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The effects of short term growth hormone administration and resistance training on the histopathology and mutation of the *BRAF* gene (T1799A) in thyroid tissue of the brown rat (*Rattus norvegicus*)

Behnam Roozbeh[1], Mahtab Moazzami[1]*, Amir Rashidlamir[1], Zahra Moosavi[2], and Ali Javadmanesh[3]

[1]Department of Exercise Physiology, Faculty of Sport Sciences, Ferdowsi University of Mashhad, Azadi Square, Mashhad, Iran

[2]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Azadi Square, Mashhad, Iran

[3]Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Azadi Square, Mashhad, Iran

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ABSTRACT

High levels of growth hormone accelerate mitosis rate but decrease the apoptosis process in its target organs. These events might cause the initiation of different cancer types. Thus, the main aims of this study were assessing the effects of short term growth hormone administration and resistance training on the histopathology and detection of the *BRAF*-V600E mutation in the thyroid tissue of male *Rattus norvegicus* brown rats. Thirty-two rats were randomly divided into four groups. After 8 weeks of the experiment (i.m), thyroid tissue and blood samples of saline (CS), resistance training+saline (RS), growth hormone (2 mg/kg) (GI) and resistance training+growth hormone (2 mg/kg) (RG) were taken to evaluate histopathology, the *BRAF* T1799A mutation of thyroid tissue, and circulating levels of IGF-1 and IGFBP-3. The protocol of training consisted of rats climbing a ladder while carrying weights (3 sets/5 reps). Microscopic evaluation of thyroid tissue did not show any histopathological changes, and there were no mutations in the studied region of the *BRAF* sequence. Serum IGF-1 concentration was significantly lower in the RS group than in other groups ($P<0.05$). However, serum IGFBP-3 concentration did not change significantly in the RS group. Moreover, serum IGF-1 and IGFBP-3 concentrations were significantly higher in the GI and RG groups than in the others ($P<0.05$). In conclusion, the decrement of serum IGF-1 concentration and IGF-1/IGFBP-3 ratio after resistance training might decrease the risk of cancer. Furthermore, short term growth hormone administration, with and without resistance training, might increase the risk of cancer through the high levels of serum IGF-1 concentration and IGF-1/IGFBP-3 ratio in male rats.

Key words: *BRAF* gene (T1799A); cancer, growth hormone; resistance training; thyroid

*Corresponding author:
Assoc. Prof. Mahtab Moazzami, Department of Exercise Physiology, Faculty of Sport Sciences, Ferdowsi University of Mashhad, Azadi Square, Mashhad, P.O.Box: 91775-1163, Iran, Phone: +98 051 3880 3454; E-mail: Mahtab.Moazami@yahoo.com; Moazami@um.ac.ir

Introduction

Health science has faced major challenges in the management of mutation in all diseases. Nowadays, many hormonal drugs affect the thyroid system, and common therapeutic methods often result in unsatisfactory results. There is increasing interest in this topic, due to the overall increase in thyroid disorder in industrialized countries (PELLEGRITI et al., 2013), but there is still much to learn concerning the effect of hormonal therapy and training on the thyroid system.

Growth hormone (*GH*) is secreted by the pituitary gland, which is controlled by hypothalamic hormones. Due to the frequency of GH receptors in practically all cells and tissues, GH induces a variety of actions in all cells (ALLARD and DUAN, 2018). One of the main actions of GH is stimulation of insulin growth factor-1 (IGF-1) and synthesis of its binding proteins. There are at least six types of IGF-Binding Proteins ($IFGBP_S$) that transport IGFs in the blood and other fluids of the body to their receptors (IGF-1R). IGF-1 is transported primarily by IGFBP-3 which protect the IGF-1 from proteolytic degradation and extend its half-life. The amount of free IGF-1 in the blood is of great importance for cell proliferation. $IGFBP_S$ can oppose actions to IGF-1, partly by binding to IGF-1 and preventing it from attaching to its membrane receptors (ALLARD and DUAN, 2018). The $IGFBP_S$ regulates IGFs' bioavailability up to 50-fold higher than IGF-1R (GHANIPOOR-SAMAMI et al., 2018). Some studies have indicated that high levels of IGF-1 concentration, low levels of IGFBP-3 concentration and/or increased IGF-1/IGFBP-3 ratio are associated with an increased risk of several types of carcinoma (FENG et al., 2017; SOUBRY et al., 2012). It has been shown that both acute and chronic resistance exercise increases intramuscular IGF-1 levels (OGASAWARA et al., 2013). Contrarily, the effects of resistance training on circulating IGF-1 and IGFBP-3 levels are inconsistent (ELIAKIM et al., 1998; NINDL et al., 2004; NINDL et al., 2010; NISHIDA et al., 2010).

