



Research Article

A Long-Term Toxicology Study of the Nutraceutical 2036 Specialty-Gaoqing Recipe Administered to Rats

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Abstract

2036 Specialty-Gaoqing recipe (2036S-GQR) is a nutraceutical that content docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), grape seed proanthocyanin extract, and bilberry anthocyanins extract. All these ingredients have been proven having various health benefit and 2036S-GQR has been widely and long use in China. However, the toxicity of long-term administration has not yet been reported. The present study explores the long-term toxicity of 2036S-GQR in Wistar rats following the OECD 407 and 408 guidelines. Three dose group was orally administered 2036S-GQR 0.6, 1.2, 2.0 g/kg body weight six day per week for 26 weeks respectively, whereas control group provided distilled water. The investigation of food intake, body weight, hematological, biochemical, and histopathological data was performed in 12, 26 weeks and at the end of recovery period (30 weeks) which was used to assess the toxic effects. The result displays no evident sign of toxicity, no clinically significant change emerges in all measuring data and observation in all dose group, demonstrating 2036S-GQR is harmless to the body under the arranged dose.

Keywords: Nutraceuticals; Docosahexaenoic acid; Eicosapentaenoic acid; Proanthocyanin; Anthocyanins; Adverse effects

Introduction

The 2036 Specialty-Gaoqing recipe (2036S-GQR) is a health-care product has been marketed and used extensively in China for a long time, comprising four staple components, the docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), grape seed proanthocyanin extract, and bilberry anthocyanins extract. DHA and EPA are a kind of ω 3 long-chain polyunsaturated fatty acids, which is rich in seafood but could not synthesis in human body, many efforts have confirmed the health promoting function for disease prevention of DHA and EPA, such as cancer, diabetes, inflammation, immune diseases and cardiovascular diseases [1-4]. Based on this, the World Health Organization, Food and Agriculture Organization, American Heart Association and the 2015–2020 Dietary Guidelines for Americans unanimously recommended consuming 250–500 mg of DHA and EPA daily to prevent diseases and for better health care [5,6]. The flavonoid compounds proanthocyanin and anthocyanins are present in various plants; according to available reports, they have similar health effects, including anti-oxidation, anti-inflammation, anti-infection, and anti-cancer effects [7,8]. However, numerous people were fell below the recommend intake of DNA and EPA [9], whereas proanthocyanin and anthocyanins were unable to be accurate statistic due to extensive source and the difficulty of data collection, most of the countries merely consumed anthocyanins less than 50 mg per day around the world and proanthocyanin intake was about 100-200 mg per day in Europe, higher anthocyanin intake seem to reduce cardiovascular disease than the lower intake countries [10,11].

As the demand of health increasing, the phenomenon of taking nutraceuticals long-term to reduce the risk of suffering from diseases is become popular around the world [12]. Many nutraceuticals tend to contain natural ingredient, such as herbal extract, amnio acid or fatty acid of animal origin, these components are often regard as safe and uninjurious due to they are derived from nature [13], however the toxicity of long-term using should not be neglected, because these ingredients often have pharmaceutical activity which should be defined as a drug but not food [14], some adverse reaction like hepatotoxicity and nephrotoxicity will occur [15]. Moreover, a considerable proportion of marketed nutraceuticals mix different ingredients to increase potential efficacy, which probably increases the difficulty of safety investigation [16]. For the purpose of providing safety use guideline, it is indispensable to assess the toxicity threshold and the specific damage of nutraceuticals. Therefore, our present study evaluated the long-term toxicity of 2036S-GQR product in Wistar rats. The results of our study will be beneficial to the safe use of 2036S-GQR, and

may provide higher safety reference thresholds for each of the four components in this product.

