Effects of royal jelly and tocotrienol rich fraction in obesity treatment of calorie-restricted obese rats: a focus on white fat browning properties and thermogenic capacity

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Abstract

Background: Obesity has reached an alarming rate worldwide. Promoting thermogenesis via increasing brown adipose tissue (BAT) function or white adipose tissue (WAT) browning has been propounded as a new approach to fight against obesity. The goal of the study was to evaluate the effect of Royal Jelly (RJ) and tocotrienol rich fraction (TRF) on BAT activation and WAT browning during calorie restriction diet (CRD) in obesity model.

Methods: In this experimental study 50 obese Wistar rats were randomly divided into 5 groups and received one of the following treatments for 8 weeks: High-fat diet (HFD), CRD, RJ+CRD, TRF+CRD, RJ+TRF+CRD. Effects of RJ and TRF, individually and their combination on body weight and the expression of key thermoregulatory genes in WAT, BAT were examined by quantitative real-time (qRT-PCR). Morphological alterations were assessed by hematoxylin and eosin staining.

Results: RJ (-67.21g ±4.84g) and RJ+TRF (-73.29g ±4.51g) significantly reduced weight gain relative to the CRD group (-40.70g ±6.50g, P<0.001). Compared with the CRD group, RJ and RJ+TRF remarkably enhanced the uncoupling protein1 (UCP1) expression in WAT (5.81, 4.72 fold, P<0.001) and BAT (4.99, 4.75 fold, P<0.001). The expression of PR domain containing 16(PRDM 16), cAMP response element-binding protein1 (CREB1), P38 mitogen-activated protein kinases (P38MAPK), Bone morphogenetic protein8B (BMP8B) increased significantly following RJ and RJ+TRF treatment (P<0.001).However, the expression levels of CCAAT/enhancer-binding protein beta (CEBPβ) and Bone morphogenetic protein7 (BMP7) did not change remarkably. Multilocular beige cells in WAT and compacted dense adipocytes in BAT of RJ and RJ+TRF received groups were observed. TRF did not demonstrate substantial effects on the expression of mentioned thermoregulatory genes and brown fat-like phenotype.

Conclusion: Our results suggest that Royal Jelly promotes thermogenesis and browning of WAT, contributing to an increase in energy expenditure. Thus, Royal Jelly may give rise to a novel dietary choice to attenuate obesity.

1-introduction

The expanding worldwide obesity arises from the complex interactions among environmental factors, genetic context, and individual behaviors. Nonetheless, the disproportion in energy intake and energy expenditure thought to be the most determining aspect of obesity [1, 2].

Although calorie restriction is the primary intervention in obesity management, it seems to be an incompetent approach in long term, since metabolic adaptations accrue in response to energy limitation, which may result from a reduction in thermogenesis, resting energy expenditure or other energy expenditure constituents [3–5].

Unlike WAT which is the main site of excess energy, brown adipose tissue is a primary site for adaptive thermogenesis. The thermogenic capacity of brown adipocytes relies on the high expression of UCP1 and

high mitochondrial content. When activated, UCP1 mediates chemical energy dissipation through dissociation of mitochondrial substrate oxidation from ATP production, thus generating heat [6, 7]. Hence, BAT function and activation have substantial potential attention from a therapeutic perspective, owing to its vital function in obesity control.

In addition to classical BAT, a phenomenon called “browning or beigeing procedure” has been demonstrated which includes the development of brite adipocytes in classical WAT [8–10]. It is suggested that some stimulants like cold exposure, β-adrenergic receptor stimuli, exercise, PPARs agonists, pharmacological agents, and some food components may develop brite or beige adipocytes [2, 11, 12]. Browning of WAT protects against obesity, through increasing energy consumption which leads to a negative energy balance [2, 9, 13]. It is speculated that PRDM16, BMPs, and C/EBPβ are the master regulators, which interactions of these factors participate in the UCP1 gene regulation ultimately contributing to BAT activation or WAT remodeling [14–17]. Identification of the food components that can induce the browning represents an attractive potential way in obesity treatment. RJ is a yellowish-white, multifunctional creamy material that is secreted from the hypopharyngeal and mandibular glands of nurse honeybees [18]. The main components of RJ are HDEA and HDAA, which main biological activities of RJ attribute to them [19, 20]. RJ has multiple biological functions including antioxidant, antitumor, antiaging, antihypercholesterolemic, anti-inflammatory, antimicrobial, hypoglycemic, radio-protective, gastro-protective, hepato-protective and vasodilative effects [21]. RJ presented a remarkable decrease in body weight and abdominal fat depots and increase skeletal muscle mass in HFD-induced obese rats [22]. Nevertheless, the exact effects of RJ on the regulation of thermogenesis and browning of white adipose tissue have not been distinguished and the procedure by which RJ ameliorate obesity is not exactly appreciated yet. Vitamin E is a lipid-soluble nutrient, composed of two biologically active TP and T3 subclasses with eight analogs each including α-, β-, γ- and δ-forms [23]. Most vitamin E studies have concentrated on TPs, and very little is known about T3s. T3s main food resources are rice bran, oat, wheat germ, palm oil, and annatto oil. T3s have been claimed to possess various physiological activities including neuroprotective, anticancer, antiangiogenesis, anti-tumor, cardiovascular-protective, hypocholesterolemic and anti-inflammatory properties [24, 25]. However, T3s effect on obesity management and its related metabolic challenges have been less assessed compared with saturated matched TPs, and the chief mechanisms of action in obesity regulation are unknown yet [23]. Gamma-tocotrienol is the most common T3 isomer and is physiologically more available than other isomers [26]. Concerning the unsuccessful methods of obesity control and the adverse consequence of long term calorie restriction on energy expenditure and thermogenesis, applying functional foods with properties of thermogenesis improvement would be profitable. According to our knowledge, the potential effects of royal jelly and γ-tocotrienol in white fat browning and thermogenesis induction during calorie restriction diet has not been examined yet. Thus, the objective of this study was to evaluate the effect of royal jelly, γ-tocotrienol and their combinations on the induction of genes involved in the beige phenotype appearance in WAT and also BAT activation via molecular involved mechanisms in obesity models of rats during CRD.

2-method

2-1-Animals and treatments

In this experimental investigation, fifty-five Male Wistar rats (3 weeks old) weighing 50–70 g were purchased from Pasteur Institute (Tehran, Iran). All rats were kept individually in stainless steel cages under the standard condition temperature of 22–25°C and relative humidity 55 ± 5%, with a 12-hour light/dark cycle (7:00–19:00hr), allowed free access to water and a normal chow diet for one week. All experimental procedures performed on animals complied with the National Institutes of Health guide for the care and use of laboratory animals [27]and approved by the Ethics Committee of Iran University of medical science (ethic code: IR.IUMS.FMD.REC 1396.9321324003). All efforts were done to decrease the number of animals used and the suffering of rats.

