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The effects of milk protein concentrate supplementation on metabolic parameters, adipocytokines, and body composition in obese women under weight-loss diet: Study protocol for a randomized controlled trial

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ABSTRACT

Introduction Obesity impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus. Evidence suggests that high-protein diets help to increase weight loss and protect against weight gain. Milk protein concentrate (MPC) is a dairy product with a high protein content with the ratio of casein and whey protein similar to skim milk. This trial aims to evaluate the effect of MPC supplementation in obese women under a weight-loss diet.

Methods and Analysis We will conduct a 2 months' open-label, parallel-group, randomized controlled trial to determine the effect of MPC supplementation on levels of glycemic and lipid profile, leptin, adiponectin, appetite, waist circumference, body mass index(BMI), and body composition in 44 premenopausal obese women on a weight-loss diet.

Ethics and dissemination This protocol, approved by Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The trial results will be published in peer-reviewed journals.

Trial registration number Iranian Registry of Clinical Trials IRCT20201223049804N1.

Strengths and limitations of the study

- This is the first study that investigates the effect of supplementation of the MPC in adjunct with a weight loss diet on improving metabolic parameters, serum levels of adipocytokines and body composition in obese women.
- The results of this trial will help obese women to reduce weight and improve their cardiometabolic health.
- A limitation of this study is that other adipokines affecting energy homeostasis in the body will not be measured due to restrictions in funding.

INTRODUCTION

Obesity is defined as the excessive accumulation of fat in the body (BMI ≥30 kg/m2) and is associated with dysregulation of glucose and lipoprotein metabolism (1). In particular, it has been shown that visceral fat, as an independent factor, impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus (2). It is reported that additional production of pro-inflammatory cytokines by macrophages in visceral adipose tissue leads to insulin resistance (3). Inflammatory cytokines produced by adipose tissue prevent the binding of insulin to its receptors, thereby induce insulin resistance in target tissues (4). According to the World Health Organization (WHO) in 2016, more than 1.9 billion adults were overweight and 650 million were obese (13% of the world's adult population) (5). Also, the prevalence of obesity in Iranian adult females and males were reported at 29.3% and 13.6%, respectively (6). Probably due to differences in sex hormones, unknown molecular mechanisms, and genetics, women have a greater risk of obesity compared with men (7).

Adipose tissue is an important metabolic organ that is crucial for insulin sensitivity and energy homeostasis in the body (8). This tissue produces two hormones leptin and adiponectin that called adipocytokines and influence lipid and glucose metabolism. According to studies with large populations, the ratio of leptin to adiponectin is an indicator of insulin sensitivity in the body (9, 10). Leptin regulates appetite, energy expenditure, and food intake by binding to its receptors in the hypothalamus and helps to control weight (11). In obese individuals, leptin levels increase and lead to the development of obesity and insulin resistance (12). Conversely, adiponectin acts as an insulin-sensitizer and improves lipid profile by increasing tissue fat oxidation. Nevertheless, decreased levels of adiponectin have been reported in obese individuals (13).

Obesity is a complex disease caused by the interaction of genetics, epigenetics, economic, physiological, social, and environmental factors (lifestyle, diet, and physical activity) (14, 15). It has been shown that the models of diet-induced obesity due to their similarity to human obesity, are often used in metabolic studies (16). Fat-rich diets play a role in the induction of obesity, impaired glucose homeostasis and as well as insulin resistance, and hyperlipidemia (17). Studies have shown that regular intake of dietary protein, especially dairy protein, may have beneficial effects on weight control and metabolic syndrome (18, 19).

Complete dairy protein or milk protein concentrate (MPC) is a relatively new dairy product with high protein content (70% protein) that is made by combining three methods including diafiltration, ultrafiltration, and spray-drying from pasteurized skim milk (31% protein). MPC contains both casein and whey protein, and the ratio of these two proteins is similar to skim milk (4:1 or 80:20). This ingredient is used in dairy products such as cheeses and yogurts (20). A study in obese rats showed that MPC reduced body weight and fat accumulation more than casein and whey protein alone. This may be due to the longer digestion process and greater satiety effects than the other two proteins (18). Also, MPC as a source of protein with a high biological value that contains all kinds of essential and non-essential amino acids can prevent muscle wasting (21). Another study reported that intraduodenal infusion of MPC significantly improved the effects of sitagliptin including glycemic and short-term food intake suppression. The results of this study confirm the hypothesis that the consumption of dairy protein may be useful as a complementary therapy to improve the hypoglycemic and reduction of food intake effects of GLP-1-based pharmacotherapies (22). Furthermore, a low-energy diet (LED) helps to the improvement of systemic dysmetabolism by inducing weight loss (23), has positive effects on adiponectin concentration (24), and is associated with decreased serum levels of leptin in obese individuals (25).

Based on the above impacts, the present study hypothesizes that the use of MPC along with a weight loss diet will be effective in the improvement of some metabolic parameters, serum levels of adipocytokines, and body composition against the lack of effect. To the best of our knowledge, there is no study to investigate the effects of MPC along with a weight loss diet in obese women. Since, the present study aims to investigate a supplementation of the 30 gram MPC in adjunct with a weight loss diet will be useful in improving metabolic parameters, serum levels of adipocytokines, and body composition in obese women.

METHODS AND ANALYSIS

Study Design

We will conduct 8 weeks open-label, parallel-group, randomized controlled trial with a superiority framework. The proposed clinical trial will be conducted at the Nutritional Research Center, Department of Nutrition, the Ahvaz Jundishapur University of Medical Science for 8 weeks to assess the efficacy of the 30 mg MPC in adjunct with weight loss diet in obese women. (Figure 1) (Figure 2)

Aims and study hypotheses

The primary aim of the current trial is to examine the effect of 8 weeks of MPC supplementation on levels of leptin, adiponectin, waist circumference, BMI, and body composition in obese women on a weight-loss diet. The secondary aim is to determine the effect of 8 weeks of MPC supplementation on glycemic and lipid profile, insulin, and appetite. It is hypothesized that 8 weeks supplementation of the MPC in adjunct with weight loss diet will improve measures of investigation.

Participants

44 premenopausal obese women aged 18 years or more referred to the diet clinic in the city of Ahvaz meeting the inclusion criteria will be recruited in this trial after obtaining their written consent. The following inclusion criteria will be applied: aged 18 years and older, BMI range of 30 to 40 kg/m2, absence of menopause, lactation, pregnancy, and food allergies, not having eating disorders, particularly binge eating disorder (BED), bulimia, not having cancer, hepatic, renal, thyroid and gastrointestinal disorders, no surgery for weight loss, no weight loss over the past 6 months, no taking herbs and drug that reduce appetite and weight and vitamin-mineral supplements. The subjects with any of the following criteria will be excluded: become pregnant during the study, unwilling to continue, changes in diet during the study period, and no consumption of powders exceed 10% of total administered powders.

Patient and public involvement

Patients were not involved in the development and design of the study protocol. The public was not involved. Study results will not be disseminated to participants specifically. However, if participants are interested in the results of the research, they will receive any information, the manuscript and published research on this topic in the future.

Sample size

The required sample size is calculated based on data from a previous human study by Faghih et al(26), which assessed the effects of milk's cow consumption in adjunct with a weight loss diet on weight and fat loss. A mean difference in weight of 1.6 kg between the two groups is aimed to be detected for a specified α of 0.05 and a study power of 80%. Based on the proposed formula for parallel clinical trials(27), we reached a sample size of 20 participants in each group. Assuming a possible drop-out rate of 10%, 22 patients will be enrolled in each group.

Randomization

The subjects will be randomly stratified according to age and BMI using a permuted block randomization procedure by Random Allocation Software (RAS). The ratio of intended numbers of participants in each of the comparison groups will 1:1. They will be assigned to one of the two study groups: (Fig 1)

(1) Standard weight loss group (n=22) (control group)

(2) MPC supplementation weight loss group (n=22) (intervention group)

A member of the research team who is not involved in assessing the outcome of the study will be responsible for generating the allocation sequence and will allocate participants to the sequence.

