Thyroid problems in pediatric oncology: damage, prevention and consequences
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The effect of cervical X-irradiation on activity index of thyrocytes and plasma TSH; a pre-clinical model for radiation-induced thyroid damage


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Abstract

Because radiotherapy in the head and neck region is necessary in the treatment of childhood cancer, possibilities to prevent damage to the thyroid gland must be investigated. We developed a model in which radiation-induced effects can be investigated in a way that these effects can be quantified, using thyroid dysmorphology and plasma TSH.

Thirty-five Wistar rats, 5 weeks old, were X-irradiated on the cervical region, with a single dose varying from 0 to 20 Gy. After 6 weeks TSH, T₄ and T₃ were determined and thyroid glands were processed for histological examination, by two independent pathologists. A histological classification scale was developed, using follicular size, colloid density and cell height of thyrocytes to measure hyperplasia and hypertrophy. By the sum of these scores a cell-activity index was calculated, which was related to plasma TSH concentration. Also numbers of PAS-positive droplets and epithelial desquamation were counted. Interobserver reliability was assessed.

Good to very good reliability was found for scores of follicular size, colloid density and cell height. Significant increase of cell-activity index was found after 10, 15 and 20 Gy. The plasma TSH concentration was positively correlated to the cell-activity index increasing with radiation-doses up to 15 Gy. The number of desquamated cells was significantly increased after radiation doses > 10 Gy, with moderate reliability.

In conclusion, this model using cell activity index of thyrocytes together with plasma thyrotropin concentrations and desquamation of cells can be used for interpretation and future (pre-clinical) studies of prevention of radiation-induced thyroid damage.
Introduction

Radiotherapy is and it is likely to remain an important option in the treatment of malignant diseases in head and neck region. This treatment may have damaging effects on the thyroid gland. These effects can be quantified as changes in thyroid function, thyroid morphology or by changes at the molecular level.

In animal studies on the thyroid gland, most work was directed towards the morphological effects of radiation. The generalized changes reported may be considered in several stages: 1: cellular degeneration and necrosis with follicular disruption, 2: vascular degeneration and thrombosis, 3: acute and chronic inflammation, 4: fibrous organization and 5: (partial) epithelial regeneration. Only minor changes occur in the low dose region (up to 5 Gy or low dose 131I), whilst total destruction of the tissue is seen after exposure to higher doses (200 Gy of 131I). For the development of radiation-induced thyroid tumours, the dose-response curve for rats has its maximum after 0.3-1.0 MBq of 131I or 11 Gy of X-rays. In humans, it has been demonstrated that the risk for radiation-induced tumours is linearly increased already from 0.1 Gy onwards. Most X-radiation-induced thyroid carcinomas are papillary (85%), but also follicular tumors (10%) and medullary or undifferentiated carcinomas occur. A higher prevalence of RET/PTC arrangements has been found in radio-iodine and X-radiation induced papillary thyroid carcinoma (PTC) compared to sporadic PTC.

For children (and young adults) to prevent the occurrence of radiation-induced hypothyroidism and thyroid malignancies, new possibilities must be investigated. This demands a model to investigate radiation-induced effects in a way that these effects can be quantified.

It has been demonstrated that irradiated rats who underwent hypophysectomy did not develop any thyroid tumours, while in contrast in irradiated rats with high concentrations of circulating TSH (e.g. after the administration of goitrogens) the number of tumours was increased. Apparently, the effects induced by radiation are dependent on the concentration of circulating TSH. Also a low dietary iodine has been associated with an increased occurrence of thyroid tumours after radiation, however, recently it was shown that the mutagen needed for tumour formation was radiation (x-radiation) more than iodine deficiency or excess. The induction of neoplasia by radiation has extensively been studied in the rat, showing an increased
radio-sensitivity for juvenile, neonatal and fetal thyroids.