The study protocol consisted of a two-phase 1) obesity induction period 2) treatment period. (Fig1) After one week of acclimatization, fifty rats were administered a HFD to induce the obesity model and five rats received normal chow diet as the control group for HFD receiving rats. All rats had free access to food and water in this phase of the study. Semi-purified HFD consisted of standard chow powder mixed with milk butter (40% w/w). The compositions of the diets used in the study are shown in Table 1. HFD was prepared every two days freshly in the form of pellets and kept at 4 $^{\circ}$C to maintain nutrients. We weighted animals every week. At the end of the 17th weeks, the mean weight of HFD administered rats increased significantly compared to normal chow diet consuming rats (443.28g ± 46.62g vs 396.24g ±28.79g $P<0.05$), indicating that HFD induced obesity model was accomplished. At the second phase, rats were randomly allocated to one of 5 groups(n=10/group) using a randomized block procedure which matched for body weight and treated for 8 weeks as follows 1) RJ group receiving lyophilized RJ powder (100 mg/kg/day) orally dissolved in CRD 2) TRF[1] group receiving TRF (85mg/kg/day) orally dissolved in CRD 3) RJ+TRF group receiving both 100 mg/kg/day lyophilized RJ powder and 85mg/kg/day TRF orally dissolved in CRD 4) CRD group; without any supplementation as control for RJ, TRF and RJ+TRF groups and **5)** HFD group; HFD without any supplementation as control for CRD group.

CRD had the same macronutrient composition as HFD (Table1), but the calorie content was 30% lower than the ad libitum intake of HFD. RJ and TRF were added to CRD and the food was weighed, then given to rats every day in certain time schedules (between 9:00-9:30 AM).HFD was fed ad libitum and given to rats every day.

Lyophilized Royal jelly powder was purchased from Bulk Supplements Co, Ltd, (Henderson, USA) containing 6% of 10-HAD. TRF was kindly provided by ExcelVite Co, Ltd (Perak, Malaysia).High-performance liquid chromatography determined that TRF contained α-tocotrienol (12%), β-tocotrienol (2%), γ-tocotrienol (19.3%) and δ-tocotrienol (5.5%) together with α-tocopherol (11.9%). The doses and duration of treatments were selected based on the previously reported oral no-observed-adverse-effects and the sample size was decided based on similar work done before [23, 28].

2-2-Sample collection

At the end of the study, all rats were anesthetized with intraperitoneal injection of xylazine (xylazine 2%, 20 mg ml−1, Alfasan, Woerden, Netherlands) and ketamine (ketamine 10%, 100 mg ml−1, Alfasan, Woerden, Netherlands) after overnight fasting and interscapular BAT, inguinal WAT and hypothalamus were quickly removed, rinsed gently with phosphate-buffered saline (PBS) solution, and kept in RNA later Stabilization Solution (Qiagen, Inc. Germantown, Maryland, USA)for RNA isolation.

2-3- Quantitative real-time PCR

All tissues were homogenized gently. Total RNA was extracted from each tissue using Trizol (Thermo Fisher, Waltham, Massachusetts, USA) according to the manufacturer's protocol. The quality and quantity of extracted RNA were determined spectrophotometrically, measuring relative absorbance ratio at A260/280 and A260/230 (NanoDrop One/Once, Thermo Fisher Scientific Inc, Wilmington, Delaware, USA).

The extracted RNA was converted to cDNA[2] using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, USA) with 1 µg of mRNA according to the manufacturer's protocol. The real-time reverse transcription-polymerase chain reaction (RT-PCR) was done using a fluorescence thermal cycler (Light Cycler system; Roche Diagnostics, Mannheim, Germany) system using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and gene-specific primers for CREB1[3], P38MAPK[4], BMP7[5], BMP8B [6] , C/EBP β, PRDM16, UCP1, and β-actin. The primer sequences were designed through the reported sequences of Primer Bank NCBI, summarized in Table 2 and obtained from Metabion **international AG (**Steinkirchen, Germany). Delta-delta method was used to calculate the relative mRNA expression of the target gene and normalized to β-Actin as a reference gene [29].PCR was done under the following conditions: 95 °C for 10 min, 95 °C for 10 s, and 60°C for 10 s for 45 cycles with 100% ramp rate under standard conditions. Triplicate Ct values were calculated for each sample.

2-4-Histological assay

Immediately after removal, adipose tissue specimens were fixed in 10% buffered formalin with the change of formalin every 2 days for 7days. The samples were then dehydrated through different solutions of alcohol and then paraffin-embedded. Tissues were cut by rotary microtome in thin sections (5 µm). H&E [7]methods were used and observed by an optical microscope (magnification, X 400).

2-5-Statistical Analysis

The normality of data was assessed by the one-sample Kolmogorov-Smirnov test. All data were represented as the mean ± SEM. ANOVA[8] was done to test the differences between groups. Tukey's post hoc was performed to analyze the multiple comparisons.

The results of gene expression were presented as the fold changes using $2^{-\Delta\Delta CT}$method [29].

IBM SPSS Statistics 23 (IBM SPSS Statistics, Armonk, USA) was applied to analyze all data.

Figures were visualized using the Prism software, version 8·0 (GraphPad, CA, USA). A significant level was considered as P-value <0.05.

[1] Tocotrienol rich fraction

[2] Complementary DNA

[3] cAMP response element-binding protein1

[4]P38 mitogen-activated protein kinases

[5] Bone morphogenetic protein7

[6] Bone morphogenetic protein8B

[7] Hematoxylin and eosin

[8] One-way analysis of variance

3- Results

3-1- Effects of CRD, RJ, TRF and mixed treatments on weight changes

All fifty-five rats completed the intervention for 8 weeks and included in the analysis. After 8 weeks of the experiment, as expected the final mean body weight of CRD-fed obese rats was significantly lower than that of HFD-fed obese rats. (CRD, 404.24g ±8.65g vs HFD, 493.28g ± 8.23 g, P<0.001) (Fig. 2A). The promoting effects of RJ, TRF and combined interventions in weight changes (relative to baseline weights) of CRD-fed obese rats were depicted in fig.2 B. As cleared, RJ treatment reinforced the weight-reducing effects of CRD and strongly decreased the weight of rats (RJ group, -67.21g±4.84g vs CRD group, -40.70g±6.50g, p<0.001).Moreover, RJ+TRF treatments considerably decreased weight (-73.29g±4.51 g, p< 0.001). However, TRF did not significantly reduce the weight (- 44.40g±3.35 g, p≥0.05).