Materials and methods

Experimental animals

Adult male and female Wistar rats (160-190g, 6-weeks-old) were obtained from the Charles River Laboratory, Beijing, China. Animals were housed two per cage under the following conditions; a specific and constant temperature (20-25°C), humidity (40–70%), and a 10-h light/14-h dark cycle in the animal center. A regular pellet diet and water were provided ad libitum. All rats were acclimatized for 7d before proceeding with the experiments. All studies involving rats were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Shenzhen University Medical School (IACUC, AEW-202300015)

Materials

The 2036S-GQR capsules were acquired from Holistol International Co., Ltd., Hong Kong, China (License No. B071203R), and mainly comprised 60% DHA, 10% EPA, 5% grape seed proanthocyanin extract, and 25% bilberry anthocyanins extract. All raw materials were supplied by Guangzhou Baoxing Bio-Technologies Co., Ltd., Guangdong, China. DHA and EPA are produced from natural fish oil through esterification, washing, dehydration, distillation, purification, filtration, and other processes. The grape seed was extracted using 50–80% alcohol or water, column chromatography was used to collect the eluent, then concentrated and dried to obtain the proanthocyanin extract powder. Fresh bilberry was extracted using 75% alcohol and then filtered to produce the anthocyanin extract. The anthocyanin extract was converted into powder using concentration and dehydration. The four ingredients will be fully mixed to formed oily mixture, and then wrapped in soft capsules.

Experimental management

All animals were divided into four groups, each group contained fifteen male and fifteen female rats and were orally (gavage) administered the abstracted oily mixture in 2036S-GQR capsule. Each group was given doses of 0 (control), 0.6, 1.2, 2.0 g/kg of 2036S-GQR once daily. After continuous treatment for 26 weeks, 2036S-GQR was substituted with the equivalent amount of distilled water in recovery period to explore the reversibility, persistence, and delayed onset of drug toxicity for 4 weeks. Standard food and water were provided ad libitum. The general condition of the rats will be observed twice a day in administration period, 4 hours before and 4 hours after in each administration, while the pre-experimental period and recovery period will be observed once a day. Morbidity and mortality were constantly recorded during the whole research period. All the toxicity studies were conducted

under the guidance of the Organization of Economic Cooperation and Development (OECD) guidelines 407.

Food intake and body weight measurement

Food intake and body weight were measured once a week during the entire research period. Food intake was determined by subtracting the leftover food quantity from the quantity supplied the previous week.

Hematological and biochemical analysis

At 12 and 26 weeks of treatment ten rats (5 animals/sex) and remainder rats at the end of recovery were anesthetized using 4% chloral hydrate (8 ml/kg). Abdominal venous blood sample (2 ml) was collected from each rat and placed into ethylenediaminetetraacetic acid tubes for hematological analyses and dry tubes for biochemical analyses. Hematological parameters included total red blood cell (RBC) count, total leukocyte (WBC) count, hemoglobin (HGB) level, platelet count, lymphocyte ratio, neutrophil ratio, monocyte ratio measured by hematological analyzer (Nihonkohden Corporation, Tokyo, Japan) and prothrombin time (PT). Activated partial thromboplastin time (APTT) was measured using a platelet agglutination and coagulation analyzer (Steellex Corporation, Beijing, China). Serum was collected and stored at 20°C after centrifugation at 2500 rpm for 15 min to test the biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, total protein (TP), blood glucose (GLU), triglyceride (TG), total bilirubin, total cholesterol (T-CHO) and creatinine (CREA). The biochemical parameters were determined using an automatic biochemical analyzer (OLYMPUS Corporation, Tokyo, Japan).

Gross necropsy and organ weights relative to bodyweight measurement

After collecting abdominal venous blood samples, all animals were scarified by bloodletting and perform gross necropsy by an expert who was blinded to the study. Measurements of absolute organ weights of the heart, liver, spleen, lungs, kidneys, adrenal glands, brain, thymus, testis, epididymis, prostate, uterus, and ovaries were carried out immediately after dissection to avert dehydration, then the organ weights relative to bodyweight (the ratio of organ to body weight) was determined.

Pathological examination

The organs and tissues, including the heart, liver, spleen, lungs, kidneys, adrenal glands, brain, cerebellum, brainstem, aorta, stomach, intestine, colon, mesentery lymph node, thymus, thyroid, testis, epididymis, prostate, uterus, and ovaries were visually observed and weighed prior to being fixed in 10% neutral buffered formalin, and being embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E), histopathological changes were observed using a light microscope to detect tissue integrity and injuries (Leica DM3000, Leica Corporation, Frankfurt, Germany).