Due to GH's anabolic and lipolytic actions, use and abuse of this hormone are widespread among professional and unprofessional athletes (ANDERSON et al., 2018). On the other hand, there is increasing evidence that the GH-IGF1 axis could influence cancer risk in patients with GH deficiency who are treated with GH, or patients who have high levels of GH secretion inherently (DABROWSKA et al., 2014; UCHOA et al., 2013; WOLINSKI et al., 2017). It has been stated that both GH and IGF-1 have mitogenic and anti-apoptotic properties (BOGUSZEWSKI et al., 2016). In addition, it has been shown that elevated serum GH and IGF-1 levels are associated with colorectal, breast, prostate and thyroid cancer (DAGDELEN et al., 2014; ENDOGENOUS et al., 2010). Thyroid function and morphology are affected by GH-IGF-1 axis. Due to the abundant IGF-1 receptors that are expressed in thyroid cells, IGF-1 is known as a proliferative factor for thyrocytes (MALAGUARNERA et al., 2011; SMITH et al., 2012). The prevalence of nodular goiter in acromegaly is high, and the risk of developing cancer is increased (CURTO et al., 2015). However, the effects of GH administration on thyroid tissue in athletes who abuse this hormone to enhance their performance and appearance are unknown.

In addition, after IGF-1 binds to its receptor (IGF-1R) it activates two main signaling pathways: 1) the Ras-Raf-ERK/MAPK pathway and 2) PI3K/AKT pathway. The MAPK pathway is a classical intracellular signaling pathway that plays an essential role in cell functions, such as proliferation, differentiation, apoptosis, and survival, and when aberrantly activated, tumorigenesis (DENDULURI et al., 2015). Serine/threonine-protein kinase B-Raf (*BRAF*) mutation is shown to be the main cause of aberrant activation of the MAPK pathway in cancer. T1799A point *BRAF* mutation is the most common (more than 90%) of all the mutations found in the *BRAF* gene. This mutation has been illustrated to occur frequently in thyroid cancer (CHENG et al., 2018).

No data are available from the literature concerning the risk of developing thyroid nodules or tumors during GH administration. In order to gain more knowledge on this matter, our purpose was to investigate whether GH injection with and without resistance training can affect thyroid histopathology and *BRAF*-V600E mutation, and what the responses are of the circulating levels of IGF-1 and IGFBP-3.

Materials and methods

Animals. Healthy adult male brown rats (*Rattus norvegicus* 245 ± 20 g; 12 weeks old) were purchased from the Pasteur Research Center (Karaj, Iran). The rats were kept in an air-conditioned animal room under a 12 h light:dark cycle, in standard environmental conditions (23 ± 1 °C, 55 ± 5% humidity), with free access to tap water and a commercial dry pellet diet. The rats were housed in polypropylene cages lined with pine wood husk, which was changed every day. Experimental protocols were approved by the Ethics Review Committee of Medicine, Ferdowsi University of Mashhad (Ethic code: IR.MUM.FUM.REC.1396.12).

Experimental design. After 8 weeks of the experiment (i.m), thyroid tissue and blood samples of saline (CS), resistance training+saline (RS), growth hormone (2 mg/kg; Genotropin, Germany) (GI) and resistance training+growth hormone (2 mg/kg) (RG) were taken to evaluate histopathology, the *BRAF* T1799A mutation of thyroid tissue, and circulating levels of IGF-1 and IGFBP-3.