Intervention

All subjects will follow a hypocaloric diet of 800 kcal below estimated energy requirements, which will be designed by a trained dietitian. Energy needs will be estimated by Mifflin Jeor St equation(28). In the control group, the percentage of macronutrients will be 55%, 30%, 15% for carbohydrate, fat, and protein; respectively(29). For the intervention group, an isocaloric weight loss diet with MPC supplements will be prescribed. Each of the intervention group participants will receive 30-gram MPC powders daily lasting 8 weeks. MPC powders will be supplied by Pegah dairy industries Co., Tehran, Iran. The MPC powders will be provided in a sachet form. Each sachet will be contained 30-gram MPC (105 kcal, 0.4 g of lipid, 6 g of carbohydrate, and 20 of protein). Considering the calorie of each MPC sachet (105kcal), 905 calories below estimated energy needs will be regarded for the intervention group. Participants in the intervention group will be instructed to add one sachet to 250 ml cold water and consume it immediately, every morning on an empty stomach(30). To check compliance, subjects will be requested to record the time and date of powder intake. Moreover, to ensure that the intervention

group participants regularly consume the powders, they will be contacted every three days by a dietitian, or if it was not possible to call them, they would be followed through SMS. Also, serum creatinine will be considered as a biochemical marker to practically assess compliance in this study. To create a variety in the diet while maintaining the general principles of diet, all subjects will be given a dietary exchange list and a diet according to their food habits(31). The study subjects will be asked not to change their dietary habits and physical activity during the study (8 weeks).

The assigned study intervention may need to be modified or discontinued by trial investigators for various reasons, including gastro-intestinal upset, allergic reactions, and withdrawal of participant consent. We will call the patients weekly and ask them about occurring any adverse events following MPC consumption. If the reported adverse events are correlated with the MPC consumption, participants will be asked to stop taking MPC supplements, and also, they will be immediately referred to a specialist for therapy.

Outcomes

The primary outcomes consist of levels of adiponectin and leptin, and the anthropometric status. Secondary outcomes will be fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status. All these factors will be measured at baseline and end of the study. We would manage multiplicity issue with regard to multiple primary outcomes using Bonferroni correction.

Assessment of dietary intake, Anthropometric parameters, and physical activity

A sociodemographic questionnaire will be recorded at the beginning of the study. Dietary intake will be evaluated by 3 days 24-hr recall questionnaires (two weekdays and one weekend day) at

the beginning, middle, and end of the study. Total energy and macronutrient intake will be calculated by Nut IV (the Hearst Corporation, San Bruno, CA). A trained dietitian will evaluate anthropometric variables such as waist circumference, body weight, height, and BMI after overnight fasting. At the baseline and the end of the study, waist circumference (WC) will be measured in a standing position using a tape with an accuracy of 1.0 cm at above the iliac crest, just below the lowest rib margin and at the end of normal expiration. Bodyweight will be measured with the accuracy of 100 gr using a Seca scale at the baseline and end of the study. Stature will be measured in a relaxed position by a Seca stadiometer with an accuracy of 0.5 cm. BMI will be calculated as body weight (kg) divided by the square of height(m), the beginning and end of the study. Direct segmental multi-frequency bioelectrical impedance method (Inbody 270, Biospace, Korea) will be used to calculate body composition including total body fat and fat-free mass percentage.

To evaluate the physical activity levels, the International Physical Activity Questionnaire (IPAQ) will be used at the baseline and the end of the trial via interviewing, and the results will be expressed as metabolic equivalent hours per week (METs hr/wk). The Persian translation of the short form IPAQ has been validated by Moghaddam et al. (Cronbach's alpha=0.7 and test-retest reliability coefficient=0.9)(32).

Assessment of Appetite

The Council on Nutrition Appetite Questionnaire (CNAQ), which was adopted by Wilson et al(33), will be used for measuring appetite. This questionnaire contains 8 single domain items, scales of each item is ranged from 1 to 5. Thus, the total score ranges from 8 to 40 points. A score of less than 28 is cause for concern. The validity of this questionnaire has been investigated in Iran (Cronbach's alpha=0.77)(34).

Assessment of biochemistry variables

At baseline and end of the study, 10 ml of venous blood samples (in regular tubes) will be collected after 10-12 hours of overnight fasting. Fasting blood glucose and lipid profile (total cholesterol, Low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride) will be evaluated by the enzymatic method with kits from Pars-Azmoon (Tehran, Iran). Insulin levels will be measured by chemiluminescent immunoassay. Homeostasis model assessment – insulin resistance (HOMA-IR) will be calculated by the following formula: fasting glucose(mg/dl) × fasting insulin(μu/ml)/405. ELISA kits will be used to determine serum leptin and adiponectin levels. All data will be entered electronically at the participating site where the data originated. Original study forms will be entered and kept on file at the participating site. Participant files are to be stored in numerical order and stored in a secure and accessible place and manner. Participant files will be maintained in storage for a period of 3 years after completion of the study. All Principal Investigators will be given access to the cleaned data sets.

Statistical analysis

Data analysts will be blinded after assignment to interventions. All statistical analyses will be performed using the IBM SPSS Statistics software (Version 23) (IBM SPSS Statistics, Armonk, USA). The normality of the variables will be confirmed using the Kolmogorov-Smirnov test. A chi-square test will be applied to compare the categorical data between treatment groups at the baseline. Independent sample t-test and Mann-Whitney test will be used to compare parametric continuous and nonparametric data between the groups, respectively. Paired sample t-test or Wilcoxon signed-rank test will be applied to compare data within the groups. Post-intervention differences in responses between intervention and control groups will be tested by analysis of

covariance (ANCOVA) with use of the baseline measurements of the outcome variables as covariates. The percent change of each variable will be also computed by the formula [(E − B)/B × 100], where E is the end of intervention values and B is the baseline values. The intention-to-treat (ITT) method will be applied for data analysis, which considers all participants in the trial and ignores anything that happens after randomization such as misallocation and noncompliance. A p-value of less than 0.05 will be considered to be statistically significant.

Ethics and dissemination: This protocol, approved by Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The monitors from Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness. Written informed consent will be obtained from participants before participation in the research project by researchers. All participant information will be stored in locked file cabinets in areas with limited access. This investigation was registered on Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1).

DISCUSSION

Epidemiological studies show that the intake of milk and dairy products is inversely associated with a lower risk of metabolic disorders and cardiovascular diseases (35). Differences in the digestion kinetics of whey and casein proteins facilitate the stimulation of gastric hormones that delay gastric emptying, thus increasing feelings of fullness and attenuation of food particle breakdown and release in the small intestine. Whey and casein proteins differentially affect postprandial blood glucose and satiety mechanisms, with relevance for type 2 diabetes and obesity(30). It seems that complete dairy improves body composition and insulin sensitivity to a

greater extent than whey or casein alone. In a diet-induced obese rat model, administration of complete dairy protein reduced weight gain and body fat mass accumulation more so than intake of whey or casein alone(18). The intraintestinal presence of specific bioactive components, whole proteins, and select amino acids found within MPC is linked with insulin and gut peptide secretions, as well as suppression of food intake(36-38).

This is the first study that investigates the effect of supplementation of the MPC in adjunct with a weight loss diet on improving metabolic parameters, serum levels of adipocytokines and body composition in obese women. Results of this trial will help us understand the effectiveness of MPC along with a weight loss diet to improve metabolic parameters, serum levels of adipocytokines, appetite, and body composition compared with controlled participants. In addition, the results of this trial will help obese women to reduce weight and improve their cardiometabolic health. One of the limitations of this study is that other adipokines affecting energy homeostasis in the body will not be measured due to restrictions in funding.

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Authors contributions: All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

Competing interests statement: The authors declare that they have no competing interests.

Patient consent for publication: Not required.

Availability of data and materials: The datasets generated and/or analyzed during the current study are not publicly available due to the privacy of subjects, but are available from the corresponding author on reasonable request.

Abbreviations: BMI: Body Mass Index; CNAQ: Council on Nutrition Appetite Questionnaire; MPC: Milk Protein Concentrate; IPAQ: International Physical Activity Questionnaire.

