Original Research

Thyroid hormone concentrations in systemic circulation and ovarian follicular fluid of cows

Fazl A Ashkar[1], Pawel M Bartlewski[1], Jaswant Singh[2], Pritpal S Malhi[2], Kathleen M Yates[1], Tarvinder Singh[1] and W Allan King[1]

[1]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON; [2]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada
Corresponding author: W Allan King, Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1. Email: waking@uoguelph.ca

Abstract

The aim of this study was to determine and compare the concentrations of total (T) and free (F) fractions of thyroid hormones (T_3-triiodithyronine and T_4-thyroxin) in peripheral circulation and follicular fluid of cows in relation to ovarian follicular status *in vivo* (Experiment 1), and in the follicles from the slaughterhouse ovaries (Experiment 2). In Experiment 1, estrus was synchronized in 15 cows using two Estrumate® (cloprostenol sodium) injections (250 mg cloprostenol intramuscular), the time of ovulation (Day 0) was confirmed by ultrasonography, and ovarian antral follicles were ablated on Day 5. The ensuing superovulatory treatment consisted of eight Folltropin®-V injections (50 mg intramuscular) administered twice daily from Day 6 to Day 9, followed by two injections of Estrumate® (Day 10 am and pm) and a single dose of Lutropin Alfa® (Day 11; 750 IU intramuscular). On Day 5, both TT_3 and FT_3 concentrations were greater ($P < 0.05$) in serum than follicular fluid from dominant (DFs) or subordinate antral follicles (SFs), and TT_4 concentrations were greater ($P < 0.05$) in DFs compared with SFs. Serum concentrations of FT_4 were greater ($P < 0.05$) on Day 12 than on Day 5, and TT_4 concentrations in follicular fluid collected on Day 12 were higher than those in DFs and SFs on Day 5. In Experiment 2, there were no differences ($P > 0.05$) in thyroid hormone concentrations between the largest and all remaining antral follicles visible on the surface of the ovary ($n = 20$ ovaries). We concluded that: (i) physiological status of bovine antral follicles (i.e. dominant versus subordinate) may impinge on the accumulation of TT_4 in follicular fluid; and (ii) hormonal ovarian superstimulation increases circulating levels of FT_4 and follicular fluid content of TT_4.

Keywords: thyroid hormones, ovary, antral follicles, ovarian superstimulation, ultrasonography, cow

Experimental Biology and Medicine 2010; **235**: 215–221. DOI: 10.1258/ebm.2009.009185

Introduction

Thyroid hormones govern the control of growth, differentiation and metabolism in nearly all somatic tissues.[1] Due to their general metabolic and permissive effects, thyroid hormones are essential for normal ovarian activity in mammalian species. Abnormal thyroid hormone levels have been reported to lead to infertility or reduced reproductive function.[2,3]

Studies that manipulated systemic thyroid hormone levels to produce a hypothyroid state in dogs have resulted in decreased sensitivity of ovarian theca and granulosa cells to gonadotropins.[4] Triiodothyronine (T_3) has been shown to synergize with follicle-stimulating hormone (FSH) to induce differentiation of granulosa cells in porcine follicles,[5,6] and the treatment with thyroxin (T_4) enhanced antral follicular development and ovulation rates in rats.[7]

One of the speculated roles of thyroid hormones currently under review is the direct regulation by thyroid hormones of cholesterol metabolism (steroidogenesis) within the ovarian follicular cells.[8,9] Spicer *et al.*[10] evaluated the effects of thyroid hormones at the level of follicular cells and it was shown that T_3 and T_4 positively contributed to luteinizing hormone (LH)-induced androstenedione production by bovine theca cells. Additional results also suggested that T_4 alone has a positive impact on FSH-induced progesterone production by bovine granulosa cells.[10] The exact mechanism by which thyroid hormones regulate steroidogenesis in ovarian follicles remains unknown.

Since the 1920s it was known that the iodine concentration in mammalian species was higher in the ovary than in every other organ except for the thyroid gland

ISSN: 1535-3702

itself.[11] The mechanism whereby the Graafian follicles accumulate iodine in the follicular fluid can be attributed to the functioning of the sodium-iodide symporter (NIS) in ovarian follicular cells.[12] Moreover, thyroid hormone receptor mRNA has been found in human and porcine granulosa cells.[13,14] The existence of NIS along with the presence of thyroid hormone receptors in ovarian follicular cells suggests possible roles in folliculogenesis and oocyte development. Oocyte maturation is dependent upon follicular fluid composition as its hormonal milieu creates an optimal physiological environment for the oocyte.

