GENOTOXICITY OF TRIIODOTHYRONINE: EFFECTS ON *Salmonella typhimurium* TA100 AND HUMAN LYMPHOCYTES *in vitro*

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There is increasing evidence that substances which are normally present in human or animal bodies may, under the certain circumstances, exhibit deleterious effects on genetic material, therefore acting as endogenous mutagenic agents. Since hormones represent one of the best studied endogenous mutagens, some research focused on the possible role of thyroid hormone in mutagenesis and carcinogenesis. Indeed, thyroid hormones accelerate aerobic metabolism and production of reactive oxygen species (ROS) and, therefore, may exhibit mutagenic effects in various test systems on mammalian cells. However, possible mutagenic effects on prokaryotic DNA has not been investigated so far. Hence, the aim of this research was to compare the sensitivity of TA 100 *Salmonella typhimurium* with and without metabolic activation with S9 fraction, and human lymphocytes to possible genotoxic effects of triiodothyronine (T_3). Therefore, we used the reverse mutation assay on *S. typhimurium* (Ames test) and *in vitro* Comet assay in isolated peripheral blood human lymphocytes. In both tests-systems a broad spectrum of T_3 concentrations was applied. The obtained results showed absence of genotoxic effects of T_3 in bacterial reverse mutation assay and very profound genotoxic effects in human lymphocytes at concentrations higher than 15 µM. We only observed cytotoxic effects in bacterial system at very high T_3 concentrations

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(300 and 500 µM). In conclusion, T_3 was unable to increase the level of reverse mutations in Ames test both with and without S9 mix. Therefore, it seems that ROS production in mitochondria may be the primary cause of DNA damage caused by T_3 in mammalian cells.

Keywords: Comet assay, DNA damage, human lymphocytes, triiodothyronine, TA100 *Salmonella typhimurium*

INTRODUCTION

Hormones, as endogenous substances, are *a priori* considered as non-carcinogenic and non-mutagenic, although there is an increased experimental and epidemiological evidence for their role in carcinogenesis (ĐELIĆ, 2002; KABBARAH *et al*., 2006; SCHIEL *et al.*, 2006). Hormones may be involved in the processes of malignant transformation both at the level of initiation and promotion of carcinogenesis (OTHMAN *et al.*, 2014; CAVALIERI *et al*., 2016). The high level of T_3 hormone is positively associated with the risk of thymus neoplasia (BOYD *et al*., 2016), prostate cancer, benign prostatic hyperplasia (LEHRER *et al*., 2002) and breast cancer (TOSOVIC *et al.,* 2012). According to HERCBERGS (1996), thyroid hormones (TH) - triiodothyronine (T_3) and its prohormone thyroxine (T_4) are promoters of carcinogenesis through their important role in cell differentiation, growth, and metabolism. Later reports explained such effects of thyroid hormones by their involvement in promoting tumor induced angiogenesis (PINTO *et al.*, 2011) and increasing cancer cell proliferation (TSUI *et al.*, 2008).

On the other hand, internally produced substances can cause instability of human cancer by increased oxidative DNA damage that can not be remedied by DNA repair mechanisms (JACKSON and LOEB, 2001). In *in vivo* conditions, MAGSINO *et al.* (2000) has shown an increment of reactive oxygen species (ROS) production in mononuclear cells and polymorphonuclear leukocytes by provoking a hyperthyroid state in euthyroid healthy volunteers by giving them orally 60 µg/d of T_3 for 7 days. Indeed, imbalanced prooxidant-antioxidant status and increased oxidative DNA damage are observed in patients with Graves hyperthyroidism (RYBUS-KALINOWSKA *et al.* 2008; TSAI *et al.,* 2009). Ability of TH to cause oxidative DNA lesion was evaluated in *in vitro* Comet assay in human lymphocytes (DJELIC and ANDERSON, 2003, ŽUKOVEC-TOPALOVIĆ *et al.*, 2015) and sperm (DOBRZYŃSKA *et al.*, 2004), as well as in rodent lymphocytes *in vivo* (DESIBIO *et al*., 2013). Increased generation of reactive oxygen species (ROS) in hyperthyreoid state can be explained by T_3 and T_4 possibility to undergo redox cycling due to the phenolic group presence in their structure (DJELIĆ *et al.*, 2007) and by increased mitochondrial oxygen consumption (MESSARAH *et al.*, 2010), mainly by T_3 (CHENG *et al.*, 2010).

