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Allicin-rich garlic extract alleviates high-fat diet-induced complications in rats: A nutrigenomic study

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Abstract

The remedial effects of garlic in various metabolic complications have been attributed to a variety of organosulfur compounds such as allicin, diallyl sulfides, alliin and allyl trisulfides. The present study was designed to prepare an aqueous extract of garlic with stable allicin and its biological evaluation on tissue-specific nutrigenomic effects in the prevention of High-fat diet (HFD)-induced weight gain and related complications in Sprague-Dawley (SD) rats. Aqueous Allicin-rich garlic extract (ARGE) was prepared and characterized for stability of allicin using HPLC. For biological evaluation, animals were fed with either normal pellet diet (NPD), HFD, HFD along with ARGE (1ml/kg p.o.) and ARGE per se (1ml/kg p.o.) for 16 weeks. Chronic administration of ARGE prevented HFD-induced weight gain, adipose tissue hypertrophy, insulin resistance and improved overall glucose homeostasis. Transcriptional analysis of different tissues highlighted that ARGE promoted browning, improved glucose metabolism and appetite regulation. Overall, this study presents ARGE as a potential nutraceutical for the prevention of obesity and related comorbidities.

Key words: ARGE, allicin-rich garlic extract; TRPA1; obesity; BAT, brown adipose tissue; WAT, white adipose tissue; DADS, diallyl disulfides; DATS, diallyl trisulfides; insulin resistance; WAT browning; gut hormones.

INTRODUCTION

Global obesity is increasing tremendously in recent years [1]. Obesity and overweight are major risk factors for the development of many non-communicable diseases, including – type 2 diabetes, cardiovascular diseases, hypertension and cancers [2,3]. Effective and safe treatments for the suppression of obesity and related disorders are limited. Although, there are many FDA-approved drugs in the market but due to their limited safety profile, use of dietary food constituents with potential to prevent the development of obesity

has emerged as a novel strategy to tackle the disease [4,5].

Garlic (*Allium sativum*), belongs to a group of herbaceous and ornamental plants (subfamily *Alloioideae*) with nutritional, medicinal and pro-health benefits. Dietary composition of garlic includes carbohydrates, proteins, dietary fibers, amino acids, fat, vitamins, minerals and water [6,7]. Besides these, garlic also contains various organosulfur compounds like allicin, ajoene, diallyl disulfides (DADS), diallyl trisulfides (DATS), allyl methyl trisulfides and S-methyl cysteine (alliin), which

not only give characteristic pungent smell to it, but are also responsible for most of the beneficial effects associated with the consumption of garlic [8]. Many experiments and clinical studies have shown anti-microbial, anti-inflammatory, anti-oxidant, hypolipidemic, hypoglycemic and immunomodulatory effects of garlic [7,9–12]. Oral administration of raw garlic in high-fat diet (HFD)-induced obese mice and rats has been reported to prevent weight gain, epididymal fat accumulation and hyperlipidemia [13–15]. Processed garlic such as methanolic extract, garlic powder and garlic oil also have the potential to combat obesity [14,16,17].

Allicin, one of the bioactive components of garlic has numerous biological functions including anti-obesity effects. Allicin administration enhances beiging/browning of white adipose tissue, modulates gut microbiota and recently has been found to enhance whole-body energy metabolism via brown adipose tissue (BAT) activation [18,19]. Allicin is absent in the raw garlic and is only produced upon crushing of the garlic clove from its odorless precursor alliin through the enzymatic reaction of enzyme alliinase [20]. Allicin has very reactive thiol groups, immediately converting or degrading it into diallyl disulfides in the presence of air [21]. Aqueous extract of garlic, however, if stored at lower temperature, has stable allicin [22]. Recently, it has been shown that higher doses of allicin (500 μ M) induce autophagy and mitophagy in cultured BAT adipocytes and excessive garlic intake could impair BAT functions [19]. Earlier studies suggested protective effects of allicin in high-fat diet-induced complications at relatively higher doses such as 50, 100 and 200 mg/kg/day [23] and 100 mg/kg/day [18], therefore we tried to evaluate the preventive effects of allicin-rich garlic extract at lower dose (~ 2 mg/kg/day) in present study. Transient receptor potential ankyrin (TRPA1) is a calcium ion channel, primarily known for its role in pain sensation. This channel has been investigated for other physiological roles in the gastrointestinal tract including gastric motility, satiety and insulin secretion from the pancreas [24–26]. Allicin is a potent agonist of the TRPA1 [27], providing a new direction for the gastro-modulatory effects of garlic extract via TRPA1 activation.

Despite available literature on anti-obesity effects of garlic, there are no previously published reports regarding the nutrigenomic effects of freshly prepared allicin-rich garlic extract in HFD-induced obesity. Therefore, the present study was designed to study the multiple tissue-specific nutrigenomic effects of aqueous allicin-rich garlic extract (ARGE) prepared from fresh garlic against HFD-induced changes in rats.

MATERIAL AND METHODS

Preparation of ARGE

ARGE was prepared freshly using a previously standardized protocol [28]. Briefly, garlic cloves were washed, peeled and squeezed through kitchen garlic press. Phosphate buffer saline (PBS) was then added to the garlic paste in a ratio of 500 μ l PBS to 1 g of garlic paste. This mixture was then incubated at 4 °C for 30–40 min with continuous agitation followed by centrifugation at 6000 rpm for 10 min at 4 °C. Supernatant was discarded and pellet was re-centrifuged at 13,000 rpm for 15 min at 4 °C. The clear supernatant was collected, filtered and used for high performance liquid chromatography (HPLC) analysis and biological evaluation.

