (1985)

Collins, J. C., A. G. Morell, R. J. Stockert: Biotin is required for expression of the asialoglycoprotein receptor (ASGP-R) in HepG2. J. Cell. Biol. 105: 330–335 (1987)

Cooke, B. A., F. H. De Jong, H. J. van der Molen, F. F. G. Rommerts: Endogenous testosterone concentrations in rat testis interstitial tissue and seminiferous tubules during in vitro incubation. Nature (London) 237: 255–256 (1972)

Dakshinamurti, K., L. Chalifour, R. P. Bhullar: Requirement for biotin and the function of biotin in cells in culture. Ann. N. Y. Acad. Sci. 447: 38–55 (1985)

Dakshinamurti, K., C. Cheah-Tan: Liver glucokinase in the biotin deficient rat. Can. J. Biochem. 46: 75–80 (1968)

Dakshinamurti, K., P. R. Desjardins: Lipogenesis in biotin deficiency. Can. J. Biochem. 46: 1261–1267 (1968)

Deacon, A. C., J. G. Dawson: Enzymatic assay of total cholesterol involving chemical or enzymatic hydrolysis – A comparison of methods. Clin. Chem. 25: 976–984 (1979)

Delost, P., T. Terroine: Les troubles endocriniens dans la carence en biotine. Arch. Sci. Physiol. 10: 17–51 (1956)

Ewing, L. L., B. Zirkin: Leydig cell structure and steroidogenic function. In Recent Progress in Hormone Research, vol. 39, Greep, R. D. Ed., Academic Press (1983), pp. 599–635

Folch, J., M. Lees, G. H. Sloane-Stanley: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497–509 (1957)

Liu, D. S. H., R. Ekstrom, J. W. Spicer, A. Richardson: Age-related changes in protein, RNA and DNA content and protein synthesis in rat testes. Exp. Gerontol. 13: 197–205 (1978)

Lowry, O. H., N. J. Rosebrough, A. L. Farr, J. Randall: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275 (1951)

Marshall, G. R., E. J. Wicklings, D. K. Lüdecke, E. Nieschlag: Stimulation of spermatogenesis in stalk-sectioned rhesus monkeys by testosterone alone. J. Clin. Endocrinol. Metabol. 57: 152–159 (1983)

Monroe, S. E., A. F. Parlow, A. R. Midgley Jr.: Radioimmunoassay for rat luteinizing hormone. Endocrinology 83: 1004–1012 (1968)

Pomerantz, D. K., G. F. Jansz: Evidence for intratesticular factors which mediate the response of Leydig cells to disruption of spermatogenesis. In: Endocrinology and Physiology of Reproduction, Leung, Armstrong, Ruf, Moger, Friesen, Eds., Plenum Press, New York (1987), pp. 233–241

Rea, M. A., G. R. Marshall, G. F. Weinbauer, E. Nieschlag: Testosterone maintains pituitary and serum FSH and spermatogenesis in gonadotropin-releasing hormone antagonist-suppressed rats. J. Endocrinol. 108: 101–107 (1985)

Sharpe, R. M.: Paracrine control of the testis. Clin. Endocrinol. Metabol. 15: 185–207 (1986)

Sharpe, R. M.: Testosterone and spermatogenesis. J. Endocrinol. 113: 1–2 (1987)

Shaw, J. H., P. H. Phillips: Pathological studies of acute biotin deficiency in the rat. Proc. Soc. Exp. Biol. Med. 51: 406–407 (1942)

Singh, I. N., K. Dakshinamurti: Stimulation of guanylate cyclase and RNA polymerase II activities in HeLa cells and fibroblasts by biotin and its analogs. Mol. Cell. Biochem. 79: 47–55 (1988)

Skinner, M. K., I. B. Fritz: Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. Mol. Cell Endocrinol. 40: 115–122 (1985)

Terroine, T.: Physiology and biochemistry of biotin. In Vitamins and Hormones, vol. 18, Harris and Ingle, Ed., Academic Press (1960), pp. 1–42

Wood, H. G., R. E. Barden: Biotin enzymes. Ann. Rev. Biochem. 46: 385–413 (1977)

Yuen, B. H., Y. S. Moon, E. K. Mincey, D. Li: Adrenal and sex steroid hormone production by a virilizing adrenal adenoma and its diagnosis with computerized tomography. Am. J. Obst. Gynaecol. 145: 164–169 (1983)

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