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EVALUATION OF THE GENOTOXIC EFFECTS OF THYROXINE USING *IN VIVO* CYTOGENETIC TEST ON SWISS ALBINO MICE

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Thyroid hormones enhance aerobic metabolism favoring oxidative stress which may lead to covalent damage of various molecules including DNA. Previous investigations revealed that thyroid hormones induce DNA damage on human lymphocytes and sperm in the in vitro Comet assay. However, cytogenetic evaluation of genotoxic effects of thyroxine gave equivocal results: increase of sister chromatid exchanges, and no incerase of micronuclei in cultured human lymphocytes. Therefore, the aim of the present study was to further evaluate the possible genotoxic effects of thyroxine using in vivo cytogenetic test on Swiss albino mice.

Three experimental concentrations of thyroxine were used (0.1 mg/kg, 0.5 mg/kg and 2.5 mg/kg). The mice were divided into several groups depending on the duration of the treatment with thyroxine. Thus, we treated mice for 1, 3, 7 and 10 days. Positive (N-methyl-N'-nitro-N-nitrosoguanidine) and negative controls were also formed for the same time periods. Cytogenetic endpoinds (numerical and structural aberrations, chormosome gaps and breaks) were analysed in bone marrow cells from femures.

The results obtained in this investigation showed that thyroxine has not induced chromosome damage or aberrations. This is in agreement with our previous analysis of micronuclei in human peripheral blood lymophocytes treated with thyroxine. On the other hand, we observed a decrease of mitotic index especially in animals treated for a longer period of time with the highest dose of thyroxine. Therefore, it can be concluded that thyroxine does not induce genotoxic effects which could be detected by cytogenetic analysis.

Key words: chromosome aberrations, genotoxicity, mouse, thyroxine

INTRODUCTION

Thyroid hormones play essential roles in the processes of metabolism, growth and development in most vertebrate tissues (Lazar, 2003). Although there

are abundant literature data concerning the molecular mechanisms of thyroid hormone action in various biochemical and physiological processes (Goglia *et al*., 1999), little is known about the possible genotoxic and mutagenic effects of thyroid hormones and/or their metabolites.

However, it is well established that thyroid hormones increase aerobic metabolism in mitochondria which causes intense production of reactive oxygen and nitrogen species, leading to the condition of oxidative stress (Venditti *et al*., 2003; Fernandez *et al*., 2005). In addition, binding of thyroid hormone to specific nuclear receptors in target cells induces enzymes related to redox processes and the total rate of oxygen consumption is enhanced (Oppenheimer *et al*., 1996). Reactive oxygen species (ROS) induced by thyroid hormones may cause lipid and protein oxidation in rat hepatocytes (Tapia *et al*., 1999). Moreover, experimentally induced hyperthyroidism is accompanied by an increased rate of O_2 consumption and enhanced microsomal oxidative capacity and generation of ROS in the rat liver (Fernandez *et al*., 2003). Interestingly, creation of oxidative stress is even more potentated by thyroid hormone-induced decrease of superoxide dismutase (SOD) and catalase activities, as well as by the reduced glutathione content in the liver cells (Fernandez *et al*., 1988). This depression of key antioxidative mechanisms favors oxidative stress in the liver (Tapia *et al*., 1999).

Overwhelming evidence indicates that oxidative stress can lead to DNA damage and mutations (Sakai *et al*., 2006; Lankoff *et al*., 2006). Indeed, there are some indices that thyroid hormones can induce oxidative stress accompanied by DNA damage in human lymphocytes (Djelić and Anderson, 2003) and sperm (Dobrzynska *et al*., 2004) evaluated by the Comet assay *in vitro*. On the other hand, thyroxine exhibited only weak clastogenic effects expressed as an increased sister chromatid exchange, without a significant increase in micronucleus frequencies in cultured human peripheral blood lymphocytes (Djelić *et al*., 2006). It should be noted, however, that these equivocal data were obtained using whole blood cultures, whereas DNA damage in the Comet assay was observed on purified human lymphocytes. Namely, it is possible that enzymes catalase and gluthathione peroxidase which are normally present in erytrocytes in whole blood cultures can reduce the effects of reactive oxygen species (Andreoli *et al.*, 1999) which may be produced under the influence of thyroid hormones.