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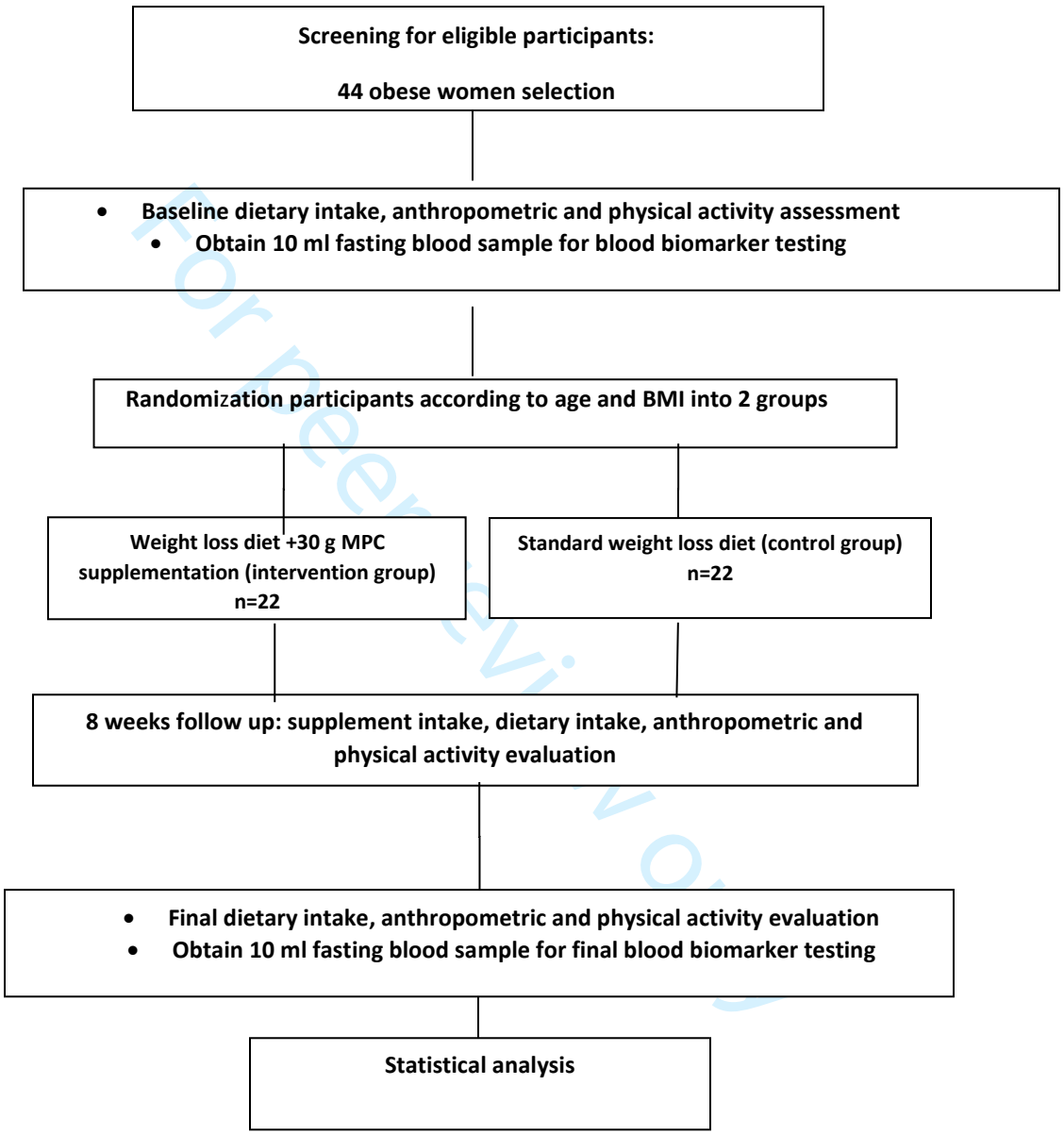

Figure 1. Protocol flow diagram

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT**	$-t_1$	0	*Week 1*	*Week 3*	*Week 6*	*Week 8*	*etc.*	t_x
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
Randomization		X						
Allocation		X						
INTERVENTIONS:								
[Intervention A]			←	—	—	→		
[Intervention B]			←	—	—	→		
ASSESSMENTS:								
Demographic variables	X	X						
primary outcome variables: levels of adiponectin and leptin, and the anthropometric status.			X	X	X	X	etc.	X
Secondary outcome variables: fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status.			X	X	X	X	etc.	X

Figure 2. Template of recommended content for the schedule of enrolment, interventions, and assessments. LDL-C: Low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: Homeostasis model assessment – insulin resistance.

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____1_____
	2b	All items from the World Health Organization Trial Registration Data Set	N/A: This investigation was registered on Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1)
Protocol version	3	Date and version identifier	_____6,11____
Funding	4	Sources and types of financial, material, and other support	_____10-11
Roles and	5a	Names, affiliations, and roles of protocol contributors	_____11_____

responsibilities	5b	Name and contact information for the trial sponsor	12: The sponsor is the same as the funder(Ahvaz Jundishapur University of Medical Sciences).
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	_12_
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_12_: Medical Ethics Committee of Ahvaz University of Medical Sciences by selecting an observer for each study supervises the RCT.
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	_____3-5_____
	6b	Explanation for choice of comparators	5
Objectives	7	Specific objectives or hypotheses	5-6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5,7
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7-8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A: This study don't include Rescue Medication or Prohibited Concomitant Medications
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8-9
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	SPIRIT figure
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7

Methods: Assignment of interventions (for controlled trials)

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	____7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	__7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	_____8-9___
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____6-7_____
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	____9-10_____

	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____9-10_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	_____9-10_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A: For this study is not Data Monitoring Committee.
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A: For this study is not Data Monitoring Committee.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___8_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	The monitors from Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness.
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	__6
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A: We will not have a Modification of the Protocol.
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	____12_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A: We have not ancillary study.
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	12, consent form
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____11
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____consent form
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___consent form

	31b	Authorship eligibility guidelines and any intended use of professional writers	13, We will not have any intended use of professional writers. In our university we are not allowed to add authors other than study researchers to the article.
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	13
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Uploaded as supplementary file
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

The effects of milk protein concentrate supplementation on metabolic parameters, adipocytokines, and body composition in obese women under weight-loss diet: Study protocol for a randomized controlled trial

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The effects of milk protein concentrate supplementation on metabolic parameters, adipocytokines, and body composition in obese women under weight-loss diet: Study protocol for a randomized controlled trial

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ABSTRACT

Introduction Obesity impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus. Evidence suggests that high-protein diets help to increase weight loss and protect against weight gain. Milk protein concentrate (MPC) is a dairy product with a high protein content with a ratio of casein and whey protein similar to skim milk. This trial aims to evaluate the effect of MPC supplementation in obese women under a weight-loss diet.

Methods and Analysis We will conduct a 2months open-label, parallel-group, randomized controlled trial to determine the effect of MPC supplementation on levels of glycemic and lipid profile, leptin, adiponectin, appetite, waist circumference, body mass index(BMI), and body composition in 44 premenopausal obese women on a weight-loss diet.

Ethics and dissemination This protocol, approved by the Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The trial results will be published in peer-reviewed journals.

Trial registration number Iranian Registry of Clinical Trials IRCT20201223049804N1.

Strengths and limitations of the study

- We will conduct a 2months open-label, parallel-group, randomized controlled trial to determine the effect of 30-gram MPC supplementation on levels of glycemic and lipid profile, leptin, adiponectin, appetite, waist circumference, body mass index(BMI), and body composition in 44 premenopausal obese women on a weight-loss diet.
- A limitation of this study is that other adipokines affecting energy homeostasis in the body and incretin hormones will not be measured due to restrictions in funding.
- Another limitation of this study is that all of the subjects will only female gender.

INTRODUCTION

Obesity is defined as the excessive accumulation of fat in the body (BMI ≥30 kg/m2) and is associated with dysregulation of glucose and lipoprotein metabolism (1). In particular, it has been shown that visceral fat, as an independent factor, impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus (2). It is reported that additional production of pro-inflammatory cytokines by macrophages in visceral adipose tissue leads to insulin resistance (3). Inflammatory cytokines produced by adipose tissue prevent the binding of insulin to its receptors, thereby induce insulin resistance in target tissues (4). According to the World Health Organization (WHO) in 2016, more than 1.9 billion adults were overweight and 650 million were obese (13% of the world's adult population) (5). Also, the prevalence of obesity in Iranian adult females and males was reported at 29.3% and 13.6%, respectively (6). Probably due to differences in sex hormones, unknown molecular mechanisms, and genetics, women have a greater risk of obesity compared with men (7).

Adipose tissue is an important metabolic organ that is crucial for insulin sensitivity and energy homeostasis in the body (8). This tissue produces two hormones leptin and adiponectin called adipocytokines which influence lipid and glucose metabolism. According to studies with large populations, the ratio of leptin to adiponectin is an indicator of insulin sensitivity in the body (9, 10). Leptin regulates appetite, energy expenditure, and food intake by binding to its receptors in the hypothalamus and helps to control weight (11). In obese individuals, leptin levels increase and lead to the development of obesity and insulin resistance (12). Conversely, adiponectin acts as an insulin-sensitizer and improves lipid profile by increasing tissue fat oxidation. Nevertheless, decreased levels of adiponectin have been reported in obese individuals (13).

Obesity is a complex disease caused by the interaction of genetics, epigenetics, economic, physiological, social, and environmental factors (lifestyle, diet, and physical activity) (14, 15). It has been shown that the models of diet-induced obesity due to their similarity to human obesity, are often used in metabolic studies (16). Fat-rich diets play a role in the induction of obesity, impaired glucose homeostasis and as well as insulin resistance, and hyperlipidemia (17). Studies have shown that regular intake of dietary protein, especially dairy protein, may have beneficial effects on weight control and metabolic syndrome (18, 19).

