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Original Research Article

Modulation of Autophagy and Mitochondrial Dynamics Gene Expression by Turmeric and Mangosteen Peel Extract

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Abstract

High fat diet (HFD) induces oxidative stress and mitochondrial dysfunction which culminates in fatty liver disease. Autophagy and mitochondrial dynamics are affected by HFD. Turmeric and mangosteen have potential roles as antioxidants and regulators of mitochondrial function in the liver. The study aims to examine the effect of turmeric and mangosteen peel extract on autophagy and mitochondrial dynamics in the liver after HFD induction. Five groups of animals (n=5) as used: negative control, positive control (HFD), turmeric (HFD + 270 mg/kg BW turmeric extract), mangosteen (HFD + mangosteen 270 mg/kg BW peel extract), and fenofibrate (HFD + 15 mg/kg BW fenofibrate). HFD was given for 7 weeks, continued by another 7 weeks plus treatment. Liver sections were extracted to conduct semi-quantitative PCR. Autophagy (LC3, p62), mitophagy (Pink1, Parkin, Bnip3), mitochondrial fission (Drp1, Fis1), and mitochondrial fusion (Opa1, Mfn1, Mfn2) gene expression were measured. LC3 (p=0.048), p62 (p=0.043), Pink1 (p=0.012), Bnip3 (p=0.010), Mfn1 (p=0.015), and Mfn2 (p=0.035) gene expressions were differed significantly, while Parkin (p=0.098) Drp1 (p=0.962), Fis1 (p=0.570), and Opa1 (p=0.055) gene expressions did not differ between groups. Both turmeric and mangosteen peel extract have positive effects by activating autophagy, mitophagy, and mitochondrial fusion in rat liver induced by HFD.

Keywords: autophagy, mitophagy, fusion, fission, HFD

Modulasi Ekspresi Gen Autofagi dan Dinamika Mitokondria oleh Ekstrak Kunyit dan Kulit Manggis

Abstrak

Diet tinggi lemak (DTL) menginduksi stress oksidatif, dengan disfungsi mitokondria yang berujung pada penyakit perlemakan hati. Autofagi dan dinamika mitokondria dipengaruhi oleh DTL. Kunyit dan manggis memiliki peran potensial sebagai antioksidan dan pengatur fungsi mitokondria di hati. Penelitian ini bertujuan untuk menguji pengaruh ekstrak kunyit dan kulit manggis terhadap autofagi dan dinamika mitokondria di hati setelah induksi DTL. Lima kelompok hewan (n=5) digunakan: kontrol negatif, kontrol positif (DTL), kunyit (DTL + 270 mg/kg BB ekstrak kunyit), manggis (DTL + ekstrak kulit manggis 270 mg/kg BB), dan fenofibrate (DTL + fenofibrate 15 mg/kg BB). DTL diberikan selama 7 minggu, dilanjutkan 7 minggu dengan perlakuan. Bagian hati diekstraksi untuk ekstraksi RNA total dan PCR semi-kuantitatif. Ekspresi gen autofagi (LC3, p62), mitofagi (Pink1, Parkin, Bnip3), fisi mitokondria (Drp1, Fis1), dan fusi mitokondria (Opa1, Mfn1, Mfn2) diukur. Ekspresi gen LC3 (p=0,048), p62 (p=0,043), Pink1 (p=0,012), Bnip3 (p=0,010), Mfn1 (p=0,015), dan Mfn2 (p=0,035) *ditemukan berbeda signifikan, sementara ekspresi gen Parkin (p=0,098) Drp1 (p=0,962), Fis1 (p=0,570), dan Opa1 (p=0,055) tidak berbeda signifikan antar kelompok. Ekstrak kulit kunyit dan kulit manggis memiliki efek positif dengan mengaktifkan autofagi, mitofagi, dan fusi mitokondria pada hati tikus yang diinduksi DTL.*

Kata Kunci: *autofagi, mitofagi, fusi, fisi, DTL*

INTRODUCTION

High-fat diet (HFD) is an increase in calorie intake that may disturb lipid homeostasis in the liver (Gluchowski et al., 2017). This disturbance of lipid homeostasis may cause liver steatosis, leading to non-alcoholic fatty liver disease (NAFLD) (Chao et al., 2019). Mitochondrial dysfunction and oxidative stress play major roles in NAFLD (Ramanathan et al., 2022). Mitochondrial function is maintained by the process called fusion and fission; fusion is a process of mixing damaged mitochondria for complementation, while fission is a process of building new mitochondria by segregating damaged from healthy mitochondria (Yu et al., 2020). These two processes are known as mitochondrial dynamics (Ramanathan et al., 2022; Yu et al., 2020).