Since the evaluation of cytogenetic effects of thyroxine in cultured human lymphocytes was equivocal, in the present study we aimed to investigate the possible cytogenetic changes in bone marrow cells of Swiss albino mice, after acute or subacute exposure to L-thyroxine. Therefore, this investigation should further elucidate the possbile clastogenic and/or aneugenic effects of thyroid hormones. It is particularly interesting that the effects of thyroid hormones were monitored on cells exposed *in vivo*.

MATERIALS AND METHODS

Test substance and controls. The test substance used for the evaluation of the possible genotoxic effects was L-thyroxine (CAS No 51-48-9, Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.1 M NaOH. The solvent was used as a negative control, whereas the well known mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, CAS No 70-25-7, Sigma Chemical Co., St. Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO) was chosen as the positive control.

Animals. In this investigation we used Swiss albino mice from the Institute of immunobiology and virusology „Torlak" (Belgrade). All animals were acclimatized for 7 days prior to the start of the experiments. Animals were kept under standard conditions: room temperature (21±1)°C, humidity 55-60%, food and water *ad libitum*, standard daily illumination. For each experimental and control group 4 males weighting 20-25 g were used.

In vivo cytogenetic test. Animals were divided into several groups: negative control (solvent-treated), positive control (treated with the well known mutagen MNNG) and thyroid hormone-treated animals. Three doses of T_4 (0.1 mg/kg, 0.5 mg/kg and 2.5 mg/kg were used. Animals were treated with intraperitoneal T_4 injection for 1, 3 and 7 and 10 days. After the last treatment, after 24 h, the animals were treated with colchicine (Sigma chemical Co., St. Louis, MO, USA, final concentration 4mg/kg) in order to cause metaphase arrest. Two hours after the treatment the femures in ether anesthesia were removed. Chromosome preparations were made according to the method of Evans and O'Riordan (1975) as previously described in detail (Djelić *et al.*, 1996). Bone marrow was isolated in 0.9% w/v NaCl. After centrifugation cell pellets were treated with hypotonic solution (0.075M KCl) for 20 min at 37°C. Afterwards, three repetitive cycles of fixation in methanol-acetic acid (3:1, v/v) were performed. After the last fixation we resuspended the cell pellet in 0.5 mL of supernatant to which one drop of 60% CH_3COOH was added. Finally, a few drops of cell pellet were air dried and stained with 5% Giemsa (Merck, Darmstadt, Germany). In order to determine the mitotic index at least 1000 cells per experimental animal were scored, and for chromosome lesions and aberrations 100 metaphase spreads per animal were analysed.

Statistical analysis. The statistical analysis of cytogenetic endpoints was performed by χ^2 test using the Statgraph 4.2 software. Mitotic indices were analysed by *z*-test. A *P*-value of ≤ 0.05 was considered as indicative of statistical significance for all tests used.

RESULTS

Results of the mitotic indices in thyroxine-treated animals and controls are presented in Table 1. Evidently, mitotic index is not significantly changed at all doses of thyroxine following the 24 h treatment. Treatment for 3 days caused a significant decrease ($P<0.01$) in mitotic index only at the highest experimental concentration (2.5 mg/kg). Likewise, after 7 and 10 days of treatment only the

highest concentration of thyroxine significantly ($P<0.001$) decreased MI. As expected, the positive control (10^{-6} M MNNG) caused a significant decrease of MI at all time periods. However, the suppresion of mitotic activity caused by MNNG was more profound at longer periods of treatment with thyroxine.