Quantification of Allicin in ARGE using HPLC

Allicin was synthesized according to previously published article [29]. Briefly, diallyl sulphide (80%; Sigma Aldrich Chemical Co, MO, USA), was oxidized with hydrogen peroxide in acidic medium at 0 °C. The reaction was neutralized by adding 1% sodium bicarbonate and was partitioned with dichloromethane to isolate the crude mixture in organic phase. The allicin was isolated from the crude reaction mixture using preparative thin layer chromatography. HPLC analysis was performed to determine the purity of synthesized allicin and its quantification in ARGE. Waters 600 liquid chromatography equipped with Waters 2998 photodiode array (PDA) detector and C₁₈ column (250 mm $\times$ 4.6 mm, 60 Å, 5.0 μ m, Waters Sunfire®) was used for HPLC. The mobile phase consisted of solvent A, methanol and solvent B, water. Isocratic HPLC program was as follows: MeOH:Water, 1:1 for 20 min. The flow rate was 1 ml/min. All the experiments were performed in triplicate. Nuclear magnetic resonance (NMR) and Mass spectroscopic techniques were performed to characterize the synthesized and purified allicin. 1 mg/ml solution of allicin in methanol was used for MS spectrometry and 10 mg of purified allicin was dissolved in 600 μ l of CDCl₃ to acquire NMR data. For stabilization studies, ARGE was lyophilized immediately after its preparation and then stored at different temperature conditions (- 80 °C, 4 °C and room temperature (RT) for varying time period), followed by HPLC analysis for determination of allicin in ARGE using same conditions as mentioned above.

Analysis of total phenolic content

The total phenolic content of ARGE was estimated spectrophotometrically using Folin-Ciocalteu reagent and gallic acid as standard [30]. Briefly, 100 μ l of ARGE was mixed with 2.5 ml of Folin-Ciocalteu reagent (0.2 N), followed by addition of 2 ml of sodium carbonate solution (75 g/l). The reaction mixture was incubated for 120 min in dark conditions and optical density was

measured at 760 nm (SpectraMax i3x, Molecular Devices, USA). Results were expressed as Gallic acid equivalents (GAE), in µg per ml of the sample.

Animals

Male Sprague-Dawley (SD) rats (6-8 weeks old) were procured from IMTech Center for Animal resources and Experimentation (iCARE), Chandigarh, India. Rats were housed in Animal Experimentation facility (AEF) at National Agri-food Biotechnology Institute (NABI), Mohali, India and kept in a pathogen-free environment at $25 \pm 2^\circ\text{C}$, with 12 h light-dark cycle. All the rats had free access to food and water. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC), NABI (approval no. NABI/2039/CPCSEA/IAEC/2019/12) and experiments were performed according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Rats (CPCSEA) guidelines.

Diets and Interventions

Normal pellet diet (NPD) was purchased from Hylasco Biotechnology Pvt. Ltd., Hyderabad, India. HFD was prepared in-house using standard composition with slight modifications. Composition of HFD used was: NPD (36.5%), lard (35%; local market, Chandigarh, India), casein (25%; Loba Chemie, Mumbai, India), vitamin and mineral mix (6%; Virbac Animal Health India, Mumbai, India); DL-methionine (0.3%; Himedia Laboratories Pvt. Ltd., Mumbai, India); and sodium chloride (0.1%; Sigma-Aldrich, Saint Louis, MO).

After 1 week of acclimatization, animals were divided into following groups using weight-based randomization: control group ($n = 4$) fed with NPD, HFD group ($n = 4$) fed with HFD, HFD+ARGE (2 mg/kg/day p.o. (~ 2 mg/kg/day allicin) + HFD; $n = 4$) and ARGE group (2 mg/kg/day p.o. (~ 2 mg/kg/day allicin) + NPD; $n = 4$). Animal body weights were recorded weekly.

Oral glucose tolerance test (OGTT)

Rats have fasted overnight (12 hours) before oral administration of glucose (2 g/kg body weight) during 14th week of study. Blood glucose concentrations were measured using tail snip method at 0 (before treatment), 30, 60, 90 and 120 minutes after glucose or sodium pyruvate administration using Glucocard (Arkray Factory Inc., Shiga, Japan).

Intraperitoneal pyruvate tolerance test (IPPTT)

Rats were fasted overnight (12 hours) before intraperitoneal injection of sodium pyruvate (1g/kg body weight) during 15th week of study. Blood glucose concentrations were measured using tail snip method at 0 (before treatment), 30, 60, 90 and 120 minutes after

glucose or sodium pyruvate administration using Glucocard (Arkray Factory Inc., Shiga, Japan).

Insulin tolerance test (ITT) and assessment of insulin resistance

During the 16th week of the study, overnight fasted rats were intraperitoneally injected with human recombinant insulin (0.5 IU/kg body weight). Blood glucose concentrations were measured using tail snip method at 0 (before treatment), 30, 60, 90 and 120 minutes after insulin administration using Glucocard (Arkray Factory Inc., Shiga, Japan). Further, Insulin resistance was calculated using Homeostasis Model Assessment Insulin Resistance (HOMA-IR) using basal glucose (mmol/l) and fasted serum insulin level (µIU/ml) and insulin sensitivity was assessed by using Quantitative insulin sensitivity check index (QUICKI).