Compensation for disturbance of mitochondrial dynamics is regulated by autophagy (known as macro-autophagy), a non-selective degradation of damaged organelles to recycle its component; and mitophagy, a selective degradation of damaged mitochondria (Haeussler et al., 2020). Autophagy and mitophagy are induced by mitochondrial dysfunction that occurs in the progression of NAFLD caused by HFD (Haeussler et al., 2020; Korovila et al., 2021; Ramanathan et al., 2022). Inhibition of mitochondrial dynamics caused by lipid disturbance could be reversed by autophagy induction, marked by LC3 and p62 gene expression changes (Gunadi et al., 2020; Ramanathan et al., 2022). Mitophagy is enabling cells to avoid producing reactive oxygen species (ROS) in oxidative stress conditions and stimulating lipid droplet breakdown, followed by beta-oxidation, thus preventing the occurrence of NAFLD (Ramanathan et al., 2022). The genes involved in mitochondrial dynamics and mitophagy are the Pink1, Parkin, Bnip3, Drp1, and Fis1, which play a more important role in mitochondrial fission, and Opa1, Mfn1, Mfn2 which play a more important role in mitochondrial fusion (Yu et al., 2020).

Recent studies have shown that several drugs such as fenofibrate, could be used to prevent the progression, severity, and extent of NAFLD (Mahmoudi et al., 2022; Oscarsson et al., 2018; Yaghoubi et al., 2017). Fenofibrate promotes fatty acid oxidation and demonstrates antioxidant and anti-inflammatory properties (Mahmoudi et al., 2022). Unfortunately, it also has some adverse effects like weight loss, enhance triglyceride content in the liver, and causes hepatic toxicity (Khorolskaya et al., 2020; Mahmoudi et al., 2022). Therefore, alternative herbal ingredients which have the same properties and protective to the liver are needed to be explored. Turmeric and mangosteen peel extract are known for their antioxidant, antihyperlipidemic, and anti-inflammatory properties (Alhusain et al., 2022; Feng et al., 2019; John et al., 2021; Suttirak & Manurakchinakorn, 2014). Curcumin in turmeric and α-mangostin in mangosteen pericarp also have roles in regulating mitochondrial function and reducing mitochondrial dysfunction (Sathyabhama et al., 2022; Tsai et al., 2016).

The biomolecular mechanism regarding the effect of turmeric and mangosteen peel extract in modulating autophagy and mitochondrial dynamics is still on the knees of the gods. Therefore, this study aims to explore the effects of turmeric and mangosteen peel extract on gene expression of autophagy, mitophagy, mitochondrial fusion, and fission in the liver of male Wistar rats induced by an HFD.

MATERIAL AND METHODS

Animals for The Experiment

Twenty-five Wistar rats (8 weeks old, 210-250 g), male, and healthy, were supplied by PT Biofarma, Bandung, Indonesia. Animals were kept in cages (5 animals per cage) under standard humidity and temperature in the animal lab of Maranatha Biomedical Research Laboratory, Bandung, Indonesia. Two weeks of adaptation period allowed the animals to orient themselves to the animal lab environment. Standard chow pellets (48.3% carbohydrate, 24.9% protein, 9.7% fat, 10.98% water, 6.13% cinder, 0.8% calcium, and 0.1% NaCl) were provided ad libitum for the negative control group (-). The high-fat diet consisting of 26.3% carbohydrate, 26.2% protein, 37.7% fat, 10.98 water, 6.13% cinder, 0.8% calcium, and 0.1% NaCl was given for 7 weeks. The treatment was continued with 7 weeks of HFD in the positive control group (+), HFD and 270 mg/kg BW/day of turmeric extract in the turmeric group, HFD and 270 mg/kg BW/day of mangosteen peel extract in mangosteen group, HFD and 15 mg/kg BW/day of fenofibrate in fenofibrate group. Animal utilization protocols were performed according to the Use and Animal Care guidelines. The ethical was approved by the Faculty of Medicine Maranatha Christian University Ethical Committee with an ethics reference number 148/KEP/XII/2022. At the end of the study, the animals were euthanized, and the liver sections were collected and stored at -80ºC refrigerator for molecular analysis.