Table 1. Mitotic indices of Swiss albino mice treated with thyroxine for 1, 3, 7 and 10 days

Treatment	No of analysed cells	MI (%) (min-max)	Mean MI±S.D. (%)
Negative control, 24 h	4536	4.15-6.11	5.13±0.71
0.1 mg/kg, 24 h	4008	5.21- 5.71	5.47±0.18
0.5 mg/kg, 24 h	4014	4.13-6.04	5.34±0.72
2.5 mg/kg, 24 h	4055	4.05-4.81	4.61±0.32
Positive control, 24 h	4427	1.94-2.44	2.04±0.22***
Negative control, 3 days	4001	5.10-6.52	5.81±0.50
0.1 mg/kg, 3 days	4507	4.27-5.81	4.89±0.63
0.5 mg/kg, 3 days	4088	5.06-6.45	5.60±0.61[a]
2.5 mg/kg, 3 days	4021	3.02-3.96	3.63±0.36**
Positive control, 3 days	4092	1.13-1.98	1.58±0.32***
Negative control, 7 days	4613	3.78-6.36	5.37±0.96
0.1 mg/kg, 7 days	4079	4.86-6.07	5.40±0.50[b]
0.5 mg/kg, 7 days	4150	4.14-5.66	4.98±0.62
2.5 mg/kg, 7 days	4003	2.85-3.84	3.41±0.36***
Positive control, 7 days	4224	1.08-1.77	1.41±0.26***
Negative control, 10 days	4028	4.55-5.92	5.27±0.51
0.1 mg/kg, 10 days	4201	4.19-5.10	4.83±0.34
0.5 mg/kg, 10 days	4096	4.24-5.20	4.46±0.34
2.5 mg/kg, 10 days	4074	2.38-3.27	2.82±0.44***[c]
Positive control, 10 days	4030	0.94-1.87	1.28±0.46***[d]

MI – mitotic index, S.D. – standard deviation; **P<0.01; ***P<0.001 (*z*-test)
[a] one animal died on the 3rd day of treatment
[b] one animal died on the 2nd day of treatment
[c] one animal died on the 6th day, and one animal died on 9th day of treatment
[d] two mice died on 8th and 9th day of treatment, respectively

As for chromosome lesions and aberrations, generally there was no significant effect of thyroxine, at all concentrations and time periods. The most common aberration was aneuploidy, probably because it can also occur as an artefact during chromosome preparation. Therefore, these results are in

accordance with our previous investigations of genotoxic effects of thyroxine showing no effect on micronucleus induction in human peripheral blood lymphocytes *in vitro*. Only the positive control (MNNG) gave a significant ($P<0.001$) rise of overall aberrations, when comapred to the negative control. Also, it should be noted that the increased frequency of chromosome aberrations was correlated with MNNG treatment duration (Table 2).

Table 2. Cytogenetic endpoints in bone marrow cells of Swiss albino mice treated with thyroxine for 1, 3, 7 and 10 days

Treatment	Observed cytogenetic changes	CA/100 cells
Negative control, 24 h	2ctg 3ane	1.25
0.1 mg/kg, 24 h	2ctg 1acf 1cf 2ane 1pol	1.75
0.5 mg/kg, 24 h	2ctg 1cf 3ane	1.50
2.5 mg/kg, 24 h	1csg 2cf 3ane	1.75
Positive control, 24 h	5ctg 2csg 9ctb 1csb 2acf 8ane	7.50***
Negative control, 3 days	1ctg 1ctb 2ane	1.00
0.1 mg/kg, 3 days	1ctg 1ctb 2ane 1pol	1.25
0.5 mg/kg, 3 days	1cf 3ane[a)]	1.33
2.5 mg/kg, 3 days	1ctb 2cf 4ane	1.75
Positive control, 3 days	3ctg 1csg 6ctb 2csb 4acf 2cf 12ane	8.25***
Negative control, 7 days	1ctb 2ctg 3ctb 1ane	1.75
0.1 mg/kg, 7 days	2ctb 1csg 1ane 1af [b)]	2.00
0.5 mg/kg, 7 days	1ctb 1af 2ane 1pol	1.25
2.5 mg/kg, 7 days	1ctg 1ctb 2ane 1pol	1.25
Positive control, 7 days	10ctg 3csg 4ctb 1csb 5acf 1cf 18ane	11.50***
Negative control, 10 days	2ctg 1ctb 2ane	1.25
0.1 mg/kg, 10 days	1ctb 3ane	1.00
0.5 mg/kg, 10 days	2ctb 1af 1cf 3ane 1pol	2.00
2.5 mg/kg, 10 days	1ctb 1ane[c)]	1.00
Positive control, 10 days	11ctg 6csg 4ctb 2csb 3af 15ane 2pol[d)]	25.50***