Glucose-stimulated insulin release

Rats were fasted for 12 hours, followed by withdrawal of blood in fasted conditions. Glucose was given orally (2 g/kg p.o.) to the fasted rats. After 30 min of glucose administration, blood was withdrawn from rats. Serum was separated and stored at -80°C until further analysis of insulin levels using ELISA (Ray Biotech, Norcross, GA, USA).

Estimation of serum ghrelin levels

Rats were fasted for 12 hours (dark cycle) followed by collection of blood samples. Blood was allowed to clot for at least 30 minutes before centrifugation at 2000 rpm for 20 minutes. Serum was collected and stored at -80°C until further analysis of fasted serum ghrelin using ELISA (Ray Biotech, Norcross, GA, USA).

Histological Analysis

After animal sacrifice, visceral white adipose tissue (vWAT) and liver were stored in 10% v/v buffered formalin for further processing. Small pieces of these tissues were then serially dehydrated using ethanol (25%, 50%, 75%, 90% and 100% for 2 h each) after which tissues were processed in xylene for 2 h (twice) followed by paraffin embedding for 2 h (60°C). Tissue sections of 5 µm were used for H&E staining. Briefly, sections were de-paraffinized using xylene (2 min, twice), followed by rehydration using ethanol (100% to 25%, 2 min each). Tissue sections were then stained in Hematoxylin and Eosin (Hi-media Laboratories, Mumbai, India) for 2 min and 30 sec, respectively, washed thrice in PBS. Tissue sections were again serially dehydrated in ethanol and treated in xylene before mounting for microscopic analysis. Images were captured using Leica microscope and analyzed using ImageScope (version 12.1.0.5029, Aperio Technologies Inc).

RNA extraction and gene expression analysis

All the tissues (stomach, ileum, colon, liver, hypothalamus, vWAT, and BAT) were excised during sacrifice and immediately stored in RNA later (Sigma-Aldrich, USA) for the gene expression analysis. RNA was extracted from tissues using Trizol-Chloroform-Isopropanol method. Briefly, tissues were homogenized in Trizol, followed by chloroform-based phase separation using high-speed centrifugation (12 000 rpm, 15 min at 4 °C). RNA was then precipitated using chilled isopropanol (centrifugation: 12 000 rpm, 15 min at 4 °C) and RNA pellet was washed with chilled 75% ethanol (centrifugation: 12 000 rpm, 15 min at 4 °C). RNA pellet was eluted in nuclease-free water. Qualitative analysis was done using 1.2% agarose gel electrophoresis to check the integrity of RNA bands. Total RNA quantification was done using Nanodrop at 260/280 nm and cDNA was synthesized using revert-aid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA). Gene expression analysis was performed using quantitative Real-time PCR (qPCR) employing iTaq Universal SYBR Green Supermix (Bio-Rad, California, United States). qPCR was performed on CFX96 Touch Real-Time PCR Detection System (BioRad) under following conditions: initial denaturation – 95 °C, 2 min, [denaturation – 95 °C, 5 s; annealing/extension – 60 °C, 30 s] x 40 cycles, final extension – 60 °C, 5 min and melt curve analysis between 60 °C – 95 °C with 0.5 °C/5 s increment. Data were analyzed using $\Delta\Delta C_t$ method [31] and β -actin was used as a housekeeping gene for normalization. The list of primers used is given in **Supplementary Table 1**.

Indirect Calorimetry

Total energy expenditure, respiratory quotient, spontaneous physical movement and feed intake was measured using calorimetric cages simultaneously. All the rats were acclimatized for these cages equipped with commercially available grid panels for at least 24 hours before the actual measurements. After acclimatization, 24 hours (12 h nocturnal: 12 h day time) data were recorded using OxyletPro™ System (Panlab, Harvard Apparatus, Spain) and analyzed using Panlab Metabolism software. Rats were housed individually in these chambers.

Statistical Analysis

All the values are expressed as mean $\pm$ SEM. Data was analyzed using GraphPad Prism Software (GraphPad, San Diego, California, USA). Multiple groups' comparison was done using One-way or Two-way ANOVA followed by specific post-hoc tests. $P \leq 0.05$ was considered statistically significant. Specific statistical tests used are mentioned in each figure legends.