Procedures for RNA Extraction and PCR

RNA from each liver section was extracted in consonance with the manufacturer's references using Trisure solution (Bioline, United Kingdom). The quality of the RNA was examined using spectrophotometry (Multiscan Go) at 260/280 nm absorbance. GAPDH was used as a control gene. MyTaq One-Step RT-PCR Kit (Bioline, United Kingdom) was used to perform semiquantitative PCR, followed by electrophoresis and visualization of PCR band using BluePad. Image J was then used to measure the PCR band (Gunadi et al., 2020). The primer sequences were provided in the table below.

Table 1. Primers Sequences

Gene	Primer Sequences	Product (bp)
LC3	GGTCCAGTTGTGCCTTTATTGA GTGTGTGGGTTGTGTACGTCG	153
p62	CTAGGCATCGAGGTTGACATT CTTGGCTGAGTACCACTCTTATC	116
Pink1	TGCAATGCCGCTGTGTATGA TCTGCTCCCTTTGAGACGAC	113
Parkin	GTTTGTCCACGACGCTCAAC CCCGGTATGCCTGAGAAGTC	350
Bnip3	GAAGCGCACAGCTACTCTCA TCCAATGTAGATCCCCAAGCC	142
Drp1	CGCTGATCCCGGTCATCAAT ACTCCATTTTCTTCTCCTGTTGT	247
Fis1	GTGTTGCGTGTTAAGGGATGA AAATTGCGTGCTCTTGGACA	250
Opa1	GATGACACGCTCTCCAGTGAAG CTCGGGGCTAACAGTACAACC	178
Mfn1	TGACTTGGACTACTCGTGCG GTGGCCATTTCTTGCTGGAC	133
Mfn2	CAGTGTTTCTCCCTCAGCTATG TAGGGCCCAGGAACCTATT	121
GAPDH	GTTACCAGGGCTGCCTTCTC GATGGTGATGGGTTTCCCGT	177

Data Analysis using Statistical Methods

The data analysis of gene expression was performed using SPSS software. Data was written as mean ± SEM (standard error mean) for quantitative measurements. Normality and homogeneity tests were conducted before measuring the differences between groups using ANOVA, followed by post hoc LSD.

RESULT

The comparison of autophagy gene expression between groups after 7 weeks of HFD was examined in this study. Gene expression was compared to GAPDH as a control gene. The result of autophagy (LC3, p62) gene expression was shown in table 2 below.

Table 2. The relative ratio of autophagy gene expression

Gene Expression	Negative Control (Mean ±SEM)	Positive Control (Mean ±SEM)	Turmeric (Mean ±SEM)	Mangosteen (Mean ±SEM)	Fenofibrate (Mean ±SEM)
LC3	0.800 ± 0.046	0.749 ± 0.024	0.885 ± 0.048	0.904 ± 0.036	0.860 ± 0.027
p62	0.769 ± 0.036	0.891 ± 0.109	0.667 ± 0.035	0.676 ± 0.036	0.655 ± 0.032

The differences were compared using One Way Anova, and the result showed significant differences with p = 0.048 for LC3 and p = 0.043 for p62 gene expression. For ensuring which groups have significant differences, post hoc LSD was used and the results showed significant differences in LC3 gene expression between positive control and turmeric (p = 0.019) and mangosteen (p = 0.008); and p62 gene expression between positive control and turmeric (p = 0.013), mangosteen (p = 0.016), and fenofibrate (p = 0.009). This finding showed a significant increase of autophagy in turmeric and mangosteen groups, supported by the increase of LC3 and decrease of p62 gene expression, while in positive control showed decrease of LC3 accompanied by an increase of p62 which showed a tendency of autophagy process inhibition. PCR band the graphical result is shown in figure 1 below.