Statistical analysis

Statistical analysis was performed in SPSS 26 (IBM Corporation, NY, USA). The data of body weight and food intake were expressed as the mean \pm standard deviations (mean \pm SD) and analyzed using one-way analysis of variance (ANOVA). The result of Hematological parameter, biochemical parameter and organ weights relative to bodyweight value were compared using one-way ANOVA followed by Dunnett's t-test. Statistical significance was labeled *P < 0.05, **P < 0.01.

Results

General observation and mortality

No mortality was observed in any of the treatment groups or control group throughout the experimental period and no abnormal changes occurred, whether in behavior or physiological activities, in all dose groups.

Body weight and food consumption

The mean body weight of male and female rats increased steadily in dose groups and control group without any significant difference in mean body weight between the dose groups and control group throughout the experimental period (Fig. 1). Food consumption of the male group did not change significantly while female rats demonstrated a slightly lower level of food consumption when compared to the control group. This difference was, however, not significant and was not seen after withdrawing treatment (Table 1).

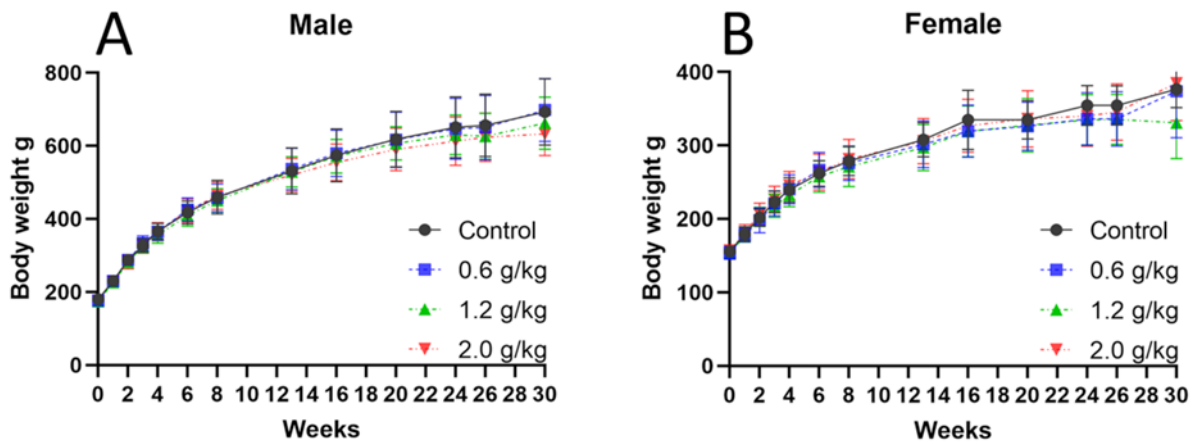


Figure 1: Effects of oral 2036S-GQR on body weight after 26 weeks of 2036S-GQR administration and 4 weeks recovery. (A) Change in body weight of males. (B) Change in body weight of females. Data presented as mean \pm SD.

Sexes	Time (weeks)	Mouse number	Control	0.6 g/kg	1.2 g/kg	2.4 g/kg
Male	1	15	150.15	151.2	148.8	147
	2	15	145.05	147.75	148.2	143.85
	3	15	129	127.05	128.4	128.1
	4	15	117.3	121.05	120.3	118.65
	6	15	101.55	103.95	100.35	100.35
	8	15	96.75	99.6	97.5	96.15
	13	10	48.5	50.7	49.5	49.4
	16	10	44	42.4	43.8	43.3
	20	10	44	43.8	44.4	45.6
	24	10	42.2	41.6	41.3	40.5
	26	10	40.4	39.5	37.6	42.9
30	5	22.7	21.9	23	23.1	
Female	1	15	144.6	136.35	141.6	144.45

	2	15	147.15	144.45	145.2	144.45
	3	15	137.7	135.75	133.35	132.3
	4	15	133.95	131.7	129.3	128.1
	6	15	119.85	117.45	116.85	115.2
	8	15	114.75	108.9	114.9	110.55
	13	10	59.2	55.9	56.6	59.3
	16	10	52.2	46.8	55.3	54.2
	20	10	51.1	48.9	52.8	49
	24	10	51.8	51.4	50.5	50.5
	26	10	50.3	50.7	52.2	50.3
	30	5	27.1	27.85	27.75	29.5

Table 1: Food consumption (g/100 g body weight) after 2036S-GQR administration.