Complete dairy protein or milk protein concentrate (MPC) is a relatively new dairy product with high protein content (70% protein) that is made by combining three methods including diafiltration, ultrafiltration, and spray-drying from pasteurized skim milk (31% protein). MPC contains both casein and whey protein, and the ratio of these two proteins is similar to skim milk (4:1 or 80:20). This ingredient is used in dairy products such as cheeses and yogurts (20). A study in obese rats showed that MPC reduced body weight and fat accumulation more than casein and whey protein alone. This may be due to the longer digestion process and greater satiety effects than the other two proteins (18). Also, MPC as a source of protein with a high biological value that contains all kinds of essential and non-essential amino acids can prevent muscle wasting (21). Another study reported that intraduodenal infusion of MPC significantly improved the effects of sitagliptin including glycemic and short-term food intake suppression. The results of this study confirm the hypothesis that the consumption of dairy protein may be useful as a complementary therapy to enhance the glycemic and food intake suppressive effects of GLP-1-based pharmacotherapies(22). Furthermore, a low-energy diet (LED) helps to the improvement of systemic dysmetabolism by inducing weight loss (23), has positive effects on adiponectin concentration (24), and is associated with decreased serum levels of leptin in obese individuals (25).

To the best of our knowledge, there is no study to investigate the effects of MPC along with a weight loss diet in obese women. Since, the present study aims to investigate a supplementation of the 30-gram MPC in adjunct with a weight loss diet will be useful in improving metabolic parameters, serum levels of adipocytokines, and body composition in obese women.

METHODS AND ANALYSIS

Study Design

We will conduct 8 weeks open-label, parallel-group, randomized controlled trial with a superiority framework. The proposed clinical trial will be conducted at the Nutritional Research Center, Department of Nutrition, the Ahvaz Jundishapur University of Medical Science for 8 weeks to assess the efficacy of the 30 mg MPC in adjunct with weight loss diet in obese women. Recruitment to this study and collecting data began in April 2022 and is expected to end in September 2022. (Figure 1) (Figure 2)

Aims and study hypotheses

The primary aim of the current trial is to examine the effect of 8 weeks of MPC supplementation on levels of leptin, adiponectin, waist circumference, BMI, and body composition in obese women on a weight-loss diet. The secondary aim is to determine the effect of 8 weeks of MPC supplementation on glycemic and lipid profile, insulin, and appetite. It is hypothesized that 8 weeks supplementation of the MPC in adjunct with a weight loss diet will improve measures of investigation.

Participants

Forty-four premenopausal obese women aged 18 years or more referred to the diet clinic in the city of Ahvaz meeting the inclusion criteria will be recruited in this trial after obtaining their informed written consent. The following inclusion criteria will be applied: aged 18 years and older, BMI range of 30 to 40 kg/m2, absence of menopause, absence of lactation and pregnancy, absence of food allergies, not having eating disorders, particularly binge eating disorder (BED), bulimia, not having cancer, hepatic, renal, thyroid and gastrointestinal disorders, no surgery for weight loss, no weight loss over the past 6 months, no taking any herbal medicine and drug that reduce appetite and weight (such as caraway extract, celery extracts, metformin, orlistate, etc.) and vitamin-mineral supplements. The subjects with any of the following criteria will be excluded: become pregnant during the study, unwilling to continue, changes in diet during the study period, and no consumption of powders exceed 10% of total administered powders.

Patient and public involvement

Patients were not involved in the development and design of the study protocol. The public was not involved. Study results will not be disseminated to participants specifically. However, if participants are interested in the results of the research, they will receive any information, the manuscript, and published research on this topic in the future.

Sample size

The required sample size is calculated based on data from a previous human study by Faghih et al(26), which assessed the effects of milk's cow consumption in adjunct with a weight loss diet on weight and fat loss. A mean difference in weight of 1.6 kg between the two groups is aimed to be detected for a specified α of 0.05 and a study power of 80%. Based on the proposed formula for parallel clinical trials(27), we reached a sample size of 20 participants in each group. Assuming a possible drop-out rate of 10%, 22 patients will be enrolled in each group.

Randomization

The subjects will be randomly stratified according to age and BMI using a permuted block randomization procedure by Random Allocation Software (RAS). The ratio of intended numbers of participants in each of the matched groups will be 1:1. They will be assigned to one of the two study groups: (Fig 1)

(1) Standard weight loss group (n=22) (control group)

(2) MPC supplementation weight loss group (n=22) (intervention group)

A member of the research team who is not involved in assessing the outcome of the study will be responsible for generating the allocation sequence and will allocate participants to the sequence.

Intervention

All subjects will follow a hypocaloric diet of 800 kcal below estimated energy requirements, which will be designed by a trained dietitian. Energy needs will be estimated by the Mifflin Jeor St equation(28). In the control group, the percentage of macronutrients will be 55%, 30%, and 15% for carbohydrates, fat, and protein; respectively(29). For the intervention group, an isocaloric weight loss diet with MPC supplements will be prescribed. Each of the intervention group participants will receive 30-gram MPC powders daily lasting 8 weeks. MPC powders will be supplied by Pegah dairy industries Co., Tehran, Iran. The MPC powders will be provided in a sachet form. Each sachet will be contained 30-gram MPC (105 kcal, 0.4 g of lipid, 6 g of carbohydrate, and 20 of protein; 20% whey protein and 80% casein). Considering the calorie of each MPC sachet (105kcal), 905 calories below estimated energy needs will be regarded for the intervention group. Participants in the intervention group will be instructed to add one sachet to 250 ml cold water and consume it immediately, every morning on an empty stomach(30). To check compliance, subjects will be requested to record the time and date of powder intake.

Moreover, to ensure that the intervention group participants regularly consume the powders, they will be contacted every three days by a dietitian, or if it was not possible to call them, they would be followed through SMS. To create a variety in the diet while maintaining the general principles of diet, all subjects will be given a dietary exchange list and a diet according to their food habits(31). The study subjects will be asked not to change their dietary habits and physical activity during the study (8 weeks).

The assigned study intervention may need to be modified or discontinued by trial investigators for various reasons, including gastrointestinal upset, allergic reactions, and withdrawal of participant consent. We will call the patients weekly and ask them about occurring any adverse events following MPC consumption. If the reported adverse events are correlated with MPC consumption, participants will be asked to stop taking MPC supplements, and also, they will be immediately referred to a specialist for therapy.

Outcomes

The primary outcomes consist of levels of adiponectin and leptin, and the anthropometric status. Secondary outcomes will be fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status. All these factors will be measured at baseline and end of the study. We would manage multiplicity issue about multiple primary outcomes using Bonferroni correction.

Assessment of dietary intake, Anthropometric parameters, and physical activity

A sociodemographic questionnaire will be recorded at the beginning of the study. Dietary intake will be evaluated by 3 days 24-hr recall questionnaires (two weekdays and one weekend day) at the beginning, middle, and end of the study. Total energy and macronutrient intake will be

calculated by Nut IV (the Hearst Corporation, San Bruno, CA). A trained dietitian will evaluate anthropometric variables such as waist circumference, body weight, height, and BMI after overnight fasting with minimum clothing. At the baseline and the end of the study, waist circumference (WC) will be measured in a standing position using a tape with an accuracy of 1.0 cm above the iliac crest, just below the lowest rib margin and at the end of normal expiration. Bodyweight will be measured with the accuracy of 100 gr using a Seca scale at the baseline and end of the study. Stature will be measured in a relaxed position by a Seca stadiometer with an accuracy of 0.5 cm. BMI will be calculated as body weight (kg) divided by the square of height(m), at the beginning and end of the study. The direct segmental multi-frequency bioelectrical impedance method (Inbody 270, Biospace, Korea) will be used to calculate body composition including total body fat and fat-free mass percentage.

To evaluate the physical activity levels, the International Physical Activity Questionnaire (IPAQ) will be used at the baseline and the end of the trial via interviewing, and the results will be expressed as metabolic equivalent hours per week (METs hr/wk). The Persian translation of the short form IPAQ has been validated by Moghaddam et al. (Cronbach's alpha=0.7 and test-retest reliability coefficient=0.9)(32).

Assessment of Appetite

The Council on Nutrition Appetite Questionnaire (CNAQ), which was adopted by Wilson et al(33), will be used for measuring appetite. This questionnaire contains 8 single domain items, scales of each item is ranged from 1 to 5. Thus, the total score ranges from 8 to 40 points. A score of less than 28 is cause for concern. The validity of this questionnaire has been investigated in Iran (Cronbach's alpha=0.77)(34).

