ELSEVIER

Journal of Chromatography B, 777 (2002) 281–287

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Thyroid gland function in ovariectomized ewes exposed to phytoestrogens

Andrzej Madej[a,*], Elisabeth Persson[b], Torbjörn Lundh[c], Yvonne Ridderstråle[a]

[a]*Department of Animal Physiology, Centre for Reproductive Biology in Uppsala, Swedish University of Agricultural Sciences (SLU), P.O. Box 7045, S-750 07, Uppsala, Sweden*

[b]*Department of Anatomy and Histology, Centre for Reproductive Biology in Uppsala, Swedish University of Agricultural Sciences (SLU), P.O. Box 7011, S-750 07, Uppsala, Sweden*

[c]*Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences (SLU), P.O. Box 7024, S-750 07, Uppsala, Sweden*

Abstract

Phytoestrogens are by definition plant-derived substances that are able to activate the mammalian oestrogen receptors. We examined the possible effects of phytoestrogens on the secretion of thyroid hormones as well as on the immunoreactivity to oestrogen receptor alpha (ERα) in the thyroid glands of ovariectomized ewes. Eight ovariectomized ewes were fed 3.5 kg of 100% red clover silage for 14 days. Blood samples were collected before and on day 14 of exposure to phytoestrogens. After 5 months, four of the ewes were re-exposed to red clover silage as described above and the other four served as controls. Blood samples were collected as above. All ewes were slaughtered at the end of the experiment and the thyroid glands were weighed and examined for macroscopical changes. Tissue samples were taken for immunohistochemistry and image analysis. Ewes exposed to red clover silage had significantly higher plasma concentrations of total T_3 and free T_3 than ewes fed hay. The cross-section area of thyroid follicles tended to be larger in ewes fed red clover silage than in the control animals. ERα immunoreactivity was stronger in thyroid glands from ewes exposed to phytoestrogens than in ewes fed hay. In conclusion, daily ingestion of 81–95 mg phytoestrogens per kg body weight for 14 days stimulated secretion of thyroid hormones and tended to increase follicle size and ERα immunoreactivity of thyroid glands of ovariectomized ewes.

Keywords: Thyroid gland function; Phytoestrogens

1. Introduction

The presence of oestrogenic substances in plant extracts was first recognised in the late 1920s [1]. Infertility in female sheep grazed on oestrogenic subterranean clover (*Trifolium subterraneum* L.) was described as part of the condition known as ‘clover disease’ [2]. Exposure of ewes to phytoestrogens produced pathophysiological and morphological changes in the reproductive tract, as well as in the pituitary, adrenal and thyroid glands [3,4]. Phytoestrogens and their metabolites interact with oestrogen receptors [5] and compete for binding sites in the brain [6] and the reproductive organs [7]. Recently it was shown that the oestrogenic potency of phytoestrogens is significant for the oestrogen receptor alpha (ERα) as well as the oestrogen receptor beta (ERβ)

*Corresponding author. Tel.: +46-18-672-114; fax: +46-18-672-111.

E-mail addresses: andrzej.madej@djfys.slu.se (A. Madej), http://www.djfys.slu.se (A. Madej).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00082-X

[8]. The demonstration of a direct effect of oestradiol on thyroid follicular cell growth [9] raises the possibility that phytoestrogens may also influence the thyroid function. In fact, a decrease in plasma free triiodothyronine (T_3) in premenopausal women fed soy isoflavones has recently been documented [10]. Forsythe [11] reported that gerbils fed soy protein had significantly higher concentrations of T_4 and TSH but significantly lower total cholesterol than gerbils fed casein. In rats fed soy protein an elevation of total T_4, T_3, free T_4 and free T_3 was seen [12]. In a previous study we have shown that feeding red clover silage to ewes causes an increase in teat size and uterine weight, as well as hyperaemia in the vulva [13]. The purpose of the current study was to examine the possible effect of oestrogenic silage on thyroid hormone secretion and localisation of ERα in thyroid glands of ovariectomized ewes.