3-2- Effects of RJ, TRF and mixed supplementation on key thermoregulatory genes expressions

To investigate the effects of CRD on thermogenesis, we first measured the expression levels of a key regulatory gene, UCP1 by RT_PCR in WAT and interscapular BAT. As anticipated, along with weight loss, CRD down-regulated the UCP1 levels by 36% and 14% in WAT and BAT respectively compare to HDF-fed rats, although there were no statistically significant (P≥0.05) (Fig.3 A). We next tested whether RJ and TRF supplementation with CRD would be able to ameliorate the aforementioned effects of CR on the UCP1 levels. Our data demonstrated that RJ added in CRD rats led to a significant elevation of UCP1 in comparison with the CRD-matched group in WAT and BAT as depicted in Fig.3 B (P<0.001). We also observed that TRF induced the UCP1 levels in both adipose tissues compared to the CRD group, although

it was not statistically significant (P≥ 0.05). Furthermore, RJ+TRF significantly induced the UCP1 levels in comparison to the CRD-matched group (P<0.001). However, the enhancing effects of RJ on UCP1 expression was superior. Next, we examined the effects of RJ and TRF on key brown fat marker PRDM16. As depicted in Fig.3C mRNA levels of PRDM16 was increased significantly about 4.65-fold and 2.80- fold in WAT and BAT in the RJ group respectively relative to CRD group (P<0.001). Whereas, TRF did not remarkably up-regulate the PRDM16 expression in none of the adipose tissues (P≥ 0.05). Moreover, gene expression of PRDM16 up-regulated significantly in the RJ+TRF group to 4.30 and 2.61 -fold in WAT and BAT respectively in comparison with the CRD group (P<0.001).To further investigate the potential mechanisms underlying the browning effects of RJ and TRF, we determined expression levels of CREB1 and CEBPβ, the master regulators of the thermogenic program. Gene expression of CREB1 increased significantly by RJ addition to 5.85 and 5 fold relative to CRD group in WAT and BAT respectively (P<0.001).However, TRF treatment did not affect the expression of CREB1 in comparison with the CRD group (P≥ 0.05). Furthermore, combinations of RJ+TRF markedly increased CREB1 expression in WAT and BAT relative to the CRD group (P<0.001, Fig.3 D).For CEBPβ, we did not observe a significant increase in mRNA levels in none of the studied groups in any adipose tissue (P≥ 0.05, Fig.3 E).

UCP1 activation can be regulated by various protein kinases. Therefore, we probed gene expression of P38MAPK in above mentioned adipose tissues. Expression of P38MAPK increased significantly in the RJ group to 5 and 3.30 -fold in WAT and BAT respectively relative to CRD group (P<0.001).Moreover, TRF did not change the expression of P38MAPK notably (P≥ 0.05). Furthermore, the combination of RJ+TRF contributed to a significant increment of the P38 MAPK mRNA level in both adipose tissues in comparison with the CRD group (P<0.001, Fig.3F).

The elevated expression of major browning markers, including UCP1 and PRDM16 as well as regulators like CREB1and P38MAPK, suggest that promising effects of RJ on white adipocyte beiging and also classic brown adipocytes activation.

3-3- Effects of RJ, TRF and mixed supplementation on BMPs pathway

Considering that BMPs are signaling molecules that regulate the thermogenic program and function of classic brown adipose tissue, we measured the expression level of BMP8B and BMP7 in WAT, BAT hypothalamus. Our findings revealed that RJ significantly elevated BMP8B expression levels (5, 2.79 and 6 folds) in WAT, BAT, and hypothalamus respectively relative to CRD group (P<0.001, Fig.4A). Moreover, TRF did not show significant upregulation of BMP8B in any of the aforementioned tissues (P≥0.05). Intriguingly, RJ+TRF increased BMP8B expression level significantly by 4.2, 3 and 5 fold in WAT, BAT and hypothalamus, respectively compared with CRD group (P<0.001).

BMP7 plays an important role in whole energy homeostasis, adipogenesis, and energy expenditure. However, we founded that RJ, TRF and their combination did not alter the expression of BMP7 mRNA levels in this study (P≥ 0.05, Fig.4 B).

3-4-Histological Results

As illustrated in Fig.5A in CRD-fed rats, white adipocytes appeared smaller than HFD-fed rats with unilocular adipocytes (Fig.5 B). There was no evidence of WAT beiging in CRD and HFD –fed rats. Notably in RJ treated group we found small, multilocular beige adipocytes in WAT, implying that RJ enhanced the formation of beige adipocytes in WAT, as confirmed by RT-PCR results of UCP1 and other thermoregulatory genes up-regulations. (Fig.5C) Whiles in TRF group WAT changes were not considerable (Fig.5D). In the RJ+TRF group manifestation of some multilocular adipocytes among white adipocytes was noticed (Fig.5E). However, it is less obvious than RJ treated group. Our findings indicated that the RJ intake may lead to browning of WAT.

Interscapular BAT in the CRD group is observable with some white adipocytes nearby (Fig.6 A), whilst in the HFD group, we observed more unilocular white adipocytes along with typical brown adipocytes, indicating the brown to white adipose tissue transition (Fig.6 B). In the RJ and RJ+TRF group, BAT is distinguished with more reddish-brown appearance, increased capillaries, greater compacted brown adipocytes with multilocular lipid droplets compared to CRD group (Fig.6 C and 6E), whereas BAT in the TRF group was less compact with white reddish morphology and more connective tissues (Fig.6D). Following molecular findings, interscapular BAT in rats receiving RJ and RJ+TRF was activated, whilst this effect was not noticeable in the TRF group.

4 - Discussion

The rapid changes in lifestyle and dietary patterns in modern life over the last decades bring about the alarming pandemic of obesity. Although Calorie restriction (CR) through confining energy intake, is the most common lifestyle dietary intervention to combat weight gain, it is ineffective in the long-term, since thermogenic adoptions as defensive mechanism diminish the energy expenditure to stop energy depletion. Therefore, stimulation of BAT development in WAT (so-called browning) or increasing BAT function to enhance energy expenditure would be a promising approach to manage adiposity. To the best of our knowledge, it is the first research investigated the browning and thermogenesis properties of Royal jelly and γ- tocotrienol in the obesity model of rats during CRD. In this study, we demonstrated that RJ decreased adiposity, induced brite phenotype in WAT and activated BAT thermogenic program during CRD through significant up-regulation of UCP1 an indicator protein of brown adipocyte concomitant with a raised expression of PRDM16, a principal modulator of BAT development and P38MAPK, BMP8B and CEREB1 as other thermogenic components.

During 8 weeks of CRD, we noted an insignificant decrease in about 36% and 14% in UCP1 expression in WAT and BAT concomitant with weight loss in comparison to HFD-fed obese rats. This might be an adaptive response (whitening) to limited calorie intake or a negative energy balance and confirmed with manifestations of white adipocytes among brown adipose tissue in CRD-fed rats in histological analysis. Reduction in UCP1 expression throughout CR is in line with other previously done studies [4, 5]. However, in the present investigation due to the short duration of the study, it might do not reach a significant level. Therefore, we investigated the molecular changes in thermogenic machinery following the addition of RJ and γ- tocotrienol to CRD-fed rats. Our results showed that 8 weeks of administration of RJ

