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IMPROVING THE MITOCHONDRIAL FUNCTIONS OF MALE RATS PITUITARY GLAND USING HYDROXYTYROSOL

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ABSTRACT : Hydroxytyrosol (HT) ((3,4-Dihydroxyphenyl)ethanol) compound, a natural polyphenol is well-known for health improving antioxidants characteristics. These characteristics are mainly attributed to the ability of HT to deplete the reactive oxygen species (ROS) and improve the endogenous antioxidant of the systems. Furthermore, increasing the intake of oxygen and hydroxytyrosol together enhances the oxidative role of HT to produce energy. Therefore, we are trying to understand the improvements in the mitochondrial functions of male rats pituitary gland due to HT. Thus, 60 male Wister rats, about 3-4 months old and 240±15 gr of weight were divided randomly into three equal groups. The animals were fed on ordinary pelleted diet and subjected to the same ventilation and living conditions. The first group, so-called control (C) was not given any treatment. The second group (T1) and the third group (T2) were subjected to daily oral administration of HT, 10 and 50 mg/kg respectively. Our results shows that there is a significant increase (P≤0.05) in Pitx1 gene expression and the western blot of complex1 (NADH-dehydrogenase) in T1 and T2 groups. Such significant increment supports the positive impact of HT on the mitochondrial functions.

Key words : Hydroxytyrosol, male rats, mitochondria, pituitary.

INTRODUCTION

The mitochondrial functions strongly affect the health of the living body. For instance, researchers have proved thattype two diabetes mellitus (T2DM) mainly results from the metabolic syndrome (MetS) due to mitochondrial dysfunction (Stump *et al*, 2003; Petersen *et al*, 2003). Furthermore, insulin resistance and obese controlled by the mitochondrial function could also be modified by the impact of oxidative stress and the biogenesis of mitochondria (Ritz and Berrut, 2005). In the meanwhile, Morino *et al* (2005) have demonstrated a direct correlation between the mitochondrial oxidative capacity and mitochondrial genome expression. In other words, the inhabitance of the mitochondrial genome expression results from the reduction in the mitochondrial oxidative capacity.

One suggested solution to overcome the negative issues presented in the mitochondria is the use of hydroxytyrosol (HT) ((3,4-Dihydroxyphenyl)ethanol). HT is a natural polyphenol that is majorly extracted from extra virgin olive oil and it is also well-known for its effective health improve antioxidants characteristics. These characteristics are attributed to the ability of hydroxytyrosol to deplete reactive oxygen species (ROS) and improve endogenous antioxidant (Stupans *et al*, 2002). Not only that but also HT is famous for improving the mitochondrial oxidative metabolism, acid content of the mitochondrial deoxyribonucleic (Hao *et al*, 2010; Zheng *et al*, 2015a), membrane potential (Bali *et al*, 2014), membrane density (Hao *et al*, 2010) and gene expression of proteins like PGC-1α and Nrf2 that support the respiration and biogenesis of mitochondria (Hao *et al*, 2010; Zhu *et al*, 2010). Furthermore, increasing the intake of oxygen with the use of hydroxytyrosol indicates the ascended oxidative role of HT to produce adenosine triphosphate (ATP) (Cao *et al*, 2014; Hao *et al*, 2010). The strengthening of HT to the mitochondria activity (Granados-Principal *et al*, 2014; Zheng *et al*, 2015b) is attributed to the enhancing of the mitochondrial expression of the complex subunit (Cao *et al*, 2014; Zheng *et al*, 2015b) and protein (Zhu *et al*, 2010).

Thus, we focused on the pituitary homeobox 1 (Pitx-1) gene since it is synthesized in all cells of the pituitary. Pitx-1 is also responsible for building pituitary-originated hormones, namely gonadotropins,prolactin, and pro-opiomelanocortin (Lamonerie *et al*, 1996). As Jeong *et al* (2004) have shown,Pitx-1 may take two approaches to activate gene expression in pituitary. The first approach is by building DNA directly and the second way is through

protein-protein interaction. For instance,Pitx-1 can activate mGnRHR gene via the first approach.

Keeping the above mentioned facts in mind, the aim of this project is to improve the functions of the mitochondria in male Wister rats using hydroxytyrosol. Researchers thought that hydroxytyrosol is able to increase the mitochondrial functions via enhancing the mitochondrial complex subunit expression and activity (Cao *et al*, 2014). Therefore, we investigated the protective effect of hydroxytyrosol on pituitary tissues via assessing the gene expression levels of mRNA-Pituitary homobox1 (Pitx1) and on the mitochondrial functions by assessing the complex1 NADH-dehydrogenase.