Several factors such as environmental temperature, season, diet, circadian rhythm and age are responsible for the fluctuations in thyroid hormone levels.[9,15] Difference in systemic and follicular concentrations of thyroid hormones can also be due to ovarian physiological status. Iodine uptake is lower before puberty, post-menopause and during pregnancy, and higher during follicular growth.[1,16] The deiodination of T_4 by ovarian 5′monodeiodinase (5′-MD) system in follicular fluid of the mare was reported to be highest during the periovulatory period.[11]

The cattle industry is one of the biggest global livestock industries and the need for efficient breeding is paramount. Also, the ovaries and ovarian structures in cattle and humans are of similar size and morphology.[17] Recent ultrasonographic studies have revealed that cows have growth patterns of antral follicles and develop pathological ovarian conditions similar to those described in women, allowing the cattle to serve as a model for ovarian function and aberrations in humans.[18] However, information on circulating concentrations and follicular fluid content of both thyroid hormones in cattle is sparse, and there have been no systematic studies that relate the ovarian follicular status to thyroid hormone levels in this species. Therefore, the main objective of the present study was to determine and compare the concentrations of total (T) and free (F) fractions of thyroid hormones in peripheral circulation and follicular fluid of cows, in relation to antral follicular status *in vivo* (i.e. between dominant and subordinate follicles, and follicles after ovarian superstimulation). *In vitro* embryo production involves the removal of ovaries from the reproductive tract of slaughtered cows and oocyte collection after transporting the ovaries to the laboratory. Therefore, the additional aim of this study was to determine follicular fluid content of the thyroid hormones, in relation to follicular size, in the bovine ovaries obtained in the local abattoir.

Materials and methods

Experiment 1

The Animal Care Committees at the University of Saskatchewan approved all procedures performed on live animals in the present study. Animals employed in this experiment were housed in the field research station in Goodale near Saskatoon, SK (latitude-52°07′ North; longitude-106°38′ West). Animals were housed in a single outdoor corral with an easy access to indoor facilities. All cows ($n = 15$) aged 1–4 years were at least 45 days postpartum, and had not received any hormonal treatments after calving. All animals were non-lactating, non-pregnant, and had an ultrasonographically detectable corpus luteum (CL) at the beginning of the experiment (Experiment 1). The body condition scores of the 15 animals at the outset of the study were between 2.5 and 3.5[18]; the system used scored all animals 1 to 5, from emaciated to fat (1 – emaciated, 2 – thin, 3 – average, 4 – heavy and 5 – fat).

Estrus was initially synchronized using two intramuscular injections of Estrumate® (Schering Canada Inc, Pointe-Claire, PQ, Canada; 250 mg cloprostenol/cow), and the time of ovulation (Day 0) was confirmed by ultrasonography (Figure 1). To confirm the time of ovulation and monitor the development of ovarian antral follicles, transrectal ovarian ultrasonographic examinations were performed every 24 h for the duration of the study by the same experienced operator using a B-mode ultrasound echo camera connected to a 7.5-MHz linear-array transducer (Aloka SSD-900; Instruments for Science and Medicine, Vancouver, BC, Canada). Follicle diameters, relative location of follicles $\geq$4 mm in diameter and corpora lutea were recorded, as described previously.[18,19]

Ultrasound-guided follicle ablation was performed on Day 5 and the superovulatory treatment commenced on Day 6. The treatment consisted of eight Folltropin®-V injections (porcine FSH; Bioniche Animal Health Canada Inc, Belleville, ON, Canada) given twice daily (from Day 6 pm to Day 9 pm). A total dose of 50 mg NIH-FSH-P1 of porcine FSH/100 kg body weight was administered over the four days (i.e. eight treatments of 6.25 mg NIH-FSH-P1 of Folltropin®-V/100 kg/cow intramuscular). A multiple-dose Folltropin®-V treatment was followed by two injections of Estrumate® (Day 10 am and pm; 250 mg cloprostenol/cow intramuscular) and a single dose of Lutropin Alfa (EMD Serono Canada Inc, Oakville, ON, Canada; 750 IU/cow intramuscular) administered in the afternoon of Day 11. Lutropin Alfa® is a biosynthetic form (recombinant DNA origin) of the naturally occurring human luteinizing hormone (hLH). All intramuscular injections were given in the semi-membranosus muscle. Jugular blood samples were drawn and follicular fluid samples obtained, by ultrasound-guided follicle aspiration, on Days 5 and 12. On Day 5, an ostensibly dominant follicle and all subordinate follicles $\geq$4 mm in diameter were ablated, whereas on Day 12 follicular fluid was collected from the largest four follicles detected by ultrasonography. Following transvaginal ultrasound-guided follicle aspirations, follicular fluid samples were centrifuged at 1500 *g* for 10–15 min and then frozen at −20°C until assayed. Please see Figure 1 for further details of experimental design and procedures.

