Triiodothyronine improves age-induced glucose intolerance and increases the expression of sirtuin-1 and glucose transporter-4 in skeletal muscle of aged rats

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Abstract. To evaluate the potential beneficial impact and to clarify the underlying mechanisms of triiodothyronine (T₃) on glucose intolerance in aged rats. Rats were divided into adult group, aged group, and T₃-treated aged group (T₃-aged). T₃ was administered at a dose of 8 µg/kg body weight for 2 weeks. In comparison to adult group, aged rats presented significant higher levels of fasting insulin and homeostatic model assessment of insulin resistance (HOMA-IR). Glucose area under the curve (AUC), and peak glycemia, estimated from oral glucose tolerance curve, were significantly increased along with decreased mRNA expression of skeletal muscle sirtuin-1, glucose transporter-4 (GLUT-4) and uncoupling protein-3 (UCP-3) in aged versus adult group. T₃ administration significantly decreased the serum levels of fasting insulin, HOMA-IR, glucose AUC, and peak glycemia in T₃-aged versus aged rats. Skeletal muscle mRNA expression of sirtuin-1 and GLUT-4 were increased, whereas UCP-3 was not changed by T₃ administration. T₃ administration improved glucose intolerance, and decreased insulin resistance in aged rats. This was associated with upregulation of skeletal muscle sirtuin-1 and GLUT-4 which could mediate such beneficial effect.

Key words: Triiodothyronine — Aging — Glucose intolerance — Skeletal muscle — Sirtuin-1

Introduction

Glucose tolerance increasingly deteriorates with advancing age leading to high prevalence of impaired glucose tolerance and type 2 diabetes in elderly (Moreno et al. 2010). Such impairment is primarily due to decline in insulin sensitivity associated with diminished glucose utilization by peripheral tissues (Akintola and van Heemst 2015). Because skeletal muscle quantitatively is the main site of insulin responsive glucose uptake and metabolism (DeFronzo et al. 1981), the age-related maladaptive events in skeletal muscles may contribute partly to the observed insulin resistance (IR) in elderly populations.

Silent information regulator-1 (sirtuin-1, SIRT-1), NAD+-dependent protein deacetylase, is found in many tissues and is considered as a key metabolic regulator because it causes deacetylation of a wide range of protein substrates (Haigis and Sinclair, 2010). It has a crucial role in glycemic control via activating pathways involved in cellular energy metabolism, glucose uptake, and glucose production inhibition (Hardie 2013). Recent researches have concluded that during aging, decreased expression of SIRT-1 in skeletal muscle fundamentally mediates IR (Pardo and Boriek 2011), whereas, increased SIRT1 expression restrains age associated-diabetes (Yoshino et al. 2011), and enhances insulin sensitivity (Banks et al. 2008). Moreover, both human (Houmard et al. 1995), and animal studies (Mohamed et al. 2014) have reported a decline in the skeletal muscle glucose transporter-4 (GLUT-4) with aging. GLUT-4 is a glucose-transport protein that increases the cellular uptake of glucose and, therefore involves the whole body glucose homeostasis (Canto and Auwerx 2010). Additionally, uncoupling protein-3 (UCP-3), one of the family of inner mitochondrial membrane proteins that uncouple mitochondrial respiration (Koshkin et al. 2004), is highly expressed in skeletal muscles and is able to increase glucose utilization rate (Krook et al. 1998). The levels of this protein have been also shown to decline with age (Kerner...
et al. 2001). Early treatment of age-related IR is crucial for alleviating or preventing the hyperglycemic complications and decreasing the costs of medical care.