In clinical practice, when evaluating thyroid damage after radiation-exposure, circulating plasma TSH is determined and ultrasound imaging of the thyroid is done for evaluation of the thyroid function and thyroid structure, respectively. When suspicious lesions are found, histological evaluation can be performed. For interpretation of thyroid histology, data on the concentration of circulating TSH is essential because the concentration of circulating TSH itself directs the size and the shape of the thyrocytes. Moreover, TSH is an indication for the output of all functioning thyrocytes together. For these reasons, when performing pre-clinical studies on prevention of thyroid damage, knowledge on the relation between radiation dose, plasma TSH levels and histology of the thyroid gland is essential. Information on circulating TSH is, however, often not provided in morphological studies on the thyroid gland after radiation exposure. Furthermore, most in vivo studies concerning radiation-induced functional defects of the thyroid gland are focused on the consequences of radio-iodide, instead of external-beam irradiation and especially long term effects are lacking.

Our aim was to develop an in vivo pre-clinical model, to find the most effective dose of irradiation, in which we could quantify the biological effects of external irradiation on the thyrocytes using the most sensitive marker of thyroid dysfunction, the plasma TSH concentration. The visible microscopic damage in the thyrocytes may then be coupled to the marker that reflects the damage of the thyroid gland. This model can be used to study ways of prevention of radiation induced thyroid damage in the rat. For this goal, we developed an activity index of thyrocytes which is correlated to the plasma TSH concentrations and to radiation dose.

Materials and Methods
2.1 Animals and thyroid radiation procedure
Thirty-five male Wistar strain rats, four weeks of age, were divided in 5 groups. Each group was housed together with tap water and food ad libitum (Hope Farms, irradiated breeding and maintenance diet for rats, mice and hamster, Woerden, The Netherlands) and subjected to a 12-h light and 12-h darkness cycle. Mean temperature was 22 °C, relative air humidity 55-60 %.

After two weeks of acclimatisation, the study was started. Group 1 received sham
irradiation, group 2: 20 Gy, group 3: 15 Gy, group 4: 10 Gy and group 5: 5 Gy. Irradiation was given from the ventral side with animals placed in a supine position. An X-ray generator (Siemens stabiliplan 2-machine) was operated at 250 kV, filtered with 0.5 mm Cu. Source to skin distance was 40 centimetres. Dosing speed was 1.3860 Gy per minute. The radiation field was marked by light, with the centre pointed to the thyroid gland, the upper boundary being the lower boundary of the skull/middle ear, just below the pituitary region \(^{26}\) (determined by marking the middle ear boundary by X-ray in a previous Wistar rat). Field size was 2 by 3 centimetres. During irradiation, animals were given inhalation-anaesthesia, 4% fluothane and 96% air, followed by a maintenance dose of 2% fluothane and 98% air. All blood samples were drawn by tail vena-puncture under inhalation anaesthesia, using 4% fluothane and 96% air. All animals were sacrificed 6 weeks after irradiation.

All experiments were approved by the Animal Ethical Committee of the Academic Medical Centre and were performed according to the Dutch law for animal experiments.

2.2 Evaluation

2.2.1 Clinical evaluation

All rats were scored daily by examination of body-mass, activity, fur (shiny/fade/hairs upright) and skin (loss of hair/redness/wounds) on place of irradiation.

2.2.2 Thyroid function

For determination of thyroid function, weekly blood samples were collected for measurement of total T\(_4\) concentrations. At sacrifice of the animals, concentrations of plasma TSH, T\(_4\) and T\(_3\) were determined. Of rats irradiated with 5 Gy, no plasma T\(_3\) measurements were obtained due to too small blood sample size.

T\(_4\) and T\(_3\) were determined by an in-house radio immuno-assay, intra-assay variation of T\(_4\): 2 - 4 % and T\(_3\): 3 - 4 %, inter-assay variation T\(_4\): 3 - 6 %, and T\(_3\): 7 - 8 %. The detection limits for T\(_4\) and T\(_3\) are 5 nmol/L and 0.3 nmol/L, respectively. TSH was determined by a chemiluminescent immuno-assay adapted for rat TSH (Immulite, Diagnostic Products Corp., Los Angeles, CA), intra-assay variation TSH: 2 - 4 %, inter-assay variation TSH: 3 - 4 % and detection limit TSH: 0.01 mU/L.
2.2.3 Histological evaluation

At sacrifice, thyroid gland, pituitary gland, hypothalamus, lungs and thymus were removed and stored in a buffered 4% formaldehyde solution for histological evaluation. Both thyroid lobes were removed en bloc together with the adjacent trachea to prevent surgical damage of the tissue. Routinely paraffin-embedded, HE and PAS (for glycoproteins) stained sections were examined for radiation effects. To determine the interobserver reliability, all examinations were done twice, by two independent blinded veterinary pathologists.