In the 1st week of familiarization, the rats were trained to climb a 1 m high ladder with 2 cm grid steps and an 85 ° grade, with weights tied to their tails. The rats were placed at the bottom of the ladder and were forced to climb by touching when necessary. The animals were trained once a day, 5 days per week, for 8 weeks. Each training session consisted of 3 sets of 5 repetitions, with a one-minute rest interval between the reps and 2 minutes between the sets. This method was repeated until either the rat finished the sets entirely or failed to climb after 3 stimulations. The intensity of the training protocol during the first week was 50% of each rat's body weight. The weight that was carried by these rats was gradually increased at the beginning of each week (10% per week) ladder (LEE et al., 2003).

Measurement of IGF-1 and IGFBP-3. Blood was obtained via the tail vein for assay of the serum IGF-1 and IGFBP-3. Samples were collected in the morning before treatment. The serum was separated by centrifugation (3,000 rpm for 15 min), kept at -20 °C and assessed by ELISA kits (Hangzhou Eastbiopharm, Elisa Kits, CAT.NO: CK-E30653-E91558), as recommended by the manufacturer.

Histopathological analysis. The thyroid was removed for histology and fixed in 10% buffered neutral formaldehyde for 72 h.

Tissue samples were processed into paraffin thin sections (5-7 μm), perpendicular to the longest axis of the, stained with hematoxylin and eosin (H&E) and examined by light microscopy (Olympus BX60, Japan) (PERRY et al., 2016).

Detecting of BRAF (T1799A) mutation. DNA was extracted from 100 mg of thyroid tissue using a NEXprep^{tm} Tissue DNA Mini Kit (NEX Diagnostics, South Korea) according to the manufacturer's protocol. DNA integrity was assessed by agarose gel electrophoresis. The purity and quantity of the extracted DNA were evaluated by Epoch microplate spectrophotometer (BioTek Instruments, USA). Specific primers adjacent to the rat's *BRAF*-V600F position were designed by primer premier 5 (Premier Biosoft International, USA). Primer sequences were
5' CACAAAATAGATCCAGACAACTGTTC-3'
for forward and
5'ATGAAGACCTCACGGTAAAAATAGG-3'
for reverse primer.

A 103 bp fragment from exon 17 of the *BRAF* gene located on chromosome 4 (Accession number: NC_005103.4) was amplified by standard polymerase chain reaction (PCR) and sequenced from both strands to detect the T1799A mutation.

High-resolution melt (HRM) assay was conducted using the LightCycler® 96 real-time PCR System (Roche Life Science, Switzerland). Every reaction was carried out in 10 μL volume and contained approximately 50 ng of DNA diluted in 5X HOT FIREPol® EvaGreen® qPCR Mix Plus HRM master mix (Solis BioDyne, Estonia) and 200 nM of each primer, with the following PCR program and melting conditions: 95 °C for 15 min; 40 cycles of 95 °C for 15S, 60 °C for 20S and 72 °C for 30S, followed by a high resolution melt of 70 °C - 90 °C (0.05 °C/S, 20 reading/ °C). Data were acquired and analyzed using the accompanying High-Resolution Melt software (Roche Life Science, Switzerland).

Statistical analysis. The Shapiro-Wilk Test was applied to examine whether measurements were appropriate for normal dispersion. The data were analyzed by SPSS software (SPSS Inc., Armonk, IL, USA) (Version 20). These were then subjected to analysis of variance (ANOVA) within and among groups followed by the post hoc Tukey test. The results were presented as Mean ± SEM (n = 7). Levels P<0.05 were considered significant.

Results

Table 1 shows the results of mean serum IGF-1, IGFBP-3 concentrations and IGF-1/IGFBP-3 ratio. A significant decrement in the serum IGF-1 concentration (P = 0.04) and the IGF-1/IGFBP-3 ratio (P = 0.001) in the RS group compared to the other groups was observed in this study. However, we did not observe any changes in the serum IGFBP-3 concentration (P = 0.93) in the RS group. Furthermore, the serum IGF-1 (P = 0.001, P = 0.001) and IGFBP-3 concentration (P = 0.003, P = 0.011) and IGF-1/IGFBP-3 ratio (P = 0.001, P = 0.001) in GI and RG groups were significantly increased compared to the other groups.