2. Materials and methods

2.1. Animals

The local Animal Ethics Committee in Uppsala, Sweden approved the experimental design and animal care in this study. Eight Swedish Finewool Landrace ewes, all of which had lambed in January, were ovariectomized in May through a mid-ventral laparotomy under general anaesthesia. They were kept in individual, adjacent boxes under natural light conditions until the end of the experiment. The ewes were given ad libitum access to non-oestrogenic hay (mainly Timothy grass) and 100 g of concentrate (39% barley, 39% oats, 11% soy, 7% rape-seed, and 4% other additions) per day except during experimental feeding periods. They had free access to mineral licks and good quality drinking water at all times.

2.2. Phytoestrogens

Red clover (*Trifolium pratense*) silage was used as the source of plant oestrogens. High-performance liquid chromatography was used to determine the oestrogen content of the red clover silage [14], which contained a total of 1.74 g phytoestrogens (1.01 g formononetin, 0.03 g daidzein, 0.61 g biochanin-A, and 0.08 g genistein) per kg wet weight. Each ewe was fed 3.5 kg silage, corresponding to a daily intake of 6.1 g phytoestrogens (of which 3.5 g was formononetin). No detectable levels of phytoestrogens were found in the hay or concentrate samples.

2.3. Experimental design

2.3.1. Experiment I

At the end of October, all ewes ($n=8$, mean body weight of 64 kg) were pretreated for 6 days with feed consisting of incremental increases (20%, 30%, 50%, 50%, 75%, 75%) of red clover silage, to allow the rumen microbial populations to adapt. After the final pretreatment, 24 h later, the ewes were fed 3.5 kg of 100% red clover silage for 14 days. Thereafter, a gradual reduction of the red clover silage content over a 6-day-period was performed and feeding with hay and concentrate continued.

2.3.2. Experiment II

Five months after the first experiment the ewes were randomly redistributed to two groups. Group I ($n=4$, mean body weight of 74 kg) was re-exposed to red clover as described above. Group II ($n=4$, mean body weight of 75 kg) was kept on hay and served as controls. All ewes were slaughtered at the end of the experiment and thyroid glands were collected, weighed and examined for gross changes and immediately cut into pieces and immersed in fixative for morphological studies.

2.4. Blood collection

In experiments I and II blood samples were collected through a catheter in the jugular vein at 08:00 and 09:00 h on day 14 of 100% red clover silage exposure. Blood samples from all animals in experiment I were also collected 2 days before any exposure to red clover silage. In experiment II blood samples from ewes fed hay were collected at the same occasion as animals fed red clover silage. Plasma samples were immediately separated after blood collection by centrifugation in heparinised vacutainer tubes and then stored at $-20\,^{\circ}C$ for subsequent hormonal analyses.

2.5. Hormone assays

Plasma concentrations of total T_4, total T_3, free T_4 and free T_3 were determined using commercially available Coat-a-Count kits (Diagnostics Products Corporation, Los Angeles, CA, USA).

Total T_4 was quantified according to the manufacturer's instructions. Serial dilution of ovine plasma produced inhibition curves parallel to the standard curve of total T_4. The sensitivity of the T_4 assay was 3.2 nmol/l. The inter-assay coefficient of variation for quality control sample was 3.4% (mean=42.5 nmol/l, n=3). The corresponding intra-assay coefficient of variation was below 10% for concentrations of T_4 up to 309 nmol/l.

Total T_3 was quantified according to the manufacturer's instructions. Serial dilution of ovine plasma produced inhibition curves parallel to the standard curve of total T_3. The sensitivity of the T_3 assay was 0.14 nmol/l. The inter-assay coefficient of variation for quality control samples was 5.9% (mean=1.8 nmol/l, n=4). The corresponding intra-assay coefficient of variation was below 10% for concentrations of T_3 up to 9.22 nmol/l.

Free T_4 was quantified according to the manufacturer's instructions. The sensitivity of the T_4 assay was 0.16 pmol/l. The inter-assay coefficient of variation for quality control samples was 10% (mean=7.5 pmol/l, n=2). The corresponding intra-assay coefficient of variation was below 10% for concentrations of T_4 up to 127.4 pmol/l.

Free T_3 was quantified according to the manufacturer's instructions. The sensitivity of the T_3 assay was 0.3 pmol/l. The inter-assay coefficient of variation for quality control samples was 9% (mean=1.3 pmol/l, n=2). The corresponding intra-assay coefficient of variation was below 10% for concentrations of T_3 up to 72.2 pmol/l.