To our best knowledge, the mutagenicity of TH was not studied in bacterial short-term test systems before, although these tests can provide considerable information about cellular mechanisms of mutagenesis (VUKOVIĆ-GAČIĆ *et al.,* 2006).Mutagenic properties of natural and synthetic hormones were studied using *S. typhymurim's* reverse mutation test (Ames test) and almost all obtained results were negative, both with and without metabolic activation (SIDDIQUE *et al.*, 2005). However, there are some experimental data showing mutagenic effects of the hormones in the Ames test (YAMAGUCHI, 1981; LUTZ *et al.*, 1988). Interestingly, in YAMAGUCHI (1981) study, a mutagenicity of adrenaline was only observed in *S. typhimurium* TA 100 not other used strains, respectively, TA 1535, TA 1536, TA 1537, TA 1538. Similary, LUTZ *et al.* (1988) has used different strains of *S. typhimurium*, TA 98, TA100 and TA102 in his study of trenbolone, synthetic androgen hormone, mutagenicity. As explained by LUTZ *et al.* (1988),

trenbolone can penetrate bacterial cells and react with DNA only in the TA100 strain leading to a slight but not significant increase the number of revertants.

Therefore, we choose to examine a mutagenic potency of T_3 hormone in a concentration which is 10^3 to 10^5 fold higher than the physiological level of T_3, with or without metabolic activation with a *Salmonella*/ microsome TA100 strain reverse mutation assay. In addition, we evaluated genotoxicity of T_3, in concentrations: 0.005, 0.025, 0.15, 1.5, 5.0, 15, 50, 100, 200 and 500 μM, in the *in vitro* Comet assay in human lymphocytes.

MATERIALS AND METHODS

Chemicals

The 3,3',5-trijodo-L-thyronine sodium salt (T_3 hormone) (CAS No, 194585, Galenika Belgrade, Serbia) was freshly dissolved in PBS (Torlak, Belgrade, Serbia). The stock solution of T_3 hormone (500 μM) was diluted for an Ames test to obtain: 5, 15, 50, 100, 200 and 300 μM and for a Comet assay to obtain: 1.5, 5.0, 15, 50, 100 and 200 μM.

Bacterial and eukaryotic cells

S. typhimurium's strain TA100 was used for mutagenicity testing of a wide range of T_3 concentrations and strain TA98 was used for the evaluation of enzyme activity of the S9 fraction in co-treatment with ethidium bromide (EtBr). While TA98 were used for determining the frame shift, TA100 was used to determine the base pair exchange type of mutations.

For the Comet assay, human lymphocytes were obtained and immediately used by venepuncture from 5 healthy male donors (25 - 35 years of age). All of them were healthy at the moment of blood sampling and non-smokers. Donors who reported alcohol consumption, medicinal usage, exposure to diagnostic X-rays and severe aerobic physical training were excluded. Human blood sampling was performed in accordance with the Declaration of Helsinki and approved by the Ethical Committee for clinical research at the Faculty of Pharmacy in Belgrade Serbia (846/2). Informed donor consent was also obtained.

Media, growth conditions and S9 fraction preparation

S. typhimurium reverse mutation assay, including media preparation, was performed by the methods described by MARON and AMES (1983). Bacteria were grown in minimal glucose medium (MG) medium at 37°C for 48 hours.

S9 fraction was isolated from the liver of Albino Wistar male rats (170-180 g) induced with phenobarbital (Sigma-Aldrich, Cas No. 50-06-6) and β-naphtho flavones (Sigma-Aldrich, Cas No. 6051-87-2). S9 mixture contained 4% (v/v) S9 fraction, 33 mMKCl, 8 mM MgCl$_2$, 5 mM glucose-6-phosphate (Sigma-Aldrich, CasNo. 54010-71-8)and 4 mM NADP (Sigma-Aldrich, CAS No. 1184-16-3) in 0.1 M phosphate buffer pH 7.4 (MARON and AMES, 1983). Preparation of S9 mixture obtained from the livers of Albino Wister male rats was approved by the Ethical Committee of the Faculty of Biology in Belgrade, University of Belgrade Serbia (EK-BF-2015/26).

