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Modulation of age related protein expression changes by gelam honey in cardiac mitochondrial rats

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ABSTRACT

Aging is characterized by progressive decline in biochemical and physiological functions. According to the free radical theory of aging, aging results from oxidative damage due to the accumulation of excess reactive oxygen species (ROS). Mitochondria are the main source of ROS production and are also the main target for ROS. Therefore, a diet high in antioxidant such as honey is potentially able to protect the body from ROS and oxidative damage. Gelam honey is higher in flavonoid content and phenolic compounds compared to other local honey. This study was conducted to determine the effects of gelam honey on age related protein expression changes in cardiac mitochondrial rat. A total of 24 Sprague-Dawley male rats were divided into two groups: the young group (2 months old), and aged group (19 months old). Each group were then subdivided into two groups: control group (force-fed with distilled water), and treatment group (force-fed with gelam honey, 2.5 g/kg), and were treated for 8 months. Comparative proteomic analysis of mitochondria from cardiac tissue was then performed by high performance mass spectrometry (Q-TOF LCMS/MS) followed by validation of selected proteins by Western blotting. Proteins were identified using Spectrum Mill software and were subjected to stringent statistical analysis. A total of 286 proteins were identified in the young control group (YC) and 241 proteins were identified in the young gelam group (YG). In the aged group, a total of 243 proteins were identified in control group (OC), and 271 proteins in gelam group (OG). Comparative proteome profiling identified 69 proteins with different abundance ($p < 0.05$) in OC when compared to YC, and also in YG when compared to YC. On the other hand, 55 proteins were found to be different in abundance when comparing OG with OC. In the aged group, gelam honey supplementation affected the relative abundance of 52 proteins with most of these proteins showing a decrease in the control group. Bioinformatics analysis showed that the majority of the affected proteins were involved in the respiratory chain (OXPHOS) which play an important role in maintaining mitochondrial function.

1. Introduction

Aging is defined as a decline in defence and repair of the body system which leads to physiological dysfunction, homeostasis imbalance, and consequently cell death (Campisi, 2013). These processes of aging also include the accumulation of aged cells (Lopez-Otin et al., 2013) as the body is incapable to the cell resuscitation process (Collado et al., 2007; Onyema et al., 2012). Aging is also known to contribute to pathological conditions such as cardiovascular disease (Minamino and Komuro, 2007). Reactive oxygen species (ROS) has been shown to be involved in the pathophysiology of various diseases (Conklin, 2002). Excess accumulation of ROS shifts the balance between oxidants and antioxidant, forming oxidative stress, thus causing structural and functional damage to DNA, lipids and proteins (Sohal, 2002). The major production site of ROS is the mitochondria (Murphy, 2009; Brand and Nicholls, 2011). Mitochondria have recently received extensive attention due to their importance in cellular function and known causative role in many pathophysiological conditions such as cardiovascular diseases and aging (Gregersen et al., 2012).

The accumulation of free radicals can attack and damage important macromolecules in the mitochondria including DNA (Van Houten et al., 2006). Apart from that, ROS is also able to inhibit the activity of oxidation and reduction in the electron transport chain (ETC) particularly complexes I, II and III (Ghezzi and Zeviani, 2012). This will lead to a decline in electron transportation and eventually cause mitochondrial dysfunction (Correa et al., 2008). These occurrences conform with the mitochondrial theory of aging by Harman in 1972 which proposed that the basic mechanism of mammalian aging is related to mitochondria

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dysfunction due to increasing oxidative damage (Harman, 1972).

Numerous studies have shown the importance of a diet equipped with antioxidants (Zamora-Rosa et al., 2013; Benzie and Strain, 2015). It can protect the mitochondria from oxidative stress, maintain genomic and mitochondrial structure, and consequently increase life expectancy (Gioscia-Ryan et al., 2014; Romano et al., 2013). Antioxidants present naturally in herbs, plants used in traditional medicine, spices, and honey (Wada and Ou, 2002). In the present study, gelam honey was used as a source of antioxidants as it contained flavonoids and phenolic compounds that are known to possess antioxidant properties that can protect aging cells from oxidative damage. This study was therefore carried out to determine the effects of gelam honey supplementation on age related protein expression changes in the mitochondria of aging rats using proteomics and bioinformatics. Studies targeting specific organelles offer the advantage of reduced sample complexity along with information about the spatial and functional relevance of the identified proteins.

2. Material and methods

2.1. Animals and treatment

Briefly, 12 Sprague-Dawley male rats were divided into two groups: the young group (2 months old), and aged group (19 months old) (Fig. 1). Each group was then subdivided into two groups: control group (force-fed with distilled water), and gelam group (force-fed with gelam honey), and were treated for 8 months before being sacrificed for mitochondria isolation and proteomic assay. The rats were obtained from Laboratory Animal Resource Unit, Faculty of Medicine, The National University of Malaysia (UKM). The experimental protocol and animal care was approved by UKM Animal Ethics Committee (UKMAEC: FP/BIOK/2014/ZAKIAH/16-JULY/600-JULY-2014-JUNE-2016-NAR-CAT2). The control group was given water 2.5 ml/kg body weight while the gelam group was supplemented with 2.5 g/kg body weight of gelam honey. The dose of gelam honey used was equivalent to one teaspoon of honey as consumed by human. All rats had free access to commercial rat pellets (Gold Chain, Malaysia) and plain water ad libitum.