RESULTS

Chemical characterization of Allicin in ARGE

NMR and Mass spectroscopy (Electron spray ionization) were performed to characterize the purified allicin. 1 mg/ml solution of allicin in methanol was used for MS spectrometry and 10 mg of purified allicin was dissolved in 600 μ l of $CDCl_3$ to acquire NMR data. The structural data obtained were as follows: 1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 3.72 - 3.90 (m, 4H, $-CH_2-$), 5.20 - 5.84 (m, 4H, $CH_2=$), 5.89 - 6.01 (m, 2H, $=CH-$); ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 35.02 (3b, SCH_2CH), 59.86 (3a, $CHCH_2SO$), 119.06 (1b, $CHCH_2$), 124.02 (1a, CH_2CH), 125.82 (2b, CH_2CHCH_2), 132.89 (2a, CH_2CHCH_2) (**Figure 1 A, B**). MS (ESI) calc. for $(C_6H_{10}OS_2) [M+Na]^+$ 185.26, found $m/z = 185.92$ (**Figure 1C**). The spectral data was compared with the reported literature and confirmed as allicin [29]. HPLC chromatogram of synthesized allicin depicted the presence of single peak at retention time 9.77 min (**Figure 1D**) with purity of 97.19%. Further, HPLC profile of ARGE depicted the presence of allicin at Rt. 9.77 min (**Figure 1D**) which was confirmed by peak spiking with pure allicin and it was estimated that ARGE contained 1.96 ± 0.01 mg/ml allicin (**Figure 1D**). ARGE was lyophilized to assess the stability of allicin at different time intervals and temperature conditions. HPLC analysis of lyophilized extract kept at - 80 °C showed the highest stability of allicin (**Figure 1E and 1F**). Garlic contains numerous polyphenols, responsible for high antioxidant properties of garlic. Therefore, total phenolic content (**Figure 1G**) was measured in ARGE using Folin-Ciocalteu method. ARGE contained 66.84 ± 0.215 μ g/ml total phenolic content.

Chronic administration of Allicin-rich garlic extract prevents HFD-induced weight gain, adiposity and improves glucose homeostasis

Coadministration of HFD with ARGE for 16 weeks prevented HFD-induced weight gain in rats (**Figure 2A**). ARGE administration also prevented diet-induced increase in visceral fat mass (**Figure 2B**). Histological examination of visceral fat using H&E staining showed that ARGE could prevent diet-induced adipocyte hypertrophy (**Figure 2C**). Digital image analysis also supported the above data of adipocyte hypertrophy in HFD-fed animals (**Figure 2D**). HFD feeding increased the distribution frequency of larger adipocytes in rats, which were less when ARGE was coadministered to the animals (**Figure 2E**).

To determine the effects of ARGE on whole-body metabolism, indirect calorimetry was measured. Total activity in all groups showed a clear circadian rhythm, with higher activity during the nocturnal phase (**Fig-**

Figure 1. Quantification of Allicin in ARGE. **(A)** Proton NMR (¹H - NMR) of pure allicin, **(B)** Proton NMR (¹³C - NMR) of pure allicin, **(C)** Mass spectra of pure allicin, **(D)** Overlay HPLC chromatogram of allicin and ARGE, **(E)** Stability characterization of allicin in ARGE, **(F)** Allicin quantification in ARGE at different time intervals and temperature conditions, **(G)** Total phenolic content. Data have been represented as Mean ± SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test. *p < 0.05 versus samples stored at - 80 °C, **p < 0.01 versus samples stored at - 80 °C, ***p < .001 versus samples stored at - 80 °C.

ure 2F). ARGE administration led to enhanced activity during the daytime (Figure 2F). However, ARGE supplementation does not affect respiratory quotient, total energy expenditure and food intake (Data not shown).

ARGE administration improved glucose clearance as observed in OGTT (Figure 3A). HFD-fed animals showed hyposensitivity towards insulin, as insulin-induced drop in blood glucose levels was lower in these animals (Figure 3C). AUC analysis of OGTT and ITT showed high level of blood glucose in HFD-fed animals (Figure 3B and 3D). However, no significant differences in any group were observed in IPTT (Figure 3E and 3F). To further evaluate the effect of ARGE on

insulin sensitivity, glucose-stimulated insulin release was measured (Figure 3G). A significantly high level of serum insulin was found in HFD-fed animals in fasted state (Figure 3G), whereas glucose-induced increase in serum insulin concentration was low in HFD-fed animals (Figure 3H). Administration of ARGE improved glucose-induced serum insulin levels, suggesting improved insulin sensitivity (Figure 3G and 3H). To evaluate the insulin resistance in HFD-fed rats, QUICKI index and HOMA-IR were calculated. Lower value in QUICKI index and higher HOMA-IR in HFD-fed rats corresponds to insulin resistance (Figure 3I and 3J), and ARGE administration prevented the insulin resistance.

Figure 2. Effect of chronic administration of ARGE on body weight gain and adiposity in HFD-fed rats. Rats were fed either with NPD (control group) or HFD, along with daily oral administration of ARGE (1 ml/kg body weight p.o.; ~ 2 mg/kg/day allicin) to the HFD + ARGE and ARGE *per se* group. **(A)** Weekly body weights and change in body weight gain in 16 weeks (n = 4), **(B)** vWAT mass (n = 4), **(C)** Representative images of hematoxylin and eosin (H&E) stained sections of vWAT (20X magnification, scale: 100 μm, n = 3 rats per group, 5 images per group), **(D & E)** Adipocytes size and distribution frequency of H&E stained sections of vWAT, **(F)** Animals were kept in the metabolic cages for 12 hours during daytime and 12 hours during nighttime for the measurement of different parameters using indirect calorimetry. Total activity during daytime and nighttime is shown. Data have been represented as Mean ± SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test and student's unpaired t-test. *p < 0.05 versus Control, **p < 0.01 versus Control, ***p < .001 versus Control; #p < 0.05 versus HFD, ##p < 0.001 versus HFD, ###p < 0.001 versus HFD.

