Study Protocol and Statistical Analysis Plan

A prospective open controlled study of creatine combined with curcumin in the intervention of early cachexia in upper gastrointestinal tumors.

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Nanjing Drum Tower hospital

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Summary

Project Name	A prospective open controlled study of creatine combined with curcumin in the intervention of early cachexia in upper gastrointestinal tumors
Purpose of the study	（1）To investigate whether creatine combined with curcumin can improve the inflammatory state, correct the disorder of nutrient metabolism and improve the nutritional status in the early cachexia stage of upper gastrointestinal neoplasms. （2）To investigate whether creatine combined with curcumin can improve the quality of life and prognosis of patients with early cachexia in upper digestive tract tumors.
Study Design	The effect of creatine combined with curcumin on inflammatory state, nutritional metabolism, nutritional state and prognosis of patients with early cachexia of upper digestive tract tumors was investigated in a prospective open controlled study
Total number of cases	152 cases
Criteria	Inclusion Criteria: (1) Clinical diagnosis of advanced tumors of the upper digestive tract with untreatable or postoperative recurrence of esophageal and gastric cancer III-IV (2) Meet the diagnostic criteria of early cachexia (anorexia and metabolic changes, involuntary body mass reduction ≤5% within 6 months) (3) Radiotherapy, chemotherapy, or immunotherapy in our hospital (4) Understand and fill in a variety of rating scales (5) Informed consent, voluntary participation in this study
	Exclusion Criteria: (1) Neoadjuvant chemotherapy patients (2) Intestinal obstruction or gastrointestinal bleeding (3) Severe heart, lung and renal insufficiency (4) Coagulopathy (5) Clinical diagnosis with diabetes and other metabolic diseases (6) The expected survival time is less than 1 month (7) With cognitive dysfunction or poor coordination (8) Allergy to creatine or curcumin (9) With a history of drug abuse (10) Doctors or researchers deem unsuitable for study participation
Treatment	1. Patients were enrolled for baseline investigation： (1) Disease status: disease stage, complications, treatment, treatment stage, drug

plan	use. (2) General investigation: dietary investigation, digestive tract symptoms, height, weight. (3) Nutritional assessment: NRS2002 and PG-SGA scores, grip strength, body composition, grip strength, deltoid skin fold thickness, upper arm muscle circumference. (4) Examination and examination: blood routine, biochemical set, IL-6, TNF-α, lactic acid, serum insulin, prealbumin, transferrin, chest, and abdominal CT, PETCT, etc. (5) Functional Assessment of Anorexia/Cachexia Therapy（FAACT）; (6) QoL Evaluation Scale: ADL scale。 After informed consent, patients were divided into groups A and B according to their wishes. Group A: According to the national industry health standard "Dietary Guidance for Patients with Malignant Tumors 2017", basic nutrition treatment, energy 30 ~ 35kcal/kg, protein 1.0-2.0g/kg, diet deficiency, total nutrition and/or whey protein oral nutrition supplement or tube feeding enteral nutrition. Group B: Since basic nutrition in group A, creatine 5g/d and curcumin 4g/d were added orally for one month. 2．follow-up Weekly telephone follow-up, 2 weeks of routine outpatient follow-up, general condition survey, nutritional intake, nutritional assessment, routine examination, anorexia/fluid therapy functional assessment, QoL rating scale, 4 weeks of effectiveness evaluation, 3 months of survival survey.
Evaluation of curative effect	Efficacy evaluation indicators (primary efficacy indicators and secondary efficacy indicators) Main research Indicators (1) Nutritional serum indexes: blood routine, biochemical complete set, prealbumin, transferrin (2) Body composition index: skeletal muscle index and body composition in L3 section of CT scan (3) Inflammatory metabolism indicators: serum insulin, IL-6, TNF-α, lactic acid, PETCT to evaluate the metabolism Secondary study indicators (1) Physical measurements: body weight, grip strength, triceps skin fold thickness, upper arm circumference diameter (2) Nutritional assessment: energy and protein intake, PG-SGA score (3) Functional Assessment of Anorexia/Cachexia Therapy（FAACT）