2.2. Mitochondrial enrichment and protein extraction

Following 8 months of treatment, the rats were sacrificed. The cardiac tissue was removed and washed with ice-cold 1.15% NaCl pH 7.2 (Sigma, St Louis, USA), and was immediately frozen in liquid nitrogen. Mitochondria isolation was performed as described in the procedure from mitochondria isolation kit (AMSBIO, UK). Briefly, tissue was weighed (100–200 mg) and cut into smaller pieces. It was then homogenized using Ultra Turrax T25 Homogenizer (IKA Labortechnik, Germany) and centrifuged at 600 $\times g$ for 10 min at 4 °C using a Bench Top Refrigerated Centrifuge Sorvall RC-5B. The supernatant was recovered and centrifuged again at 12, 000 $\times g$ for 15 min at 4 °C. The resulting pellet is the crude mitochondria fraction. To obtain purified mitochondria, the pellet was resuspended in isolation buffer and was centrifuge again at 600 $\times g$ and 12, 000 $\times g$ for 10 and 15 min respectively. All the procedures were carried out on ice. Validation of isolated mitochondria was performed by using transmission electron microscopy.

2.3. Sample preparation for mass spectrometry analysis (one-dimensional SDS-PAGE)

Mitochondrial protein, 50 µg from each rats within each group were individually subjected to 1D SDS-PAGE using 12% gradient gels to separate proteins, stained with Coomassie blue and cut into 20 equal pieces as described by Wong et al. (2016). Annotated bands were then in-gel digested with Trypsin Gold (Promega, USA). The digested samples were cleaned using Zip Tip (Millipore, UK) before being analysed by Q-TOF LC-MS/MS.

2.4. Mass spectrometry identification of proteins

Mass spectrometry analysis was carried out to detect differentially expressed protein in the mitochondria samples. Analysis was performed in triplicate for each fraction. Peptide separation was performed using a Nano-LC 1260 directly connected to an Accurate Mass Q-TOF 6550 (Agilent, USA) with a Chip-Cube interface Nano-ESI ion source. In the chip column, peptides were first enriched using an enrichment column before being separated on a separation column (C18 reverse phase, 300 Å, 150 mm, 5 µm) with a 5–80% gradient of solvent B (0.1% formic acid in acetonitrile) for 24 min with a flow rate of 0.4 µl/min. Each mass data acquisition (8 spectra per second from 200 to 3000 m/z) was followed by collision induced dissociation of the twenty most intense ions. MS/MS data were then acquired within the 50–3200 m/z range. To identify the proteins, the mass spectra obtained from the analysis were searched against the Swiss Prot rat (*Rattus norvegicus*) database using the Spectrum Mill (Wong et al., 2016).

Scaffold (Proteome Software Inc., Portland) was then used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at > 1.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at > 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters and the exclusively unique peptides of each protein were identified.

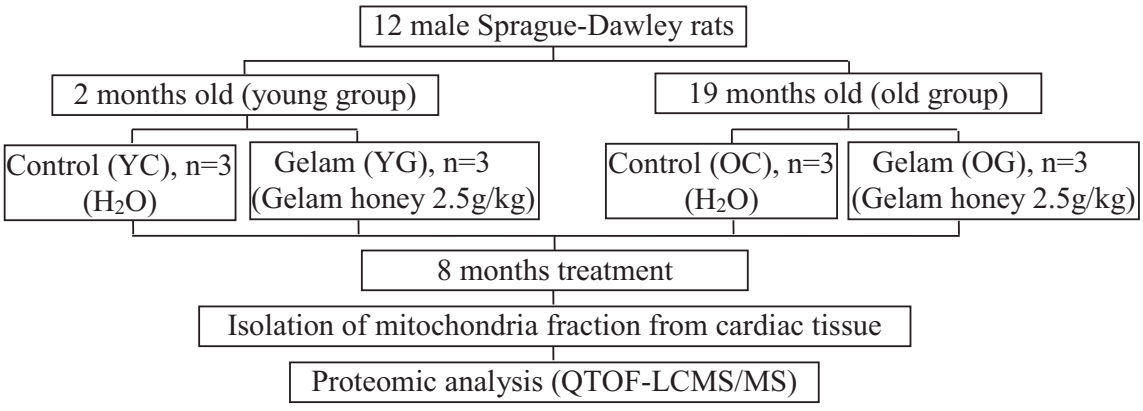

Fig. 1. The flowchart of the research study.