Data represents weekly food consumption of all the rats in each group relative to their body weight. Results of all dose groups were compared to the control.

Hematological parameters

Hematological parameters of control and dose groups are shown in Fig. 2. These were measured at 12 and 26 weeks, and at the end of the recovery in both male and female rats. No significant difference was observed between treatment and control groups.

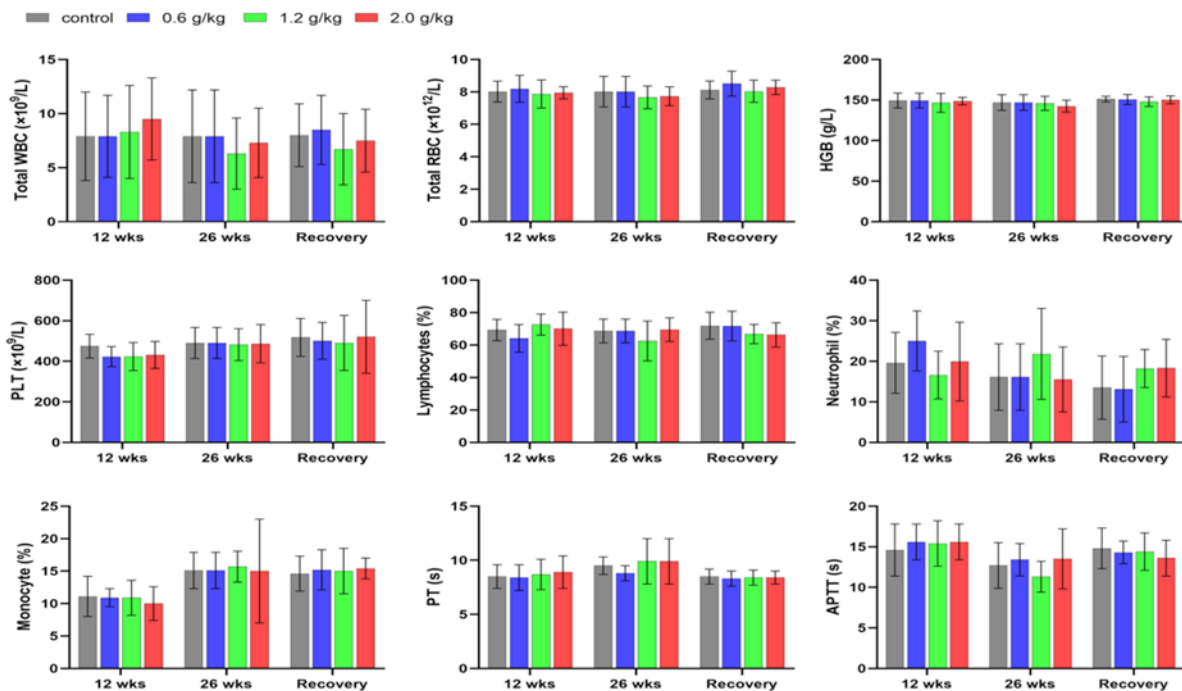


Figure 2: Hematological parameters comparing each index to the control group.

Values are given as mean \pm SD. * $p < 0.05$ and ** $p < 0.01$. ‘Recovery’ represents the end of recovery (30 weeks)

Biochemical parameters

T-CHO levels in the 0.6 g/kg (1.28 ± 0.19 vs. 1.78 ± 0.53 , $p < 0.01$) group, 1.2 g/kg (1.36 ± 0.26 vs. 1.78 ± 0.53 , $p < 0.05$) group and 2.0 g/kg (1.20 ± 0.20 vs. 1.78 ± 0.53 , $p < 0.01$) groups were significantly lower, at 12 weeks, compared with the control group but other parameters showed no significant differences. After 26 weeks of administration, only the 2.0 g/kg group demonstrated a significant decrease in T-Bil (0.1 ± 0.2 vs. 0.7 ± 0.8 , $p < 0.05$) and T-CHO (1.21 ± 0.35 vs. 1.78 ± 0.53 , $p < 0.01$) levels whereas the other parameter was similar to the control group. Urea (6.75 ± 1.60 vs. 5.44 ± 0.57 , $p < 0.05$) levels of the control group tended to increase at recovery period, but no significant differences were observed (Fig. 3). However, the changes in T-CHO, T-Bil and urea levels only appeared in individual serum biochemical indexes and without dose correlation. These indicators were higher than the normal laboratory range but none of these changes were related to any gross findings or pathological examination.