	(4) QoL evaluation scale: ADL score (5) Survival time survey: 1, 3 months survival rate (6) Evaluation of tumor efficacy: Compared with the baseline at the time of enrollment, tumor remission and progression were compared
	Safety evaluation index Creatine and curcumin taken orally by the patients in this study were both food grade: creatine is a sports nutrition food at home and abroad, which can increase muscle synthesis; Curcumin is the main component of curry, often used as a food additive, there are a variety of health food sales of curcumin abroad. After large dose of creatine supplementation (more than 25g/ day), muscle stiffness, spasm and other adverse phenomena may occur; Large doses of curcumin supplementation may cause nausea and even vomiting and diarrhea, with no toxic effects below 8g daily. Both creatine and curcumin have been used orally in cancer patients in domestic and foreign studies without adverse reactions reported. The creatine (5g/ day) and curcumin (4g/ day) consumed in this study belong to the safe dosage range according to the oral dosage in domestic and foreign literatures and the dosage in the product instructions, so there are generally no side effects. For safety reasons, researchers are still closely monitoring patients for possible side effects.
Method of statistics	Statistical analysis was performed using GraphPad Prism version 18.0 (La Jolla, CA, USA). Student's t-test was used for comparison between groups, and one-way analysis of variance (ANOVA) was used for other data. All data are presented as mean ± SEM. $p<0.05$(*) indicates statistical significance. $p<0.01$(**) indicates that the difference is more statistically significant.
Duration of study	Jun 1,2023 to Dec 31,2025

1.Background

1.1 The purpose and significance of the research

Cancer cachexia (CAC) is a common complication of various advanced malignant tumors, which seriously affects the quality of life of patients, reduces the sensitivity and tolerance of the body to treatment, and even directly reduces the survival time of patients [1, 2]. The incidence of cachexia in digestive system tumors is significantly higher than other tumors [3].

The pathogenesis of cachexia is mainly that a series of mediators produced by tumor tissues directly or indirectly promote inflammation, resulting in increased catabolism of the body and inhibition of anabolism [4]. Although nutritional therapy is the basic treatment of tumor cachexia intervention measures, a common clinical problem is that even if the body is provided with very sufficient energy and protein, the body still does not respond, unable to enter the normal anabolic process, continuous loss of skeletal muscle and weight loss cannot be avoided, and so far there is no effective drug to correct the metabolic disorder of tumor cachexia [5, 6]. The main purpose of this study is how to make nutrition play a normal role in the synthesis of cachexia, keep the skeletal muscle and body weight not lost or less lost, and reverse the development of cachexia.

To achieve this goal, inflammation should be solved first. Many studies have found that curcumin, a plant polyphenolic compound, can effectively inhibit inflammation in tumor mice. Our group's previous studies found that curcumin can prevent the lipolysis of colon cancer mice with cachexia. In terms of anabolism, previous studies have found that creatine supplementation can promote muscle synthesis in mice, improve lean body mass and muscle mass, and partially prevent fat decomposition. Some published clinical studies have also confirmed the positive effects of oral curcumin or creatine alone on patients with cachexia [7, 8], but other studies have found that although oral curcumin alone can improve internal environment such as inflammation, the effect of lean body mass increase is not ideal [9]. Based on this, we speculate that the combination of the two may play a better and more stable synergistic effect.

In this study, creatine combined with curcumin was intended to intervene in patients with upper digestive tract tumors complicated with cachexia since adequate nutrition, and it was also considered that once the tumor cachexia developed to the advanced stage, both clinical and nutritional treatment would be difficult to achieve effect [2], so the study object was limited to the early cachexia. The main

purpose of this study is to determine whether the combination of the two can inhibit the inflammatory state and improve the metabolic state of the tumor cachexia, to make the basic nutrition play a role, improve the nutritional status, and eventually reverse the tumor cachexia. This will have extremely important clinical significance for improving the tolerance and response rate of the clinical treatment, improving the quality of life of the tumor patients, and even extending the survival period of the patients.