MATERIAL AND METHODS

Experimental animals

Sixty male Wisterrats,age between three to four months and weight of about 240±15 gr were randomly divided into three equal groups, 20 rats per each group. All three groups had daily been fed on basal diet and given normal water for two months. The first group, control group (C), had been given no HT along the entire time of the experiment. However, the second group (T_1) and third group (T_2) were subjected to daily oral administration of HT with 10 mg/kg and 50 mg/kg, respectively. Furthermore, the living conditions of all three groups were also controlled via dividing each main group into five sub-groups, housing each sub-group in an isolated cage, controlling the temperature of the laboratory to be within 25 ± 2^0C and finally maintaining twenty-four-hour cycle which includes twelve-hour light and twelve-hour darkness.

Preparation of hydroxytyrosol

A 50-gram container of Hydroxytyrosol was obtained from Transhuman Technologies, UK. The low (10mg/kg) and high (50 mg/kg) doses of HT were daily prepared by dissolving the required amount in distal water at room temperature and keeping it in dark bottle.

Total RNA extraction

Total RNA was extracted from pituitary samples using Accuzol® reagent kit, Bioneer, Korea. The extraction has been done according to instructions of the company. These instructions include homogenizing 10mg of pituitary tissue by adding 1000 ìl of TRIzol® reagent. Next, we added 200ìl of chloroform to each tube, shook it for about 15 seconds, incubated the mixture on ice for fifteen minutes, and finally centrifuged at 12,000 rpm and 4°C for fifteen minute. Later, we transferred the resultant supernatant into new Eppendorf tubes and added 500ì l isopropanol. The new mixture was also mixed, incubated and centrifuged (at 12,000 rpm and 4°C) for 10 minutes. After that, we discarded the second supernatant, and added 1ml of 0.8 Ethanol concentration solution, mixed via the vortex, centrifuged at 12,000 rpmand 4°C for five minutes.We discarded the third supernatant and left the RNA pellet to dry in air. Finally, we dissolved the RNA pellet using 100ìlfree nuclease water per sample in order to extracted RNA, which was stored at -20°C.

We used Nanodrop spectrophotometer (THERMO, USA) to assess and measure the extracted RNA, which is purified from any traces of genomic DNA using DNase I enzyme kit. The purification procedure is performedusing 10 µl of total RNA 100 ng/µl, 1 µl of DNase I enzyme, 4 µl of 10X buffer, and 5 µl ofDEPC water according to Promega Company, USA. Next, we incubated the mixture for 30 minutes at 37°C. Then, we inactivated the DNase enzyme action by adding 1 ìl stop reaction solution and incubating the mixture for 10 minutes at 65°C. The RNA samples were treated with DNase-I enzyme to synthesis cDNA according to AccuPower® Rockt Script RT PreMix kit that provided from Bioneer Company, Korea. The substances used in this step are (8 µl) total RNA 100 ng/µl (1 µl) random Hexamer primer, and (1 µl) DEPC water.

This RT PreMix was placed in Accu Power Rocket Script RT PreMix tubes that contains lyophilized reverse transcription enzyme. Then, the tubes were undergone a complete dissolution through the vortex and spinning down. The result was cDNA converted from RNA via thermocycler.

The quantification of pituitary homobox1 (Pitx1) gene was performed using quantitative Real-Time PCR (qPCR). The corresponding gene expression analysis was calculated via Livak method through ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). The qPCR reaction was done on a RealTime PCR system (BioRad, USA) using SYBER Green dye qPCR master mix. The master mix is then used in detection and amplification oftarget gene (Pitx1) andhousekeeping gene (â-actin). Primers, illustrated in Table 1 were designed using NCBI-Gene Bank database and Primer 3 design online. The following primers were used in our research: Ptx1 gene (Forward) TTTCACAAGCCAGCAGTTGC and (reverse) TCTCCTCTCTCATGCTCATGTC; â actin (Forward) AACACGGCATTGTCACCAAC and (reverse) TTTTCACGGTTGGCCTTAGG.

The qPCR master mix was prepared for Pixt1 target gene and â-actin housekeeping gene in accordance with AccuPowerTM 2XGreen Star qPCR master mix kit,

Bioneer, Korea. The instructions are illustrated in Table 1.

Then, the components of the qPCR master mix reaction were placed in qPCR white tube strips, mixed for 3 minutes, and placed in Miniopticon Real-Time PCR system, BioRad, USA. The thermocycler conditions are shown in Table 2.