(100 mg/kg/day) to obese rats underwent CRD enhanced the UCP1 expression levels in both adipocytes. Consistent with these findings, Yoneshiro et al reported 5% lyophilized RJ powder in HFD-induced obese rats, enhanced UCP1 mRNA expression in BAT but not in WAT without modifying food intake suggesting the possible augmentation of thermogenesis in BAT and energy expenditure [30]. It is suggested that RJ attenuated the adverse effects of CR on thermogenesis via increasing BAT activity and WAT remodeling. However, TRF (85 mg/kg/day) added in CRD -fed rats was not efficient in the up-regulation of UCP1 and also other thermogenic regulators in adipose tissues. However, some studies revealed the positive thermogenic effects of other vitamin E analogs (α-tocopherol and δ tocopherol) in UCP1 gene induction in WAT of rats and also mouse preadipocytes [31, 32]. TRF used in our study comprises all isoforms of tocotrienols together with α- tocopherol. It is speculated that α- tocopherol interact with tocotrienols and represses their activity and also other isomers of tocotrienol in TRF may produce a synergistic or antagonistic impact with γ- tocotrienol and affect the outcomes. Thus, a pure isomer of γ- tocotrienol may provide different results. We confirmed combined RJ + TRF treatment in CRD obese rats significantly induced the expression of hallmark protein of thermogenesis, UCP1, and almost other thermogenic genes mRNA in both adipose tissues. Bearing in mind that TRF has not remarkable effect on UCP1 and other regulators of thermogenic program expression, the UCP1s induction is attributed to RJ treatment in the RJ + TRF group. Hence, we mechanistically explored the thermoregulatory and browning potency of RJ in the present investigation.

Our results showed that RJ added in CRD-fed obese rats caused a significant reduction in body weight to a greater extent relative to CRD alone (-67.21g ± 4.84 g vs -40.70g ± 6.50 g). It is in agreement with Yoneshiro et al's investigation, which reported that 5% of RJ restrained HFD-induced obesity and diminished the white adipose tissue collection in young mice without moderation in food intake [30]. The present study could not demonstrate the remarkable effect of TRF on weight loss of CRD-fed obese rats. Furthermore, Wong et al reported 120 mg/kg/day TRF for 8 weeks did not change body weight gain in HFD-fed Wistar rats [24]. In contrast, in young C57BL/6J mice supplementation of HFD with 0.05% γT3 for 4 weeks ameliorated HF diet-mediated obesity [33]. Different genetic backgrounds of studied animals and supplementation of pure γT3 versus TRF are possible major contributing factors in achieving inconsistent outcomes.

Recent studies find out new transcriptional components such as PRDM16, C/EBPβ, and CEREB1, which control BAT development and promote brown adipogenesis in inducible WAT. PRDM16 induces thermogenic program by interacting with PPARγ, C/EBP-β, PGC-1α, and PPARα in the regulatory promoter site of the UCP1 gene [10, 34, 35]. Our data revealed that RJ treatment in CDR-fed obese rats induced PRDM16 and CREB1 mRNA levels but not C/EBPβ mRNA in both WAT and BAT. It is identified that HDEA and HDAA, the main functional compounds in RJ are responsible for its biological activity [20]. HDEA and HDAA act as agonists of temperature-sensitive TRPs channels specially TRPA1 in sensory neurons of the gastrointestinal tract, provoke thermogenesis via a β-adrenergic receptor-mediated pathway in classic brown and inducible white adipocytes and simulate cold-induced nonshivering thermogenesis[13]. Therefore, the TRP-SNS-UCP1 pathway activity is the proposed mechanism for thermoregulatory effects or RJ in this study. Figure 7 demonstrated the suggested molecular mechanisms of RJ effect on

thermogenesis induction and browning of WAT. Despite the increasing trends, we did not find significant effects of TRF treatment on PRDM16, C/EBPβ, and CEREB1 induction during 8 weeks of the experiment. The reason why the expression of key thermoregulatory components was not up-regulated remarkably by TRF currently remains unclear, but the inhibitory effect of α-tocopherol on the absorption of γ-tocotrienol and suppression of its activity, insufficient doses of TRF or limitations of treatment duration may be the fact.

The downstream molecular signaling in the TRP-SNS-UCP1 pathway mainly includes discharging of norepinephrine from the sympathetic nerve terminals, provoking the tissue which mostly acting through β3-adrenergic receptors and finally PKA activation. Phosphorylated PKA leads to P38MAPK phosphorylation which in turn activates CREB1 and PGC1-α co-activator, ultimately result in transcription of UCP1 [17]. The proposed pathways are depicted in Fig. 7. The P38MAPK signaling has been defined as one of the important pathways trigger beiging and thermogenic machinery in various models [10]. Our results robustly demonstrated the potency of RJ but not TRF to induce the P38MAPK mRNA level in white and brown adipose tissues of CRD-fed rats. Therefore, these data support the theory of agonistic activity of RJ at TRPA1 channels in the TRP-SNS-UCP1 pathway.

BMPs are components of the TGF-b superfamily acting as extracellular signaling proteins and influence the adipogenesis in WAT and development of BAT. BMP7 promotes the differentiation of brown preadipocytes to mature brown adipocyte through PRDM16, PGC-1α induction and increased expression of UCP1 by P38MAPK dependent pathways [16]. BMP8Bs are signaling molecules. They are mostly expressed and active in mature BAT and also CNS.Therefore BMP8B has both central and peripheral actions which it's a consonant performance in CNS and BAT regulates thermogenesis and energy balance through increased the response to noradrenaline in mature BAT and enhancement of p38MAPK/CREB signaling [15, 36]. In the brain, BMP8B increases the level of sympathetic activation of thermogenesis. Our data demonstrated that RJ but not TRF added to CRD induced the expression of BMP8B, in BAT, WAT, and hypothalamus. Although the molecular mechanism by which RJ induced the BMP8B is not known yet, it is suggested that RJ may cooperate with BMP8B to increase the response to noradrenaline in adrenergic receptors and trigger the thermogenic program (Fig. 7). Despite the increment of the BMP7 mRNA level after RJ treatment, it did not reach a significant level. Longer duration of intervention with more doses may give rise to considerable results.

5- Conclusion

Here, our results suggest that RJ induced thermogenic gene expression and activation of BAT and brown-like phenotype emergence in WAT which is called browning or beigeing. Hence, RJ regulates adaptive thermogenesis by increasing the expression of thermogenic genes. Moreover, our data demonstrated that RJ treatment lowered body weight in comparison to CRD alone and prevent the thermogenesis decline or even cessation which occur usually in CR.

These findings suggest an important role for RJ in obesity treatment; moreover, these outcomes expand our vision toward dietary compounds and fat browning factors and propose a new approach in the treatment of obesity through the browning process of adipose tissue. It was the first study that assessed the effect of RJ and TRF in CRD –fed obese rats, however, there are a few limitations on it. We did not use genetically modified rats to firmly confirm the involvement of RJ in TRP-SNS-UCP1 axis. Therefore, Further Studies using TRPs or UCP1 knockout models or treatments with β-adrenergic blockers seem highly desirable to support the proposed pathway. We did not find a striking effect of TRF on BAT thermogenesis and/or browning of WAT since all isoforms of tocotrienols also α-tocopherol are available in TRF. Therefore interpreting the results would be difficult, owing to the possible interactions of tocopherol and tocotrienols. Additional studies with single γ-tocotrienol are required to fill these knowledge gaps.