The histopathological appearance of thyroid tissues was normal in all groups (Fig. 1). In thyroid tissues, microscopic examination did not show any significant differences in follicles, cells and the other tissue structures compared to the control group. The size of the thyroid gland and hormone secretions were the same in all groups. High-quality DNA was extracted (Fig. 2A) and a 103 bp fragment from exon 17 of the *BRAF* gene was amplified specifically (Fig. 2B). HRM results revealed no mutation in *BRAF* exon 17 (Fig. 3), which was confirmed by two-way sequencing.

Table 1. Statistical analysis of Mean serum IGF-1, IGFBP-3 concentrations and IGF-1/IGFBP-3 ratio

Parameter	Group	Mean ± SD		F Value	P - Value
IGF-1 (ng/mL)	CS	293.38 ± 52.5		32.48	0.001
	RS	220.62 ± ± 38.1	*		
	GI	451.88 ± 57.9	* #		
	RG	412.25 ± 60.1	* #		
IGFBP-3 (ng/mL)	CS	374.62 ± 66.4		7.61	0.001
	RS	388.38 ± 29.0			
	GI	463.00 ± 36.0	* #		
	RG	451.62 ± 41.4	* #		
IGF-1/IGFBP-3	CS	0.78 ± 0.014	*	416.42	0.001
	RS	0.56 ± 0.018	*		
	GI	0.97 ± 0.021	* #		
	RG	0.91 ± 0.016	* # $		

Control plus saline injection group (CS), resistance training plus saline injection group (RS), Growth hormone injection group (GI) and resistance training plus Growth hormone injection group (RG). * Significant difference with C group, # significant difference with RS group, $ significant difference with GI group. Significant difference P<0.05.