Evaluation of mutagenicity of T₃ in Ames test

For evaluation of the mutagenic potential of T_3 two experimental procedures were applied: A) co-incubation of T_3 concentrations (5, 15, 50, 100, 200, 300 and 500 μM) with *S. typhimurium* TA100 without the S9 fraction; B) pre-incubation of T_3 concentration (5, 15, 50, 100, 200, 300 and 500 μM) and the S9 fraction for 30 min on 37ºC, followed by incubation, and the final addition of *S. typhimurium* strain TA100.

The enzyme activity of the S9 fraction in co-treatment with EtBr in concentration 50, 75 and 100 μg per plate, was evaluated in the *S. typhimurium* strain TA98. In all above mentioned experimental procedures the overnight culture of *S. typhimurium* TA100 strain or TA98 strain was washed by centrifugation and resuspended in the same volume of 0.01 M MgSO₄. Samples (0.1 ml) were added to 3 ml of molten top agar containing 0.05 mM histidine and biotin and poured in duplicates onto MG plates with different concentrations of T_3 (5, 15, 50, 100, 200, 300 and 500μM) with or without S9 mix (0.3 ml). PBS was used as a negative control. After incubation at 37°C for 48h the number of *His⁺* revertants was determined and the presence of the bacterial background lawn on all plates was confirmed.

Isolation of lymphocytes

Isolation of lymphocytes from whole blood was performed with a Ficoll-Paque medium. After centrifugation at 1900 g for15 min, the lymphocytes formed a layer directly above the Ficoll-Paque. The isolated lymphocytes were washed twice in RPMI 1640 medium, each wash was followed by a centrifugation of 10 min at 1800 g. Finally, the supernatant was removed as carefully as possible without disturbing the pellet. An aliquot of 1 mL of RPMI 1640 was added and the pellet was resuspended.

The Comet assay

An alkaline Comet assay was performed according to SINGH *et al.* (1988) and TICE *et al.* (2000) technique, with slight modifications. For every treatment in the Comet assay, human lymphocytes from 5 donors were used. In this study the genotoxic effects of 3,3 ',5-triiodo-L-thyronine sodium salt (T_3) dissolved in PBS to experimental concentrations 0.005, 0.025, 0.15, 1.5, 5.0, 15, 50, 100, 200 and 500 μM were investigated. We used 100 μM hydrogen peroxide as the positive control (Galafarm, Skopje, Macedonia), and the solvent (PBS) for T_3 was the negative control.

Prior to the Comet assay, the viability of lymphocyte cells was evaluated after incubation for 30 min with the tested compounds using the Trypan blue dye assay.

Microscope slides were pre-coated with 1% normal melting point agarose and were dried at a room temperature for at least 48 h. After centrifugation (5 min at 2000 rpm), 100 μl of cell suspension was mixed with 100 μl of 1% low melting point agarose (LMPA). 90 μL of the suspension was rapidly pipetted onto the first agarose layer and spread using a coverslip, and put in the fridge to solidify. After removal of the coverslip, the 90 μL of 0.5% LMPA was added as the third layer, spread using a coverslip and allowed to solidify at 4°C for 5 min. Afterwards, the slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO) overnight at 4°C. After lysis, the slides were placed in a horizontal gel electrophoresis tank to allow DNA unwinding in cold (4°C) alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 30 min. Electrophoresis were done at 4°C under electric current of 25 V and 300 mA for 30 min. All these steps were

performed under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. The slides were then neutralized with 400 mM Tris-HCl (pH 7.5) three times for 5 min. Then, the slides were fixed with cold methanol, dried and stored. Before analysis, the slides were rehydrated with ice cold distilled water and stained with 50 μL of 20 μg/mL EtBr.