CA – chromosome aberrations; ctg – chromatid gap; csg – chromosome gap; ctb – chromatid break; csb – chromosomal break; acf – acentric fragment; cf – centric fusion; ane – aneuploidy; pol – polyploidy

***$P<0.001$ (χ^2-test)

[a)] one animal died on the 3rd day of treatment

[b)] one animal died on the 2nd day of treatment

[c)] one animal died on the 6th day, and one animal died on 9th day of treatment

[d)] two mice died on 8th and 9th day of treatment, respectively

Finally, it has to be mentioned that animal deaths were observed. Namely, one animal in group treated with 0.5 mg/kg of T_4 for 3 days died on the third day of treatment. Also, one animal in the group treated for 7 days died on day 2 of treatment with 0.1 mg/kg T_4, and two animals died (on days 6 and 9 respectively) in the group treated for 10 days with 2.5 mg/kg. As for the positive control, we observed that two mice died on the 8th and 9th day of treatment with MNNG.

DISCUSSION

There is abundant literature data about the genotoxic and mutagenic effects of environmental pollutants (Pitarque *et al*. 2002; Stanimirović *et al*., 2005). Most of the environmental genotoxins result from intense development of chemical and other industries. Therefore, they increase in number each year. However, there is an increasing evidence that some substances which are normally present in the human or animal bodies may, under certain circumstances, exhibit genotoxic effects, therefore acting as endogenous mutagenic agents (Lutz, 1990; Totsuka *et al*., 2005). Probably the best studied endogenous mutagenic agents are oestrogenic hormones (Anderson *et al*., 2003; Schallreuter *et al.*, 2006).

The principal molecular mechanism of the genotoxic effects of oestrogens comprises their metabolic conversion to catecholoestrogens and subsequent redox cycling creating the condition of oxydative stress (Liehr, 2001; Li *et al*., 2004). Interestingly, there are some experimental indices that phenolic groups of some non-steroidal hormones (adrenaline, thyroid hormone) and neurotransmitters (dopamine, noradrenaline) may undergo redox cycling accompanied by generation of ROS favoring oxidative stress (Moldeus *et al.*, 1983; Djelić and Anderson, 2003; Dobrzynska *et al.*, 2004). Therefore, the induction of oxidative stress may be the key mechanism of genotoxic effects of both steroidal and non-steroidal hormones with phenolic groups.

There is an experimental confirmation that thyroid hormones can induce DNA damage in human lymphocytes (Djelić and Anderson, 2003) and sperm (Dobrzynska *et al,* 2004) measured by the Comet assay. Namely, the DNA damage measured by tail moment was significantly reduced in cells concomitantly treated with thyroid hormone and the antioxidant enzyme catalase. On the other hand, thyroxine induced SCEs in whole blood cultures, whereas there was no increase in micronuclei (MN) (Djelić *et al.*, 2006). It is generally acknowledged that SCE test is an indicator test of genotoxic exposure, whereas MN test represents a mutagenicity test for detection of chromosome aberrations. Therefore, the results of MN test should be considered of higher significance than the results of SCE test. In previous investigations we considered that thyroxine has not exhibited mutagenic effects in cultured human lymphocytes. Anyhow, since the molecular mechanism(s) of SCE formation and their biological significance remain unclear, we undertook this investigation to evaluate possible chromosome alterations under *in vivo* conditions. The negative results obtained in this *in vivo* investigation are in complete accordance with the absence of mutagenic effects in the MN assay on human lymphocytes *in vitro*. This is quite logical, because MN assay detects acentric fragments and/or whole