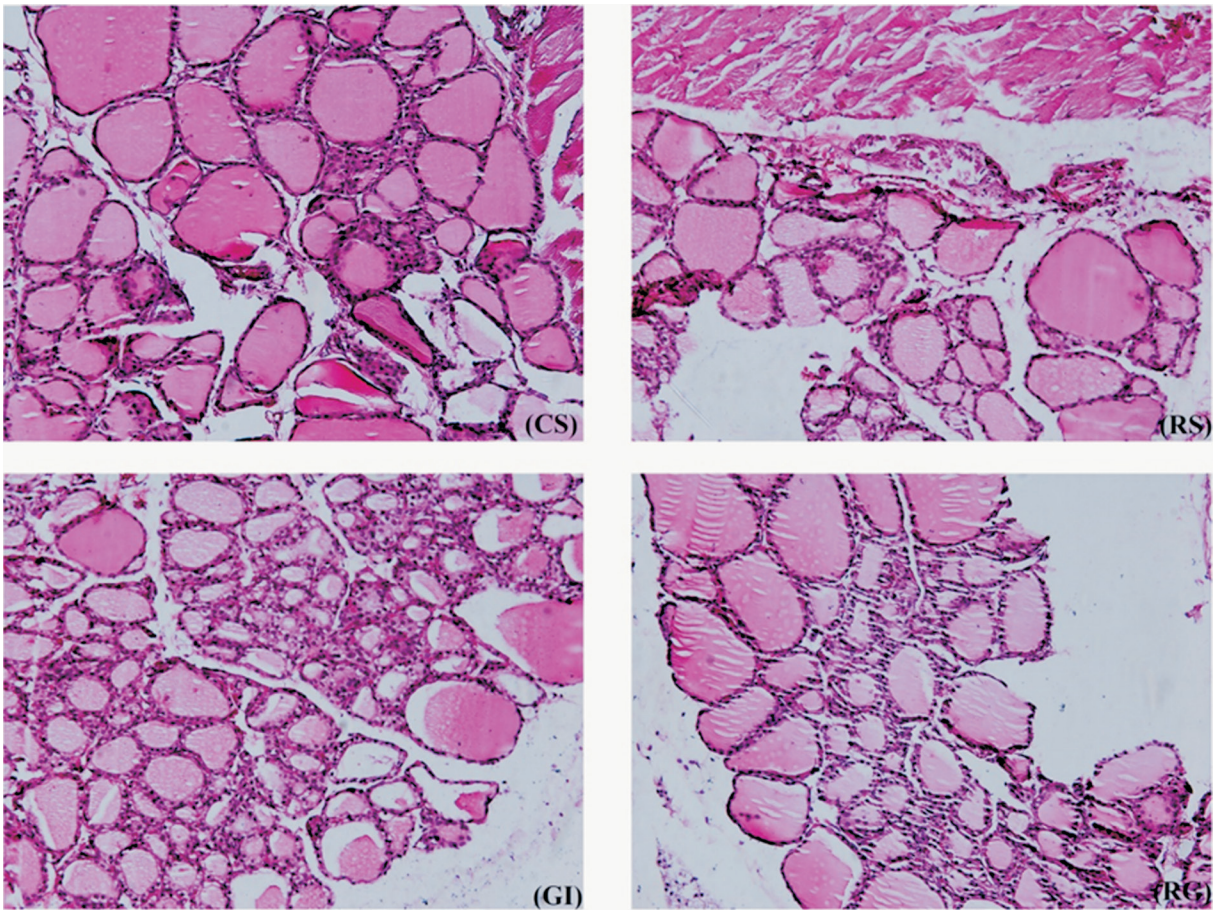

Fig. 1. Histopathological features of stained thyroid tissues (X200). Control plus saline injection group (CS), resistance training plus saline injection group (RS), Growth hormone injection group (GI) and resistance training plus Growth hormone injection group.

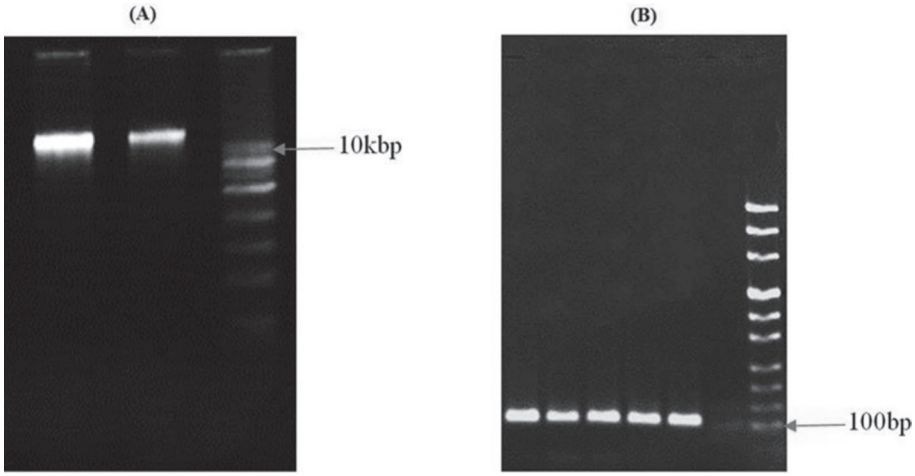

Fig. 2. Agarose gel electrophoresis of extracted DNA from thyroid tissue of rats (A) and 103 bp PCR products (B) of *Ratus norvegicus* BRAF exon 17