Scoring of comets

Slides were examined at 400× magnification with a fluorescent microscope (Leica, UK) and image analysis software (Comet Assay IV Image Analysis system, PI, UK). From each replicate slide, 50 nuclei were scored (a total of 100 nuclei per donor) and a tail intensity-percentage of tail DNA (Ti) was used to evaluate the extent of DNA migration.

Statistical analysis

The results of the Ames test, are expressed as mean values of three independent experiments with two replicates. Statistical analysis of the Comet assay results was performed using Statistica 7.0 Software. Mean values of Ti for each group and each of 5 donors in genotoxic and antigenotoxic treatments were calculated. Afterwards, groups of 5 values were formed in both treatments and compared using *t*-test. A difference at $P< 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Mutagenic potential of T_3 in the Ames test

According to available literature the mutagenicity of thyroid hormones was not evaluated before in the Ames test. Prior to testing mutagenic effects of T_3 in the concentration range 5-500 μM/plate in TA 100 *Salmonella typhimurium* with and without metabolic activation, we evaluated the enzyme activity of the S9 fraction in TA98 and TA100. The number of spontaneous revertants with or without the S9 fraction was in the expected range for strains, 30-50 for TA98 and 120-200 for TA100 (MARON and AMES, 1983). A significant, more than 2 fold, increase in the number of revertants occurred after treating the TA98 strain with the S9 fraction in the presence of 50, 75 and 100 μg/plate of EtBr (Table 1). On the other hand, none of the T_3 concentrations were mutagenic in TA100, in the absence and presence of S9 metabolic activation in both experimental procedures (Table 2). No increase in the number of revertants occurred for PBS (negative control). However, in our study, we observed a slight decrease of the bacterial lawn at concentrations ≥300 μM, which lead to the conclusion that cytotoxic effects are present. Our results show the absence of mutagenic effects of T_3 at all concentrations, which is consistent with all previous negative results of hormones in the Ames test (SIDDIQUE *et al.*, 2005). It is possible that the ambiguous results of trenbolone mutagenic effects in the Ames test in LUTZ *et al.* (1988) study, originated from an absence of a twofold increase of revertants in groups challenged with trenbolone and/or contamination of the test substance. Similarly, controversial results of YAMAGUCHI (1981) probably arise from the fact that in his study, 120 was referent number of TA 100 *Salmonella typhymurium* revertants, which is not consistent with wide accepted range 120-200 (MARON and AMES, 1983). Although we also used *S. typhimurium* TA100 in this investigation, there was an absence of mutagenic effects of T_3. Therefore, we

conclude that T$_3$ was unable to exhibit mutagenic effects on *S. typhimurium*TA100, both with and without metabolic activation using S9 mix.

Table 1. Evaluation of enzyme activityof the S9 on TA98 Salmonella typhimurium in cotreatment with EtBr

tested supstances		Number of revertants/plate (mean ± SE)	
		TA 98	
		without S9	With S9
	0[a]	12,50±2,50	18,50±6,50
EtBr[b]	50	20,00±1,00	317,00±49,00
	75	19,00±3,00	177,00±16,00
	100	21,00±6,00	133,00±12,00

The efficacy of the of S9 (Albino Wistar liver microsomal liver fractions induced by pheno-barbital and β -naphtho flavones) wasassed in cootretment with [b]EtBr (ethidium bromide, μM/plate) in accordance to MARON and AMES (1983);[a]0- the number of spontanious revertants of TA98 *Salmonella typhimurium;*

Table 2. Mutagenic potential of T$_3$ in concentration range of 5-500 μM/plate in TA 100 Salmonella typhimurium

tested supstances		Number of revertants/plate (mean ± SE)		
		TA 100		
		without S9	with S9	pre-incubated with S9
	0[a]	179 ± 48	259 ± 54	
	S[b]	210 ± 81	266 ± 75	
T$_3$[c]	5	218 ± 5	315 ± 12	293 ± 7
	15	160 ± 42	230 ± 43	232 ± 16
	50	155 ± 49	228 ± 66	242 ± 17
	100	194 ± 69	272 ± 86	252 ± 5
	200	194 ± 58	252 ± 77	278 ± 10
	300	188 ± 71 [d]	270 ± 87 [d]	270 ± 38 [d]
	500	179 ± 82 [d]	258 ± 62 [d]	229 ± 35[d]