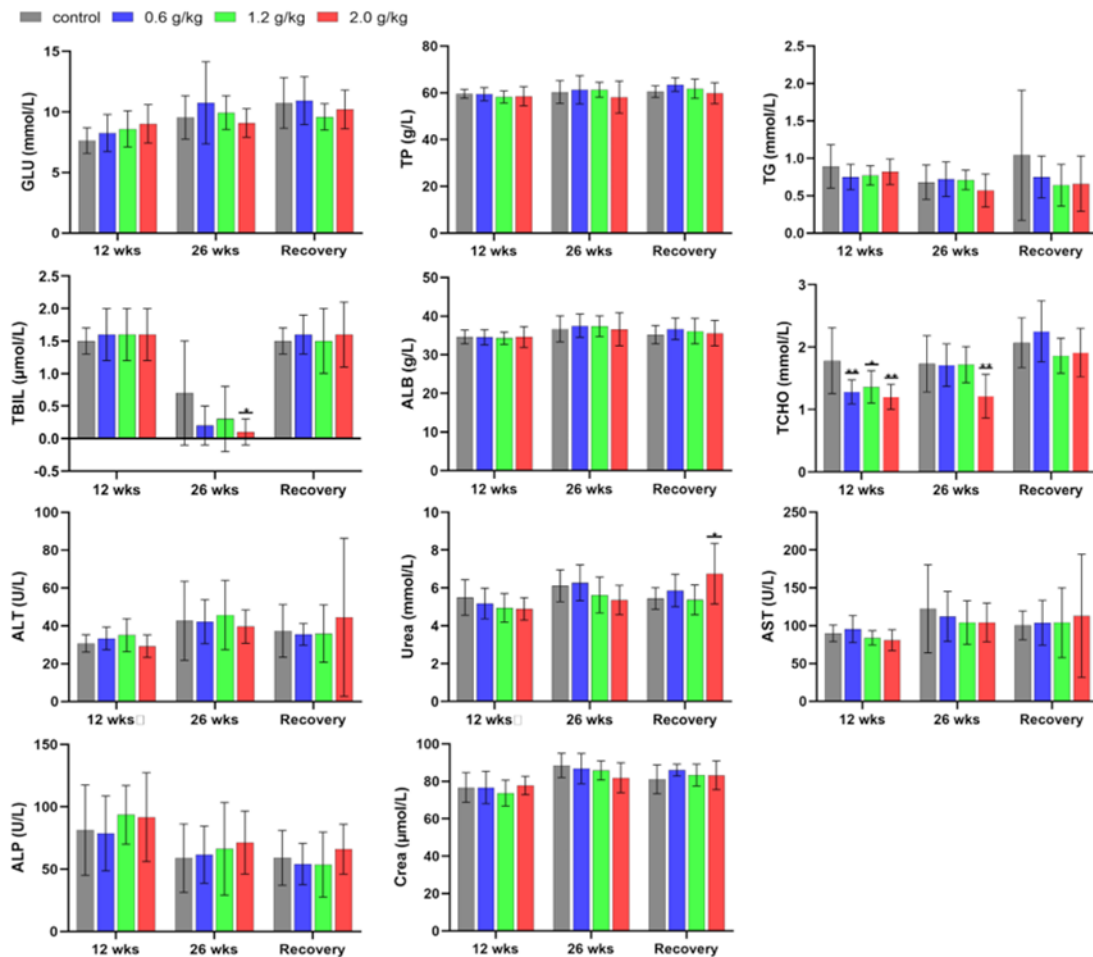


Figure 3: Biochemical parameters of rats after 2036S-GQR administration compared to the control group.