Assessment of biochemistry variables

At baseline and end of the study, 10 ml of venous blood samples (in regular tubes) will be collected after 10-12 hours of overnight fasting. Fasting blood glucose and lipid profile (total cholesterol, Low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride) will be evaluated by the enzymatic method with kits from Pars-Azmoon (Tehran, Iran). Insulin levels will be measured by chemiluminescent immunoassay. Homeostasis model assessment – insulin resistance (HOMA-IR) will be calculated by the following formula: fasting glucose(mg/dl) × fasting insulin(μu/ml)/405. ELISA kits will be used to determine serum leptin and adiponectin levels. All data will be entered electronically at the participating site where the data originated. Original study forms will be entered and kept on file at the participating site. Participant files are to be stored in numerical order and stored in a secure and accessible place and manner. Participant files will be maintained in storage for a period of 3 years after completion of the study. All Principal Investigators will be given access to the cleaned data sets.

Statistical analysis

Data analysts will be blinded after the assignment to interventions. All statistical analyses will be performed using the IBM SPSS Statistics software (Version 23) (IBM SPSS Statistics, Armonk, USA). The normality of the variables will be confirmed using the Kolmogorov-Smirnov test. A chi-square test will be applied to compare the categorical data between treatment groups at the baseline. Independent sample t-test and Mann-Whitney test will be used to compare parametric continuous and nonparametric data between the groups, respectively. Paired sample t-test or Wilcoxon signed-rank test will be applied to compare data within the groups. Post-intervention differences in responses between intervention and control groups will be tested by analysis of covariance (ANCOVA) with the use of the baseline measurements of the outcome variables as covariates. The percent change of each variable will be also computed by the formula [(E − B)/B

× 100], where E is the end of intervention values and B is the baseline values. The intention-to-treat (ITT) method will be applied for data analysis, which considers all participants in the trial and ignores anything that happens after randomization such as misallocation and non-compliance. A p-value of less than 0.05 will be considered to be statistically significant.

Ethics and dissemination: This protocol, approved by the Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The monitors from the Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness. Written informed consent will be obtained from participants before participation in the research project by researchers. All participant information will be stored in locked file cabinets in areas with limited access. This investigation was registered on the Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1).

DISCUSSION

Epidemiological studies show that the intake of milk and dairy products is inversely associated with a lower risk of metabolic disorders and cardiovascular diseases (35). Differences in the digestion kinetics of whey and casein proteins facilitate the stimulation of gastric hormones that delay gastric emptying, thus increasing feelings of fullness and attenuation of food particle breakdown and release in the small intestine. Whey and casein proteins differentially affect postprandial blood glucose and satiety mechanisms, with relevance for type 2 diabetes and obesity(30). It seems that complete dairy improves body composition and insulin sensitivity to a greater extent than whey or casein alone. In a diet-induced obese rat model, administration of complete dairy protein reduced weight gain and body fat mass accumulation more so than intake

of whey or casein alone(18). The intraintestinal presence of specific bioactive components, whole proteins, and select amino acids found within MPC is linked with insulin and gut peptide secretions, as well as suppression of food intake(36-38).

This is the first study that investigates the effect of supplementation of the MPC in adjunct with a weight loss diet on improving metabolic parameters, serum levels of adipocytokines, and body composition in obese women. Results of this trial will help us understand the effectiveness of MPC along with a weight loss diet to improve metabolic parameters, serum levels of adipocytokines, appetite, and body composition compared with controlled participants. In addition, the results of this trial will help obese women to reduce weight and improve their cardiometabolic health.

Acknowledgments: We thank Ahvaz Jundishapur University of Medical Sciences for funding this trial.

Authors contributions: FH and VA designed the research protocol. FH, VA, and ME were involved in the set-up of the original intervention study and follow-up study. FH, ME, MM, HB Sh, ShM ShI, and VA were involved in the writing of the article and carefully reviewed the article. FH acted as guarantor.

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Competing interests statement: The authors declare that they have no competing interests.

Patient consent for publication: Not required.

Availability of data and materials: The datasets generated and/or analyzed during the current study are not publicly available due to the privacy of subjects, but are available from the corresponding author on reasonable request.

Abbreviations: BMI: Body Mass Index; CNAQ: Council on Nutrition Appetite Questionnaire; MPC: Milk Protein Concentrate; IPAQ: International Physical Activity Questionnaire.

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Figure 1. Protocol flow diagram

Figure 2. Template of recommended content for the schedule of enrolment, interventions, and assessments. LDL-C: Low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: Homeostasis model assessment – insulin resistance.

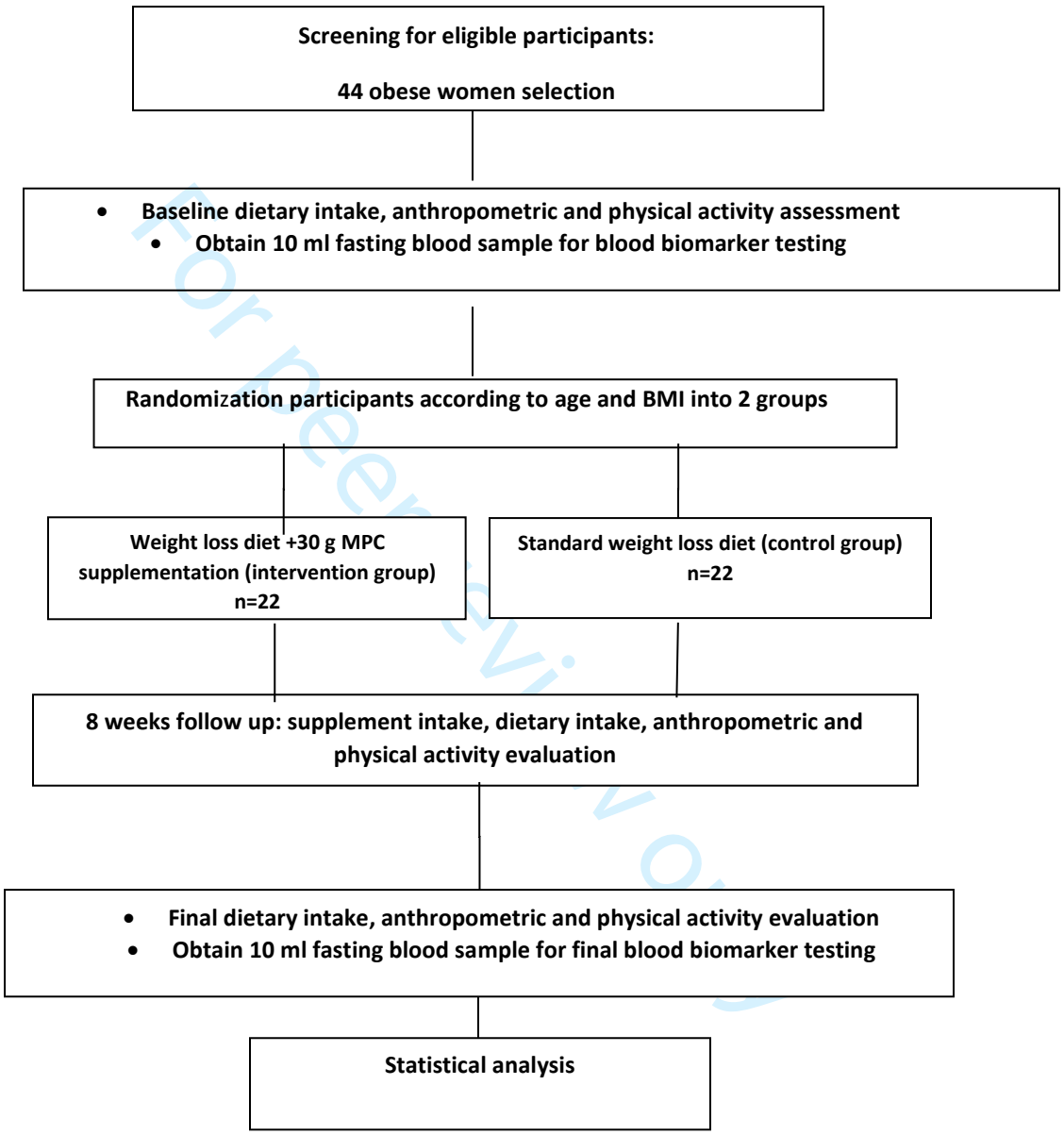

Figure 1. Protocol flow diagram

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT**	$-t_1$	0	*Week 1*	*Week 3*	*Week 6*	*Week 8*	*etc.*	t_x
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
Randomization		X						
Allocation		X						
INTERVENTIONS:								
[Intervention A]			←	—	—	→		
[Intervention B]			←	—	—	→		
ASSESSMENTS:								
Demographic variables	X	X						
primary outcome variables: levels of adiponectin and leptin, and the anthropometric status.			X	X	X	X	etc.	X
Secondary outcome variables: fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status.			X	X	X	X	etc.	X

Figure 2. Template of recommended content for the schedule of enrolment, interventions, and assessments. LDL-C: Low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: Homeostasis model assessment – insulin resistance.