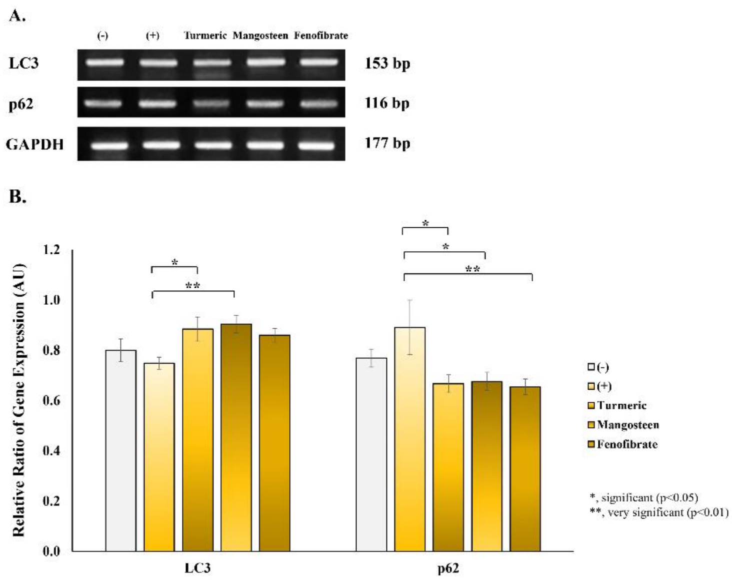

Figure 1. PCR band and graphical results of autophagy gene expression after treatment with HFD

Furthermore, mitochondrial dynamics (mitophagy, mitochondrial fission and fusion) was examined in the liver after 7 weeks of HFD in treatment groups. Relative ratio of mitophagy (Pink1, Parkin, Bnip3) gene expression was obtained as shown in Table 3 below.

Table 3. The relative ratio of mitophagy gene expression

Gene Expression	Negative Control (Mean ±SEM)	Positive Control (Mean ±SEM)	Turmeric (Mean ±SEM)	Mangosteen (Mean ±SEM)	Fenofibrate (Mean ±SEM)
Pink1	0.905 ± 0.048	0.822 ± 0.024	0.996 ± 0.030	0.957 ± 0.030	0.927 ± 0.019
Parkin	0.889 ± 0.071	0.728 ± 0.056	0.929 ± 0.057	0.915 ± 0.067	0.957 ± 0.045
Bnip3	0.735 ± 0.045	0.643 ± 0.045	0.837 ± 0.024	0.777 ± 0.026	0.782 ± 0.025

Statistical analysis using One Way ANOVA also indicated significant differences in Pink1 (p = 0.012) and Bnip3 (p = 0.010), while no significant difference in Parkin (p = 0.098) gene expression between groups. Post hoc LSD results indicated that positive control and turmeric groups differed in Pink1 (p = 0.001) and Bnip3 (p = 0.001), and positive control and mangosteen groups also differed in Pink1 (p = 0.007) and Bnip3 (p = 0.013) gene expression. As a comparison with therapy for dyslipidemia, there was a significant differences between positive control and fenofibrate in Pink1 (p = 0.029) and Bnip3 (p = 0.010). These results showed that turmeric, mangosteen, and fenofibrate increased mitophagy (Pink1, Bnip3) toward baseline level (the same or above the negative control level) after 7 weeks treatment with HFD. Although it showed no significant difference in Parkin gene expression, but there were a tendency of its increasement in turmeric, mangosteen, and fenofibrate groups compared to positive control group. PCR band and the graphical result of mitophagy gene expression were shown in Figure 2 below.

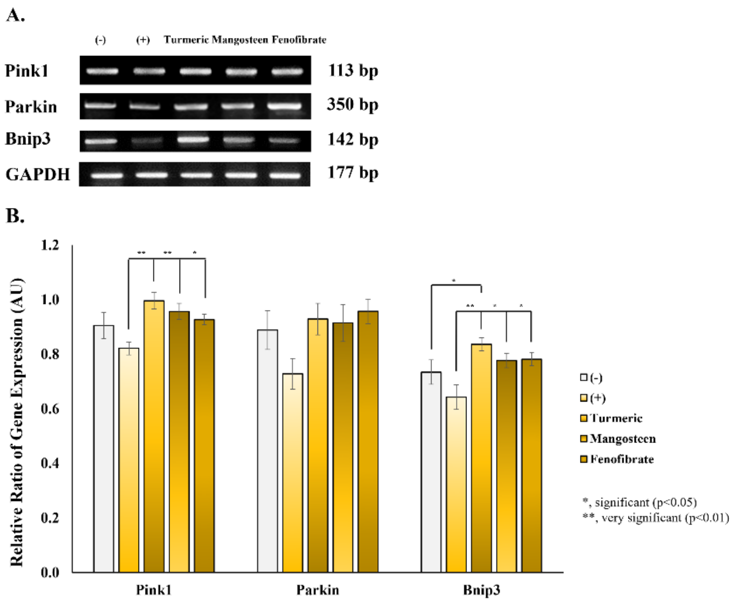

Figure 2. PCR band and graphical results of mitophagy gene expression after treatment with HFD

We then examined the mitochondrial fission (Drp1, Fis1) gene expression, and the result was obtained as shown in Table 4 below.