Values are given as mean \pm SD. * $p < 0.05$ and ** $p < 0.01$. ‘Recovery’ means represents the end of recovery (30 weeks)

Gross necropsy and organ weights relative to bodyweight

No lesions or abnormalities were found in gross necropsy of body organs and cavities at 12 and 26 weeks of 2036S-GQR treatment and at recovery period. No significant change in organ weights relative to bodyweight was detected in the treatment or control group (Fig. 4) except the testis. The organ weights relative to bodyweight of the testes showed significant difference in the 1.2 g/kg (left testis 0.32 ± 0.03 vs. 0.38 ± 0.04 , $p < 0.05$; right testis 0.33 ± 0.03 vs. 0.39 ± 0.03 , $p < 0.05$) group and 2.0 g/kg (left testis 0.31 ± 0.04 vs. 0.38 ± 0.04 ,

$p < 0.05$; right testis 0.31 ± 0.03 vs. 0.39 ± 0.03 , $p < 0.01$) group when compared to the control group at 12 weeks, which was considered incidental and showed no correlation in the pathological examination that followed.

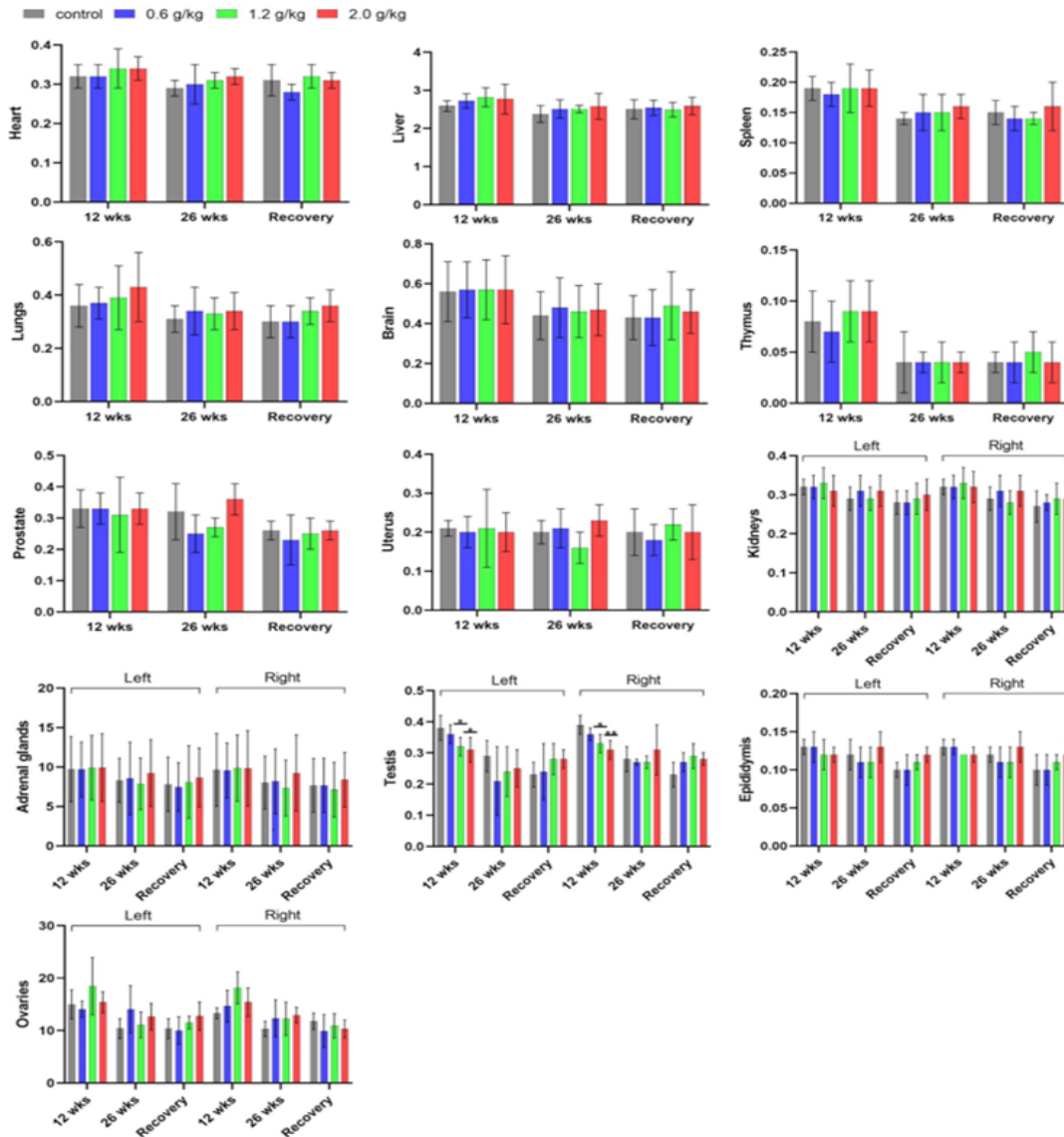


Figure 4: Organ weights relative to bodyweight (g/100 g body weight) of all rats after 2036S-GQR administration.