Experiment 2

In Experiment 2, bovine ovaries ($n = 20$) collected at the local abattoir (Guelph, ON, Canada) were used to aspirate follicular fluid from the largest and all remaining follicles visible on the surface of the ovary. The ovaries ($n = 20$) were placed in a flask containing conditioned phosphate buffer saline (PBS) at 37°C, transferred to the laboratory within an hour and then used for follicle aspiration performed at 29°C. The largest follicles from each ovary

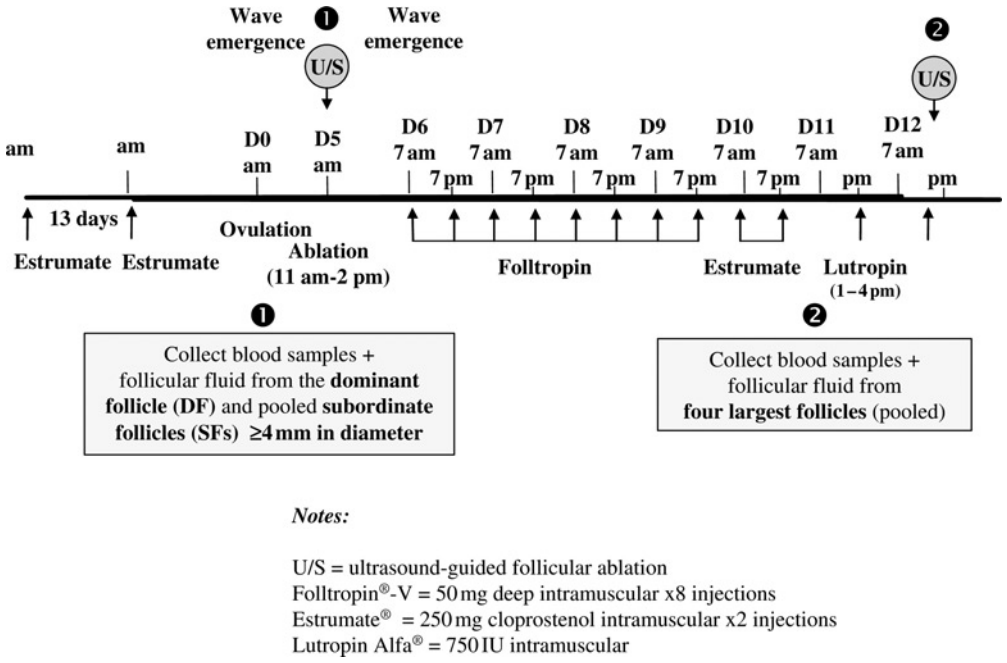

Figure 1 Experimental design (Experiment 1)

(diameter: 12–22 mm) were aspirated with a syringe and a 16-gauge needle. Follicular fluid from all remaining follicles visible on the surface of the ovary was aspirated by a vacuum pump through a 16-gauge needle into 15-mL glass tubes. Following aspirations, follicular fluids were processed as described for Experiment 1 above. In Experiment 2, no attempt was made to determine the physiological status of the follicles (i.e. growing versus atretic antral follicles) or to assess the stage of the cycle at which the ovaries were collected.

Hormone assays

Thyroid hormone levels were measured by validated radioimmunoassays (RIAs) in the Diagnostic Center for Population and Animal Health, Michigan State University, USA. The manufacturer's information for the TT_4, TT_3 and FT_3 assays can be obtained from the Clinical Assays^{TM}, Gammacoat M Total T_4, Free T_4 or Free T_3 ^{125}I RIA Kit (DiaSorin Inc, Stillwater, MN, USA). The assay for total T_4 utilized a competitive RIA procedure that was performed entirely in 12 × 75 mm polypropylene tubes coated with a highly specific monoclonal antibody to T_4. The assay has minimal cross-reactivity with other iodothyronines and iodotyrosines except for D-thyroxine, with which it has 92% cross-reactivity. Sensitivity of the assay was 3 nmol/L. Intra- and inter-assay coefficients of variation (CVs) were <10%.