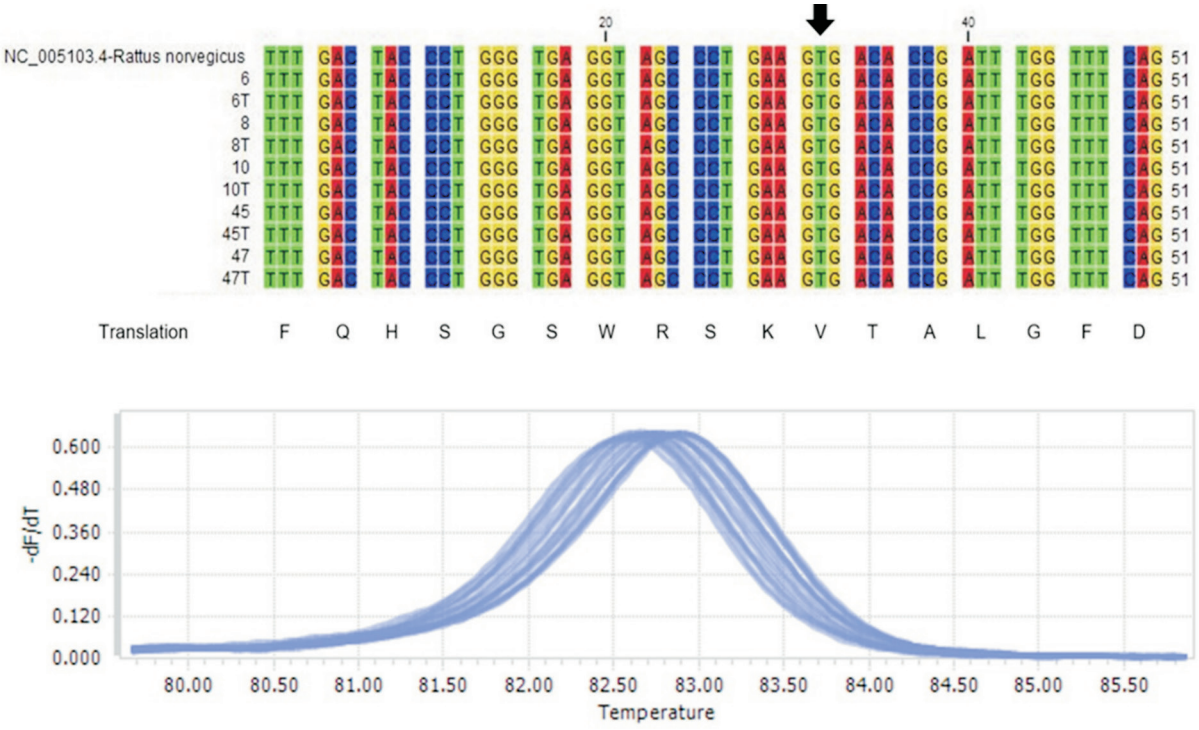

Fig. 3. (Top) Sequencing results and sequence alignment of number of samples with the *Ratus norvegicus* reference sequence for BRAF exon 17 (partial). The arrow shows the position of T1799A (V600E) mutation; (Bottom) Melting peaks of samples after HRM analysis showed no detectable mutation in the studied region.

Discussion

In this study, GH injection with and without resistance training and consequently elevated IGF-1 levels caused no histopathological and functional changes on normal thyroid cells. It has been well established that GH and IGF-1 are associated with tumorigenesis (DENDULURI et al., 2015). Moreover, it is believed that a chronic excess of GH and IGF-1 plays a crucial role in the pathogenesis of thyroid carcinoma in acromegaly, by stimulating mitosis and inducing anti-apoptotic functions (WU et al., 2018). Additionally, acromegaly has been related to goiter as well as benign and malignant tumors (KALDRYMIDIS et al., 2016). Similarly, administration of recombinant GH and IGF-1 has been found to promote tumorigenesis in vivo (BOGUSZEWSKI et al., 2016). The results of the present study showed that circulating levels of IGFBP-3 increased after GH injection, which reduced IGF-1 bioactivity. This might be a reason why there were no histopathological changes, since the time required for malignant transformation is inversely correlated with IGF-1 bioactivity (SHANMUGALINGAM et al., 2016). As far as we know, this study is the first to examine the chronic short-term effects of GH injection on normal thyroid tissue. However, further study is needed to evaluate the long-term effects of GH injection on the thyroid and other tissues.