Table 4. The relative ratio of mitochondrial fission gene expression

Gene Expression	Negative Control (Mean ±SEM)	Positive Control (Mean ±SEM)	Turmeric (Mean ±SEM)	Mangosteen (Mean ±SEM)	Fenofibrate (Mean ±SEM)
Drp1	0.787 ± 0.105	0.857 ± 0.071	0.797 ± 0.097	0.834 ± 0.065	0.794 ± 0.038
Fis1	0.568 ± 0.065	0.640 ± 0.030	0.532 ± 0.042	0.602 ± 0.048	0.571 ± 0.040

Statistical analysis using One Way ANOVA indicated no significant differences in Drp1 (p = 0.962) and Fis1 (p = 0.570) gene expression between groups. These results showed a tendency of mitochondrial fission to decrease in turmeric, mangosteen, and fenofibrate, until they reach the baseline (the same level as negative control), especially in turmeric and fenofibrate group. PCR band and the graphical result of mitochondrial fission gene expression were shown in figure 3 below.

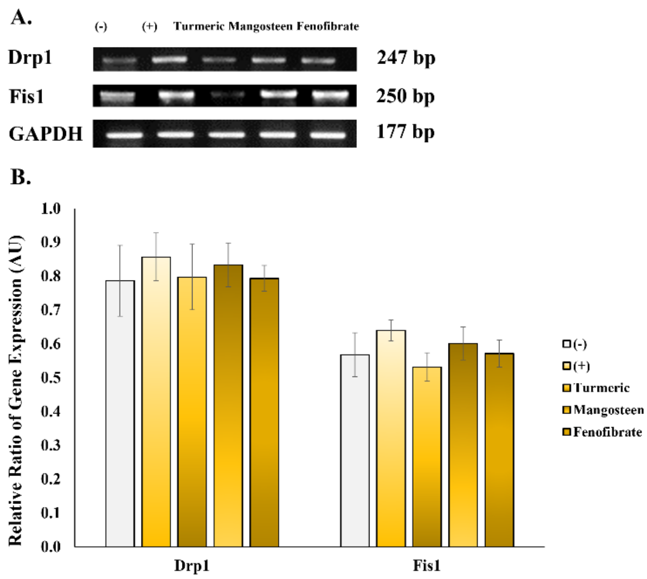

Figure 3. PCR band and graphical results of mitochondrial fission gene expression after treatment with HFD

Lastly, the mitochondrial fusion (Opa1, Mfn1, Mfn2) gene expression, and the result was obtained as shown in Table 5 below.

Table 5. The relative ratio of mitochondrial fusion gene expression

Gene Expression	Negative Control (Mean ±SEM)	Positive Control (Mean ±SEM)	Turmeric (Mean ±SEM)	Mangosteen (Mean ±SEM)	Fenofibrate (Mean ±SEM)
Opa1	0.939 ± 0.059	0.774 ± 0.036	0.891 ± 0.026	0.842 ± 0.025	0.852 ± 0.025
Mfn1	0.843 ± 0.030	0.701 ± 0.045	0.848 ± 0.021	0.775 ± 0.015	0.782 ± 0.030
Mfn2	0.861 ± 0.025	0.761 ± 0.037	0.853 ± 0.026	0.864 ± 0.014	0.882 ± 0.025

Statistical analysis using One Way ANOVA indicated significant differences in Mfn1 ($p = 0.015$) and Mfn2 ($p = 0.035$) gene expression, while no significant difference in Opa1 ($p = 0.055$) gene expression between groups. Post hoc LSD results indicated that positive control and turmeric groups differed in Mfn1 ($p = 0.002$) and Mfn2 ($p = 0.024$), while positive control and mangosteen groups differed in Mfn2 ($p = 0.013$) gene expression. As a comparison with therapy for dyslipidemia, it found significant difference between positive control and fenofibrate in Mfn2 ($p = 0.005$) gene expression. These results indicated an increase of mitochondrial fusion toward the baseline level (the same level as negative control), especially in turmeric group compared to other treatment groups. PCR band and the graphical result of mitochondrial fusion were shown in Figure 4 below.