2.6. Morphological studies

The thyroid glands were transversely cut into 3–4 mm thick slices and fixed in either Bouin's solution or in phosphate-buffered 2.5% glutaraldehyde, pH 7.2. After rinsing in phosphate buffer, the samples were dehydrated via increasing ethanol concentrations. The Bouin-fixed tissue was embedded in paraffin for immunohistochemistry and the glutaraldehyde-fixed tissue in water-soluble resin (Technovit 7100, Heraeus Kulzer GmbH, Werheim, Germany) for image analysis.

2.6.1. Immunohistochemistry

Tissue sections (4 μm thick) were deparaffinized and rehydrated. After rinsing in PBS buffer the sections were transferred to 0.01 *M* Na-citrate buffer, pH 6.0 and treated for 10 min at 750 W in a microwave oven. Following rinses with PBS buffer, pH 7.4, endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 in PBS buffer and endogenous biotin with avidin–biotin blocking kit (Vector SP 2001, Vector Laboratories, Burlingame, CA, USA) for 15 min, each followed by rinsing in PBS buffer. Incubation with diluted normal horse serum was carried out for 30 min. Thereafter, incubation for 90 min with a mouse monoclonal antibody against ERα (C-311, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:25 was carried out. According to the manufacturer this antibody reacts with bovine ERα and does not react with ERβ or other steroid receptors. Negative controls were obtained by exchanging the primary antibody for normal mouse IgG diluted 1:100 (Vectastain, Vector Laboratories). Prior to and after a 30-min incubation with a biotinylated secondary horse anti-mouse IgG antibody diluted in normal horse serum (Vectastain, Elite ABC, Vector Laboratories), the sections were rinsed three times for 5 min with PBS buffer pH 7.4 containing 0.1% Triton X. Thereafter, the sections were incubated for 30 min with a horseradish peroxidase–avidin–biotin complex (Vectastain, Elite ABC, Vector Laboratories) and rinsed as above. The peroxidase activity was visualised by treating the sections with diaminobenzidine tetrachloride (DAB) (Saveen Biotech AB, Malmö, Sweden) in PBS buffer containing hydrogen peroxide for 5 min, followed by rinsing with distilled water. The sections were dehydrated and mounted in Pertex, some of them after first having been weakly counterstained with hematoxylin. Photographs were taken under identical conditions using a Nikon Microphot-FXA.

2.6.2. Image analysis

Sections, 2 μm thin, were cut of the resin-embedded tissue on a microtome using glass knives and

stained with hematoxylin/eosin. The slides were coded and randomly analysed by the same person using an image analysis system (Easy Image Analysis, Bergström Instrument AB, Stockholm, Sweden). All follicles appearing in the field, when moving the section straight across the gland from one edge to the other, were analysed. Thus the areas of between 400 and 600 thyroid follicles from each animal were measured. The average area from each animal was used for further statistical calculations.

2.7. Statistical analysis

Statistical analysis of the data was made using the General Linear Model procedure from the Statistical Analysis System program package (Release 6.12, 1996, SAS Institute, Cary, NC, USA). Within-treatment variation between ewes as an error term and the test for differences in least squares means were used when checking for differences between the red clover silage and hay groups. Hormonal values are presented as least squares means (LSmeans)±S.E.M.

Table 1
Thyroid hormone concentrations (LSmeans±S.E.M.) in eight ovariectomized ewes fed hay and then exposed to phytoestrogens during experiment I

	$n=8$	
	Hay	Oestrogenic silage
Total T_4 (nmol/l)	84.2±9.1	85.5±8.5
Total T_3 (nmol/l)	2.1±0.3	3.3±0.3*
Ratio T_4/T_3	42.1±4.1	30.0±3.8*
Free T_4 (pmol/l)	12.8±1.4	15.9±1.3
Free T_3 (pmol/l)	3.3±0.5	5.6±0.5*

*$P<0.05$ vs. control group.

Table 2
Thyroid hormone concentrations (LSmeans±S.E.M.) in ovariectomized ewes fed hay and red clover silage during experiment II

	Control $n=4$	Oestrogenic silage $n=4$
Total T_4 (nmol/l)	73.8±8.4	102.1±8.4†
Total T_3 (nmol/l)	1.6±0.2	2.9±0.2*
Ratio T_4/T_3	47.1±4.5	36.7±4.5
Free T_4 (pmol/l)	15.5±0.9	18.5±0.9†
Free T_3 (pmol/l)	2.1±0.5	4.5±0.5*

†$P=0.06$ and *$P<0.05$, respectively, vs. control group.