chromosomes left behind during the nuclear division and are visible as small additional nuclei. Moreover, our experimental results on Swiss albino mice are particularly valuable showing that thyroxine did not induce chromosome mutations under the *in vivo* conditions. Also, we monitored effects of thyroxine during subacute treatment of up to 10 days and there was no significant change in chromosome aberrations compared to the negative control.

Finally, our results of both *in vivo* and *in vitro* cytogenetic analysis of the genotoxic effects of thyroxine disagree with findings that thyroid hormones produce profound DNA damaging effects in human lymphocytes (Djelić and Anderson, 2003) and sperm (Dobryznska *et al*., 2004). This discrepancy may have been caused because the Comet assay was performed on purified lymphocytes. Namely, erythrocytes in whole blood cultures contain catalase and glutathione peroxidase, which eliminate hydrogen peroxide (Andreoli *et al*., 1999; Karaca *et al.*, 2006). These antioxidant enzymes can reduce the effects of ROS created primarily by the action of thyroid hormones on mitochondria. We also assume that under the *in vivo* conditions antioxidant defence mechanisms (Benzie, 2000) could have reduced the genotoxic effects of thyroxine. In addition, it must be emphasised that the Comet assay has a much higher sensitivity than standard cytogenetic analysis (Tice *et al*., 2000).

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EVALUACIJA GENOTOKSIČNIH EFEKATA TIROKSINA PRIMENOM *IN VIVO* CITOGENETIČKOG TESTA NA SWISS ALBINO MIŠEVIMA

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SADRŽAJ

Tireoidni hormoni podstiču aerobni metabolizam favorizujući oksidativni stres koji može da dovede do kovalentnih oštećenja različitih molekula uključujući i DNK. U prethodnim istraživanjima otkriveno je da tireoidni hormoni indukuju oštećenja molekula DNK u humanim limfocitima i spermi u *in vitro* Komet testu. Međutim, citogenetička evaluacija genotoksičnih efekata tiroksina dala je kontradiktorne rezultate: povećanje razmena sestrinskih hromatida bez porasta učesalosti mikronukleusa u kulturama humanih limfocita. Stoga je cilj istraživanja u ovom radu bio da dodatno ispitamo moguće genotoksične efekte tiroksina koristeći *in vitro* citogenetički test na Swiss albino miševima.

Upotrebljene su tri eksperimentalne koncentracije tiroksina (0,1 mg/kg, 0,5 mg/kg and 2.5 mg/kg). Miševi su podeljeni u nekoliko grupa zavisno od dužine tretmana tiroksinom: 1, 3, 7 i 10 dana. U istim vremenskim periodima miševi su tretirani pozitivnom (N-metil-N'-nitro-N-nitrozogvanidin) i negativnom kontrolom. Analizirani su citogenetički parametri (numeričke i strukturne aberacije hromozoma, gapovi i prekidi na hromozomima) u ćelijama kostne srži izolovanim iz femura.

Rezultati dobijeni u ovom istraživanju ukazuju da tiroksin ne indukuje hromozomske prekide i aberacije, što je u saglasnosti sa našim prethodnim zapažanjima na humanim limfocitima u kulturi. Istovremeno, primetili smo smanjenje mitotskog indeksa, naročito kod životinja tretiranih u dužem vremenskom periodu sa visokim dozama tiroksina. Prema tome, može se zaključiti da tiroksin ne indukuje genotoksične efekte koji mogu da se detektuju citogenetičkim analizama.