1.2 Research progress

1.2.1 Pathogenesis of cachexia

Tumor cachexia is a multifactorial syndrome with three most important features: persistent skeletal muscle loss (with or without loss of adipose tissue), failure to be completely alleviated by conventional nutritional support, and functional impairment [10]. Weight loss and fatigue caused by skeletal muscle loss are the core manifestations of the cachexia, and excessive protein decomposition (especially muscle protein) is an important pathophysiological change. The main clinical manifestations include weight loss, muscle atrophy, fatigue, loss of appetite, anorexia, fullness, anemia, edema, hypoproteinemia and so on. Tisdale MJ believed that when the weight loss of tumor patients was greater than 30% of the stable body weight, death began and could not be avoided [11]. About 60%~80% of tumor patients may develop cachexia, which can occur in any process of tumor development, and about 20% of malignant tumor patients die from cachexia [12].

The pathogenesis of tumor cachexia is currently believed to be the result of complex and multiple factors, including anorexia and digestive tract obstruction caused by tumor, which lead to insufficient nutrient intake. A series of mediators produced by tumor itself lead to body inflammation, and directly or indirectly lead to metabolic disorders of the three nutrients, especially catabolism is significantly enhanced, anabolism is inhibited, etc. [2-4]. Various approaches ultimately accelerate the breakdown of skeletal muscle protein and inhibit its anabolism in tumor patients.

Antitumor therapy and nutritional support are difficult to play a role in advanced stage of the cachexia [13]. Therefore, early intervention depends on accurate identification and scientific staging of cachexia. At present, it is widely divided into three stages by the Professional Committee of Tumor

Nutrition and Supportive Therapy of the Chinese Anti-Cancer Association in 2015, referring to the European clinical guidelines for tumor cachexia and the Chinese malnutrition standards: early stage, stage of cachexia and refractory stage [10, 14].

1.2.2 New progress in the treatment of cachexia

At present, the most studied intervention measures for cachexia include nutrition intervention, drug intervention, exercise intervention and multidisciplinary intervention mode. For the treatment of tumor-related cachexia in clinical trials [15], it has been found that many drugs may be effective against the cachexia, and these drugs basically have the following three functions [1]: reducing tumor-related inflammation; Harnessing the body's anabolic potential to combat consumption and hypercatabolic states; Stimulate appetite. However, the clinical effect of drug therapy is not stable, the individual difference is large, and the side effects are many.

Exercise has anti-inflammatory properties, which can fight the muscle degradation and insulin resistance caused by inflammation. Animal studies have shown that exercise influences reducing skeletal muscle degradation and body weight, reducing inflammation and oxidative stress, reducing depressive symptoms, and increasing anabolic hormones (such as IGF-1) [6]. However, to achieve a certain level of exercise and exercise duration, there are many uncontrollable restrictions on the implementation of exercise therapy in cancer patients.

Nutrition intervention to improve cachexia has shown its unique advantages and has been widely concerned by clinical medical staff. The basic requirement is to provide tumor patients with 30-35 kcal/kg of energy and 1.0-2.0 g/kg/day of protein. In addition, there are much research on branched chain amino acids, omega-3 unsaturated fatty acids, L-L-carnitine, and other nutrients [16]. Although nutritional support is the basic treatment for cachexia and can enable energy and protein intake to a certain extent and sustainable over a period, it does not fundamentally solve the catabolic problems. How to correct the inflammatory state and metabolic disorder of such patients, so that basic nutrition can enter anabolism, is the main problem to be solved in this study.

1.2.3 New progress of creatine and curcumin in the treatment of cachexia

Some special nutrients and phytochemicals have been found to have a good effect on correcting

inflammation and metabolism of tumor cachexia and improving lean body mass.