The assay for estimating FT_4 used a competitive procedure that was performed entirely in 12 × 75 mm polypropylene tubes coated with a highly specific, low affinity monoclonal antibody against T_4. The assay was performed in two steps; the first incubation (30 min) was with the sample in contact with solid phase T_4 antibody at 37 °C. Incubation time is very important to avoid stripping bound T_4 from binding proteins. The serum was then decanted and the second incubation was with the ^{125}I-T_4 in buffer. The ^{125}I-T_4 fills any remaining solid phase antibody sites so that the amount of bound radiation is inversely proportional to the amount of FT_4 in the serum. Decanting the contents of the tube separated bound and free assay fractions and the bound fraction of radiation was estimated. The remainder of the assay was standard RIA technology. The antibody had less than 0.02% cross-reactivity with related iodothyronine molecules. Sensitivity was determined to be 3 pmol/L using the 90% of specific binding (B/B_0) endpoint. The intra-assay CVs for control samples with mean FT_4 concentrations of 8, 16 and 33 pmol/L were 7%, 5% and 5%, respectively. The inter-assay CVs for control samples with mean hormone concentrations of 7, 14 and 32 pmol/L were 13%, 10% and 14%, respectively.

The assay for TT_3 was an 'in house' ^{125}I RIA method using activated charcoal in the separation step. Specific antibodies are used, which have minimal cross-reactivity with related compounds and precursors of the hormones such as iodotyrosines and tyrosine. The intra- and inter-assay CVs for the assays were all less than 10%. Sensitivity of the TT_3 assay is 0.25 nmol/L.

The assay for estimating FT_3 used a ^{125}I-T_3 derivative that does not bind significantly to the natural binding proteins in serum. In addition, a high affinity antibody was used that binds both the derivative and T_3. These two T_3 compounds allow for a classic equilibrium RIA to be performed without interference from binding proteins and bound T_3. The assay antibody was bound to the wall of 12 × 75 mm polypropylene tubes for a simple solid-phase separation of bound assay fractions from free fractions by decantation; the remainder of the assay was a standard RIA procedure. The antibody has less than 0.02% cross-reactivity with related iodothyronine molecules. Sensitivity was determined to be

1.2 pmol/L at 90% B/B_0. The intra-assay CVs were 14%, 14% and 6% at FT_3 concentrations of 3, 6 and 13 pmol/L, and the inter-assay CVs were 14%, 20% and 14% at hormone concentrations of 2, 4 and 11 pmol/L, respectively.

Statistical analyses

In separate statistical tests (General Linear Model procedure for analysis of variance [ANOVA]; SimgaStat®3.0 for Windows®, Systat Software Inc, Richmond, CA, USA), the differences in total and unbound thyroid hormone concentrations were analyzed between: in Experiment 1 (i) serum and follicular fluid samples obtained at various time points (i.e. Days 5 and 12); (ii) dominant (DFs) and subordinate antral follicles (SFs), and large antral follicles present after ovarian superstimulation; and in Experiment 2 (iii) the largest and all remaining follicles aspirated from the slaughterhouse bovine ovaries. For the purpose of statistical comparisons, the concentrations of the hormones lower than the assay detection limit were assigned a value equal to the assay sensitivity. For each fraction of thyroid hormones except for TT_3 (non-detectable levels in follicular fluid), the Pearson Product Moment (correlation analysis) was carried out between circulating and follicular fluid concentrations of the hormone, using paired samples from each animal (Experiment 1); the samples obtained on Day 5 and Day 12 were analyzed in separate analyses. Significant statistical differences were regarded as P value <0.05. The Bonferroni test was used as a post-ANOVA test to determine differences between individual means. All data are presented as mean $\pm$ SEM.

Results

Results of hormonal analyses performed in Experiment 1 are summarized in Table 1. FT_3 concentrations in FF samples obtained on Days 5 and 12 were all less than the assay detection limit. On Day 5, circulating TT_4 concentrations and follicular fluid levels of TT_4 measured in DFs were both greater ($P < 0.05$) compared with those in SFs of the first follicular wave after ovulation.

Both TT_3 and FT_3 concentrations were greater ($P < 0.05$) in serum than in follicular fluid from DFs or SFs, and circulating FT_4 concentrations were greater ($P < 0.05$) than those in follicular fluid from SFs. Circulating concentrations of TT_4, TT_3 and FT_4 were strongly ($r = 0.70–0.84$) and positively correlated to follicular fluid levels of the hormones in DFs and SFs of cows (Table 2).

On Day 12, serum concentrations of FT_4, TT_3 and FT_3 were greater ($P < 0.05$) compared with those in follicular fluid. There was a strong ($r = 0.64–0.85$) positive correlation between TT_4, TT_3 and FT_4 concentrations in blood serum and follicular fluid aspirated from the four largest antral follicles present (Table 2). Serum concentrations of FT_4 were greater ($P < 0.05$) on Day 12 compared with Day 5 (Table 1). Follicular fluid content of TT_4 on Day 12 was also greater ($P < 0.05$) than on Day 5 (Table 1). In Experiment 2, there were no differences ($P > 0.05$) in the concentrations of total and free fractions of thyroid hormones between the largest and all remaining antral follicles aspirated (Table 3).