Declarations

Ethics approval

All experimental procedures performed on animals complied with the National Institutes of Health guide for the care and use of laboratory animals [27]and approved by the Ethics Committee of Iran University of medical science (ethic code: IR.IUMS.FMD.REC 1396.9321324003).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Conflict of interest

The authors have declared no conflict of interest.

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Author Contributions:

F.Sh and MR.V supervised the work, designed research and overseeing the study implementation. N.MA and P.I contributed to experiment performing, developed the hypothesis, and drafted the manuscript. M.AJ analyzed and interpreted data. N.R and MR. A advised in RT-PCR experiments. Sh. A assisted in technical

experiments and laboratory works. L.R participated in histological examinations. F.F participated in manuscript editing and reviewing. All authors approved the final draft.

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Tables

Table 1: Composition of the experimental diets.

Dietary Composition(g/kg)	Chow	HFD	CRD
Carbohydrate	536.2	335.125	335.125
Fiber	42	26.25	26.25
Protein	260.8	163	163
Lipid	40	400	400
Calcium	9.5	5.93	5.93
Phosphorus	6.5	4.06	4.06
Salt	5	3.125	3.125
Moisture	50	31.25	31.25
Ash	50	31.25	31.25
Energy density (kcal/g)	3.6	5.6	5.6

HFD: high-fat diet; CRD: calorie restriction diet

Table 2: Sequences of primers used for qRT-PCR in study.

Gene	Forward	Reverse
CREB-1	CTACAATATGCACAGACCACT	GAGGACGCCATAACAACTCCA
p38MAPK	GACCTAAAGCCCAGCAACCTC	CGTAGCCGGTCATTTCGTCA
C/EBP β	ACACGGGACTGACGCAAC	AAACATCAACAGCAACAACCC
PRDM16	CCAAAACCGTGTGATAAGGTC	GGGTATTTGGCACATTAACAAC
BMP7	TTCCTCACCGACGCCGACA	AAGATCAAACCGGAACTCTCGAT
BMP8B	TCGAGCACCACTAGCGACT	GTTGCCACTGTCATCCGTCA
UCP-1	TTCTTTTCTGCGACTCGGAT	GCCCAATGGTGTTTAGCATC
β-actin	TCAGGTCATCACTATCGGCAA	TTACGGATGTCAACGTCACAC

Figures

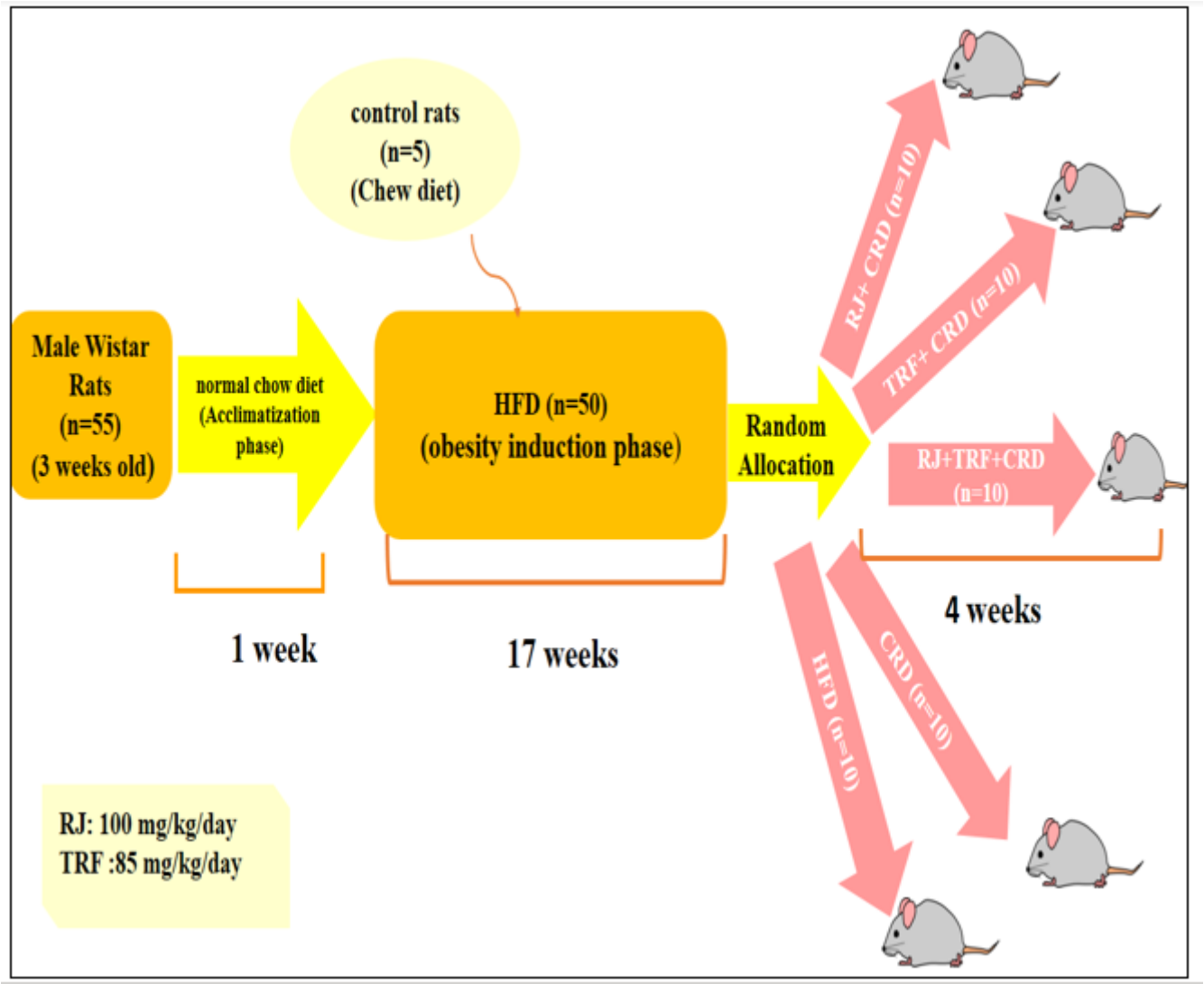

Figure 1

Scheme of study design.