Western Blot Enzyme detection

Western blot was performed for detection and quantification target enzyme (complex 1 NADH: ubiquinone oxidoreductase) and normalized with housekeeping gene β-actin enzyme. This method was carried out according to method perviously described by Zheng *et al* (2015b). Western blot method was done according to company instructions (Elabscience, USA), which are well explained in ESI file.

RESULTS AND DISCUSSION

Relative Expression Of pituitary homobox1 Gene

The results of the current study showthat there was a significant (P≤ 0.05) increase in the fold change of the gene expression level in treated groups, (T_1) and (T_2). In contrast, there was a significant decrease in the fold change of the gene expression level in the control (C) group (P≤ 0.05). We have also seen that group T_1 and T_2 shows higher significant difference (P<0.05) when they are compared with the control group. Moreover, by comparing between the two treatment groups, the results showed that the significant difference of T_2 overcomes T_1 with the values 7.786±0.947 pg/ml and 4.990±0.464 pg/ml, respectively. These results are illustrated in the Table 3.

The real time quantitative PCR (RT-qPCR) technique has been used to investigate the gene expression in the pituitary gland of the male rats by measuring the amplification rate of the target gene (pituitary homobox1 Gene(pitx1)) and housekeeping gene (â actin), as shown in Figs. 1 and 2, respectively. Furthermore, we have also conducted a statistical analysis to calculate the fold change of the two genes as shown in Fig. 3. It should be mentioned that the RT-qPCR method depends on the value of the threshold cycle (Ct), which is the value of the intersection between the reaction line and the threshold line. The threshold line is a horizontal level separates between a clear detection of the gene (above the line) and a vague detection (under the line). The reaction line is the curved course of the amplification of the gene that starts from zero, takes almost a straight line, crosses the threshold line at the Ct point and finally reaches the top to continue at a plateau level to the end of the cycles of the RT-qPCR, the curves in Figs. 1 and 2.

Table 1 : qPCR master mix preparation.

qPCR master mix	Volume (µL)
cDNA template (100ng)	2.5
Forward primer (10pmol)	1
Reverse primer (10pmol)	1
qPCR Master Mix	10
DEPC water	5.5
Total	20

Table 2 : MiniopticonReal-Time PCR thermocycler conditions.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\Extension Detection (scan)	60 °C	30 sec	

Table 3 : Effect of hydroxytyrosol on gene expression pitx1 gene in adult males Wister rats.

Groups	Fold change mRNA transcript level
C	1.141±0.199 A
T1	4.990±0.464 BC
T2	7.786±0.947 BC
$LSD_{0.05}$	1.797

Table 4 : Relative protein expression of complex1.

Groups	Relative expression of complex1
C	40.67 ± 6.64 A
T1	123 ± 16.5 BC
T2	236.67 ± 29.29 BC
$LSD_{0.05}$	55.20

Returning to Figs. 1 and 2, where they show the number of florescent detections versus the number of cycles for the target gene and reference gene, respectively. In addition, the color code of the detections was set to be blue for the control group (C), green for the first treatment (T_1) and red for the second treatment (T_2). From the figures, the Ct value of the target gene spreads over a wide range of cycles from 28 to 38, while the reference gene shows a narrow range from 32 to 34 cycles. The value of Ct associates the duplication rate of the gene. In other words, high value of Ct represents a low concentration of the protein, while the low value of Ct signifies a high concentration of the gene. Therefore, the wide window of Ct for pitx1 gene reflects the variation of the concentration in the samples. This variation is considered normal since the collected sample may differ in volume, concentration, animal, and collection method; although we tried hard to minimize the number of variables.