Discussion

The primary goal of the present study was to determine the concentrations of total and unbound fractions of thyroid hormones in ovarian follicular fluid of cows and, whenever possible, compare them with circulating levels of the hormones. The comparison of systemic and intrafollicular concentrations of thyroid hormones would give us a better understanding of the transport of these hormones and their potential role in ovarian function. The T_4:T_3 ratio in blood, determined in various animal species of veterinary interest, varies from 15:1 to 44:1, with a notable exception of cows, in which a T_4:T_3 ratio approximating 67:1 has been reported.[16,20] This contrasts with our present observations in cows where the estimated T_4:T_3 ratio in peripheral circulation ranged from 32:1 to 39:1. The reason for the discrepancy between those earlier and our present estimates remains uncertain.

Serum concentrations of TT_3 and FT_3 were greater than those in the follicular fluid of dominant and subordinate follicles shortly after deviation of the dominant follicle of the first follicular wave post-ovulation (Table 1). Following the administration of superovulatory doses of gonadotropic hormones, serum concentrations of FT_4, TT_3 and FT_3 were also greater compared with those in follicular fluid (Table 1). The results of correlation analyses indicated that circulating and follicular fluid concentrations of TT_4, FT_4 and TT_3 tended to change in parallel (Table 2). Although the exact cellular mechanism for thyroid hormone transport from blood to various target organs is not completely understood, thyroid hormones appear to enter ovarian

Table 1 Summary of concentrations of total (T) and free (F) fractions of thyroid hormones (T_3-triiodithyronine and T_4-thyroxin; mean $\pm$ SEM) in peripheral serum and follicular fluid of cows in Experiment 1 ($n = 15$)

Thyroid hormone fraction and ratios/ time of sampling and source	TT_4 (nmol/L)	TT_3 (nmol/L)	TT_4:TT_3	FT_4 (pmol/L)	FT_3 (pmol/L)	FT_4:FT_3
Day 5 serum	47.3 ± 2.7^{ab}	1.5 ± 0.08^{a}	32:1	15.6 ± 0.9^{b}	1.5 ± 0.1	12:1
Day 5 FF (DFs)	40.3 ± 2.2^{b}	1.1 ± 0.06^{bc}	36:1	13.6 ± 0.9^{bc}	ND	–
Day 5 FF (SFs)	31.2 ± 3.3^{c}	0.8 ± 0.2^{c}	39:1	12.6 ± 1.1^{c}	ND	–
Day 12 serum	55.5 ± 3.9^{a}	1.5 ± 0.09^{a}	36:1	18.4 ± 1.2^{a}	1.7 ± 0.1	11:1
Day 12 FF	48.7 ± 3.7^{a}	1.3 ± 0.09^{b}	39:1	12.9 ± 0.8^{bc}	ND	–

Within columns, means denoted by different letters are significantly different ($P < 0.05$)
ND, not detected (hormone concentrations below the assay sensitivity); FF, follicular fluid; DFs, dominant follicles; SFs, subordinate follicles

Table 2 Summary of correlations between serum and follicular fluid (FF) concentrations of total (T) and free (F) fractions of thyroid hormones (T_3-triiodithyronine and T_4-thyroxin) determined in Experiment 1 ($n = 15$ cows)

Variables analyzed/ hormone fraction	TT_4	TT_3	FT_4
Day 5 serum versus Day 5 FF (DFs)	$r = 0.77$	$r = 0.80$	$r = 0.84$
	$P < 0.001$	$P < 0.001$	$P < 0.001$
Day 5 serum versus Day 5 FF (SFs)	$r = 0.83$	$r = 0.80$	$r = 0.70$
	$P < 0.01$	$P < 0.05$	$P < 0.05$
Day 12 serum versus Day 12 FF	$r = 0.85$	$r = 0.71$	$r = 0.64$
	$P < 0.001$	$P < 0.01$	$P < 0.05$

DFs, dominant follicles; SFs, subordinate follicles

follicular fluid by the same mechanism(s) by which they pass the blood barrier in other tissues.[11] It is presently assumed that there exist specific protein transporters, the monocarboxilate transporter (MCT) family, which facilitate thyroid hormone passage through capillary endothelium.[21] Specific binding sites for T_3 and T_4 have been identified in plasma membrane, cell nuclei and mitochondria of diverse organs, and it is possible that the entry of thyroid hormone molecules into the cells is driven by the equilibrium between the extracellular and intracellular thyroid hormone binding proteins.[1]

On the basis of our present results (Experiment 1), it is feasible that a selective transport mechanism may be involved in trafficking thyroid hormones across the apical and basal plasma membrane of ovarian follicular cells. Ritchie *et al.*[22] demonstrated that overexpression of System L, a plasma membrane transporter in *Xenopus* oocytes, increased both cytoplasmic and nuclear delivery of thyroid hormones from external circulation. Our present observations on differences between circulating and follicular fluid levels of thyroid hormones appear to support a theoretical supposition for such a cell-surface transport in bovine ovarian follicles.