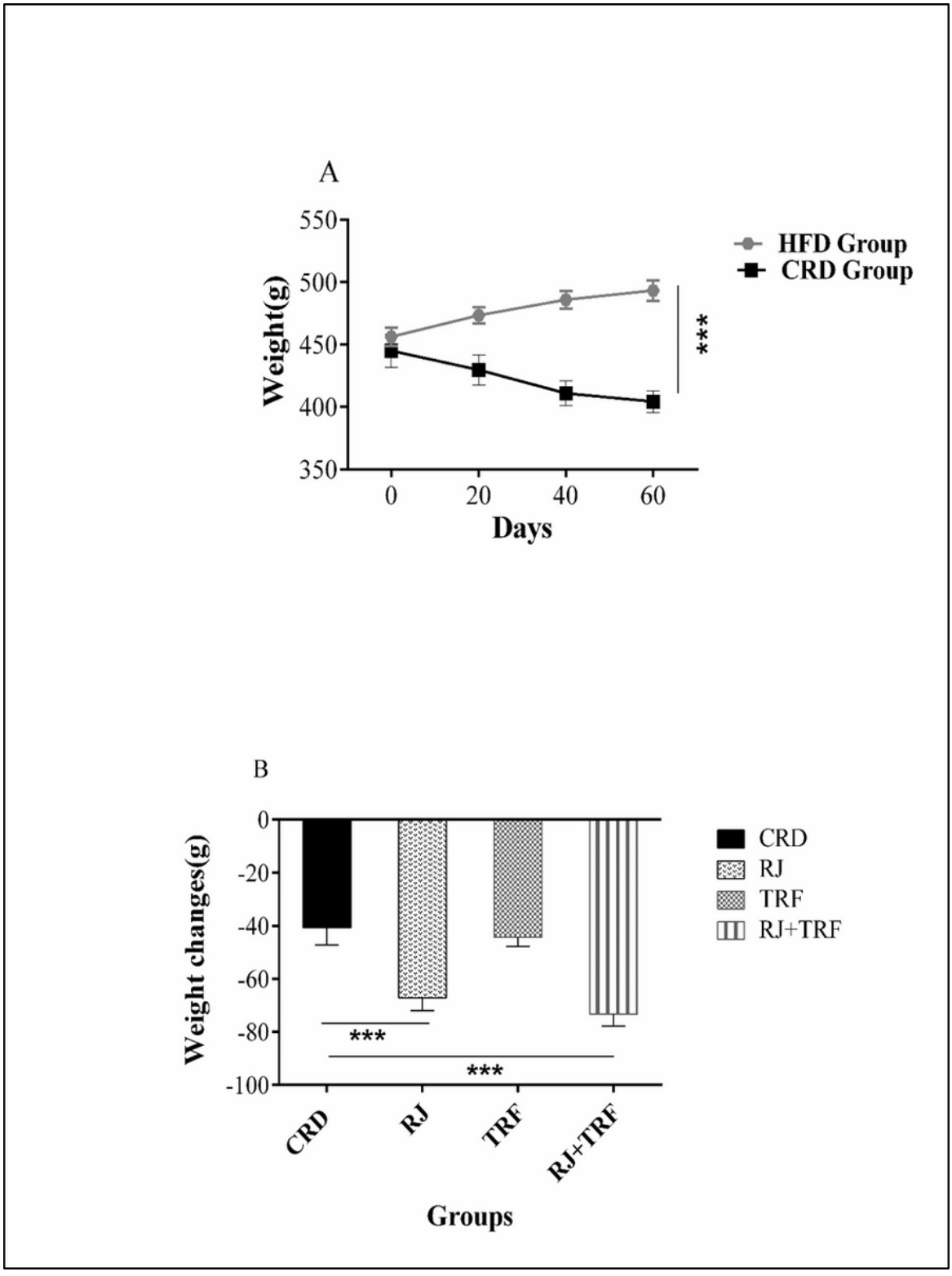

Figure 2

(A) Comparison of body weight after 8 weeks of treatment in the CRD vs. HFD group. (B). Bodyweight changes in RJ, TRF and TRF+RJ groups vs. CRD group. Data are expressed as mean ±SEM, (n=10). ***P<0.001 by one-way ANOVA.

Figure 3

(A, B) Effect of CRD after 8 weeks of intervention, on the expression of UCP1 vs. HFD in WAT and BAT, quantified by qRT-PCR. (C-F) Effect of RJ, TRF and TRF+RJ treatment on expression of brown fat-specific and thermogenic genes vs. CRD in WAT and BAT quantified by qRT-PCR. Data are presented as the mean ±SEM (n=10). ***P<0.001 by one-way ANOVA.