Thyroid hormones have an important physiological role in glucose metabolism of almost all body cells. These functions are mediated by activating the nuclear transcription of large numbers of genes that further stimulate the synthesis of protein enzymes, structural proteins, and transport proteins (Brenta 2010). Although both hyperthyroidism and hypothyroidism have been related to disturbances in insulin signaling and glucose metabolism (Teixeira et al. 2012), exogenous thyroid hormone administration in IR and glucose intolerance conditions have recently attracted considerable attention with favorable outcomes. Accumulating recent reports have shown that exogenous triiodothyronine (T3) administration can decrease glycemia and improve insulin signaling in diabetic (Prieto-Almeida et al. 2018) and obese rats (Panveloski-Costa et al. 2018). It has been also demonstrated that T3 is able to enhance insulin-stimulated glucose transport and glycolysis in skeletal muscles (Moreno et al. 2011). Interestingly, Vazquez-Anaya et al. (2017) reported an improvement of glucose intolerance and IR by thyroxine (T4) treatment in obese rats. Vazquez-Anaya and colleagues attributed these beneficial effects to the capability of T4 to upregulate the expression of SIRT-1 and UCP-2 in skeletal muscle. On the other hand, a number of studies have failed to show a protective effect of thyroid hormone against glucose intolerance. Lambadiari et al. (2011) have investigated the effect of variations in thyroid hormone plasma levels (within the normal euthyroid range) on insulin sensitivity in humans and they recorded a positive relationship between high levels of thyroid hormone and IR. The authors added, thyroid hormone plays a role in mediating metabolic derangement early in diabetes. Similarly, in another human study Jing et al. (2014) recorded an increase in the prevalence of impaired fasting glucose and glucose intolerance in association with high free T3 levels. This has led to some uncertainty regarding the appropriate recommendation for thyroid hormone use and further studies are required to elucidate the valuable impact of thyroid hormone on glucose intolerance. Thus, the present work was designed to evaluate the potential beneficial effect and the underlying mechanisms of triiodothyronine on glucose intolerance in aged rats.

Materials and Methods

Animals

Seven young adult (3–4 month old, body weight 190 ± 10 g) and 14 aged (20–24 month old, body weight 405.2 ± 22.4 g) male Wister rats were obtained from Helwan Farm, Cairo, Egypt. Rats were maintained under regular 12 h/12 h day/night cycle in the Medical Research Center, Faculty of Medicine, Ain Shams University. Rats were fed standard rat chow and allowed free access to food and water. All experimental procedures were carried out according to the guidelines of FMSAU, REC (Faculty of Medicine, Ain Shams University, Research Ethics Committee, Cairo, Egypt) which conforms to the Guide for the Care and Use of Laboratory Animals published by United States National Institute of Health.

Experimental design

Rats were divided randomly into three groups: 1) adult group (n = 7; rats received intraperitoneal (i.p.) injection of the hormone vehicle); 2) aged group (n = 7; rats received i.p. of the hormone vehicle), and 3) T3-aged group (n = 7; triiodothyronine-treated aged group – rats received 3,3',5-triiodo-L-thyronine) (Sigma, St. Louis, MO, USA) which was dissolved in 1 N of NaOH/isotonic saline and given i.p. at a dose of 8 µg/kg body weight per day for 2 weeks) (Vazquez-Anaya et al. 2017). Body weight was measured at the beginning (BW1) and at the end (BW2) of the experiment. On 13th day an overnight fasting started at 9 p.m. On the 14th day an oral glucose tolerance test (OGTT) was performed in overnight fasted rats at 9 a.m. then rats were allowed free access to food and water and at 9 p.m. the overnight fasting started again. On the 15th day, at 9 a.m. the rats were weighed and injected intraperitoneally with sodium thiopental (40 mg/kg). A midline abdominal incision was made, the abdominal aorta was exposed and cannulated with a polyethylene catheter, and a blood sample was collected in a plastic tube. Blood was allowed to coagulate, at room temperature, centrifuged at 3000 rpm for 15 min and serum was stored at –20°C, till used for estimation of serum levels of free triiodothyronine (FT3), fasting glucose and insulin levels. The gastrocnemius muscle was extracted for determination of GLUT-4, UCP-3, and SIRT-1.

Oral glucose tolerance test

OGTT was performed in overnight fasted rats as previously described by (Okada et al. 2017). Rats received 1 g/kg glucose by gavage and rats’ blood samples were taken intermittently from the tails and glucose levels were measured with a glucometer (Glucostar, Medland Co., Middle East. Gulf & Africa).