Another factor to consider with regards to the difference in circulating and intrafollicular contents of TT_3 and FT_3 in cyclic cows is the level of 5′-MD activity in granulosa cells and its potential role in regulating the intermediate metabolism of thyroid hormones within the follicle. Removal of iodide from T_4 would subsequently increase the level of the biologically active T_3 in follicular fluid.[23] The breakdown of T_4 to free iodide molecules may also contribute, in addition to the existence of the NIS system, to the overall high levels of iodine observed in the mammalian ovary.[11] Lastly, both aforementioned mechanisms may co-exist in ovarian antral follicles: a selective transport mechanism at the level of the follicular cell surface and fluctuations in the deiodinase activity within granulosa and thecal cells may potentially regulate TT_3 and FT_3/T_4 levels in individual follicles.

Total T_4 levels were greater in the dominant compared with subordinate follicles of the first wave post-ovulation, suggesting a possible role of T_4 hormone in the establishment and/or maintenance of the dominance status of bovine antral follicles. Sato and Jiang[7] noted an increase in the total number of ovulations in induced hypothyroid rats after T_4 treatments, indicating that thyroid hormones are essential for maturation and terminal development of large antral follicles selected for ovulation. Biswas *et al.*[24] reported that thyroid hormones exerted a direct effect on murine granulosa cells, increasing their proliferation and release of progesterone along with other cholesterol derivatives. Differences in the vasculature between dominant and subordinate follicles may also account for the regulation of thyroid hormone transport into the individual follicles. Under the influence of follicular estrogens, dominant follicles are highly vascular and would therefore be exposed to a greater amount of circulating thyroid hormones than their subordinate counterparts.[7]

Whether or not the mammalian ovary is able to utilize iodide and amino acid precursors to *de novo* produce T_4 and/or T_3 is still equivocal. Although there is evidence for the presence of thyroid stimulating hormone receptors (TSH-Rs) in gonads and other extra-thyroid tissues of a non-mammalian vertebrate, the striped bass,[25] until recently similar evidence has been lacking for any mammalian species. Using immunohistochemical techniques, Mutinati *et al.*[26] have shown that both small and large luteal cells in mature bovine CL contain TSH-Rs and thyroglobulin, suggesting the existence of luteal synthesis of thyroid hormones, which may act in an autocrine and paracrine way. Glutathione peroxidase activity has also been detected in CL of the ewe; however, the evidence for its expression in growing and static ovine follicles has not been provided using histochemical staining.[27]

In the present study, thyroxine levels increased after the superstimulatory doses of gonadotropins had been administered. In contrast to these results, Muller *et al.*[28] observed a decrease in FT_4 levels after a controlled ovarian hyperstimulation in humans. It is important, however, to note that patients who participated in clinical trials were enrolled in IVF programs and may have had infertility problems related to their thyroid condition, compromising the outcome of that study. Despite an increase in serum concentration of FT_4 after hormonal ovarian stimulation, follicular fluid concentrations of FT_4 on Day 12 were still lower than those in blood serum and they did not vary from follicular fluid levels of FT_4 determined in DFs and SFs on Day 5 after ovulation.

Hormone assays of follicular fluid aspirated from ovaries that were collected at the abattoir, revealed no difference in total and free fractions of thyroid hormones between the largest and all remaining visible follicles. In Experiment 1,

Table 3 Concentrations of total (T) and free (F) fractions of thyroid hormones (T_3-triiodithyronine and T_4-thyroxin; mean ± SEM) in follicular fluid aspirated from the largest and all remaining antral follicles in the slaughterhouse bovine ovaries ($n = 20$)