Moreover, in the current study, GH injection and consecutive elevated IGF-1 levels resulted in no mutations of the *BRAF* exon 17 gene. It has been shown that the standard Sanger sequencing method can be applied successfully to detect known somatic mutations, such as in this study, although comprehensive sequencing may be required by high throughput methods (S. H. LEE et al., 2017). After IGF-1 binds to its tyrosine receptor, it activates the GTPase Ras-Raf-ERK/MAPK pathway (DENDULURI et al., 2015). It has been shown that continuous and uncontrolled activation of the kinase pathway results in *BRAF*-V600E mutation (CARONIA et al., 2011). Studies have reported that excessive secretion of GH and

IGF-1 in patients with acromegaly caused thyroid cancer, in which activated *BRAF* gene mutations have been found (DABROWSKA et al., 2014). In benign and malignant human tumors, activating mutations of the serine-threonine kinase v-RAF murine sarcoma viral oncogene homolog B1 are prevalent and emerging as a potentially decisive biomarker (PAKNESHAN et al., 2013). Mutation of the *BRAF* gene is determined by the DNA-based method, most commonly by sequencing (CAPPER et al., 2011). Over 90% of *BRAF* mutations are transversions from thymine to adenine (T1799A), leading to a Glu for Val substitution at a mutational hotspot at amino acid position 600 (referred to as *BRAF* V600E). This substitution emulates the phosphorylation of amino acid residues T599 and S602, and induces a change in the activation segment, resulting in kinase activity of *BRAF* and consequently phosphorylation of downstream targets (HUSSAIN et al., 2015). Exclusively high *BRAF* V600E mutation rates have been detected in papillary thyroid carcinoma (40-70%) (S. E. LEE et al., 2017). Inconsistent with a crucial role in thyroid cancer initiation, *BRAF*-V600E has been shown to induce transformed features in thyroid follicular cells in culture (MELILLO et al., 2005) and thyroid carcinoma formation in transgenic mice (IVA et al., 2018). In this study, GH administration increased both IGF-1 and IGFBP-3 levels, which decreased IGF-1 bioavailability. Additionally, in previous studies that reported *BRAF* gene mutation or incidence of cancers in acromegaly patients, or people who were administered GH, the exposure time to high levels of GH was longer (five years or more) (UCHOA et al., 2013; WOLINSKI et al., 2017). Thus, these could be the reasons that we did not observe any histopathological changes or *BRAF* gene mutation in thyroid tissue. However, it is possible that mutation occurred in other tissues. Moreover, the examination of gene expression may help to explain more details about the effects of hormone administration.

In our study, resistance training decreased circulating levels of IGF-1 and the IGF-1/IGFBP-3 ratio, but circulating levels of IGFBP-3 did not change significantly. Over the years, the results of studies have demonstrated that high circulating levels of IGF-1 and low circulating levels of IGFBP-3 are associated with the risk of colorectal, breast, prostate and thyroid cancers (CLAYTON et al., 2011). Contrarily, epidemiological data show that physical activity is associated with a decreased risk in the incidence of many common cancers (FRIEDENREICH et al., 2010; GONCALVES et al., 2014; SAX et al., 2014). The decreased circulating levels of IGF-1 in the current study might be due to the anabolic role of IGF-1. It has been suggested that physical activity might increase uptake of IGF-1 into peripheral tissues and the central nervous system (NISHIJIMA et al., 2010). Studies have shown that, chronic exercise training causes an increase (STEIN et al., 2018), no change (VITIELLO et al., 1997) and a decrease (NISHIDA et al., 2010) in the circulating level of IGF-1, therefore are inconsistent. The results of previous studies were also inconsistent regarding the effects of exercise training on circulating levels of IGFBP-3 (BORST et al., 2001; MANNERKORPI et al., 2017). NINDL et al. (2010) showed a significant increase in IGFBP-3 levels after resistance exercise and a decrease to the baseline during the following 13 hours. NISHIDA et al. (2010) reported that after 6 weeks of aerobic training, circulating levels of IGF-1 decreased by 9% and IGFBP-3 levels did not change significantly. CHICHARRO et al. (2001) demonstrated that 3 weeks of endurance competition did not alter circulating levels of IGFBP-3, but decreased IGF-1 levels. The disparity in the results of the above studies might be due to the exercise training models, the duration of training and age, and the participants' training background and nutritional status (NINDL et al., 2010).