Curcumin is a plant polyphenolic compound, which can effectively inhibit inflammation in the body, reduce the production of cytokines, inhibit the production and activation of key proteins in the ubiquitination protein degradation system, have anti-tumor effects, and further delay the process of muscle atrophy. Studies on its efficacy have been confirmed by many literatures. Metabolomics studies have found that curcumin treatment can effectively regulate the biosynthesis of branch chain amino acids such as leucine and valine, change the synthesis and degradation of ketone bodies in the body, and affect the metabolism of taurine, triglyceride, glycine, serine and threonine, confirming the intervention of curcumin on the specific metabolic pathway of tumor cachexia and its effective treatment of high metabolic state of cachexia Therapeutic potential [17]. An animal study of mice with progressive skeletal muscle atrophy from colon tumors showed that low-dose curcumin(100mg/kg) was effective in preventing weight loss and high-dose curcumin(250mg/kg) was associated with approximately 25% weight gain compared with placebo. Curcumin can inhibit the complex activity of proteasome and reduce the expression of muscle-specific ubiquitin ligase MAFbx/atrogin-1 and MURF-1 to varying degrees [18]. Our research group found that curcumin has anti-inflammatory and anti-lipolysis effects in mice with colon cancer. However, some studies have found that although curcumin can inhibit tumor growth (accounting for 31% of the total cell number), it does not improve muscle volume [19, 20]. Population studies have shown that curcumin can effectively increase the body weight of patients with bad fluid and reduce the serum TNF-α level. Curcumin can improve the prognosis of patients with colorectal cancer by increasing the expression of tumor suppressor protein P53 in tumor cells [7].

Creatine is a tripeptide composed of arginine, methionine, and glycine, which can be synthesized by the body or taken from food. Creatine is a naturally occurring amino acid derivative in the human body. Creatine supplementation can improve lean body mass and muscle function, rapidly increase muscle strength, accelerate fatigue recovery, and improve explosive power. Therefore, creatine is widely used in exercise supplement and sarcopenia treatment [21]. Studies have shown that both short-term and long-term supplementation (30 g/day for 5 years) is safe and well tolerated for healthy individuals and many patients from infants to the elderly, and that low dose creatine intakes (e.g., 3 g/day) can provide

significant health benefits [21]. Many animal studies have confirmed the beneficial effect and mechanism of creatine in the cachexia of tumor [22, 23]. However, in clinical studies, the sample size is small and high-quality evidence is lacking. A randomized double-blind controlled trial in which 30 patients with stage III or IV colorectal cancer were selected to receive creatine versus placebo showed more significant improvements in phase Angle and grip strength in the creatine supplement group, but no significant effects on body composition and body weight [24]. Another study on head and neck cancer showed a significant increase in lean body mass from baseline in the creatine supplement group, but no significant difference in the control group. In addition, skeletal muscle strength was also improved in the creatine group, but there was no statistical difference between the two groups [25]. So far, there have been few relevant studies. Although the optimal creatine supplement scheme is not yet clear, it has been reported that creatine supplement of 3-5g/d can make the body reach the saturation level of creatine [9, 26]. In addition, other studies have found that creatine may enhance the invasion and metastasis of some cancers, including colorectal cancer, pancreatic cancer, and breast cancer [27], which is a problem that researchers need to pay attention to.

To sum up, although curcumin partially increases muscle mass, studies have found that curcumin is more likely to be anti-inflammatory and prevent adipose tissue decomposition, while creatine mainly improves lean body mass and muscle function. Therefore, whether the combination of the two can play a more stable synergistic effect in the treatment of cachexia is the main content of this study.

1.3 Research to be carried out.

In this review, cachexia is a common complication of various advanced malignant tumors, which seriously affects the quality of life and survival time of patients. In view of the clinical problem of non-nutritional response in patients with cachexia, we plan to carry out a clinical case-control study on the intervention of creatine combined with curcumin in patients with cachexia. Overall, we limited the study subjects to upper digestive tract tumors and diagnosed patients with early cachexia. The main purpose of this study is to determine whether the combination of the two can play a positive and stable role in inhibiting the inflammation of cachexia and improving metabolic status, so that basic nutrition can play a role, to reduce the level of skeletal muscle consumption, maintain weight, improve quality of

life, save medical costs, and extend survival time.

2.Purpose of the study

（1）To investigate whether creatine combined with curcumin can improve the inflammatory state, correct the disorder of nutrient metabolism and improve the nutritional status in the early cachexia stage of upper gastrointestinal neoplasms.

（2）To investigate whether creatine combined with curcumin can improve the quality of life and prognosis of patients with early cachexia in upper digestive tract tumors.

3. Study design type, principle, and test procedure

(1) Study Design

Type of study design: prospective open controlled clinical trial; Non-random grouping method: According to the exclusion criteria, patients were included into group A or group B according to their wishes after informed consent; Blind level: This experiment is an open study without blind or emergency letters. Research Center: None.