The daily diet of the Mediterranean region is well

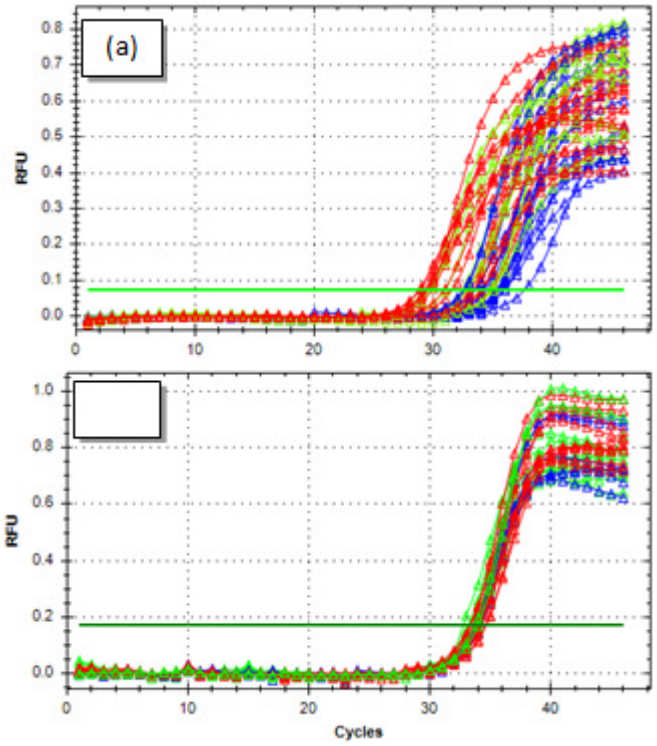

Fig. 1 : Real time PCR amplification plot for pituitary (a) homobox1Gene (pitx1) and (b) housekeeping gene (â actin) in pituitary gland tissue of male rats that show difference in threshold cycle numbers (Ct value) between treatment and control groups. Blue curves: Control group(C) was not given any treatment. Green curves stand for the first treated Group (T1) which was subjected toa low-dose of HT (10 mg/kg). The red lines refer to the second treated Group(T2) that was drenched a high-dose of HT (50 mg /kg).

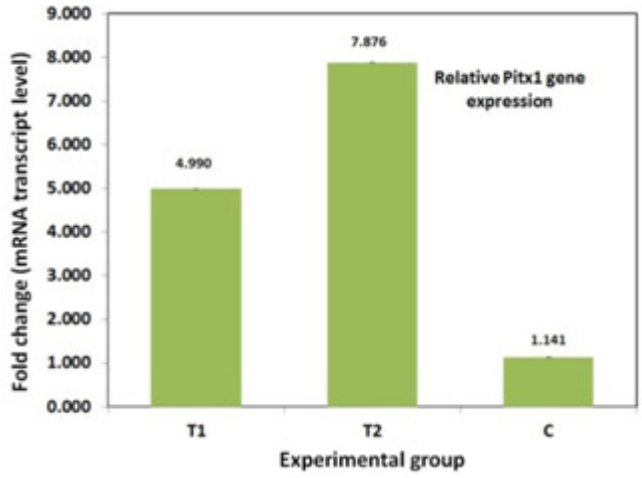

Fig. 2 : The statistical fold change variation of Pitx1 gene expression. The experimental groups are: (i) control group (C), (ii) low-dose treated group (T1) and (iii) high-dose treated group (T2).

known for being one of the best diet to follow. One reason for its excellence is the olive oil which is considered as a major ingredient and help to considerably reduce the declination of the cognition (Lourida *et al*, 2013). In spite of its importance, one can see that there is a small number

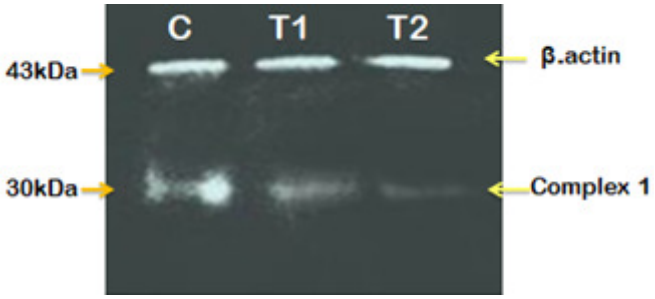

Fig. 3 : Western blot image that showed the mitochondrial Complex I (NADH Coenzyme Q oxidoreductase) in Rats pituitary tissue normalized with housekeeping â-actin protein. Where, C: control group rats, T1: low dose hydroxytyrosol 10mg/kg BW. and T2: high dose hydroxytyrosol 50mg/kg BW. Complex 1 enzyme at 30kDa and â-actin protein 43kDa.

of studies confirmed in the relation between the antioxidant character of, for instance, polyphenolsand the significant role of the diet. Thus, as far as we know, the main beneficent ingredients and their mechanisms are inaccurately characterized. One component, highly found in olive oil, is the hydroxytyrosol (HT) polyphenol. The anti-inûammatory, anti-cancer and anti-obesity character of HT had urged us to investigate the role of HT in defining the gene expression of the pituitary via pituitary homobox1 (pitx1) gene. The positive impact of HT has been also confirmed by the study of Zheng *et al* (2015), who showed that HT enriched diet would enhance the gene expression levels of activity regulated cytoskeleton associated protein (Arc), which exists in the brain of the mice, as it is compared with results of the control group (Zheng *et al*, 2015b). Moreover, Al-Meramdhi 2019 has shown very recently that HT can regulate the blood pressure by increasing the expression of Atrial Natriuretic Peptide (ANP) gene in the heart of the rats (Al Meramdhi, 2019). In the same direction, our results reveals that a diet consists of HT may also increase the gene expression of pitx1 gene in the pituitary gland of the rats which proves the beneficial property of HT.