ARGE enhances browning and energy expenditure in vWAT and BAT

To investigate the effects of ARGE on energy expenditure through WAT, qRT-PCR was performed. **Figure 4A** represents a heat map showing differential expression of various genes involved in energy expenditure as well as browning in WAT. Supplementation of ARGE to HFD-fed rats significantly increased the expression of several genes including *AdipoQ* (Adiponectin), *TMEM26* (a browning marker on beige preadipocytes), *PGC1-α* (a key regulator of energy metabolism, mitochondrial biogenesis), *PAT2*, *P2RX5* (markers for beige adipocytes) and *TBX1* (**Figure 4B**). Gene expression analysis was performed to evaluate the effect of ARGE on energy expenditure genes in BAT. The heat map in **Figure 5A** shows an overview of gene expression of various energy expenditure-related markers in mentioned groups. Since BAT is a metabolically active organ, some of the genes, such as *PPARγ*, *COX 8b* and *SHOX2* were

found to be significantly higher in both HFD group and ARGE-treated animals (**Figure 5B**). Browning markers like *TMEM26* and *TBX1* were significantly increased in ARGE-supplemented animals (**Figure 5B**).

ARGE administration prevents HFD-induced ectopic fat deposition in liver

To determine histopathological changes in liver tissues, Hematoxylin-Eosin staining was performed. We observed that ARGE prevented the HFD-induced lipid accumulation in liver (**Figure 6A**). We also examined expression pattern of gluconeogenic, glycogen metabolism and key insulin signaling genes. Transcriptional factors controlling lipogenesis and gluconeogenesis, such as *FOXO1*, *ChREBP* and fatty acid translocase gene *CD36* were increased after HFD feeding (**Figure 6B**). ARGE supplementation also upregulated the gene expression of these transcriptional factors (**Figure 6B**). Significantly higher expression levels of *CPT1*, *GYS* (gly-

Figure 3. Effect of ARGE on glucose homeostasis and serum insulin levels. Glucose homeostasis tests (OGTT, ITT, IPPTT) were performed during 13-15th week of the study. Animals were fasted for 12 hours, after which dose of 2 g/kg body weight p.o. glucose for OGTT, 0.5 IU/kg body weight i.p. insulin for ITT and 1 g/kg body weight i.p. pyruvate for IPPTT were given. Blood glucose levels were measured at 0 (before any treatment), 15 min, 30 min, 60 min, 90 min and 120 min. **(A)**, **(B)** and **(C)** Recorded blood glucose concentrations in OGTT, ITT and IPPTT (n = 4); **(D)**, **(E)** and **(F)** Area under the concentration curves of OGTT, ITT and IPPTT respectively. For serum insulin level measurement animals were fasted for 12 hours followed by 2 g/kg body weight p.o. glucose administration. Blood was collected both in fasted as well as after glucose administration; **(G)** and **(H)** Serum insulin concentrations in fasted and glucose treated animals; percentage change in serum insulin levels; **(I)** and **(J)** QUICKI and HOMA-IR indexes. Control - Normal pellet diet-fed animals; HFD + ARGE – 1 ml/kg body weight p.o. ARGE (~ 2 mg/kg/day allicin) to HFD fed animals; PP – 2 g/kg body weight p.o. glucose. Data have been represented as Mean ± SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test. *p < 0.05 versus Control, **p < 0.01 versus Control, ***p < .001 versus Control; #p < 0.05 versus HFD, ##p < 0.001 versus HFD, ###p < 0.001 versus HFD.

cogen synthase), *GCK1* (glucokinase), *ACC* (acetyl-coA carboxylase) and *UCP2* (uncoupling protein 2) were found in ARGE-treated groups, whereas gene expression of *G-6-PC* (glucose-6-phosphatase) was significantly downregulated in ARGE group, corresponding to more glycogen synthesis or less circulating glucose (**Figure 6C**).

ARGE administration regulates expression of hunger and satiety hormones

Various genes involved in the regulation of gut hormones were evaluated in stomach, ileum and colon using qRT-PCR. **Figure 7A** represents a heat map showing differential expression of various genes in stomach, ileum and colon. Ghrelin, a hunger hormone, is secreted in stomach. Gene expression analysis showed higher expression of ghrelin in HFD-fed animals, which was reversed by ARGE supplementation (**Figure 7B**). Higher level of circulating ghrelin was observed in HFD-fed rats (**Figure 7B**). ARGE supplementation also increases the gene expression of *GOAT* (ghrelin-o-acyl transfer-

ase; processing of pre-proghrelin to ghrelin) and *TRPA1* in stomach (**Figure 7C**). Hormones responsible for satiety, *GLP-1* and *CCK* showed higher gene expression in ARGE-administered animals, however non-significant (**Figure 7A**). Genes involved in the regulation of secretion of these hormones, *CRTC2* and *PCSK2* were significantly higher in HFD+ARGE group (**Figure 7D**). Gene expression of medium-chain and long-chain fatty acid sensors, *GPR84* and *GPR120* was decreased in HFD-fed animals. *TRPA1* expression was significantly higher in ARGE group in ileum (**Figure 7D**).