Thyroid hormone fraction and ratios/follicle type	TT_4 (nmol/L)	TT_3 (nmol/L)	TT_4:TT_3	FT_4 (pmol/L)	FT_3 (pmol/L)	FT_4:FT_3
Largest follicle	90.2 ± 7.9	2.0 ± 0.1	45:1	21.4 ± 1.4	2.0 ± 0.1	11:1
Remaining antral follicles	84.1 ± 8.8	1.9 ± 0.1	44:1	21.6 ± 1.5	2.2 ± 0.1	9:1

in which follicular fluid content of TT_4 was greater in DFs compared with SFs, the subordinate follicles $\geq$4 mm in diameter were aspirated, and the size advantage and hence dominant status of the largest follicle could be clearly determined. Since the reproductive status and stage of growth of individual follicles in slaughterhouse ovaries were not known, it is not surprising that there were no detectable differences in thyroid hormone levels between the largest and all other antral follicles. Both free and total thyroid hormone levels were up to two-fold higher in the follicular fluid from the slaughterhouse ovaries compared with those observed in Experiment 1. While basal TSH and thyroid hormone secretion is mainly regulated by intrinsic hypothalamic activity, different neurotransmitters (particularly catecholamines) activated during stress can significantly increase T_4 and T_3 production.[16] It is possible that transportation stress was one of the factors responsible for the elevated levels of thyroid hormones in the slaughterhouse bovine ovaries (Experiment 2).

In summary, the concentrations of both total and unbound fractions of T_3 were significantly greater in serum than in follicular fluid from antral follicles of the first follicular wave after ovulation and follicular fluid concentrations of TT_4 were greater in DFs compared with SFs. In the present study, FT_3 in follicular fluid aspirated from bovine antral follicles on Day 5 after ovulation and after hormonal ovarian superstimulation were non-detectable. Following the ovarian superstimulation, serum concentrations of FT_4, TT_3 and FT_3 were all greater compared with those in follicular fluid, and circulating FT_4 concentrations were greater than those recorded on Day 5 post-ovulation. Administration of exogenous gonadotropins also significantly increased follicular fluid content of TT_4. We speculate that the existence of differences between serum and intrafollicular concentrations of certain fractions of thyroid hormones may be indicative of their selective transport and/or intraovarian deiodination processes. We concluded that: (i) the physiological status of bovine antral follicles (i.e. dominant versus subordinate) was related to the accumulation of TT_4 in follicular fluid; and (ii) hormonal ovarian superstimulation increased circulating levels of FT_4 and follicular content of TT_4. A better understanding of the role of thyroid hormones in ovarian function may be applied to the assisted reproductive technologies including the induction of multiple ovulations and/or embryo production.

ACKNOWLEDGEMENTS

The authors thank the farm staff at Goodale experimental farm, University of Saskatchewan, for care and management of experimental animals. This study was funded by the Canada Research Chair grant (Animal Biotechnology; WAK), the Ontario Ministry of Food and Rural Affairs (WAK, PMB), and the National Sciences Engineering Research Council (NSERC) of Canada grants (JS, PMB).