Pituitary homeobox 1 (Ptx1) was discovered in the gene expression process of proopiomelanocortin (POMC) as transcription factor (Lamonerie *et al*, 1996). The homeodomain transcription factor gene Ptx1 can also affect on the transcription of all genes exist in the pituitary gland (Lanctot *et al*, 1999). Other than the pituitary, Tremblay *et al* (2000) showed that the Ptx1isexpressed in the stomodeum and mesodermal derivatives.

Al-Zamely and Al-Tamemi (2018) showed a significant regulation in NF-kappa B gene expression in the group which was exposed to a high fat diet (HFD) for 6 weeks. Such result indicates the close relationship between induced obesity and inflammatory signaling a pathway regulated by NF-KB gene. At the same time,

they also showed a decline in the same gene expression in the group treated with both hydroxytyrosol and HFD. Al-Zamely and Al-Tamemi outcomes reveal the protective role of HT gene against the oxidative stress induced by HFD as well as the decrease in the regulation of NF-KB gene expression as mentioned in the low does group. The results of the group confirm the potent antioxidant impact of HT, which reduces geneexpression of NF-kb gene even within normal feeding condition (Al-Zamely and Al-Tamemi, 2018).

Western blot analysis

Results of the current study show that there was a significant ($P \leq 0.05$) increase in relative protein expression of complex1 (NADH Coenzyme Q oxidoreductase) level in T_1 and T_2 groups, whereas there was a significant decrease in relative protein expression of complex1 level in C group ($P \leq 0.05$). Furthermore, we have seen that T_1 and T_2 present high significant differences ($P<0.05$), when it is compared with control group. A comparison between the result of the treatment groups reveals that there are high significant differences ($P<0.05$) of group T_2 relative to T_1 group with the following values 236.67 ± 29.29 pg/ml and 123 ± 16.5 pg/ml respectively, as describe in Table 4.

A recent study suggested that HT could improve mitochondrial function and reduce oxidative stress in the brain of mice (Zheng *et al*, 2015b). Additionally, a previous study reported changes in the central nervous system and peripheral molecular in diabetic mice with significant reduction in the mitochondria-related molecules including complexes subunit, TCA cycle and the antioxidant SOD2. The study concluded that these changes might contribute to the cognitive dysfunction in mice (Ernst *et al*, 2013). Morino and co-workers showed a positive correlation between the mitochondrial oxidative capacity and mitochondrial genome expression (Morino *et al*, 2005). In other words, the inhabitance of the mitochondrial genome expression results from the reduction in the mitochondrial oxidative capacity. In the present study, HT treatment could significantly improve the expression levels of mitochondrial complexes I in the pituitary gland and increase the activity of complex I, which is the major complex in the electron transport chain and oxidative phosphorylation. HT has been proven to enhance the function of the mitochondria in the pituitary gland via qPCR test and Western Blot technique. Thus, we believe that HT would overcome the negative impacts of the dysfunction of the mitochondria, and improve the mitochondrial oxidative metabolism. Such improvement is attributed to the essential contribution of HT in the mitochondrial deoxyribonucleic acid content (Zheng *et al*, 2015a; Hao *et al*, 2010), membrane potential (Bali *et al*, 2014) and membrane density (Hao *et al*, 2010). Our findings are also supported by Hao *et al* (2010) and Zhu *et al* (2010), who found that HT improves gene expression of proteins like PGC-1á and Nrf2, which also enhance the respiration and biogenesis of mitochondria.

CONCLUSION

(3,4-Dihydroxyphenyl)ethanol compound, so-called hydroxytyrosol (HT) is well-known as health improving substance. Therefore, we tried to expose the susceptibilityof the mitochondrial functions of male rats pituitary gland to HT. Thus, 60 male Wister rats, about 3-4 months old and 240±15 gr of weight, were divided randomly into three equal groups. All animals were subjected to the same ordinary pelleted diet, ventilation, and living conditions. The first, control (C), group was not given any HT. The second group (T_1) and the third group (T_2) were daily drenchedan oral administration of HT, 10 and 50 mg/kg, respectively. Our results reveal that both T_1 and T_2 groups showed a significant increase ($P \leq 0.05$) in Pitx1 gene expression and the western blot of complex1 (NADH-dehydrogenase). Thus, it can be concluded that hydroxytyrosol positively impact on the mitochondrial functions of male rats.

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