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____1_____
	2b	All items from the World Health Organization Trial Registration Data Set	N/A: This investigation was registered on Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1)
Protocol version	3	Date and version identifier	_____6,11____
Funding	4	Sources and types of financial, material, and other support	_____10-11
Roles and	5a	Names, affiliations, and roles of protocol contributors	_____11_____

responsibilities	5b	Name and contact information for the trial sponsor	12: The sponsor is the same as the funder(Ahvaz Jundishapur University of Medical Sciences).
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	_12_
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_12_: Medical Ethics Committee of Ahvaz University of Medical Sciences by selecting an observer for each study supervises the RCT.
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	_____3-5_____
	6b	Explanation for choice of comparators	5
Objectives	7	Specific objectives or hypotheses	5-6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5,7
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7-8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A: This study don't include Rescue Medication or Prohibited Concomitant Medications
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8-9
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	SPIRIT figure
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7

Methods: Assignment of interventions (for controlled trials)

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	____7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	__7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	_____8-9___
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____6-7_____
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	____9-10_____

	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____9-10_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	_____9-10_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A: For this study is not Data Monitoring Committee.
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A: For this study is not Data Monitoring Committee.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___8_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	The monitors from Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness.
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	__6
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A: We will not have a Modification of the Protocol.
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	____12_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A: We have not ancillary study.
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	12, consent form
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____11
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____consent form
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___consent form

	31b	Authorship eligibility guidelines and any intended use of professional writers	13, We will not have any intended use of professional writers. In our university we are not allowed to add authors other than study researchers to the article.
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	13
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Uploaded as supplementary file
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

The effects of milk protein concentrate supplementation on metabolic parameters, adipocytokines, and body composition in obese women under weight-loss diet: Study protocol for a randomized controlled trial

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The effects of milk protein concentrate supplementation on metabolic parameters, adipocytokines, and body composition in obese women under weight-loss diet: Study protocol for a randomized controlled trial

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ABSTRACT

Introduction Obesity impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus. Evidence suggests that high-protein diets help to increase weight loss and protect against weight gain. Milk protein concentrate (MPC) is a dairy product with a high protein content with a ratio of casein and whey protein similar to skim milk. This trial aims to evaluate the effect of MPC supplementation in obese women under a weight-loss diet.

Methods and Analysis We will conduct a 2months open-label, parallel-group, randomized controlled trial to determine the effect of MPC supplementation on levels of glycemic and lipid profile, leptin, adiponectin, appetite, waist circumference, body mass index(BMI), and body composition in 44 premenopausal obese women on a weight-loss diet.

Ethics and dissemination This protocol, approved by the Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The trial results will be published in peer-reviewed journals.

Trial registration number Iranian Registry of Clinical Trials IRCT20201223049804N1.

Strengths and limitations of the study

- We will conduct a 2months open-label, parallel-group, randomized controlled trial to determine the effect of 30-gram MPC supplementation on levels of glycemic and lipid profile, leptin, adiponectin, appetite, waist circumference, body mass index(BMI), and body composition in 44 premenopausal obese women on a weight-loss diet.
- A limitation of this study is that other adipokines affecting energy homeostasis in the body and incretin hormones will not be measured due to restrictions in funding.
- Another limitation of this study is that all of the subjects will only female gender.

INTRODUCTION

Obesity is defined as the excessive accumulation of fat in the body (BMI ≥30 kg/m2) and is associated with dysregulation of glucose and lipoprotein metabolism (1). In particular, it has been shown that visceral fat, as an independent factor, impairs metabolic function and increases the risk of cardiovascular disease and type 2 diabetes mellitus (2). It is reported that additional production of pro-inflammatory cytokines by macrophages in visceral adipose tissue leads to insulin resistance (3). Inflammatory cytokines produced by adipose tissue prevent the binding of insulin to its receptors, thereby induce insulin resistance in target tissues (4). According to the World Health Organization (WHO) in 2016, more than 1.9 billion adults were overweight and 650 million were obese (13% of the world's adult population) (5). Also, the prevalence of obesity in Iranian adult females and males was reported at 29.3% and 13.6%, respectively (6). Probably due to differences in sex hormones, unknown molecular mechanisms, and genetics, women have a greater risk of obesity compared with men (7).

Adipose tissue is an important metabolic organ that is crucial for insulin sensitivity and energy homeostasis in the body (8). This tissue produces two hormones leptin and adiponectin called adipocytokines which influence lipid and glucose metabolism. According to studies with large populations, the ratio of leptin to adiponectin is an indicator of insulin sensitivity in the body (9, 10). Leptin regulates appetite, energy expenditure, and food intake by binding to its receptors in the hypothalamus and helps to control weight (11). In obese individuals, leptin levels increase and lead to the development of obesity and insulin resistance (12). Conversely, adiponectin acts as an insulin-sensitizer and improves lipid profile by increasing tissue fat oxidation. Nevertheless, decreased levels of adiponectin have been reported in obese individuals (13).

Obesity is a complex disease caused by the interaction of genetics, epigenetics, economic, physiological, social, and environmental factors (lifestyle, diet, and physical activity) (14, 15). It has been shown that the models of diet-induced obesity due to their similarity to human obesity, are often used in metabolic studies (16). Fat-rich diets play a role in the induction of obesity, impaired glucose homeostasis and as well as insulin resistance, and hyperlipidemia (17). Studies have shown that regular intake of dietary protein, especially dairy protein, may have beneficial effects on weight control and metabolic syndrome (18, 19).

Complete dairy protein or milk protein concentrate (MPC) is a relatively new dairy product with high protein content (70% protein) that is made by combining three methods including diafiltration, ultrafiltration, and spray-drying from pasteurized skim milk (31% protein). MPC contains both casein and whey protein, and the ratio of these two proteins is similar to skim milk (4:1 or 80:20). This ingredient is used in dairy products such as cheeses and yogurts (20). A study in obese rats showed that MPC reduced body weight and fat accumulation more than casein and whey protein alone. This may be due to the longer digestion process and greater satiety effects than the other two proteins (18). Also, MPC as a source of protein with a high biological value that contains all kinds of essential and non-essential amino acids can prevent muscle wasting (21). Another study reported that intraduodenal infusion of MPC significantly improved the effects of sitagliptin including glycemic and short-term food intake suppression. The results of this study confirm the hypothesis that the consumption of dairy protein may be useful as a complementary therapy to enhance the glycemic and food intake suppressive effects of GLP-1-based pharmacotherapies(22). Furthermore, a low-energy diet (LED) helps to the improvement of systemic dysmetabolism by inducing weight loss (23), has positive effects on adiponectin concentration (24), and is associated with decreased serum levels of leptin in obese individuals (25).

To the best of our knowledge, there is no study to investigate the effects of MPC along with a weight loss diet in obese women. Since, the present study aims to investigate a supplementation of the 30-gram MPC in adjunct with a weight loss diet will be useful in improving metabolic parameters, serum levels of adipocytokines, and body composition in obese women.

METHODS AND ANALYSIS

Study Design

We will conduct 8 weeks open-label, parallel-group, randomized controlled trial with a superiority framework. The proposed clinical trial will be conducted at the Nutritional Research Center, Department of Nutrition, the Ahvaz Jundishapur University of Medical Science for 8 weeks to assess the efficacy of the 30 mg MPC in adjunct with weight loss diet in obese women. Recruitment to this study and collecting data began in April 2022 and is expected to end in September 2022. (Figure 1) (Figure 2)

Aims and study hypotheses

The primary aim of the current trial is to examine the effect of 8 weeks of MPC supplementation on levels of leptin, adiponectin, waist circumference, BMI, and body composition in obese women on a weight-loss diet. The secondary aim is to determine the effect of 8 weeks of MPC supplementation on glycemic and lipid profile, insulin, and appetite. It is hypothesized that 8 weeks supplementation of the MPC in adjunct with a weight loss diet will improve measures of investigation.