3. Results

The plasma levels of thyroid hormones in ovariectomized ewes from experiment I are presented in Table 1. Ewes exposed to red clover had significantly higher ($P<0.05$) plasma concentrations of the total T_3 and free T_3 than control animals. There were no differences between groups regarding concentrations of the total T_4 and free T_4. A decreased T_4/T_3 ratio was observed in ewes fed red clover silage (Table 1).

The plasma levels of thyroid hormones in ovariectomized ewes fed hay and oestrogenic silage during experiment II are presented in Table 2. Ewes exposed to red clover had higher ($P<0.05$) plasma concentrations of total T_3 and free T_3 than control animals. The total T_4 and free T_4 concentrations tended to be higher ($P=0.06$) in ewes exposed to red clover than in control animals. There were no differences between groups regarding the T_4/T_3 ratio.

There were no differences between groups in the body weights (75±5.5 vs 74±0.7 kg,

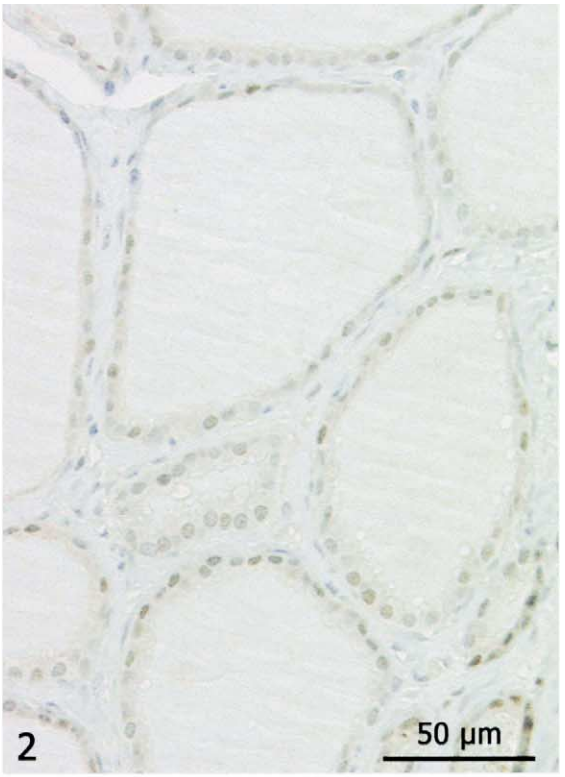
Fig. 1. Immunohistochemical localisation of ERα in the thyroid gland from an ovariectomized ewe fed hay, showing weak or moderate reaction in several cell nuclei. Weak hematoxylin counterstaining.

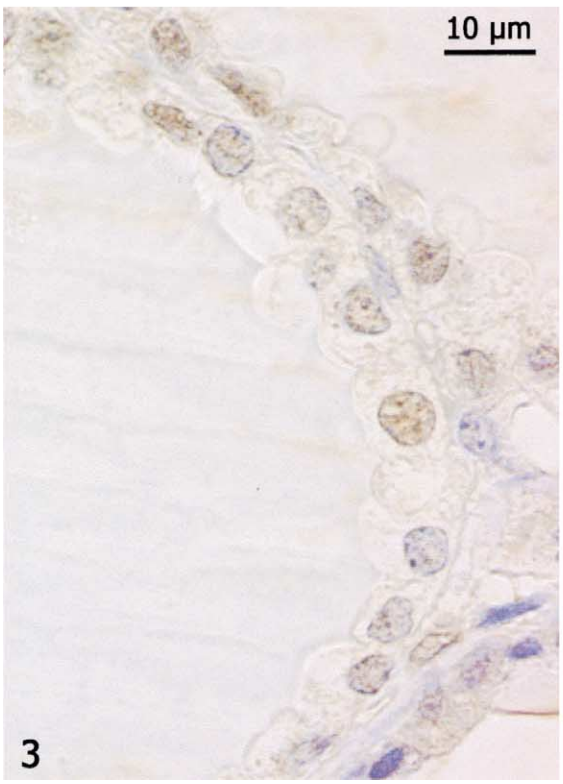
Fig. 2. Immunohistochemical localisation of ERα in the thyroid gland from ovariectomized ewe fed oestrogenic silage with intense and frequent staining of cell nuclei. Weak hematoxylin counterstaining.