The histological classification score for activity of thyroid glands was based on the predominant aspects of five criteria in two histological sections (figure 1):

1. follicular size, ranging from 1-5, having an inverse relation; the smaller the follicle, the higher the score (1=very large follicles, 2=large follicles, 3=relatively small, 4=small, 5=very small).

2. colloid density, ranging from 1-5, having an inverse relation; the lower the density of the colloid, the higher the score (1=very strong staining, 2=strong staining, 3=average staining, 4=pale staining, 5=very pale staining).

3. cell height, ranging from 1-5, having a direct relation; the higher the follicular epithelial cells, the higher the score (1=flat, 2=low cubic, 3=cubic, 4=high cubic, 5=cylindrical).

From these an activity-index was calculated, being the sum of the scores for follicular size, colloid density and cell height, reflecting hyperplasia and hypertrophy of thyroid cells. A high index indicates a high activity within the cell with a high protein turnover.

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**Figure 1: Histological classification of thyroid tissue in Wistar rats, 12 weeks of age** (1-6, PAS stain, obj. 20x, 7; PAS stain, obj 40 x, 8; PAS stain, obj 10x)

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A low index represents thyroid glands with low activity with a lot of thyroglobulin piled up.

IV) Intensity of PAS staining of thyrocytes due to endocytosis of colloid; many PAS-positive granules giving a high score (1=no droplets, 2=few droplets, 3=moderate number of droplets, 4=significant number of droplets, 5=many droplets).

V) Number of desquamated cells in the follicular lumen, ranging from 1-5; the lower the score, the less desquamation present (1=no desquamation, 2=a few follicles with one or a few desquamated cells, 3=a moderate number of follicles with one or a few desquamated cells, 4=several follicles with one or a few desquamated cells, 5=many follicles with one or a few desquamated cells).

Also, the presence of benign or malignant neoplasms was evaluated.

Histological classification of the pituitary glands was based on four criteria (high power field, objective 40 x):

1. Presence of ‘colloid droplets’ in the anterior pituitary gland, recognized as cytoplasmatic inclusions, circumspectly present in the basophilic cells, divided in three categories: very little (≤1), a moderate number (2-4) or many (≥5) present.

2. Number of basophilic cells in the anterior pituitary gland; divided in three categories: small in size or number, moderate in size or number or large in size or number.

3. Presence of pycnotic nuclei in the anterior pituitary gland, divided into three categories; sporadic (<1), moderate (2-4) or many (≥5) present.


2.3 Statistical analysis

Data was analysed using MS Excel 97 and SPSS 10.0.7 Statistics UK software. Statistical analysis was carried out using One Way ANOVA (Bonferroni for testing statistical significance) and 2-tailed, Pearson Correlation Coefficient, with a preset for significance of 0.05. For determination of the interobserver variation (α) between the two blinded pathologists, reliability analysis with Intraclass Correlation Coefficients (ICC) in a two-
way mixed effects model was performed for follicular size, cell height, colloid density, PAS+ droplets and number of desquamated cells, rated as very good if $\alpha$: 0.81-1.0; good if $\alpha$: 0.61 to 0.80; moderate if $\alpha$: 0.60 to 0.41, and fair if $\alpha$: 0.40 to 0.21.