Participants

Forty-four premenopausal obese women aged 18 years or more referred to the diet clinic in the city of Ahvaz meeting the inclusion criteria will be recruited in this trial after obtaining their informed written consent. The following inclusion criteria will be applied: aged 18 years and older, BMI range of 30 to 40 kg/m2, absence of menopause, absence of lactation and pregnancy, absence of food allergies, not having eating disorders, particularly binge eating disorder (BED), bulimia, not having cancer, hepatic, renal, thyroid and gastrointestinal disorders, no surgery for weight loss, no weight loss over the past 6 months, no taking any herbal medicine and drug that reduce appetite and weight (such as caraway extract, celery extracts, metformin, orlistate, etc.) and vitamin-mineral supplements. The subjects with any of the following criteria will be excluded: become pregnant during the study, unwilling to continue, changes in diet during the study period, and no consumption of powders exceed 10% of total administered powders.

Patient and public involvement

Patients were not involved in the development and design of the study protocol. The public was not involved. Study results will not be disseminated to participants specifically. However, if participants are interested in the results of the research, they will receive any information, the manuscript, and published research on this topic in the future.

Sample size

The required sample size is calculated based on data from a previous human study by Faghih et al(26), which assessed the effects of milk's cow consumption in adjunct with a weight loss diet on weight and fat loss. A mean difference in weight of 1.6 kg between the two groups is aimed to be detected for a specified α of 0.05 and a study power of 80%. Based on the proposed formula for parallel clinical trials(27), we reached a sample size of 20 participants in each group. Assuming a possible drop-out rate of 10%, 22 patients will be enrolled in each group.

Randomization

The subjects will be randomly stratified according to age and BMI using a permuted block randomization procedure by Random Allocation Software (RAS). The ratio of intended numbers of participants in each of the matched groups will be 1:1. They will be assigned to one of the two study groups: (Fig 1)

(1) Standard weight loss group (n=22) (control group)

(2) MPC supplementation weight loss group (n=22) (intervention group)

A member of the research team who is not involved in assessing the outcome of the study will be responsible for generating the allocation sequence and will allocate participants to the sequence.

Intervention

All subjects will follow a hypocaloric diet of 800 kcal below estimated energy requirements, which will be designed by a trained dietitian. Energy needs will be estimated by the Mifflin Jeor St equation(28). In the control group, the percentage of macronutrients will be 55%, 30%, and 15% for carbohydrates, fat, and protein; respectively(29). For the intervention group, an isocaloric weight loss diet with MPC supplements will be prescribed. Each of the intervention group participants will receive 30-gram MPC powders daily lasting 8 weeks. MPC powders will be supplied by Pegah dairy industries Co., Tehran, Iran. The MPC powders will be provided in a sachet form. Each sachet will be contained 30-gram MPC (105 kcal, 0.4 g of lipid, 6 g of carbohydrate, and 20 of protein; 20% whey protein and 80% casein). Considering the calorie of each MPC sachet (105kcal), 905 calories below estimated energy needs will be regarded for the intervention group. Participants in the intervention group will be instructed to add one sachet to 250 ml cold water and consume it immediately, every morning on an empty stomach(30). To check compliance, subjects will be requested to record the time and date of powder intake.

Moreover, to ensure that the intervention group participants regularly consume the powders, they will be contacted every three days by a dietitian, or if it was not possible to call them, they would be followed through SMS. To create a variety in the diet while maintaining the general principles of diet, all subjects will be given a dietary exchange list and a diet according to their food habits(31). The study subjects will be asked not to change their dietary habits and physical activity during the study (8 weeks).

The assigned study intervention may need to be modified or discontinued by trial investigators for various reasons, including gastrointestinal upset, allergic reactions, and withdrawal of participant consent. We will call the patients weekly and ask them about occurring any adverse events following MPC consumption. If the reported adverse events are correlated with MPC consumption, participants will be asked to stop taking MPC supplements, and also, they will be immediately referred to a specialist for therapy.

Outcomes

The primary outcomes consist of levels of adiponectin, leptin, body composition, waist circumference (WC), and BMI. Secondary outcomes will be fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status. All these factors will be measured at baseline and end of the study. We would manage multiplicity issue about multiple primary outcomes using Bonferroni correction.

Assessment of dietary intake, Anthropometric parameters, and physical activity

A sociodemographic questionnaire will be recorded at the beginning of the study. Dietary intake will be evaluated by 3 days 24-hr recall questionnaires (two weekdays and one weekend day) at the beginning, middle, and end of the study. Total energy and macronutrient intake will be

calculated by Nut IV (the Hearst Corporation, San Bruno, CA). A trained dietitian will evaluate anthropometric variables such as waist circumference, body weight, height, and BMI after overnight fasting with minimum clothing. At the baseline and the end of the study, waist circumference (WC) will be measured in a standing position using a tape with an accuracy of 1.0 cm above the iliac crest, just below the lowest rib margin and at the end of normal expiration. Bodyweight will be measured with the accuracy of 100 gr using a Seca scale at the baseline and end of the study. Stature will be measured in a relaxed position by a Seca stadiometer with an accuracy of 0.5 cm. BMI will be calculated as body weight (kg) divided by the square of height(m), at the beginning and end of the study. The direct segmental multi-frequency bioelectrical impedance method (Inbody 270, Biospace, Korea) will be used to calculate body composition including total body fat and fat-free mass percentage.

To evaluate the physical activity levels, the International Physical Activity Questionnaire (IPAQ) will be used at the baseline and the end of the trial via interviewing, and the results will be expressed as metabolic equivalent hours per week (METs hr/wk). The Persian translation of the short form IPAQ has been validated by Moghaddam et al. (Cronbach's alpha=0.7 and test-retest reliability coefficient=0.9)(32).

Assessment of Appetite

The Council on Nutrition Appetite Questionnaire (CNAQ), which was adopted by Wilson et al(33), will be used for measuring appetite. This questionnaire contains 8 single domain items, scales of each item is ranged from 1 to 5. Thus, the total score ranges from 8 to 40 points. A score of less than 28 is cause for concern. The validity of this questionnaire has been investigated in Iran (Cronbach's alpha=0.77)(34).

Assessment of biochemistry variables

At baseline and end of the study, 10 ml of venous blood samples (in regular tubes) will be collected after 10-12 hours of overnight fasting. Fasting blood glucose and lipid profile (total cholesterol, Low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride) will be evaluated by the enzymatic method with kits from Pars-Azmoon (Tehran, Iran). Insulin levels will be measured by chemiluminescent immunoassay. Homeostasis model assessment – insulin resistance (HOMA-IR) will be calculated by the following formula: fasting glucose(mg/dl) × fasting insulin(μu/ml)/405. ELISA kits will be used to determine serum leptin and adiponectin levels. All data will be entered electronically at the participating site where the data originated. Original study forms will be entered and kept on file at the participating site. Participant files are to be stored in numerical order and stored in a secure and accessible place and manner. Participant files will be maintained in storage for a period of 3 years after completion of the study. All Principal Investigators will be given access to the cleaned data sets.

Statistical analysis

Data analysts will be blinded after the assignment to interventions. All statistical analyses will be performed using the IBM SPSS Statistics software (Version 23) (IBM SPSS Statistics, Armonk, USA). The normality of the variables will be confirmed using the Kolmogorov-Smirnov test. A chi-square test will be applied to compare the categorical data between treatment groups at the baseline. Independent sample t-test and Mann-Whitney test will be used to compare parametric continuous and nonparametric data between the groups, respectively. Paired sample t-test or Wilcoxon signed-rank test will be applied to compare data within the groups. Post-intervention differences in responses between intervention and control groups will be tested by analysis of covariance (ANCOVA) with the use of the baseline measurements of the outcome variables as covariates. The percent change of each variable will be also computed by the formula [(E − B)/B

× 100], where E is the end of intervention values and B is the baseline values. The intention-to-treat (ITT) method will be applied for data analysis, which considers all participants in the trial and ignores anything that happens after randomization such as misallocation and non-compliance. A p-value of less than 0.05 will be considered to be statistically significant.

Ethics and dissemination: This protocol, approved by the Medical Ethics Committee of Ahvaz University of Medical Sciences, is in accordance with the Declaration of Helsinki (approval number: IR.AJUMS.REC.1399.795). The monitors from the Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness. Written informed consent will be obtained from participants before participation in the research project by researchers. All participant information will be stored in locked file cabinets in areas with limited access. This investigation was registered on the Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1).