However, GH injection with and without resistance training increased the circulating levels of IGF-1 and IGFBP-3 and IGF-1/IGFBP-3 ratio. Binding of IGF-1 to its receptor activates ERK/MAPK and PI3K/AKT pathways, which stimulates proliferation, and inhibits apoptosis (DENDULURI et al., 2015). According to the role of IGF-1 as cell proliferator and apoptosis inhibitor, previous studies have indicated that increased circulating IGF-1 levels, decreased circulating IGFBP-3 and/or increased IGF-1/IGFBP-3 ratio are specific biomarkers and might be involved in some cancers by increasing IGF-1 bioavailability (CLAYTON et al., 2011; ENDOGENOUS et al., 2010; SOUBRY

et al., 2012). Therefore, GH administration might increase the risk of cancer in the long term. Further study is needed to examine the effects of long term GH administration on thyroid and other tissues.

Conclusion

In conclusion, short term GH administration with and without resistance training caused no changes in the histopathology of thyroid tissue, and no mutations were detected in the studied region of the *BRAF* gene. However, this does not imply that GH administration has no side effects on thyroid tissue or risk of cancer over a longer period of administration. In addition, resistance training decreased circulating levels of IGF-1 and the IGF-1/IGFBP-3 ratio, which could be noted as a lower risk of cancer. Nevertheless, short term GH administration increased circulating levels of IGF-1 and IGF-1/IGFBP-3 ratio, which is a situation with a higher risk of cancer.

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ROOZBEH, B., M. MOAZZAMI, A. RASHIDLAMIR, Z. MOOSAVI, A. JAVADMANESH: Učinak kratkotrajne primjene hormona rasta i treninga snage na histopatološke promjene tkiva štitnjače i mutaciju gena *BRAF* (T1799A) u smeđeg štakora (*Rattus norvegicus*). Vet. arhiv 90, 403-412, 2020.

SAŽETAK

Visoke razine hormona rasta povećavaju brzinu mitoze, ali smanjuju proces apoptoze u ciljnim organima, što može uzrokovati nastanak različitih tipova raka. Stoga je glavni cilj ovoga istraživanja bio utvrditi učinak kratkotrajne primjene hormona rasta i treninga snage na histopatološke promjene tkiva štitnjače i nalaz mutacije gena *BRAF*-V600E u mužjaka smeđeg štakora *Rattus norvegicus*. Ukupno 32 štakora nasumično su podijeljena u četiri skupine koje su ovisno o primjenjenom tretmanu označene kao CS (fiziološka otopina), RS (trening snage + fiziološka otopina), GI (hormone rasta, 2 mg/kg) i RG (trening snage+hormone rasta, 2 mg/kg). Nakon osam tjedana pokusa (i.m.) uzeti su uzorci tkiva štitnjače i uzorci krvi kako bi se procijenila histopatološka svojstva, mutacija *BRAF* T1799A tkiva štitnjače te razine cirkulacijskog IGF-1 i IGFBP-3. Trening se sastojao od toga da se štakori penju ljestvama noseći težinu (3 seta vježbi, 5 ponavljanja). Mikroskopska procjena tkiva štitnjače nije pokazala histopatološke promjene i nije bilo mutacija u promatranoj regiji sekvencija gena *BRAF*. Koncentracija serumskog IGF-1 bila je znakovito manja u skupini RS nego u ostalim skupinama ($P < 0,05$), no u toj skupini nije bilo znakovite promjene u koncentraciji serumskog IGFBP-3. Štoviše, koncentracije serumskog IGF-1 i IGFBP-3 bile su znakovito veće u skupinama GI i RG nego u ostalim skupinama ($P < 0,05$). Stoga smo zaključili da bi smanjenje koncentracije serumskog IGF-1 i IGF-1/IGFBP-3 nakon treninga snage moglo smanjiti rizik od raka. Osim toga kratkotrajna primjena hormona rasta, s treningom snage kao i bez njega, mogla bi povećati rizik od raka povećavajući koncentracije serumskog IGF-1 i IGF-1/IGFBP-3 u mužjaka štakora.

Ključne riječi: gen *BRAF* (T1799A); rak; hormon rasta; trening snage; štitnjača