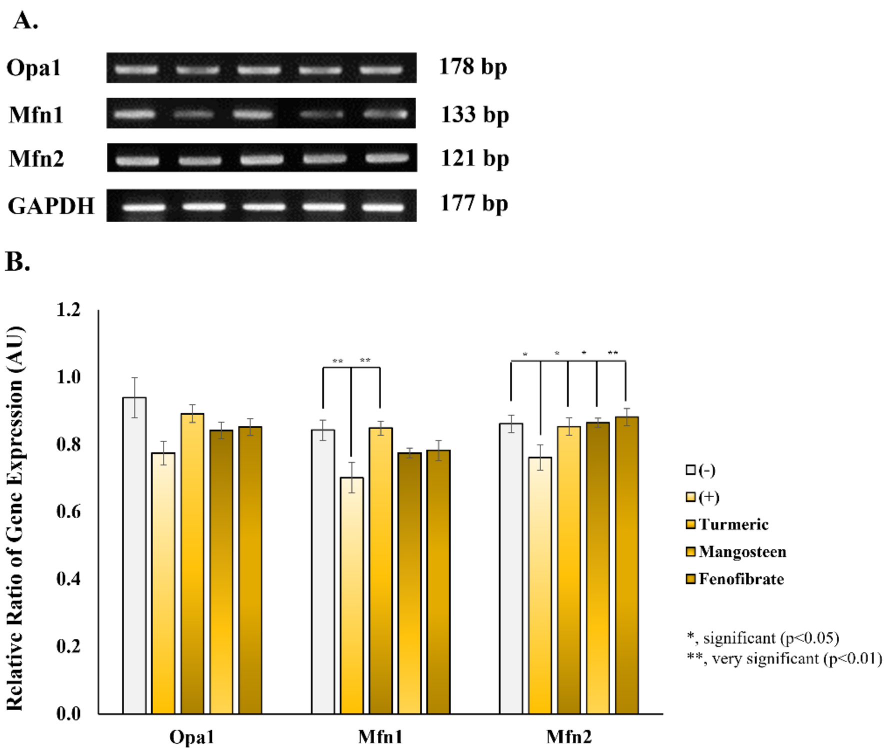

Figure 4. PCR band and graphical results of mitochondrial fusion gene expression after treatment with HFD

DISCUSSION

The liver has a cardinal role in lipid metabolism; thus, its function is essential in maintaining lipid metabolism homeostasis (Gluchowski et al., 2017). The process of lipid metabolism started with the uptake, esterification process, beta-oxidation, and fatty acid delivery, which all occur in hepatocytes (Mashek, 2013). HFD causes a lipid imbalance as the result of surplus uptake of fatty acid, lipid formation, and reduced fatty acid oxidation or impaired triglyceride or very-low-density-lipoprotein (VLDL); all of these finally lead to NAFLD (Gluchowski et al., 2017). As the ‘powerhouse’ of the cells, mitochondria have a central role in the course of NAFLD induced by HFD (Ramanathan et al., 2022).

Mitochondrial dynamic is maintained through the balance of autophagy, mitophagy, mitochondrial fission, and fusion (Yu et al., 2020). Autophagy is an organelle clearance mechanism for cell survival to achieve cell homeostasis (Anding & Baehrecke, 2017). Dysfunctional organelles, including damaged mitochondria are engulfed through autophagosomes, then fuse with lysosome to form autophagolysosomes, followed by degradation and recycling process of cellular components (Mizushima, 2018). LC3 (microtubule-associated protein light chain 3) and p62 (Sequestosome 1) are widely used as autophagy monitor, with LC3 as an autophagosome marker and p62 that binds to LC3 then degraded by autophagy (Yoshii & Mizushima, 2017). Thus, an increase of LC3 and a decrease of p62 are indicating an activation of autophagy process.

As a selective autophagy, mitophagy is controlled by Parkin and Pink1 and several mitophagy-specific receptors, such as Nix and Bnip3. In a damaged mitochondrial, Pink1 is accumulated on the cell’s surface which then recruits Parkin from inside the cell to the surface, promoting lysosome engulfment of the damaged mitochondria. Bnip3 was reported as a specific receptor that could recognize damaged mitochondria and then interact with LC3 to form autophagosomes followed by degradation (Shi et al., 2014; Yu et al., 2020). Thus, Parkin, Pink1, and Bnip3 are genes that are mostly used for monitoring mitophagy (Williams & Ding, 2018).