The mutagenicity of [c] T$_3$- Triiodothyronine(μM/plate)was evaluated in accordance to MARON and AMES (1983) with or without addition of S9 (Albino Wistar liver microsomal liver fractions induced by pheno-barbital and β -naphtho flavones); [a]the number of spontanious revertants of TA100 *Salmonella typhimurium;* [b]Solvent- PBS for T$_3$; [d] Toxicity manifested as an alteration in the background lawn

Genotoxic potential of T$_3$ in the Comet assay

A cytogenetic study in humans with an impaired thyroid status (FEDOROVA *et al*., 1992) showed that hyperthyroidism may cause chromosome and chromatid aberrations. In contrast, DJELIĆ *et al.* (2006, 2007) reported that L-thyroxine does not exhibit cytogenetically detectable genotoxic effects in lymphocytes from the whole-blood cultures. The same groups of authors reported that an increment in SCE rate was found at very high concentrations which were comparable to accidental acute poisoning with thyroxine (BERKNER *et al.* 1991). On the other

hand, the *in vitro* Comet assay, which is more sensitive than cytogenetic tests, gave positive results in genotoxicity testing of T_3 hormone on sperm (DOBRZYŃSKA *et al.*, 2004) at concentrations higher than 10 μM. Although the cytobiochemical and physiological effects of T_3 are generated in a lower concentration range (0.001-0.003 μM), the higher concentrations in previous studies were justified due to difficulties to extrapolate a longer period of exposure in a laboratory *in vitro* conditions (CEMELI and ANDERSON, 2011). Bearing in mind all the above mentioned, in our study, we chose to investigate the genotoxic effects of a wide-ranging T_3 (0.005- 200 μM) on isolated human lymphocytes, which are considered as common surrogate cells. The Trypan blue exclusion assay was used to test the viability of cells treated. Cell viability of lymphocytes after the treatment with T_3 at 500 μM and 100 μM doses for 30 min, was mostly higher >90%. In our experimental conditions, it was observed that, at lower concentrations, such as 0.005, 0.025, and 0.15 μM, T_3 did not show a significant effect on DNA damage in human lymphocytes (data not shown). Treatments with 1.5 μM and 5 μM of T_3 increased average Tail intensity values by 1.7 and 1.8 fold respectively, comparing with negative control but without statistical significance. DNA damage in lymphocytes treated with 15 μM of T_3 and all other higher concentrations gave rise to a significant DNA damage in a dose dependent manner (Figure 1). Our results are consistent with previous findings of DJELIC and ANDERSON (2003), although treatment duration in our study was 1.5 h shorter. MAGSINO (2000) in the *in vivo* study has observed an exacerbation of ROS in a hyperthyroid state, possibly due to the depression of the endogenous enzymatic antioxidant defense by increased T_3 concentration (CHATTOPADHYAY *et al.*, 2007). The results of the present study demonstrated that T_3 concentration of 1.5 μM was high enough to induce oxidative stress at level accompanied with DNA damage which was not completely repaired, and consequently was expressed as increased DNA migration in the Comet assay. Anyway, the underlying molecular mechanisms of T_3 genotoxicity require further investigations.
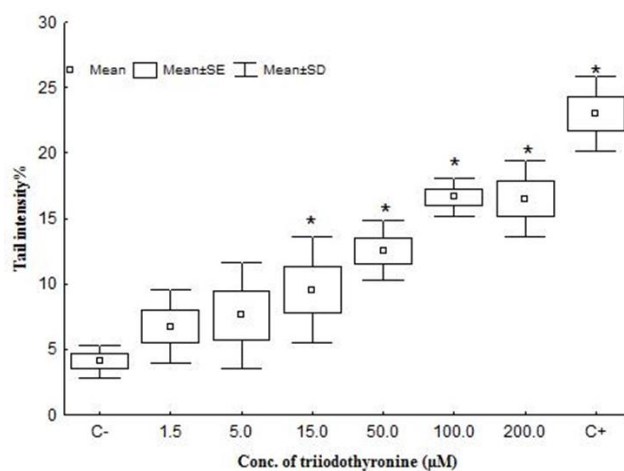