Fig. 3. Immunohistochemical localisation of ERα in cell nuclei of the thyroid gland from an ovariectomized ewe fed oestrogenic silage at higher magnification. Weak hematoxylin counterstaining.

Fig. 4. Negative control for immunohistochemical localisation of ERα in the thyroid gland from an ovariectomized ewe fed oestrogenic silage. No reaction product is visible.


1
50 μm


2
50 μm


10 μm
3


4
50 μm

means±S.E.M.), thyroid gland weights (6.8±0.7 vs 6.2±0.5 g) and relative thyroid weight (91.8±10.1 vs 84.1±6.1 mg/kg body weight). The average area of the thyroid follicles in ovariectomized ewes fed red clover silage tended to be larger than that in the control ewes (12 947±1179 μm^2 vs 10 354±1055 μm^2, $P=0.07$).

The immunohistochemical localisation of ERα showed that the receptor protein was present in both groups of animals (Figs. 1–3), but at varying frequencies and staining intensity. Individual differences were seen between animals in the same group and especially in the control animals unlabelled follicles were common. Neighbouring follicles differed in number of stained cell nuclei as well as in staining intensity. However, after considering this variation, as a whole the nuclear ERα immunoreactivity was stronger and more frequent in the thyroid glands from the ovariectomized ewes exposed to phytoestrogens than in the ewes fed hay. No specific nuclear staining was found in the negative control sections (Fig. 4).

4. Discussion

To our knowledge, this is the first report describing effects of phytoestrogens from red clover silage on thyroid hormone concentrations and ERα immunoreactivity in the thyroid gland in ovariectomized ewes. Adams [3] reported an increase in thyroid weight and height of the thyroid epithelium in ewes exposed to phytoestrogens and suggested that their thyroid metabolism may have been altered. The efficiency of production may therefore be affected in sheep grazing oestrogenic pasture. In the present study we did not observe any changes in thyroid weight which was possibly due to a relatively short exposure to phytoestrogens. However, the ingestion of oestrogenic silage resulted in increased secretion of thyroid hormones. In premenopausal women consuming usual diet plus soy protein powder containing high levels of isoflavones, the plasma concentrations of free T_3 were significantly lower than in the control group [10]. The authors concluded that this change was not of any physiological importance because no effect on free T_4 and TSH was observed. Recently, Watanabe et al. [15] reported that T_3 and T_4 increased during the follicular phase but decreased during luteal phase in young women given isoflavone supplements.

Divi et al. [16] reported that genistein and daidzein blocked the tyrosine iodination catalysed by thyroid peroxidase, thus inhibiting thyroxin synthesis. According to these authors an inhibition of thyroid hormone synthesis can induce goiter and thyroid neoplasia in rodents. Lisboa et al. [17] have studied the effects of oestrogen in rats on the activity of 5′-deiodinase (5′-ID), an enzyme responsible for the generation of T_3, which is the biologically active thyroid hormone. Treatment with a high oestradiol benzoate dose induced an increment of 5′-ID-I in the thyroid gland, which was associated with an increased level of serum TSH but normal levels of free T_3 and free T_4. It is interesting to note that the mean serum concentrations of total T_3 and T_4 were significantly higher in women with than without breast cancer [18]. On the other hand, studies in animals have shown an increase in T_4 concentrations after soy protein feeding [11,12]. The latter authors also found that in rats soy protein increased total T_3 and free T_3 compared with casein [12].

The presence of oestrogen receptor in human thyroid tissue has been demonstrated by immunohistochemistry [19], binding assay [20] and enzyme immunoassay [21]. Giani et al. [22] reported that immunohistochemical detection of ERα could be affected by the presence of high endogenous peroxidase activity in thyroid follicular cells, which could lead to the suggestion that the staining observed in thyroid tissue is not specific and related to the activity of thyroperoxidase. In the present study the endogenous peroxidase activity was blocked and there was no positive staining in the negative controls. Additionally, we have demonstrated that the nuclear immunostaining for ERα in the thyroid glands is stronger in ovariectomized ewes fed red clover silage than in control animals. Similarly, the ERα protein is present in rat thyroid follicular cells and oestradiol was shown to increase cell growth [9]. In the present study we found that the area of thyroid follicles tended to increase in ovariectomized ewes fed oestrogenic silage compared to animals fed hay. The reason for this increase needs to be elucidated in further studies. Interestingly, Khan et al. [23] found that an acute acrylamide exposure of rats caused a

significant decrease in the thyroid colloidal area and a significant increase of the follicular cell height.