**Results**

3.1 Clinical and macroscopic findings

Upon arrival, the 35 male Wistar rats had an average body-mass of 121.4 grams (range 100.7-153.9). During anaesthesia and irradiation no adverse events occurred. Several days after irradiation, animals that had received 10, 15 and 20 Gy lost hair on the site of irradiation, starting in the dorsal neck region, followed by loss of hair on the ventral side of the neck. Radiation effects varied from slightly thinner hair to baldness with red skin, wounds and crusts. Animals that had received 20 Gy were most severely ill, consumed little amounts of water and had a rough fur. Their weight gain was substantially reduced. Although the rats irradiated with 10 and 15 Gy showed weight loss and diminished water intake too, they were far less ill than the 20 Gy group. All rats recovered within three weeks after irradiation and subsequently showed some catch-up growth. At necropsy, no macroscopic abnormalities were found in thyroids, pituitary glands, lungs or thymus glands.

3.2 Thyroid function

Plasma T$_4$ concentrations were measured weekly. Mean plasma T$_4$ concentration after one week of acclimatisation, at 5 weeks of age, was 58 nmol/L (range 34 - 78). Mean plasma T$_4$ values of sham-irradiated animals 6 weeks after irradiation, at 12 weeks of age, was 85.14 nmol/L (range 75-109) versus 79.0 nmol/L (71-85) after 15 Gy and a significantly lower concentration of 67.86 nmol/L (range 66-85) after 20 Gy. As shown in figure 2, mean plasma TSH value 6 weeks after irradiation was significantly lower in the sham-irradiated group versus the rats that received 15 Gy; mean TSH of 1.51 mU/L (range 0.80-2.26) versus 4.37 mU/L (range 3.10-7.26). TSH was not significantly increased after 20 Gy. Mean T$_3$ value 6 weeks after irradiation was significantly reduced after 20 Gy (1.51 nmol/L versus 1.73 nmol/L in controls, p=0.047).
Figure 2: Mean TSH, T4, and T3 plasma concentrations in young Wistar rats, 6 weeks after X-irradiation. Significant increase of TSH is seen between 0 and 15 Gy, and 5 and 15 Gy (p=0.003 and p=0.010, One Way ANOVA, Bonferroni). A decrease of plasma T4 is found after 15 Gy (79.9 nmol/L), compared to 0 Gy (85.1 nmol/L), that becomes significant after 20 Gy (67.9 nmol/L, p=0.010). Also, plasma T3 is significantly lower after irradiation with 20 Gy (p=0.047, Mann-Whitney U).

3.3 Histo-pathology
The microscopic examination of the thyroid glands, 6 weeks after irradiation, showed clear cellular effects with increasing radiation dose. The results of histologic examinations of both observers, with corresponding interobserver correlation coefficients (α) in the legend, are given in figures 3 and 4. The correlation coefficients for follicular size, cell height and colloid density were all rated as good and very good. Their sum, expressed as activity index, was significantly increased, for both observers, after irradiation with 10, 15 and 20 Gy. The correlation coefficient for mean number of PAS+ droplets was rated as fair. Only a significant increase in number of PAS+ droplets was found for observer B after 15 Gy. For the mean numbers of desquamated cells in the follicular lumen a moderate strength of agreement was found, with significantly increased numbers for both observers after 10, 15 and 20 Gy (figure 4). No adenomas or carcinomas were found.
Radiation dose up to 15 Gy was positively correlated to plasma TSH (p=0.021), follicular size (p=0.002), cell height (p=0.029), colloid density (p< 0.000), activity index (p< 0.000) and number of desquamated cells (p< 0.000), as scored by observer A (Pearson Correlation). TSH was significantly correlated to follicular size (p< 0.000), cell height (p< 0.000), colloid density (p< 0.000), activity index (p< 0.000), but not to the number of desquamated cells, as scored by observer A (figure 5).
Histological analysis of pituitary glands showed no significant differences in presence
A rat model for radiation damage to the thyroid

Figure 3: Scores for follicle size, colloid density, cell height and their sum as activity index of thyroid cells, 6 weeks after cervical X-irradiation with 0, 5, 10, 15 and 20 Gy in young Wistar rats, for two different blinded observers (observer A and observer B). Given is the 95% confidence interval for the mean, with upper and lower boundaries, One Way ANOVA, Bonferroni). ** p ≤ 0.001, * p ≤ 0.05, compared to controls.