The balance of mitochondrial fission and fusion are important for maintaining mitochondrial health and dynamics (Adebayo et al., 2021). Drp1 and Fis1 are marker genes for mitochondrial fission, while Opa1, Mfn1, and Mfn2 are marker genes for mitochondrial fusion (Adebayo et al., 2021; Williams & Ding, 2018). Prior studies suggested that Drp1 and its receptor

(Fis1) also participate in mitophagy and there was a crosstalk between the fission and fusion machinery (MacVicar & Lane, 2014; Yu et al., 2020). Drp1 is a central regulator of mitochondrial fission, located in the cytosol, recruited by Fis1 to the mitochondrial surface for triggering mitochondrial fission via GTPase (Otera et al., 2013). Opa1, Mfn1, and Mfn2 are the mitochondrial fusion factors which also involved in the regulation of mitophagy. Impaired Opa1 cleavage could affect mitophagy, while Mfn1 and Mfn2 are activated through Pink1/Parkin dependent on mitophagy induction (Liao et al., 2017; Yu et al., 2020). Mfn2 was proven to mediate Parkin recruitment to the surface of damaged mitochondria, and Pink1 could activate Mfn2 to enhance its interaction with Parkin (Chen & Dorn, 2013).

The balance of fusion and fission of mitochondria in the liver is determining metabolic homeostasis in the liver (Yu et al., 2020). HFD induced an enhancement in mitochondrial fission (Drp1, Fis1) and depression in mitochondrial fusion (Mfn2) and mitophagy in the liver mitochondria (Lionetti et al., 2014). Wang et al suggested that disruption of the liver mitochondrial fission (by deleting the Drp1 protein in the liver) protects against obesity and metabolic deterioration induced by HFD (Wang et al., 2015). In line with previous studies, it found an enhancement in mitochondrial fission (Drp1 and Fis1) and a depression in autophagy (LC3, p62), mitophagy (Pink1, Parkin, Bnip3), and mitochondrial fusion (Opa1, Mfn1, Mfn2) in positive control which was given high-fat diets for 14 weeks.

Dietary supplementation of antioxidant and antihyperlipidemic such as turmeric and mangosteen peel could reverse the disruption of mitochondrial dynamics that occurred in HFD. In this study, it found an increase of autophagy, mitophagy, and mitochondrial fusion, accompanied by a decrease of mitochondrial fission, in the treatment group given HFD and turmeric or mangosteen peel extract, as shown in Table 2-4. In this study, the decrease of mitochondrial fission (Drp1, Fis1) is referring to the baseline level (negative control level), indicating the effect of turmeric, mangosteen, and fenofibrate in altering mitochondrial fission toward its basal level. Mitochondrial fission and fusion are interlinked processes which have critical role in maintaining cellular homeostasis(Adebayo et al., 2021), therefore an increase of fusion and a decrease of fission to its baseline level showed the effect of turmeric and mangosteen in maintaining mitochondrial dynamic. These effects were more clearly seen in the group given HFD and turmeric ethanol extract (figure 3 and 4). Curcumin has a credible role against mitochondrial dysfunction because it has phenolic and β-diketone functional groups that enhance the activities of SOD, CAT, and GPx (Sathyabhama et al., 2022). Curcumin supplementation inhibits ROS production, increasing cell survival, restoring mitochondrial membrane potential, intensifying intestinal barrier function, lowering endotoxin, and depressing hepatic TLR4/NF-κB signaling pathway (Feng et al., 2019; Sathyabhama et al., 2022). α-mangostin supplementation showed antioxidant properties by increasing SOD, GPx, and GSH activities; anti-apoptosis by improving mitochondrial membrane potential and suppressing caspase activity (Fang et al., 2016; Tsai et al., 2016).

The limitation of this study, oxidative stress, antioxidant level and inflammatory and apoptosis gene expression in the liver after HFD were not examined. Future studies is required to confirm the exact mechanism of turmeric and mangosteen in modulating autophagy and mitochondrial function in the liver after HFD.

CONCLUSION

HFD induces changes in autophagy, mitophagy, mitochondrial fission and fusion gene expression in the liver of Wistar rats. Both turmeric and mangosteen peel extract have positive effects by activating autophagy, mitophagy, and mitochondrial fusion, and returning mitochondrial fission to baseline level. Turmeric seems to have better properties in modulating mitophagy and mitochondrial dynamics in the liver after HFD treatment.

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