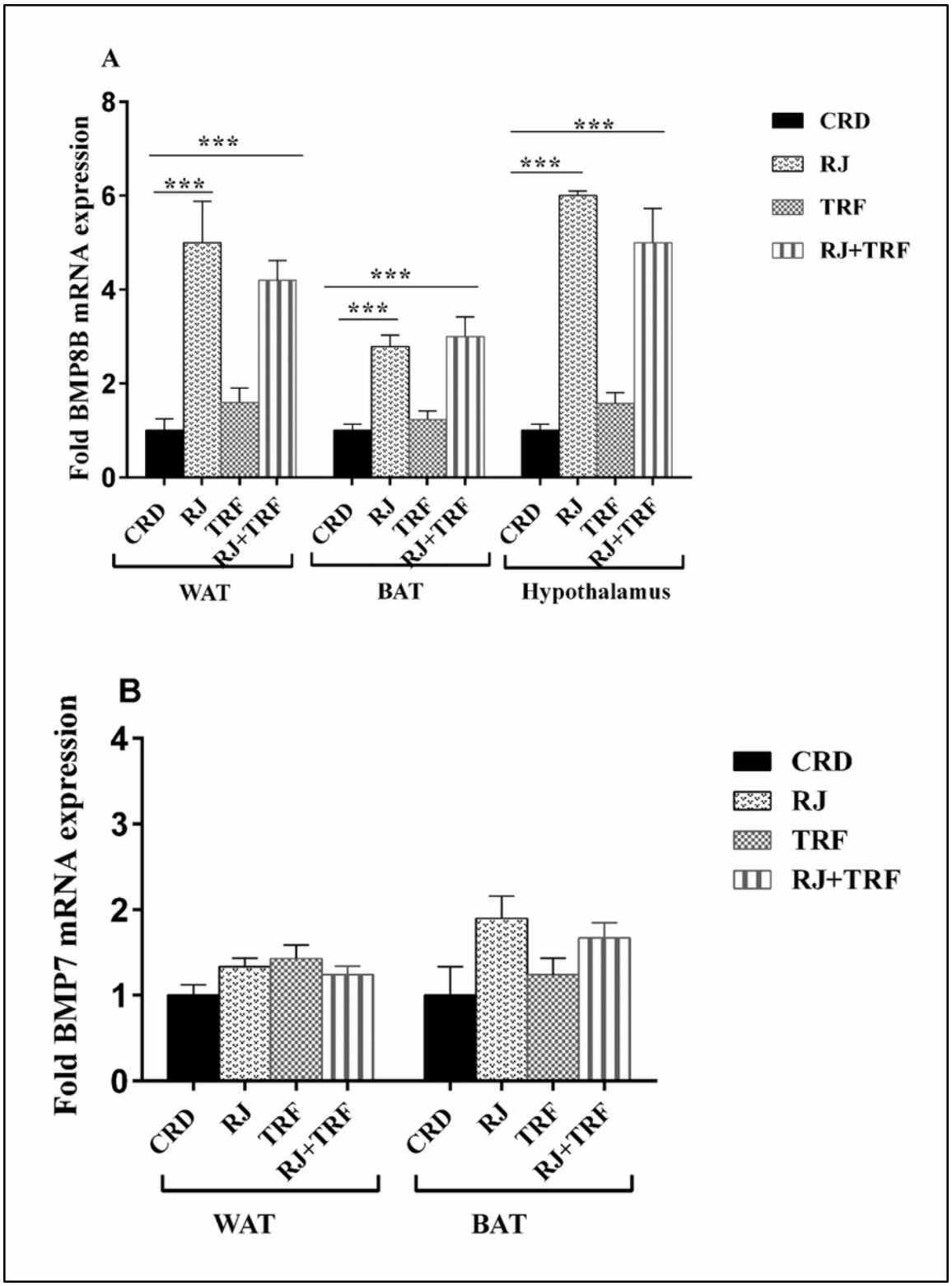

Figure 4

Effect of RJ, TRF and TRF+RJ treatment on the expression of BMP8B gene in WAT, BAT, and hypothalamus (A) and expression of BMP7 gene in WAT and BAT vs. CRD quantified by qRT-PCR. Data are presented as the mean ±SEM (n=10). ***P<0.001 by one-way ANOVA

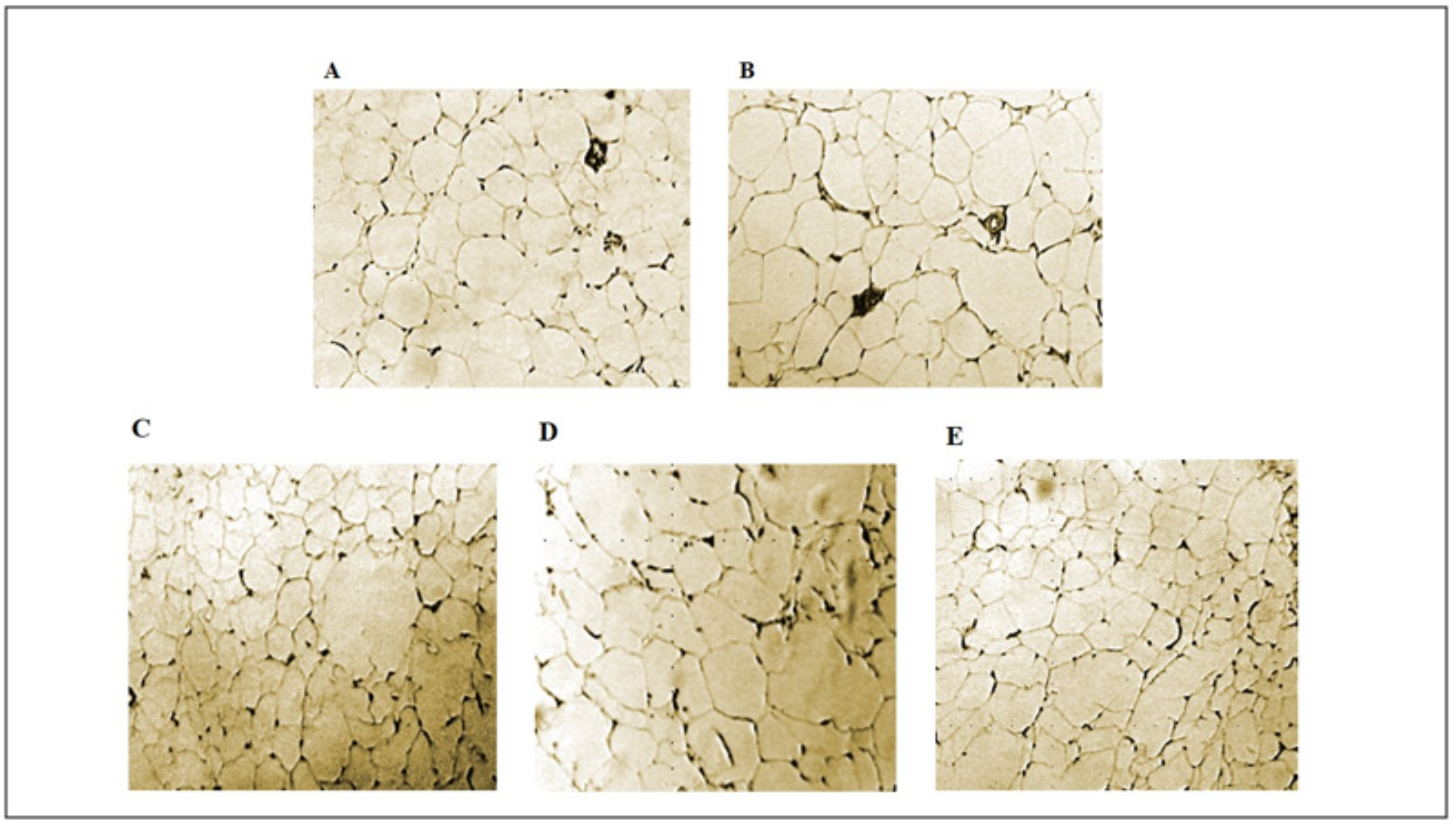

Figure 5

Hematoxylin and eosin (H&E) staining of inguinal WAT section of CRD (A), HFD (B), RJ(C), TRF (D) and RJ+TRF (E) received rats. All images were obtained at × 400 magnification.