Values are given as mean \pm SD * $p < 0.05$ and ** $p < 0.01$. ‘Recovery’ represents the end of recovery (30 weeks)

Pathological examination

At 12 and 26 weeks and at the end of recovery, a histopathological examination of the control group and 2.0 g/kg groups was conducted. All 2.0 g/kg groups had no significant pathological change such as degeneration, necrosis and apoptosis when compared to the contemporary control group. These results are displayed in Fig. 5.

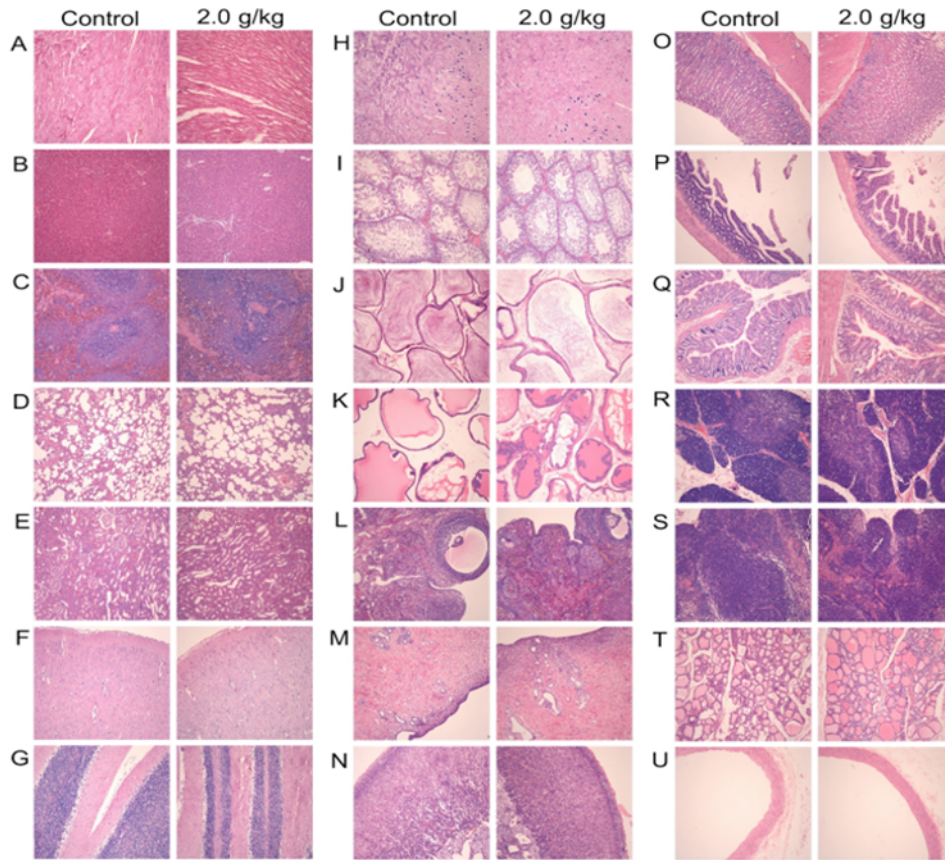


Figure 5: Histopathological organ examination after administering 2036S-GQR for 26 weeks.

All sections used H&E staining (10×). (A) Heart. (B) Liver. (C) Spleen. (D) Lungs. (E) Kidneys. (F) Brain. (G) Cerebellum. (H) Brainstem. (I) Testis. (J) Epididymis. (K) Prostate. (L) Ovaries. (M) Uterus. (N) Adrenal Gland. (O) Stomach. (P) Intestine. (Q) Colon. (R) Thymus. (S) Mesentery lymph node. (T) Thyroid. (U) Aorta.

Discussion

The objective of these studies was to evaluate the safety of 2036S-GQR, a nutraceutical widely uses in China, which contains DHA, EPA, grape seed procyanidins extract, and bilberry anthocyanins extract as main ingredients.