Figure 1. Effects of T_3 in concentration range of 1.5 - 200 μM on the DNA integrity of human lymphocytes; C[-] - negative control, C[+]- positive control (100μM hydrogen peroxide),*significance in comparison with negative control ($P < 0.05$)

It has to be mentioned that concentrations of T_3 in our investigation that gave significant DNA damage were much higher than plasma levels of T_3 in severe hyperthyroidism (DE LEO *et al.*, 2016), or accidental thyroxine poisoning (BERKNER *et al.*, 1991; MAJLESI *et al.*, 2010). Therefore, pathophysiologically elevated T_3 probably do not cause significant DNA damage during the short-time period, as in our investigation. However, we assume that prolonged exposure to elevated levels of T_3 and T_4 may pose some genetic risk, as discussed by DJELIĆ *et al.* (2006). In addition, we confirmed using the Ames test that T_3 is not a direct mutagen in TA 100 *S.typhimurium*, but it has the ability to cause DNA damage just probably by enhancing mitochondrial oxygen consumption accompanied by the production of reactive oxygen species-ROS (LANNI *et al.*, 2016). It is well-known that thyroid hormones strongly influence mitochondria as an organelle important for the production of energy necessary for various biochemical processes underlying cell growth and differentiation (VAITKUS *et al.*, 2015). Considering that antioxidant therapy has been beneficial in the therapy of hyperthyroid patients (GUERRA *et al.*, 2001), our further research will focus on finding an adequate antioxidant antigenotoxic agent that counteracts T_3 induced oxidative DNA damage.

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GENOTOKSIČNOST TRIJODTIRONINA: EFEKTI U SOJU TA 100 *Salmonella typhimuriumi* U LJUDSKIM LIMFOCITIMA *in vitro*

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Izvod

Sve je više dokaza da supstance, koje su normalno prisutne u životinjskom i ljudskom organizmu, mogu, pod izvesnim okolnostima, da ispolje štetne efekte na nasledni materijal, delujući na taj način kao endogeni mutageni agensi. S obzirom da su hormoni jedna od najbolje proučenih grupa endogenih mutagena, pojedina istraživanja bila su usmerena ka ispitivanjima dejstva tireoidnih hormona u procesima mutageneze i kancerogeneze. Zaista, tireoidni hormoni ubrzavaju aerobni metabolizam i stvaranje reaktivnih kiseoničnih vrsta (ROS), tako da dovode do mutagenih efekata u različitim test sistemima na ćelijama sisara. Međutim, mogući mutageni efekti na prokariotsku DNK do sada nisu ispitivani. Otuda, cilj ovog istraživanja bio je da uporedimo osetljivost TA 100 *Salmonella typhimurium* u prisustvu ili odsustvu metaboličke aktivacije pomoću S9 frakcije i limfocita čoveka na moguće genotoksične efekte trijodtironina (T_3).Tako smo koristili test povratnih mutacija na *S. typhimurium* (Ames-ov test) i in vitro komet test na izolovanim limfocitima periferne krvi čoveka. Širok spektar koncentracija T_3 primenjen je u oba testa. Dobijeni rezultati pokazuju odstustvo genotoksičnih efekata T_3 u testu povratnih mutacija na bakterijama i veoma izražene genotoksične efekte na limfocitima čoveka pri koncentracijama većim od 15 µM. U bakterijskom sistemu smo zapazili samo citotoksične efekte pri veoma visokim koncentracijama T3 (300 i 500 µM). Može se zaključiti da T_3 nije bio u stanju da poveća nivo povratnih mutacija u Amesovom testu kako sa tako i bez metaboličke aktivacije sa S9 mešavinom. Prema tome, izgleda da je stvaranje ROS u mitohondrijama glavni uzrok oštećenja DNK u ćelijama sisara pod dejstvom T_3.