Analytical procedures and data analysis

The glucose area under the curve was calculated by trapezoid method (Purves 1992). Peak glycemia was calculated from OGTT. The homeostatic model assessments (HOMA) for determining IR was calculated according to Zhou et al. (2006) as follow: HOMA-IR (a measure for IR) = [(fasting insulin (mU/l) x fasting glucose (mg/dl)) x 0.0555]/22.5.
Biochemical analysis

Fasting serum glucose was measured using oxidase-peroxidase method (Trinder 1969). Serum levels of insulin and FT$_3$ were analyzed using enzyme-linked immunosorbent assay ELISA (Dako, Carpinteria, CA, and Cusabio, USA, respectively) according to the manufacturer's instructions.

Detection of relative gene expression by real time PCR

Total RNA extraction

Total RNA was extracted from tissue homogenate using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instruction. The RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

Complementary DNA (cDNA) synthesis

The cDNA was synthesized from 1 μg RNA using Super Script III First-Strand Synthesis System as described in the manufacturer's protocol (#K1621, Fermentas, Waltham, MA, USA). In brief, 1 μg of total RNA was mixed with 50 μM oligo (dT) 20, 50 ng/μl random primers, and 10 mM dNTP mix in a total volume of 10 μl. The mixture was incubated at 56°C for 5 min, and then placed on ice for 3 min. The reverse transcriptase (RT) master mix containing 2 μl of 10×RT buffer, 4 μl of 25 mM MgCl$_2$, 2 μl of 0.1 M DTT, and 1 μl of Super Script® III RT (200 U/μl) was added to the mixture and was incubated at 25°C for 10 min followed by 50 min at 50°C.

Real-time quantitative PCR

Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (Step One®, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs were designed with Gene Runner Software (Hasting Software, Inc, Hasting, NY) from RNA sequences from the gene bank. All primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR was performed in a 25-μl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer and 2 μl of cDNA. Amplification conditions were: 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation for 15 s and annealing/extension at 60°C for 10 min. Data from real-time assays were calculated using the v1 7 sequence detection software from PE Biosystems (Foster City, CA). Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalized to beta actin which was used as the control housekeeping gene and reported as fold change over background levels detected in the diseased groups. The sequences of PCR primer pairs used for each gene are listed as follows: SIRT-1 (Forward primer: 5’- TTGGCACCAGTCCCTGGAAC-3’, Reverse primer 5’- CCCAGCTCCAGTCAGGAACTAT-3’); UCP-3 (Forward primer :5’- AGAACATCGCAGGGAGGAAGGA-3’, Reverse primer:5’- CACGGGGAGGGCCACCACTGT-3’); GLUT-4 (Forward primer: 5’ GAGCCTGATGCTAAATGGAG3’, Reverse primer: 5’GAGAGAGACGTCCATATGTC3); Beta actin (Forward primer :5’-TGTTTGAGACCTTCAACACC-3’, Reverse primer:5’ CGCTCATTGC-GATAGTGAT-3’).

Statistical analysis

All variables were presented as mean ± standard deviation (SD). The one-sample Kolmogorov-Smirnov test was used to test for normality of variables and all variables are normally distributed. One way ANOVA was done to determine the differences between groups and in case of significant “F” test ($p < 0.05$), further analysis was made by LSD to find inter-group significance.

Results

Body weight and serum levels of FT$_3$

As demonstrated in Table 1, the body weight of T$_3$-aged rats was not significantly different from that of aged group. The serum levels of T$_3$ were significantly higher in T$_3$-aged group compared to aged rats.

Glucose regulation and IR

In comparison to adult group, aged rats presented significantly higher levels of fasting insulin, and HOMA-IR. Also, the fasting glucose was higher in aged versus adult group, though it was statistically insignificant. T$_3$ administration in T$_3$-aged rats decreased the levels of fasting insulin, fasting glucose, and HOMA-IR compared to aged rats. All were statistically significant except for fasting glucose level (Table 1). In comparison to adult group, fasting insulin, and HOMA-IR were still significantly higher in T$_3$-aged group compared to aged rats.
Table 1. Changes in initial (BW$_1$) and final (BW$_2$) body weights, serum levels of free triiodothyronine (FT$_3$), fasting glucose, fasting insulin, and homeostatic model assessment of insulin resistance (HOMA-IR) in the three study groups