In conclusion, ingestion of phytoestrogens seemed to stimulate secretion of thyroid hormones, changes in the area of thyroid follicles and ERα immunoreactivity of thyroid glands in ovariectomized ewes.

Acknowledgements

This work was supported financially by the Swedish Council for Forestry and Agricultural Research. The authors wish to thank Mrs. Ingrid Wennerberg, Mrs. Marianne Ekwall and Mrs. Eva Werner for technical assistance.

References

[1] C.D. Burroughs, Proc. Soc. Exp. Biol. Med. 208 (1995) 78.
[2] H.W. Bennetts, E.J. Underwood, F.L. Shier, J. Dept. Agric. West. Aus. 23 (1946) 1.
[3] N.R. Adams, Res. Vet. Sci. 22 (1977) 216.
[4] N.R. Adams, Proc. Soc. Exp. Biol. Med. 208 (1995) 87.
[5] L. Markiewicz, J. Garey, H. Adlercreutz, E. Gurpid, J. Steroid Biochem. Mol. Biol. 45 (1993) 399.
[6] R.A. Mathieson, W.D. Kitts, J. Endocrinol. 85 (1980) 317.
[7] F.E. Newsome, W.D. Kitts, Anim. Reprod. Sci. 3 (1980) 233.
[8] G.G.J.M. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.-Å. Gustafsson, Endocrinology 139 (1998) 4252.
[9] T.W. Furlanetto, L.Q. Nguyen, J.L. Jameson, Endocrinology 140 (1999) 5705.
[10] A.M. Duncan, B.E. Merz, X. Xu, T.C. Nagel, W.R. Phipps, M.S. Kunzer, J. Clin. Endocrinol. Metab. 84 (1999) 192.
[11] W.A. Forsythe, J. Nutr. 116 (1986) 1165.
[12] C.A. Barth, K.E. Scholz-Ahrens, M. de Vrese M, A. Hotze, J. Nutr. Sci. Vitaminol. 36 (Suppl.) (1990) S111.
[13] A. Nwannenna, T.J.-O. Lundh, A. Madej, G. Fredriksson, G. Björnhag, Proc. Soc. Exp. Biol. Med. 208 (1995) 92.
[14] H. Pettersson, K.-H. Kiessling, J. Assoc. Off. Anal. Chem. 67 (1984) 503.
[15] S. Watanabe, K. Terashima, Y. Sato, S. Arai, A. Eboshida, Biofactors 12 (2000) 233.
[16] R.L. Divi, H.C. Chang, D.R. Doerge, Biochem. Pharmacol. 54 (1997) 1087.
[17] P.C. Lisboa, F.H. Curty, R.M. Moreira, C.C. Pazos-Moura, Braz. J. Med. Biol. Res. 30 (1997) 1479.
[18] M. Lemaire, L. Baugnet-Mahieu, Eur. J. Cancer/Part B Oral Oncol. 22 (1986) 301.
[19] S.R. Money, W. Muss, W.L. Thelmo, O. Boeckl, W. Pimpl, H. Kaindl, P. Sungler, J. Kirwin, H. Waclawicek, B.B. Jaffe et al., Surgery 106 (1989) 975.
[20] H. Miki, K. Oshimo, H. Inoue, T. Morimoto, Y. Monden, Cancer 66 (1990) 1759.
[21] R. Bonacci, A. Pinchera, P. Fierabracci, A. Gigliotti, L. Grasso, C. Giani, J. Endocrinol. Invest. 19 (1996) 159.
[22] C. Giani, D. Campani, F. De Negri, L. Martini, R. Fabbri, R. Bonacci, E.M. Ciancia, A. Gigliotti, P. Fierabracci, A. Pinchera, J. Endocrinol. Invest. 16 (1993) 37.
[23] M.A. Khan, C.A. Davis, G.L. Foley, M.A. Friedman, L.G. Hansen, Toxicol. Sci. 47 (1999) 151.