ARGE modulates anorexigenic and orexigenic gene expression in hypothalamus

We investigated the changes in the expression level of anorexigenic genes and their associated receptors in hypothalamus (**Figure 7E**). A significant reduction in the expression level of anorectic gene, *BDNF* (Brain derived neurotrophic factor) was observed in HFD-fed animals, which was reversed by ARGE supplementa-

Figure 4. Gene expression analysis of vWAT samples. Rats were fed either with NPD (control group) or HFD, along with daily oral administration of ARGE (1 ml/kg body weight p.o.; ~ 2 mg/kg/day allicin) to the HFD + ARGE group and ARGE *per se* group. At the end of the study tissues were collected; RNA was isolated using trizol: chloroform: isopropanol method followed by cDNA synthesis. Quantitative real-time PCR was performed to analyze different genes associated with energy expenditure, browning and related markers. **(A)** Heat map depicting overview of different genes in vWAT; **(B)** Genes representing major changes in vWAT related to energy expenditure and browning ($n = 3$ in control group; $n = 4$ in other groups). *PPAR γ* - peroxisome proliferator-activated receptor gamma; *CIDEA* - Cell death-inducing DFFA like effector A; *CPD* - Carboxypeptidase D; *AdipoQ* - Adiponectin precursor; *TMEM 26* - Transmembrane protein 26; *PGC1- α* - PPAR γ coactivator 1 alpha; *PAT 2* - p-type cation-transporting ATPase; *P2RX5* - Purinergic receptor P2X; *SHOX2* - Short stature homeobox 2; *NRF2* - Nuclear factor erythroid 2-related factor 2; *TFAM* - Transcription factor A mitochondrial; *TBX1* - Tata binding box transcription factor 1. Data have been represented as Mean $\pm$ SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ versus Control, ** $p < 0.01$ versus Control, *** $p < .001$ versus Control; # $p < 0.05$ versus HFD, ### $p < 0.001$ versus HFD, #### $p < 0.001$ versus HFD.

tion. We compared expression levels of two receptors related to the functionality of anorectic and orexigenic genes. There was a significant upregulation of *MC4R* genes in HFD-fed group, corresponding to the hunger-phenotype in these animals which was reversed by ARGE administration. (**Figure 7E**). We also observed an enhanced expression of *InsR* (Insulin receptor) (**Figure 7E**).

DISCUSSION

Obesity adversely affects almost every physiological function of the body and increases the risk of developing cardiovascular diseases hypertension, type 2 diabetes, hyperlipidemia, several types of cancers, kidney diseases and liver disorders [32]. Garlic is a widely consumed spice throughout the world. It contains a variety of bioactive compounds, such as organosulfur compounds (allicin, DADS, DATS, alliin, SAM), saponins, phenolic compounds and polysaccharides, responsible for the diverse effects of garlic on health [7]. Garlic in different forms has been studied for its anti-obesity

and hypolipidemic effects. Garlic oil reduced body weight gain, adipose tissue weight and prevented fatty liver in HFD-fed rats [14,15]. Similar effects on body weight gain have been observed with methanolic extract of black garlic in HFD-fed rats [16].

In present study, we employed aqueous allicin-rich garlic extract (ARGE), prepared according to a previously reported method with slight modifications [33]. Allicin has multiple pharmacological functions, including cholesterol-lowering effects [34], browning of WAT [35] and regulates energy homeostasis through BAT activation [19]. Since allicin is a highly reactive volatile compound, it decomposes rapidly in the presence of air. It was shown that aqueous extract of garlic, if stored at lower temperature (-20°C or -80°C) have more stability of allicin [22,36]. HPLC analysis of ARGE showed allicin stability even at 4°C for almost 48 hours. Degradation of allicin was much less in aqueous extract when stored at -80°C , suggesting higher stability in extract form. Also, presence of polyphenols in ARGE could prevent oxidation of allicin in extract. This allicin-rich garlic extract offers several advantages over other extracts of garlic such as to prepare methodol-

Figure 5. Effect of chronic administration of ARGE on brown adipose tissue (BAT). Rats were fed either with NPD (control group) or HFD, along with daily oral administration of ARGE (1ml/kg body weight p.o.; ~ 2 mg/kg/day allicin) to the HFD + ARGE group and ARGE *per se* group. At the end of the study tissues were collected; RNA was isolated using trizol:chloroform:isopropanol method followed by cDNA synthesis. Quantitative real-time PCR was performed to analyze different genes associated with energy expenditure, browning and related markers. **(A)** Heat map depicting overview of different genes in BAT; **(B)** Genes representing major changes in BAT related to energy expenditure and browning (n = 3 in control group; n = 4 in other groups). *PPAR γ* - peroxisome proliferator-activated receptor gamma; *CIDEA* - Cell death-inducing DFFA like effector A; *CPD* - Carboxypeptidase D; *AdipoQ* - Adiponectin precursor; *TMEM 26* - Transmembrane protein 26; *PGC1- α* - PPAR γ coactivator 1 alpha; *PAT 2* - p-type cation-transporting ATPase; *P2RX5* - Purinergic receptor P2X; *SHOX2* - Short stature homeobox 2; *NRF2* - Nuclear factor erythroid 2-related factor 2; *TFAM* - Transcription factor A mitochondrial; *TBX1* - Tata binding box transcription factor 1; *FOXC2* - Forkhead box protein C2; *DIO2* - Iodothyronine deiodinase 2; *Cox8b* - Cytochrome C oxidase subunit 8B; *ATP5b* - ATP synthase F1 subunit beta. Data have been represented as Mean $\pm$ SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test and Two-Way ANOVA followed by Bonferroni's multiple comparisons. *p < 0.05 versus Control, **p < 0.01 versus Control, ***p < .001 versus Control; #p < 0.05 versus HFD, ##p < 0.001 versus HFD, ###p < 0.001 versus HFD.

ogy as it employs use of PBS and no other chemical, safer to use and higher stability of allicin.