| Group         | Adult  | Aged   | T$_3$-Aged |
|---------------|--------|--------|------------|
| BW$_1$ (g)    | 190 ± 10 | 405.3 ± 21.9* | 405 ± 25.4* |
| BW$_2$ (g)    | 202.4 ± 11.5 | 429 ± 11.6* | 421.3 ± 18* |
| FT$_3$ (ng/dl)| 2.96 ± 0.4  | 2.44 ± 0.6  | 3.1 ± 0.7*  |
| Fasting glucose (mg/dl) | 95.1 ± 5.6 | 105 ± 13.7  | 95.4 ± 3.3  |
| Fasting insulin (ng/ml)  | 1.36 ± 0.2  | 1.84 ± 0.1*  | 1.62 ± 0.1±* |
| HOMA-IR       | 9.8 ± 2.2  | 13.6 ± 0.9*  | 10.3 ± 0.4* |

Data are expressed as mean ± SD, n = 7 per each group; * p < 0.05 vs. adult group, ± p < 0.05 vs. aged group. T$_3$-Aged, triiodothyronine-treated aged group.

Discussion

The main finding of the present study was the attenuation of glucose intolerance and IR along with enhancement of insulin sensitivity by T$_3$ administration in aged rats. Skeletal muscle levels of SIRT-1 and GLUT-4 expression were also increased and proposed as possible mechanisms mediating such favorable metabolic effects. Moreover, UCP-3 expression in skeletal muscle was not significantly affected by T$_3$ administration.

The results of the present study revealed a significant increase in HOMA-IR in aged versus adult rats, implying high IR. This finding was further supported by the significant fasting hyperinsulinemia in aged rats which seems to be a compensatory mechanism to maintain glucose homeostasis in light of high IR (Chang and Halter 2003). Additionally, the glucose clearance was slower in aged versus adult rats, so that the glucose AUC as well as the peak glycemia were significantly higher in aged group. These findings agree with previous studies (Moreno et al. 2010) and point to high IR and glucose intolerance in aged rats.

Although the results of fasting glucose were insignificant, they showed a tendency toward impairment of both fasting glucose and glucose tolerance in aged versus adult rats. The fasting glucose in aged rats (whether measured before OGTT (111.7 ± 2.8) or immediately before sacrifice (105 ± 13.7)) and the 2 h glucose obtained by OGTT (149.2 ± 9.9) approached the values of impaired fasting glucose (fasting glucose ≥ 100 mg/dl and < 126 mg/dl) and impaired glucose tolerance (2 h plasma glucose ≥ 140 mg/dl and < 200 mg/dl) (Zhou et al. 2006), which might point to impaired glucose regulation or prediabetes in aged rats.

Several evidences support the hypothesis that during aging the skeletal muscle is the principle site where the early metabolic disturbances leading to IR take place: 1) Age-associated decrease in the skeletal muscle expression of SIRT-1 (Mohamed et al. 2014), and AMP-activated protein kinase (AMPK) (Qiang et al. 2007) were found to promote IR and glucose intolerance (Jing et al. 2008; Jayanthy et al. 2017). 2) Diminished expression of GLUT-4 in aged skeletal muscle (Larkin et al. 2001) was reported to decrease the capacity of glucose uptake which lead to IR and diabetes (Kern et al. 1992; Stenbit et al. 1996). 3) Lower levels of UCP-3 in aged skeletal muscle (Kerner et al. 2001), were supposed to reduce the glucose utilization rate (Krook et al. 1998). In line with this assumption, the present results showed a downregulation of skeletal muscle levels of SIRT-1, GLUT-4, and UCP-3 mRNA expression in concomitance with significant glucose intolerance and IR in aged versus adult rats.

T$_3$ administration, in the current work, decreased levels of fasting insulin, HOMA-IR, glucose AUC, and peak glycemia in association with upregulation of skeletal muscle SIRT-1 mRNA expression. Such results suggest that T$_3$ administration improves glucose tolerance and IR in aged rats partly via increasing expression of muscle SIRT-1. This inference is in agreement with previous studies (Vázquez-Anaya et al.
Triiodothyronine improves age-induced glucose intolerance (Liu and Brent 2010; Nogueiras et al. 2012).