Group A: basic nutrition treatment, energy 30-35 kcal/kg, protein 1.0-2.0 g/kg, diet deficiency, total nutrition and/or whey protein oral nutrition supplement or tube feeding enteral nutrition supplement.

Group B: Since basic nutrition in group A, creatine 5g/d and curcumin 4g/d were orally added, and the intervention time was 1 month.

It was assumed that after 4 weeks of oral administration of creatine + curcumin in group B, combined with the existing literature on the improvement of the quality of the bad fluid, the skeletal muscle index at L3 section of CT scan in group B was 20% higher than that in control group A, and the standard deviation was 14%. Bilateral test was required, α was 0.05, the sample ratio between the two groups was 1:1(i.e., the number of cases in the two groups was equal), β=0.2. The degree of assurance (test efficacy)1-β=80% was calculated using SPSS software, then the sample size of each group was 68 cases, considering the 10% loss of follow-up rate, each group needed 76 cases, 152 patients in total.

Follow-up test item time point

Classification	Item	0	0.5m	1m	3m
Serological indicators	blood routine	√	-	√	√
	biochemical set	√	-	√	√
	prealbumin	√	-	√	√

	transferrin	√	-	√	√
Body composition index	CT scans skeletal muscle index at L3 cross section	√	-	√	√
	BIA measures muscle mass	√	-	√	√
Inflammatory metabolic indicators	Serum insulin	√	-	√	√
	IL-6	√	-	√	√
	TNF-α	√	-	√	√
	lactic acid	√	-	√	√
	PETCT（SUV）	p.r	-	p.r	-
Physical measurements	Weight	√	√	√	√
	grip strength	√	√	√	√
	skin fold thickness	√	√	√	√
	upper arm circumference	√	√	√	√
nutrition assessment	intake	√	√	√	√
	PG-SGA score	√	√	√	√
Questionnaire Survey	Functional Assessment of Anorexia/Cachexia Therapy（FAACT）	√	√	√	√
	ADL evaluation scale	√	√	√	√

4. Criteria

Inclusion Criteria: (1) Clinical diagnosis of advanced tumors of the upper digestive tract with untreatable or postoperative recurrence of esophageal and gastric cancer III-IV

(2) Meet the diagnostic criteria of early cachexia (anorexia and metabolic changes, involuntary body mass reduction ≤5% within 6 months) (3) Radiotherapy, chemotherapy, or immunotherapy in our hospital (4) Understand and fill in a variety of rating scales (5) Informed consent, voluntary participation in this study
Exclusion Criteria: (1) Neoadjuvant chemotherapy patients (2) Intestinal obstruction or gastrointestinal bleeding (3) Severe heart, lung and renal insufficiency (4) Coagulopathy (5) Clinical diagnosis with diabetes and other metabolic diseases (6) The expected survival time is less than 1 month (7) With cognitive dysfunction or poor coordination (8) Allergy to creatine or curcumin (9) With a history of drug abuse (10) Doctors or researchers deem unsuitable for study participation
Removal Criteria： (1) Do not meet the inclusion criteria and mistakenly included or meet any of the exclusion criteria. (2) Meet the inclusion criteria but did not use after inclusion or did not have any record of return visit. (3) The intervention measures used during the trial did not conform to the protocol.
Criteria for study termination： (1) With allergic reactions or serious adverse events should be stopped according to the investigator's judgment. (2) Other complications and special physiological changes occurred during the test. (3) During the experiment, the subjects needed parenteral nutrition support during the nutritional support process, and the experiment should be stopped. (4) Poor compliance. (5) Pregnancy.

5．Research methods and technical route

（1） Research food names and specifications

The creatine provided to the patient in this experimental study scheme was purchased from the sports nutrition food Decathon creatine powder (powder: 10g/ packet *10 packets/box) produced by Jiangsu Alante Nutrition Co., LTD. Purchased from Meriva Curcumin Phytosome (capsule: 1g/ capsule *120 capsules/box) from THORNE Company, USA.