ARGE supplementation prevented HFD-induced weight gain in rats. HFD feeding is also associated with adipocyte hypertrophy [37]. Histological examination revealed larger adipocytes and increased visceral adipose tissue mass in HFD-fed rats, which were attenuated by chronic supplementation of ARGE. In liver, ARGE supplementation attenuated HFD-induced ectopic fat accumulation as observed in histology, however in gene expression analysis for lipid metabolism, we did not find any significant changes. WAT, majorly responsible for energy storage in the body, could be stimulated to undergo browning or "beiging", which effectively enhances energy expenditure and prevents the development of obesity [38]. Browning of WAT is regulated by multiple transcription factors and signaling pathways. Garlic consumption has been reported to stimulate thermogenesis and decrease adipogenesis in WAT [39]. In present study, RT-PCR analysis of thermogenic genes and their transcriptional factors suggested induction of brite type phenotype in ARGE

supplemented group. *ADIPOQ* gene provides instruction for the synthesis of hormone adiponectin, a hormone exclusively synthesized by white adipocytes which stimulates fat burning and glucose utilization in liver and muscle cells [40]. A significantly higher expression of *ADIPOQ* gene after ARGE supplementation was observed. BAT is involved in the thermogenesis and regulation of energy expenditure [41]. Chronic consumption of HFD increased the gene expression of transcription factor *PPAR γ* , *PGC1- α* (responsible for mitochondrial biogenesis) and mitochondrial enzyme *COX8b*, possibly due to diet-induced thermogenesis. Since BAT is involved in excessive fat combustion in the body, chronic HFD feeding could activate a compensatory mechanism to counter excess energy consumed [42,43]. ARGE supplementation further increased the expression of browning-related mitochondrial biogenesis and energy expenditure genes (*TMEM26*, *TBX1*, *CIDEA*, *SHOX2*, *PPAR γ* , *P2RX5*) as compared to HFD group. HFD feeding is well-known inducer of peripheral insulin resistance, a progressive reduction in the responsiveness of the peripheral organs to the insulin [44].

Figure 6. Effect of chronic administration of ARGE on liver. Rats were fed either with NPD (control group) or HFD, along with daily oral administration of ARGE (1 ml/kg body weight p.o.; ~ 2 mg/kg/day allicin) to the HFD + ARGE group and ARGE *per se* group. **(A)** Representative images of H&E stained sections of liver tissues (20X magnification, scale: 100 μM); **(B-C)** Gene expression analysis of gluconeogenesis and glycogen metabolism in liver. Data have been represented as Mean ± SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ versus Control, ** $p < 0.01$ versus Control, *** $p < .001$ versus Control; # $p < 0.05$ versus HFD, ## $p < 0.001$ versus HFD, ### $p < 0.001$ versus HFD.

Oral administration of raw garlic improves insulin sensitivity in fructose-fed diabetic rats [45]. Higher serum insulin, and assessment of HOMA-IR and QUICKI indicated a state of hyperinsulinemia and systemic insulin resistance in HFD group. OGTT also reflected higher blood glucose levels in HFD-fed animals. ARGE supplementation prevented HFD-induced insulin resistance and improved glucose homeostasis. Also, gene expression analysis for glycogen metabolism in liver showed an upregulated level of *GYS2*, a rate-limiting enzyme in the synthesis of glycogen in the liver. On the other hand, glucose-6-phosphatase (G-6-PC), which catabolizes glycogen into glucose was found to be lower at gene level in ARGE supplemented group. These observations suggested that ARGE improved overall glucose homeostasis.

The gastrointestinal tract not only absorbs dietary nutrients but also controls total dietary intake. Several hormones secreted by parts of gastrointestinal tract, ghrelin, CCK, PYY, GLP-1 and GIP, play a vital role in maintaining energy homeostasis and body weight

regulation [46]. Ghrelin is the only known orexigenic or hunger hormone secreted by stomach. Several studies have reported ghrelin as a potent appetite stimulator [47,48]. Gene expression of ghrelin, as well as total ghrelin levels were drastically enhanced in HFD-fed rats whereas ARGE supplementation reversed the same. Further, ARGE administration altered the gene expression of satiety hormones such as *GLP-1* and *CCK* in ileum, with little or no effect in colon. Gene expression of *PCSK2* and *CRTC2* (genes involved in the synthesis and exocytosis of gut hormones) was significantly upregulated after ARGE supplementation. Hypothalamus is involved in the central control of feeding and energy expenditure [49]. Two distinct antagonistic populations of neurons i.e., *NPY/AgRP* (appetite stimulant) and *POMC/CART* (appetite suppressant) in hypothalamus and their receptors collectively regulate dietary intake [49]. We observed that gene expression of orexigenic hormones, *NPY/AgRP* remained unaffected in ARGE supplemented group (data not shown). By contrast, gene expression of satiety hormones *BDNF*