SIRT-1, through its dependent transduction pathways, is a well-established insulin sensitizer (Schenk et al. 2011). It modulates glucose metabolism by influencing insulin signaling (Pulla et al. 2012; Luu et al. 2013). Also, AMP-activated protein kinase (AMPK), is an energy sensor that positively modulates insulin-dependent glucose uptake (Jing et al. 2008). SIRT-1 and AMPK are reported to activate each other and also, have a crucial role in the glycemic control induced by thyroid hormones (de Lange et al. 2008). Thyroxine is able to upregulate muscle SIRT-1 expression (Vazquez-Anaya et al. 2017), and T3 can activate AMPK either directly (Lombardi et al. 2009) or indirectly via stimulating SIRT-1 (de Lange et al. 2011). Moreover, T3 administration in animal models rapidly phosphorylates and activates AMPK in muscle (Irrcher et al. 2008; Lombardi et al. 2009). The interaction between SIRT-1 and AMPK activates pathways that control cellular energy metabolism, mitochondrial biogenesis and glucose uptake and inhibit glucose production (Hardie 2013). Upregulation of SIRT-1 increases insulin-stimulated phosphoinositide 3-kinase signaling and glucose uptake, improving insulin sensitivity (Schenk et al. 2011). Also, activation of AMPK phosphorylates insulin receptor substrate-1 Ser-789 which, in turn, enhances insulin signaling (Jakobsen et al. 2001). Accordingly, the ability of T3 to upregulate muscle level of SIRT-1, herein, might be a fundamental path to modulate glucose homeostasis, improving IR.

Another important mechanism by which T3 administration, in the current study, could improve glucose tolerance was the significant increase in GLUT-4 expression in skeletal muscle of T3-aged versus aged rats. This postulate is in accord with previous studies (de Lange et al. 2008). The main cellular mechanism which decreases blood glucose after glucose load is insulin-stimulated glucose transport into skeletal muscle. The principle glucose transporter that mediates this uptake is GLUT-4, hence it is a key determinant of glucose disposal (Larkin et al. 2001). Defective expression of the gene encoding for GLUT-4 in skeletal muscle is one possible mechanism of insulin resistance (Bell et al. 1989). Decreased GLUT-4 mRNA levels was reported in streptozotocin-induced diabetes in rats (Berger et al. 1989; Garvey et al. 1989) and in insulin-resistant patients (Dohm et al. 1991). Transgenic ablation of GLUT-4 in muscle also results in insulin resistance.

![Figure 1](image-url). Oral glucose tolerance test (A), area under the curve (AUC, B), and peak glycemia (C) in the three study groups. Data are expressed as mean ± SD, n = 7; * p < 0.05 versus adult group, # p < 0.05 versus aged group. T3-Aged, triiodothyronine-treated aged group.
and impaired glucose tolerance (Zisman et al. 2000). Neufer et al. (1993) concluded that decreased rate of synthesis of skeletal muscle GLUT-4 mRNA (gene transcription) mediates insulin resistance in streptozotocin-induced diabetic animals. Moreover, diabetic rats presented severe reduc-
tion in glucose transport capacity associated with reduced skeletal muscle GLUT-4 protein content (Barnard et al. 1990). In contrast, the increase in muscle GLUT-4 protein expression enhanced insulin sensitivity in vitro (Charron and Kahn 1990). Furthermore, overexpression of GLUT-4 in muscle of genetically diabetic mice attenuates insulin resistance and promotes glycemic control by increasing both basal and insulin-stimulated glucose transport (Gibbs et al. 1995). Accordingly, these reports denote that skeletal muscle glucose transport is a rate-limiting step for whole body glucose disposal and suggest that the upregulation of GLUT-4 expression in T3-aged group might partly alleviate insulin resistance. The T3-induced upregulation of GLUT-4, herein, might be attributed to the increased muscle SIRT-1 expression which in turn activates AMPK, leading to enhancement of GLUT-4 translocation from intracellular vesicles into the cell membrane (Koistinen et al. 2003).