The recommended clinical dose of 2036s-GQR is 40mg/kg per day, equaling to consume DHA+EPA 24 mg, anthocyanins 10 mg, proanthocyanins 1.6mg per day. We set up three different dose group that is 0.6g/kg, 1.2g/kg, 2.0g/kg per day, corresponding fifteen, thirty and fifty time the recommended dose to fully study the safety of 2036s-GQR, the 2.0 g/kg was higher than 2 times the OECD guideline 408 recommended dose for long-term study to explore a wider range of long-term toxic dose since no safety concern about the component of 2036S-GQR.

Through observing the animal exist/death, the change of behaviors that can reflect basic physiology condition to assess the overall security [17], no death and abnormal behaviors change appeared in any dose group. The body weight was also a sensitive index of general healthy status, 20% of weight loss was considered as serious damage according to the OECD guidelines, during the whole study period, the body weight of treatment groups grew steadily and have no significance variation compared with control group. Slightly low food intake appear in female dose group may be ascribe to 2036S-GQR is rich in lipid but have no influence on normal physiological activity. Above all, these four basic indicators proving that 2036s-GQR have no toxicity in general and did not lead to significant change in body weight or food intake.

Blood cell is the most susceptible to toxic molecule, so the alternation of hematological indicator may signify the drug possess toxicity [18]. Our study shows that whether different kinds of blood cell count or some coagulation markers (PT/APTT), have no significant change compared to the control group, revealing that the possibility of causing hematological toxicity is minimal even long-term and high dose of administering 2036S-GQR.

Serological indicators are favorable criterion in clinical practice to reflex the body metabolism and organ function [19]. In our study, comparing to the control group, T-CHO level of all dose group after administering 2036S-GQR emerge significant decline, but after 26 weeks treatment, only 2.0 g/kg group show lower T-CHO, T-Bil level and higher urea level. T-CHO, T-Bil level may hint abnormal metabolism of sterols, impairment of liver and diseases [20,21], while the increase of urea may reflex renal failure [22]. However, these biochemical change is lack of dose relation, situated in the normal laboratory range and the liver did not appear obvious pathological change after pathological examination, the decline in the normal range has no clinicopathological significance [23]. Hence, the change of T-CHO, T-Bil and urea cannot proof 2036-GQR possess the toxicity of long-term and high dose administering.

In organ coefficient examination, we detect a descent of testicular organ coefficient in 1.2g/kg and 2.0g/kg group after administering 2036s-GQR 12 weeks, however, appropriately supplementing DHA is beneficial to spermic development and had no effect on testis relative weight [24]. Organ coefficient was also an important index to evaluate toxic action [25], its calculation was affected by weight increase, its changes can not directly relate to the toxicity, comprehensive analysis should be combined with the pathological observation and other related indicators. But following the result of no significant variation of testis in pathology observation, this change has no reference value. The DHA, EPA, procyanidins, anthocyanins could be available in many cells of body [26,27], thus, expecting primary organ like liver, kidney, spleen and heart, the pathological examination also carried on lungs, brain, cerebellum, brainstem. Testis, epididymis, prostate, ovaries, uterus, adrenal, stomach, intestine, colon, thymus, mesentery lymph node, thyroid, and Aorta. Based on pathological assessment, no abnormal structure was found of the selected organs, confirming the 2036S-GQR had no toxicity under 2.0 g/kg per day.

In pervious study, DHA, EPA and proanthocyanin of separate source has been turn out to be no acute and subacute toxicity [28-30]. Anthocyanin toxicity was less reported, but it was also considered minimally toxic [31]. Based on our current result, 2.0g/kg per day of 2036S-GQR, which also mean consuming 1400 mg/kg DHA+EPA, 500 mg/kg anthocyanins and 100 mg/kg procyanidins per day was safety according to the proportion of ingredients of 2036S-GQR. These results may further prove the

long-term safety of the respective components and increase their toxicity threshold.

In general, we provide comprehensive research and found no adverse reaction under 2.0g/kg per day for 26 weeks in rat, corresponding to 50 times the recommend dose is relatively safe for oral administration.

Conflict of interest: The authors have declared no conflict of interest.

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Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article.

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