Figure 7. Effects of chronic administration of ARGE on gene expression in the gastrointestinal tract and hypothalamus. Rats were fed either with NPD (control group) or HFD, along with daily oral administration of ARGE (1 ml/kg body weight p.o. ~ 2 mg/kg/day allicin) to the HFD + ARGE group and ARGE *per se* group. At the end of the study tissues were collected; RNA was isolated using trizol:chloroform:isopropanol method followed by cDNA synthesis. Quantitative real-time PCR was performed to analyze different genes associated with maintaining hunger and satiety in the gastrointestinal tract. **(A)** Gene expression and serum concentration of ghrelin in stomach; **(B)** Heat map depicting overview of different genes in stomach, ileum and colon; **(C)** Gene expression of *GOAT* and *TRPA1* in stomach; **(D)** Genes with major changes in ileum (n = 3 in control group; n = 4 in other groups); **(E)** Gene expression of anorexigenic hormones and their associated receptors in hypothalamus. *GOAT* - Ghrelin O-acetyl transferase; *CRTC1* - CREB regulated transcription co-activator 1; *PYY* - peptide YY; *CCK* - Cholecystokinin; *GLP-1* - Glucagon-like peptide 1; *Caln* - calmodulin; *PCSK1* - proconvertase 1; *CAMK4* - Calcium/Calmodulin dependent protein kinase 4; *CaN* - calcineurin; *GPR84* - G-protein coupled receptor; *BDNF* - Brain-derived neurotrophic factor; *CART* - Cocaine- and amphetamine-regulated transcript; *MC4R* - Melanocortin 4 receptor; *InsR* - Insulin receptor. Data have been represented as Mean $\pm$ SEM. Analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison test. *p < 0.05 versus Control, **p < 0.01 versus Control, ***p < .001 versus Control; #p < 0.05 versus HFD, ##p < 0.001 versus HFD, ###p < 0.001 versus HFD.

and *CART* was significantly higher in HFD + ARGE group. Reduction in the gene expression of *BDNF* is associated with obesity [50].

TRPA1 (transient receptor potential cation channel subfamily ankyrin, type 1) ion channel has many physiological roles including regulation of gut hormone secretion, gastric motility, insulin secretion etc. other than its primary function in nociception [24,25,51]. Since allicin is a known agonist of *TRPA1* [28], dietary modulation of *TRPA1* could also contribute to the regulation of gut hormones. In present study, gene expression of *TRPA1* in gastrointestinal tract and dorsal root ganglion neurons innervating colon was analyzed. RT-PCR analysis showed significantly higher expression of *TRPA1* in stomach and ileum of HFD + ARGE, with no change in colon as compared to HFD-fed rats, suggesting a possible role of *TRPA1*-mediated regulation of ghrelin secretion in ARGE supplemented group. In our study, we did not find any direct effect on energy expenditure after ARGE supplementation. Further studies focusing on gut hormones or protein expressions and molecular mechanism are required to validate gene expression changes.

CONCLUSION

The present study has shown that ARGE provides an easy and economical method to prepare aqueous garlic extract with stable allicin. Comparable to the previously reported anti-obesity effects of different formulation of garlic, ARGE also reduced weight gain, adipose tissue hypertrophy and liver steatosis. Additionally, nutrigenomic effects of ARGE in browning of WAT, glycogen metabolism, gut hormone secretion and their regulation, provide a novel insight into the nutrigenomic effects of ARGE, which can be exploited in the future as a nutraceutical for prevention of obesity.

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AUTHORS' CONTRIBUTION

MB and NM designed the experiments. NM, ViK, VK and JK performed the experiments. NM, PK, PM, RV, RM and SJ performed the characterization experiments. NM and MB did analysis of experiments. NM and ViK wrote the manuscript; MB and KKK reviewed and edited the manuscript.

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Ekstrakt crnog luka bogat alicinom ublažava komplikacije izazvane visokomasnom ishranom kod pacova: Nutrigenomska studija

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Kratak sadržaj

Lekovito dejstvo crnog luka pri različitim metaboličkim komplikacijama pripisuje se raznovrsnim organskim sum-pornim jedinjenjima, kao što su alicin, dialil sulfidi, aliin i alil trisulfidi. U ovoj studiji izolovani su vođeni ekstrakti crnog luka sa stabilnim alicinom i ispitivani njihovi biološki tkivno specifični nutrigenomski efekti u sprečavanju gojaznosti i povezanih komplikacija izazvanih visokomasnom ishranom (HFD) u Sprague-Dawley (SD) pacovima. Pripremljen je rastvor ekstrakta crnog luka bogat alicinom (ARGE), a stabilnost alicina okarakterisana je HPLC metodom. Za biološku evaluaciju životinje su hranjene 16 nedelja normalnom hranom, visokomasnom hranom, visokomasnom hranom zajedno sa ARGE (1 ml/kg p.o.) ili samim ARGE (1 ml/kg p.o.). Hronična administracija ARGE sprečila je gojenje izazvano visokomasnom ishranom, hipertrofiju masnog tkiva, insulinsku rezistenciju i generalno je dovela do poboljšanja glukozne homeostaze. Transkripciona analiza različitih tkiva pre svega je pokazala da ARGE promovise browning, pospešuje glukozni metabolizam i regulaciju apetita. Ova studija pokazuje da ARGE ima nutraceutski potencijal u prevenciji gojaznosti i povezanih komorbiditeta.

Ključne reči: ARGE, ekstrakt crnog luka bogat alicinom; TRPA1; gojaznost; mrko masno tkivo; belo masno tkivo; dialil disulfidi; dialil trisulfidi; insulinska rezistencija; WAT browning; hormoni creva.