UCP-3 is an inner mitochondrial membrane protein principally present in brown adipocytes, cardiac and skeletal muscles (Boss et al. 1997; Vidal-Puig et al. 1997). The potential protective effect of UCP-3 in limiting IR is not well understood, although some data suggest a role in the fatty acids metabolism (Khalfallah et al. 2000) as well as in alleviating reactive oxygen species production (Vidal-Puig et al. 2000). Krool et al. recorded a decrease in skeletal muscle UCP-3 content in the prediabetic state with impaired glucose tolerance (Krook et al. 1998). Moreover, 50% lower levels of UCP-3 were found in skeletal muscles of type 2 diabetic patient compared to healthy controls (Schrauwen et al. 2001). A direct relationship was demonstrated between the levels of UCP-3 in muscle and the rate of insulin activated glucose utilization (Krook et al. 1998). Transgenic upregulation of UCP-3 in muscles of mice was reported to decrease adiposity, fasting plasma glucose and insulin levels with increased glucose clearance rate (Clapham et al. 2000). T3 administration in the present study non significantly affects the muscle UCP-3 expression in T3-aged versus aged rats. Inconsistent with the present finding, both T3 and T2 upregulated skeletal muscle UCP-3 in hypothyroid rat models (Lanni et al. 1999; de Lange et al. 2001). Hyperinsulinemia is known to suppress the skeletal muscle UCP-3 (Harmancey et al. 2015). Additionally, SIRT-1 can repress the glucocorticoid induced UCP-3 gene expression in skeletal muscle (Amat et al. 2007). Therefore, the inability of T3 administration, herein, to upregulate UCP-3 might be explained by the high fasting insulin and/or the upregulation of SIRT-1 in T3-aged group.

Although 2 weeks of T3 administration ameliorated glucose intolerance and IR in the present study, it did not affect UCP-3 or completely normalize the fasting insulin, glucose AUC, peak glycemia, and muscle expression of GLUT-4, and SIRT-1 in comparison to adult rats. A higher dose and/or longer duration of T3 may be required to fully correct such changes.

Figure 2. Relative mRNA expression of Sirtuin-1 (SIRT-1), glucose transporter-4 (GLUT-4), and uncoupling protein-3 (UCP-3) in the three study groups. Data are expressed as mean ± SD, n = 7; * p < 0.05 versus adult group, # p < 0.05 versus aged group. T3-Aged, triiodothyronine-treated aged group.
This study has some limitations. Firstly, measurement of heart rate would be helpful to detect the potential development of exogenous hyperthyroidism. However, in the present study there was no detectable decrease in the body weight of T3-aged versus aged rats. Furthermore, the FT3 levels were not significantly different in T3-aged versus adult group. Secondly, performing insulin tolerance test would be convenient to investigate the insulin response to glucose load, particularly the insulin response to oral glucose load was reported to diminish with age (Muller et al. 1996). Thirdly, the use of sodium thiopental in anesthesia might increase insulin secretion from the pancreas (Dou et al. 2012). However, thiopental was given to the 3 study groups and serum levels of fasting insulin showed a significant increase in aged rats versus adult group, and a significant decrease in T3-aged versus aged rats, notifying a favorable impact of T3 on insulin resistance. Also, the two follow-on overnight fasting procedure might suppress GLUT-4 (Olson 2012). Nevertheless, skeletal muscle mRNA expression of GLUT-4, herein, showed a significant decrease in aged versus adult rats and a significant increase in T3-aged versus aged rats, confirming a role of T3 in upregulating GLUT-4. Fourthly, it would be very suitable to determine plasma membrane GLUT-4 protein under basal and insulin stimulated conditions because they are the best markers of muscle glucose uptake, reflecting the real muscle insulin sensitivity (Olson 2012).

**Conclusion**

This study provides evidence to a potential benefit of T3 on glucose homeostasis in the process of aging. The present data show that T3 administration in aged rats significantly improved glucose tolerance, and decreased IR. These might be attributed in part to the upregulation of skeletal muscle SIRT-1, and GLUT-4 mRNA expression. Skeletal muscle UCP-3 mRNA expression is not significantly changed. Further exploration of the proper T3 dose and/or duration, taking into consideration the risk of exogenous hyperthyroidism, could yield more promising results in the process of aging.

**Conflict of interest.** The author declares that there are no conflicts of interest.

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Triiodothyronine improves age-induced glucose intolerance

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