DISCUSSION

Epidemiological studies show that the intake of milk and dairy products is inversely associated with a lower risk of metabolic disorders and cardiovascular diseases (35). Differences in the digestion kinetics of whey and casein proteins facilitate the stimulation of gastric hormones that delay gastric emptying, thus increasing feelings of fullness and attenuation of food particle breakdown and release in the small intestine. Whey and casein proteins differentially affect postprandial blood glucose and satiety mechanisms, with relevance for type 2 diabetes and obesity(30). It seems that complete dairy improves body composition and insulin sensitivity to a greater extent than whey or casein alone. In a diet-induced obese rat model, administration of complete dairy protein reduced weight gain and body fat mass accumulation more so than intake

of whey or casein alone(18). The intraintestinal presence of specific bioactive components, whole proteins, and select amino acids found within MPC is linked with insulin and gut peptide secretions, as well as suppression of food intake(36-38).

This is the first study that investigates the effect of supplementation of the MPC in adjunct with a weight loss diet on improving metabolic parameters, serum levels of adipocytokines, and body composition in obese women. Results of this trial will help us understand the effectiveness of MPC along with a weight loss diet to improve metabolic parameters, serum levels of adipocytokines, appetite, and body composition compared with controlled participants. In addition, the results of this trial will help obese women to reduce weight and improve their cardiometabolic health.

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Authors contributions: FH and VA designed the research protocol. FH, VA, and ME were involved in the set-up of the original intervention study and follow-up study. FH, ME, MM, HB Sh, ShM ShI, and VA were involved in the writing of the article and carefully reviewed the article. FH acted as guarantor.

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Competing interests statement: The authors declare that they have no competing interests.

Patient consent for publication: Not required.

Availability of data and materials: The datasets generated and/or analyzed during the current study are not publicly available due to the privacy of subjects, but are available from the corresponding author on reasonable request.

Abbreviations: BMI: Body Mass Index; CNAQ: Council on Nutrition Appetite Questionnaire; MPC: Milk Protein Concentrate; IPAQ: International Physical Activity Questionnaire.

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Figure 1. Protocol flow diagram

Figure 2. Template of recommended content for the schedule of enrolment, interventions, and assessments. LDL-C: Low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: Homeostasis model assessment – insulin resistance.

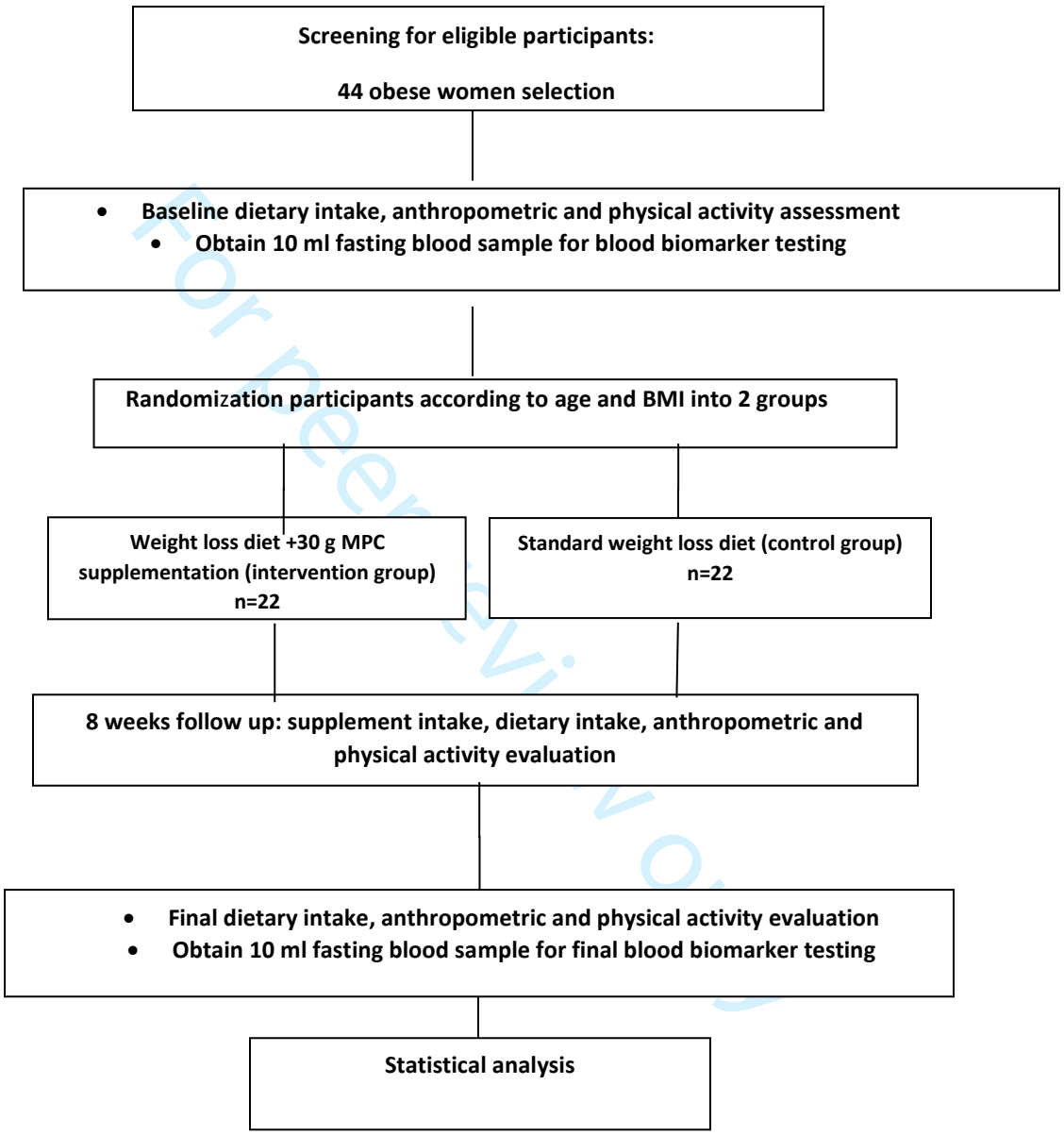

Figure 1. Protocol flow diagram

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT**	$-t_1$	0	*Week 1*	*Week 3*	*Week 6*	*Week 8*	*etc.*	t_x
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
Randomization		X						
Allocation		X						
INTERVENTIONS:								
[Intervention A]			←	—	—	→		
[Intervention B]			←	—	—	→		
ASSESSMENTS:								
Demographic variables	X	X						
primary outcome variables: levels of adiponectin and leptin, and the anthropometric status.			X	X	X	X	etc.	X
Secondary outcome variables: fasting plasma insulin and glucose levels, serum levels of lipid profiles (total cholesterol, LDL-c, HDL-c, and triglyceride), homeostasis model assessment of insulin resistance (HOMA-IR), and appetite status.			X	X	X	X	etc.	X

Figure 2. Template of recommended content for the schedule of enrolment, interventions, and assessments. LDL-C: Low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: Homeostasis model assessment – insulin resistance.

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____1_____
	2b	All items from the World Health Organization Trial Registration Data Set	N/A: This investigation was registered on Iranian Registry of Clinical Trials (Irct registration number: IRCT20201223049804N1)
Protocol version	3	Date and version identifier	_____6,11____
Funding	4	Sources and types of financial, material, and other support	_____10-11
Roles and	5a	Names, affiliations, and roles of protocol contributors	_____11_____

responsibilities	5b	Name and contact information for the trial sponsor	12: The sponsor is the same as the funder(Ahvaz Jundishapur University of Medical Sciences).
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	_12_
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_12_: Medical Ethics Committee of Ahvaz University of Medical Sciences by selecting an observer for each study supervises the RCT.
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	_____3-5_____
	6b	Explanation for choice of comparators	5
Objectives	7	Specific objectives or hypotheses	5-6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5,7
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7-8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A: This study don't include Rescue Medication or Prohibited Concomitant Medications
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8-9
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	SPIRIT figure
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6-7

Methods: Assignment of interventions (for controlled trials)

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	____7_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	__7_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	7
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	_____8-9___
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	_____6-7_____
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	____9-10_____

	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____9-10_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	_____9-10_____
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	N/A: For this study is not Data Monitoring Committee.
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A: For this study is not Data Monitoring Committee.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	___8_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	The monitors from Medical Ethics Committee of Ahvaz University of Medical Sciences will discuss the protocol in detail and identify and clarify any areas of weakness.
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	__6
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A: We will not have a Modification of the Protocol.
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	____12_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A: We have not ancillary study.
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	12, consent form
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____11
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	_____consent form
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___consent form

	31b	Authorship eligibility guidelines and any intended use of professional writers	13, We will not have any intended use of professional writers. In our university we are not allowed to add authors other than study researchers to the article.
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	13
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Uploaded as supplementary file
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	10

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.