I: Follicular size: scoring ranges from 1-5, the smaller the follicle, the higher the score, α=0.77, 95% CI: 0.67 to 0.84 II: Colloid density: scoring ranging from 1-5; the weaker the staining, the higher the score, α=0.84, 95% CI: 0.76 to 0.89 III: Cell height: scoring ranging from 1-5; the higher the follicular epithelial cells, the higher the score, α=0.69, 95% CI: 0.56 to 0.79. IV: Activity index. The activity index is calculated as the sum of scores for follicular size, colloid density and cell height. For both observers, significant increase in activity index was found after irradiation with 10, 15 and 20 Gy.
Figure 4: Number of PAS+ droplets and number of desquamated cells in follicles, 6 weeks after X-radiation in young Wistar rats, for two different blinded observers (observer A and B). Given is the 95% confidence interval for the mean, with upper and lower boundaries. ** p < 0.001, compared to controls. No significant differences and a low interobserver reliability were found for the score for PAS+ droplets, \( \alpha = 0.38 \) (95% CI: 0.10 to 0.57). For desquamated cells, \( \alpha = 0.59 \) (95% CI: 0.39 to 0.71), both observers found significant differences after radiation with 10, 15 and 20 Gy, One Way ANOVA, Bonferroni.

Figure 5: Correlation between plasma TSH level, activity index and number of desquamated cells, 6 weeks after X-radiation in young Wistar rats (2-tailed Pearson Correlation, fitted with quadratic regression line). O = activity index, \( \Delta \) = desquamated cells. A significant correlation is found between activity index and TSH, but not for desquamated cells and TSH.
of colloid droplets or number of basophilic cells (p=0.3 and p=0.3 respectively). In pituitary glands of the animals that received 20 Gy, a significantly higher amount of pycnotic nuclei was seen compared to those which received no irradiation. No abnormalities were found in the pars intermedia; status spongiosus was not detected in any posterior pituitary lobe.

No radiation effects, such as atrophy, hyperplasia, apoptosis, fibrosis or vascular damage were seen in hypothalamus, lungs or thymus.

**Discussion**

In this pre-clinical model for evaluation of the effects of X-radiation on thyrocytes in relation to plasma TSH, we found the thyroid cell-activity index positively correlated with the concentration of plasma TSH and X-radiation dose up to 15 Gy. The plasma TSH concentration at time of obtaining thyroid tissue is very useful to interpret the thyroid histology after radiation exposure, since the cellular effects seen after exposure to radiation are not only caused directly by the radiation, such as desquamation of cells, but also indirectly by stimulation of elevated plasma TSH. Furthermore, the plasma TSH is of importance because it is a tool which can be translated from the animal model to the human situation. In humans, plasma TSH concentration is used to evaluate radiation damage, as it is most easily measured and can be monitored sequentially. Most in vivo studies that describe thyroid histology after radiation exposure, however, do not relate the thyroid morphology to plasma TSH at time of sacrifice\(^{3,7,13,28-32}\).

In case of loss of function of thyroid cells, plasma TSH will increase to compensate the impaired thyroid hormone production. The concentrations of plasma T\(_4\) and T\(_3\) may still be in the normal range, though, which is often described as compensated or subclinical hypothyroidism. In fact, plasma TSH is the most sensitive marker for thyroid damage. In case of an elevated TSH, hyperplasia and hypertrophy of thyrocytes will be found, indicating active cells, compensating the loss of function of these or neighboring cells (hypothyroidism) \(^{33}\). To quantify the biological activity of the thyrocytes in our model after exposure to irradiation, we calculated an activity index of thyrocytes and correlated it to the concentration of plasma TSH and radiation dose.
The activity index provides the direct information on the thyrocytes themselves, the concentration of plasma TSH provides the information on the overall function of the thyroid cells. As was demonstrated, the activity index was a well reproducible and thus reliable score. Scoring PAS + droplets was shown not to be reliable and did not show any correlation to radiation dose, concluding that this marker is not to be used for interpretation of radiation damage. The number of desquamated thyrocytes was for both independent pathologists significantly correlated to the radiation dose but not to TSH concentration (figure 5). No other direct radiation effects were seen such as vascular degeneration or inflammation, concluding that after six weeks following radiation exposure, the compensatory reaction, calculated as activity index, is pre-dominantly present compared to direct radiation effects.