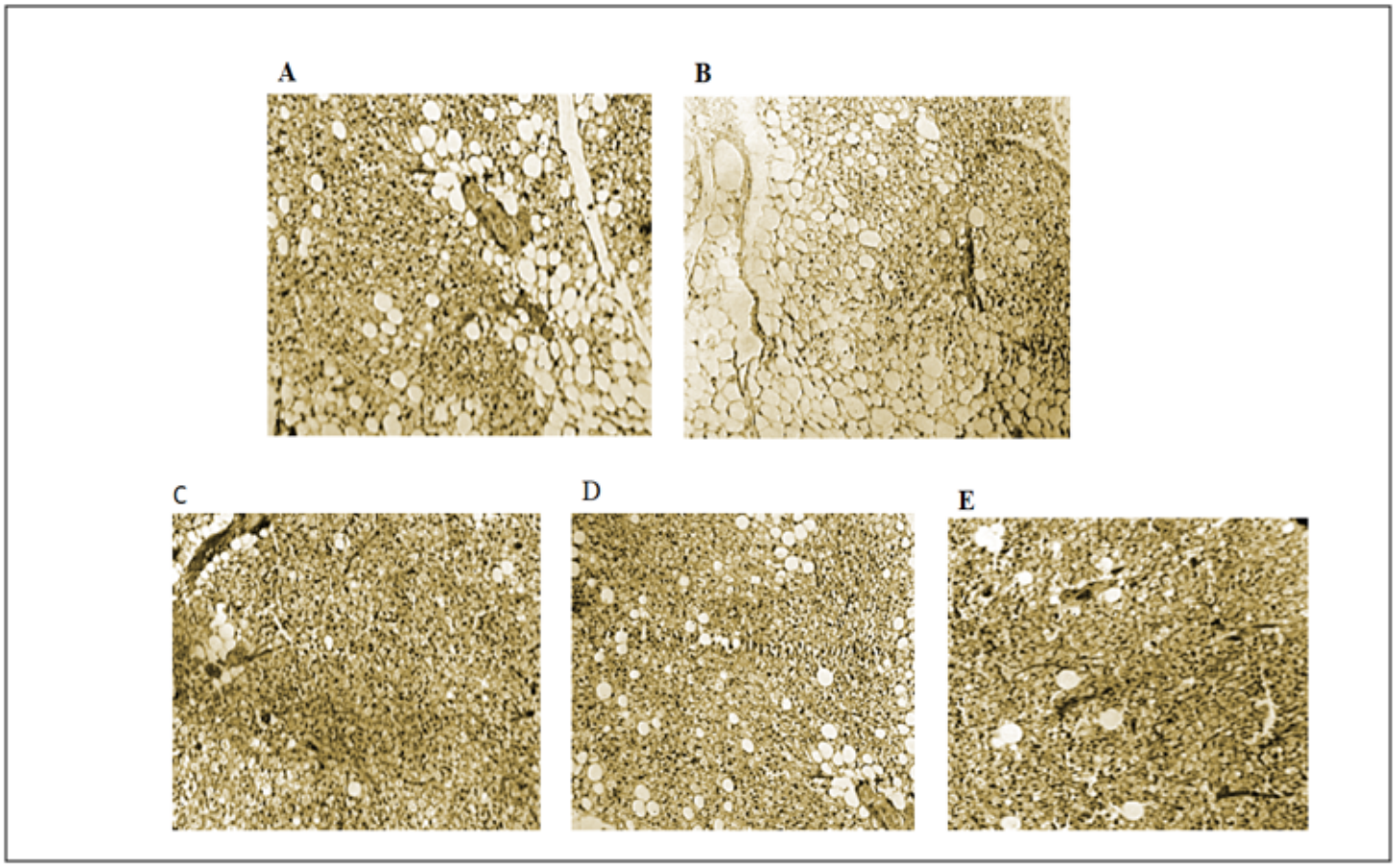

Figure 6

Hematoxylin and eosin (H&E) staining of interscapular BAT section of CRD (A), HFD (B), RJ(C), TRF (D) and RJ+TRF (E) received rats. All images were obtained at × 400 magnification.

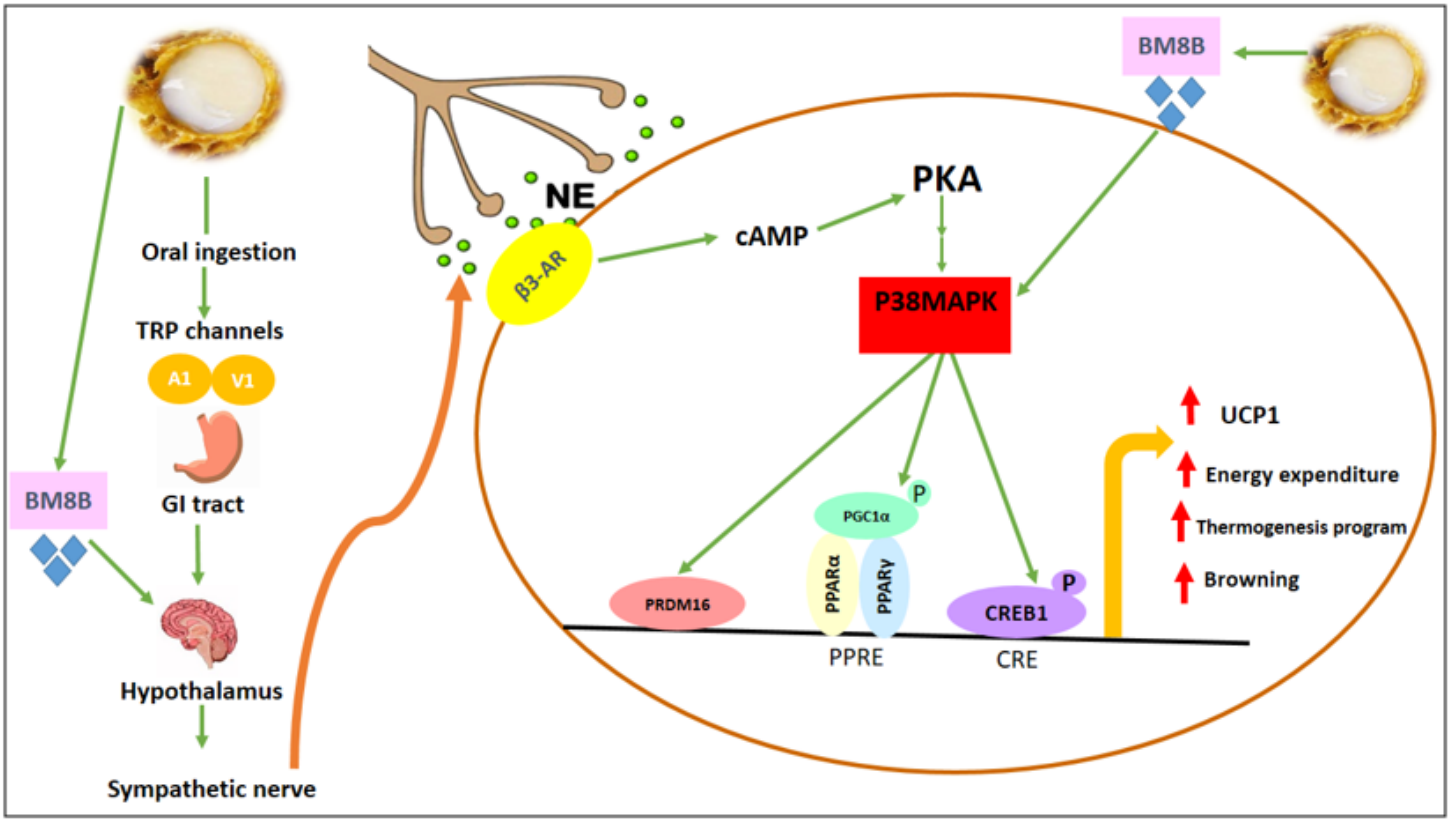

Figure 7

Suggested pathways for browning of white adipose tissue and brown fat activation by RJ. RJ with agonistic activity, activate temperature-sensitive TRP channels in the GI, triggering thermogenesis through the activation of the TRP-SNS-UCP1 axis. B3-AR (β3-adrenoceptor) , BMP8B (bone morphogenetic protein8B), cAMP (cyclic adenosine monophosphate), CREB1 (cAMP response element-binding protein1), NE (norepinephrine), PRDM16 (PR domain containing16), PPARγ (peroxisome proliferator-activated receptor-gamma), PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), PPARα (peroxisome proliferator-activated receptor alpha), P38 MAPK(P38 mitogen-activated protein kinase), PKA (cAMP-dependent protein kinase A), TRP(transient receptor potential) and UCP1 (uncoupling protein 1).