REFERENCES

1 Ingbar SH, Wieber KA. *The thyroid gland*. In: Williams RH, ed. *Textbook of Endocrinology*. Philadelphia: W.B. Sounders Company, 1981:117–243
2 Fitko R, Kucharski J, Szlezyngier B. The importance of thyroid hormone in experimental ovarian cyst formation in gilts. *Anim Reprod Sci* 1995;**39**:159–68
3 Haentjens P, van Meerhaeghe A, Poppe K, Velkeniers B. Subclinical thyroid dysfunction and mortality: an estimate of relative and absolute excess all-cause mortality based on time-to-event data from cohort studies. *Eur J Endocrinol* 2008;**159**:329–41
4 Panciera DL, Purswell BJ, Kolster KA. Effect of short-term hypothyroidism on reproduction in the bitch. *Theriogenology* 2007;**68**:316–21
5 Maruo T, Hayashi M, Matsuo H, Yamamoto T, Okada H, Mochizuki M. The role of thyroid hormones as a biological amplifier of the actions of follicle-stimulating hormone in the functional differentiation of cultured porcine granulosa cells. *Endocrinology* 1987;**121**:1233–41
6 Maruo T, Hiramatsu S, Otani T, Hayashi M, Mochizuki M. Increase in the expression of thyroid hormone receptors in porcine granulosa cells in follicular maturation. *Acta Endocrinol* 1992;**127**:152–60
7 Sato E, Jiang JY. Follicular development and ovulation in hypothyroid rdw rats. *Mol Cell Dev Biol Reprod* 2001;**8**:249–56
8 Gregoraszczuk EL, Skalka M. Thyroid hormone as a regulator of basal and human chorionic gonadotropin-stimulated steroidogenesis by cultured porcine theca and granulosa cells isolated at different stages of the follicular phase. *Reprod Fertil Dev* 1996;**8**:961–7
9 Blaszczyk B, Stankiewicz T, Udala J, Gaczarzewicz D, Lasota B, Blaszczyk P, Szymanska A, Szymanska-Pasternak J. Free thyroid hormones and cholesterol in follicular fluid of bovine ovaries. *Bull Vet Inst Pulawy* 2006;**50**:189–93
10 Spicer LJ, Alonso J, Chamberlain CS. Effects of thyroid hormone on bovine granulosa and thecal cell function in vitro: dependence on insulin and gonadotropins. *J Dairy Sci* 2001;**84**:1069–76
11 Slebodzinski AB. Ovarian iodide uptake and triiodothyronine generation in follicular fluid: the enigma of the thyroid ovary interaction. *Dom Anim Endocrinol* 2005;**29**:97–103
12 Spitzweg C, Joba W, Eisenmenger W, Heufelder AE. Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland and gastric mucosa. *J Clin Endocrinol Metab* 1998;**83**:1746–51
13 Wakim AN, Polizotto SL, Buffo MJ, Marrero MJ, Burholt DR. Thyroid hormones in follicular fluid and thyroid hormone receptors in human granulosa cells. *Fertil Steril* 1993;**59**:1187–90
14 Zhang SS, Carrillo AJ, Darling DS. Expression of multiple thyroid hormone receptor mRNAs in human oocytes, cumulus cells, and granulosa cells. *Mol Hum Reprod* 1997;**3**:555–62
15 Bitman J, Kahl S, Wood DL, Lefcourt AM. Circadian and ultradian rhythms of plasma thyroid hormone concentrations in lactating dairy cows. *Am J Physiol* 1994;**266**:R1797–R1803
16 Eiler H. *Endocrine glands*. In: Reece WO, ed. *Dukes' Physiology of Domestic Animals*. New York: Cornell University Press, 2004:621–69
17 Singh J, Adams GP. Histomorphometry of dominant and subordinate bovine ovarian follicles. *Anat Rec* 2000;**258**:58–70
18 Malhi PS, Adams GP, Pierson RA, Singh J. Bovine model of reproductive aging: response to ovarian synchronization and superstimulation. *Theriogenology* 2006;**66**:1257–66
19 Pierson RA, Ginther OJ. Reliability of diagnostic ultrasonography for identification and measurements of follicles and detecting the corpus luteum in heifers. *Theriogenology* 1987;**28**:929–46
20 Nixon DA, Akasha MA, Anderson RR. Free and total thyroid hormones in serum of Holstein cows. *J Dairy Sci* 1988;**71**:1152–60
21 Visser WE, Jansen J, Kester MH, Bruower OF, Lunsig RJ, Lundgren J, Mancilla E, Friesema EC, Visser TJ. Differences in T3 uptake and metabolism in fibroblasts of MCT8 patients reflect phenotypic variability. *Hormone Res* 2007;**68**:56
22 Ritchie JWA, Shi YB, Hayashi Y, Baird FE, Muchekehu RW, Christie GR, Taylor PM. A role for thyroid hormone transporters in transcriptional regulation by thyroid hormone receptors. *Mol Endocrinol* 2003;**17**:653–61
23 Pezzi C, Accorsi PA, Vigo D, Govoni N, Gaiani R. 5(-Deiodinase activity and circulating thyronines in lactating cows. *J Dairy Sci* 2003;**86**:152–8
24 Biswas R, Bandyopadhyay A, Guin S, Bhattacharya S. Binding of thyroid hormone to mouse granulosa-cell nuclei and its biological relevance. *J Biosci* 1993;**18**:327–35

25 Kumar RS, Ijiri S, Kight K, Swanson P, Dittman A, Alok D, Zohar Y, Trant JM. Cloning and functional expression of a thyrotropin receptor from the gonads of a vertebrate (bony fish): potential thyroid-independent role for thyrotropin in reproduction. *Mol Cell Endocrinol* 2000;**16**:1–9
26 Mutinati M, Desantis S, Rizzo A, Zizza A, Ventriglia G, Pantaleo M, Sciorsci RL. Localization of thyrotropin receptor and thyroglobulin in the bovine corpus luteum. *Anim Reprod Sci* 2009 June 6 [Epub ahead of print]
27 Al-Gubory KH, Ceballos-Picot I, Nicole A, Bolifraud P, Germain G, Michaud M, Mayeur C, Blachier F. Changes in activities of superoxide dismutase, nitric oxide synthase, glutathione-dependent enzymes and the incidence of apoptosis in sheep corpus luteum during the estrous cycle. *Biochem Biophys Acta* 2005;**1725**:348–57
28 Muller AF, Verhoeff A, Mantel MJ, de Jong FH, Berghout A. Decrease of free thyroxine levels after controlled ovarian hyperstimulation. *J Clin Endocrinol Metab* 2000;**85**:545–8

(Received June 12, 2009, Accepted August 7, 2009)