Using these four determinants (TSH, activity index, desquamated cells and radiation dose), the severity of direct and indirect radiation damage can objectively be interpreted which subsequently can be used for further studies of prevention of radiation damage in rats. Furthermore, by performing histo-pathology, it was possible to evaluate, macroscopically and microscopically, the occurrence of nodules, adenomas and carcinomas. In this short follow-up period of 6 weeks, no macroscopic abnormalities were found. One could hypothese, however, that cells with the highest metabolic and mitotic activity after radiation exposure giving the highest activity index, are most at risk to develop neoplasms. This will have to be determined in a long term follow-up study.

Current assays for evaluation of radiation induced damage to thyroid tissue include molecular genetic studies. A high prevalence of RET/PTC arrangements has been described in X-radiation induced PTC and PTC occurring after the Chernobyl accident. We did not include molecular genetic studies. Firstly, we had to examine the entire gland microscopically, because a histological aberration could be present in one lobe of the thyroid and not in the other. This is a recognised limitation. Secondly, it is not uniformly accepted that the increase of RET/PTC-1 rearrangements in malignant thyroid tumours is caused by radiation as it could not be confirmed in other studies. Furthermore, these genetic rearrangements in rats may differ from those in humans. So, although scientifically very challenging, these rearrangements cannot directly be used in clinical practice and are therefore not yet useful for evaluation of radiation damage in a model designed for prevention.
It has been demonstrated that in young animals the uptake and retention of radioiodine in the thyroid gland are increased \(^{36}\). Furthermore, just as in humans \(^{17,38}\), the younger the age, the higher the risk to develop radiation damage to the thyroid \(^{39}\). With this in mind, we chose animals four weeks of age to find the highest possible radiation effect.

Due to the fact that irradiation effects on the thyroid may not be present until many years after the exposure to radiation, it is important to foresee the possibility to study long term effects. For this reason, it is essential to irradiate the cervical region without damaging surrounding tissues like lungs, heart and thymus. Also, it is important to exclude the pituitary-hypothalamic region from the irradiation field, to prohibit occurrence of pituitary or hypothalamic hypothyroidism. Lastly, it must be possible to measure and quantify the effects in a reproducible way.

In the animals irradiated with 20 Gy, a decrease of plasma TSH and of many morphological defects were observed. In pituitary glands in 3 of 7 animals of this group, the amount of pyenotic nuclei was significantly higher (>5). This finding may indicate that the pituitary gland in these animals received stray irradiation, resulting in a decreased response of pituitary TSH. However, also in control animals a moderate amount of pyenotic nuclei was seen and no other morphological irradiation effects were found in the pituitary parenchyma or in its vascularisation. Moreover studies on irradiation of the pituitary gland mention that a radiation dose of \(\leq 20\) Gy will have hardly any effect on the thyrotrophic cells 6 weeks after irradiation \(^{40}\). Another reason for the low plasma TSH that must be considered is the presence of the sick euthyroid syndrome, because this group had been severely ill after irradiation. In the sick euthyroid syndrome, low levels of \(T_4\), \(T_3\) and TSH are found with relatively higher levels of reverse \(T_3\) \(^{41}\). Unfortunately, we were not able to measure reverse \(T_3\). Taking these facts into consideration, we concluded that the dose of 20 Gy is too high for use in this model.

In conclusion, when using X-irradiation doses up to 15 Gy, the presented model has shown to be appropriate to study the morphology of thyocytes in relation to TSH after radiation on the thyroid gland. In these animals, no damaging effects were found in surrounding tissues which makes this model suitable for studies of late radiation effects.
In the future, we will use this pre-clinical model to study thyroid damage as a result of X-radiation, focusing on the occurrence and prevention of late radiation-induced thyroid defects.

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