（2） Treatment plan

Clinical data of patients with advanced esophageal and gastric cancer combined with early cachexia were collected, abdominal CT scan was performed, and the skeletal muscle area and

density of L3 section were measured by Matlab software. Skeletal muscle index was obtained after height correction, and body composition and bone mineral density were measured at the same time. Routine examination and nutritional indexes were determined, including blood routine, biochemical set, prealbumin, transferrin; Inflammatory metabolic markers including serum insulin, IL-6, TNF-α, lactic acid, and PETCT were assessed for metabolism. NRS2002 and PG-SGA scores were performed. According to the standard body weight, dietitians formulated diets for all enrolled patients. According to the ESPEN guidelines and the national industry health standard "Dietary Guidelines for Patients with Malignant Tumors 2017", the dietary energy intake standard was 30-35kcal /kg·d, and the protein intake was 1.0-2.0g /kg·d. The deficiency was given to oral enteral nutrition supplements or whey protein supplements, or to patients requiring tube feeding. Patients were enrolled in group A control group or group B intervention group on a voluntary basis.

Group A intervention group: The above basic nutritional support was provided.

Group B control group: In addition to the above basic nutrition, oral creatine powder 5g qd, curcumin capsule 2g bid for one month.

The intake, complications and survival of the patients were followed up by telephone for 1 and 3 weeks, physical indicators, nutritional assessment, FAACT and QoL scale were followed up for 2 weeks in the hospital and outpatient department, and nutritional serum indicators, body composition indicators, inflammatory metabolism indicators, physical indicators, nutritional assessment were reviewed for 4 weeks. Evaluation of effect. The long-term survival, re-hospitalization and hospitalization expenses were investigated 3 months later. To evaluate the effect of creatine combined with curcumin on improving the nutritional status, metabolic level and clinical prognosis of alimentary tract neoplasm. Note: Weight is calculated by standard weight: Standard weight (kg) = height (cm)-105.

（3）Combination of drugs

During the study period, patients may have been treated with chemotherapy drugs and immunotherapy drugs due to tumor treatment and may have taken megestrol orally against the oxalic fluid to improve appetite. To avoid the influence of the two groups of therapeutic drugs, the diseases, and drugs of the two groups will eventually be grouped and stratified to eliminate the interference of combined drug use.

（4）Safety evaluation index

① Incidence and severity of side effects

② The rate of unplanned rehospitalization.

6．Observation of adverse events

Adverse Event (Adverse Event): An adverse medical event occurs after a patient or clinical trial subject receives a drug or a formula for a special medical use, but there is not necessarily a causal relationship.

Serious Adverse Event: An event occurs during the clinical trial that requires hospitalization, prolongates the hospital stay, causes disability, affects the ability to work, threatens life or death, or

results in congenital malformations.

Adverse reaction: an adverse reaction that is harmful rather than expected but is causally related to the application of a drug or special medical use formula food during normal application of the drug or special medical use formula food at prescribed doses. In clinical trials for a new use, all reactions that are harmful and not causally related to the use of a drug or a formula for special medical use, as expected, should be considered adverse when the dose has not been determined.

Any adverse events that occur during the test should be recorded in detail.

(1) Large dose of creatine supplement may cause muscle stiffness, spasm and other adverse phenomena.

(2) Exceeding recommended doses of curcumin can cause nausea and even vomiting and diarrhea.

None of the doses used in this study exceeded the recommended dose, so the probability of adverse events was small. Once adverse events occur, the oncologist and nutritionist will be reported immediately, and the current intervention will be suspended according to the actual condition of the patient, and the treatment measures will be developed.

7. Research on quality control and quality assurance

（1）Laboratory quality control measures

The standard operating procedures and quality control procedures for experimental observation indicators should be established in hospital laboratories.

（2）Training of researchers

Prior to the commencement of clinical trials, the investigator should be trained in the protocol.

（3）Measures to improve subject compliance

① The researcher should carefully perform informed consent so that the subjects can fully understand the test requirements and cooperate with the test.

(2) Pay attention to the number of days of experimental drugs, whether there is intermittent drug use, and monitor the compliance of subjects.

Compliance = actual number of days of medication/number of days of medication ×100%

Compliance is 80% ~ 120% compliance is good; Compliance is poor if less than 80% or more than 120%.

（4） Oversight of clinical trials

Monitoring of patients' actual nutrient intake during nutritional therapy:

Follow-up: The patients were discharged from the hospital for routine dietary guidance, with daily total energy set at 30-35kcal /kg.d and daily protein intake at 1.0-2.0 g/kg.d, and continued to receive total nutrition preparations and/or whey protein oral nutrition supplement or tube feeding enteral nutrition supplement.

Monitoring of patients' condition during the experiment:

When the patients included in the study experience changes or even adverse reactions, strict diagnosis and treatment will be taken immediately to avoid the occurrence of adverse events.

8. Data security audit

The clinical study will develop a data safety monitoring plan according to the risk. All adverse events are recorded in detail, properly handled and tracked until properly resolved or the condition is stable, and serious adverse events and unexpected events are reported to the Ethics Committee, competent authorities, sponsors and drug regulatory authorities in a timely manner in accordance with regulations; The principal investigator conducts regular cumulative reviews of all adverse events and holds investigator meetings where necessary to assess study risks and benefits; For studies with greater than minimum risk, an independent data monitor will be assigned to monitor the study data. For high-risk studies, an independent Data Safety Monitoring committee will be established to monitor the accumulated safety and efficacy data and make recommendations on whether to proceed.

9. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 (La Jolla, CA, USA). Student's t-test was used for comparison between groups, and one-way analysis of variance (ANOVA) was used for other data. All data are presented as mean ± SEM. $p<0.05$(*) indicates statistical significance. $p<0.01$(**) indicates that the difference is more statistically significant.

10．The ethics of clinical research

Clinical research will follow the relevant regulations such as the Declaration of Helsinki of the World Medical Congress. Clinical studies are conducted only after the protocol is approved by the Ethics Committee prior to the start of the study. Before each subject is enrolled in this study, the investigator is responsible to give a complete and comprehensive introduction of the purpose, procedure and possible risks of this study to the subject or his/her agent and sign a written informed consent. Subjects should be informed that they have the right to withdraw from this study at any time, and informed consent should be kept as a clinical study document for future reference. Subjects' privacy and data confidentiality will be protected during the study.

11．Research schedule

Jun 1,2023 to Aug 31,2023: The formal implementation of the project was started. 76 cases were collected, and baseline investigation was conducted for enrolled patients. The patients were not randomly enrolled, and grouped intervention was performed.

Sep 1,2023 to Dec 31,2023: Completed the follow-up of patients in the early stage, conducted statistical analysis of data, and modified the program according to the existing problems in the early stage.

Jan 1,2024 to Dec 31,2024: The remaining 76 cases were collected, and baseline investigation was carried out for enrolled patients, who were not randomly enrolled, and group intervention was performed.

Jan 1,2025 to Jun 31,2025: All patients were followed up and all data were analyzed.

Jul 1,2025 to Dec 31,2025: Write 2-3 papers; Participate in academic exchange of relevant

achievements.

12.Reference

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Scheme Signature Page

The researchers stated:

I agree to comply with the review opinions of the Ethics Committee and start the clinical trial after approval, report to the Ethics Committee in a timely manner any changes in the clinical trial activities, as well as any unexpected problems involving risks to subjects or other personnel, and carry out the trial after re-obtaining the approval of the ethical review. Follow up review and conclusion review as required by the Ethics Committee.

I agree to conduct clinical trials in strict accordance with the design and specific provisions of this protocol.

I understand that I can interrupt or terminate this clinical trial at any time if it is in the best interest of the subjects.

I agree that I will personally conduct or supervise the clinical trial and that all investigators in my organization who assist me in conducting the clinical trial understand their role in the clinical trial.

In carrying out this clinical trial, I will strictly abide by the current GCP and the Declaration of Helsinki. And promised that the whole process will be ethical, ethical and scientific requirements.

During the implementation of clinical trials, I will strictly abide by all laws and regulations related to clinical trials to protect the rights and interests of patients.

I agree to maintain adequate and accurate medical records and ensure that these medical records are readily available for inspection and inspection in accordance with relevant laws and regulations.

Signature Date