Nutritional supplements formulated to prevent cognitive impairment in animals

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**ABSTRACT**

Heat stress will cause a series of response in the living system and the most significant impact is on brain functions. The aim of this article is to develop nutritional supplements that can alleviate cognitive decline caused by heat stress. In this article, we screen functional food factors which can prevent or relieve effects on heat stress injury based on bioinformatics. 129 function factors related to the crossover targets were obtained, and a food database related to the prevention of high-temperature impairment was constructed. After a series of scoring standards combined with food classification, two formulas—nutrition fortifier formula (tyrosine and multivitamin B) and plant compound formula (quercetin, proanthocyanidin, and naringin) were investigated using animal experiments to determine their ability to prevent cognitive impairment of heat-stressed animals. Our results demonstrated that certain functional food factors and our two designed formulations significantly prevent cognitive impairment of heat-stressed animals.

Further mechanism was carried out by cell viability assay, reactive oxygen species assay, real-time quantitative PCR and Western blot. The results showed that the plant compound formula diluted 4000 times had the best relieving effect on HT22 after heat stress, and this concentration formula can significantly alleviate the elevated levels of reactive oxygen species caused by heat stress. This formula also can significantly down-regulate IL-1β, IL-6, TNF-α, IL-10, iNOS and COX-2 expression. Likewise, Western blot results showed that the formula could activate the cAMP pathway and increase the expression of phosphorylated PKA and BDNF in hippocampal cells.

**1. Introduction**

A high-temperature environment can be a typical extreme working environment for many individuals. When the ambient temperature exceeds the body’s ability to regulate itself, a series of immune responses are triggered, which we call heat stress. When exposed to heat stress for a long time, the body’s immunity decreases, which leads to the release of inflammatory mediators, causing acute inflammation and inducing the secretion of reactive oxygen species by leukocytes, followed by various pathophysiological responses, such as thermoregulation dysfunction and water and electrolyte balance disorders. The most significant impact is on brain functions, including cognition, memory, judgment, emotion, and consciousness (Hocking et al., 2001; Mazlomi et al., 2017; Pilcher et al., 2002). For example, when a person is exposed to high-temperature, their body will show impaired cognitive abilities, including perceptual discrimination, short-term memory, and central executive tasks (Cian et al., 2001; McMorris et al., 2006). In animal models, heat stress has been found to reduce acquisition speed and cause poor retention of memory tasks. Several studies have also demonstrated that heat stress profoundly impacts brain structure and function, and high-temperature environments can also cause the hippocampus to shrink (Porcelli et al., 2008; Carmen Sandi, 2004), causing hippocampus-dependent memory impairment (Jeansok J Kim and David M Diamond, 2002). Additionally, heat stress can lead to changes in neural circuits (Kim et al., 2013), loss of neurons (Rakesh Kumar Sinha, 2007; White et al., 2003), neurological deficits (Xiao et al., 2007; Yang YL and Lin MT, 1999), and accelerated brain dysfunction (Abderrezak Bouchama and James P Knochel, 2002). Furthermore, stress can

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increase inflammatory responses (Yeager et al., 2009; Cohen et al., 2012), and can also increase the circulating levels of inflammatory cytokines in the blood, such as interleukin (IL-6) and tumor necrosis factor (TNF)-α (Starkie et al., 2005). Consequently, there is evidence that systemic and central inflammation is directly related to cognitive decline (Pickering et al., 2005; Trollor et al., 2012; Wilson et al., 2002), and the impact of heat stress may be promoting these detrimental responses. In relation to these findings, Wonil Lee (Lee et al., 2015) found that heat stress can activate glial cells and induce inflammatory molecules in the hippocampus, which may cause memory loss, neuronal death, and impaired neurogenesis in adults.

The Chinese Nutrition Association report pointed out that high-temperature nutrition research should focus on functional foods such as vitamins, amino acids and phytochemicals, and medicinal and homologous food substances to prevent thermal environment-related diseases. Functional foods are a part of the daily diet. They are natural and have little or no toxic effects, so they have broad application prospects. Ingested functional foods play an essential role in preventing and treating some diseases due to the natural phytochemicals (functional factors) that play a crucial role. Therefore, some food compounds can be used as medicines and are under investigation for these beneficial effects. Many phytochemicals have been demonstrated to have beneficial effects for disease prevention and treatment of high-temperature environments. Functional factors such as tyrosine, glutamic acid, arginine can significantly reduce the body’s nitrogen loss under heat stress and improve the body’s thermal adaptability. mineral elements (mainly sodium, potassium, chromium, and selenium) can generally enhance the body heat stress ability. Likewise, natural phytochemicals can improve the body’s resistance to heat stress, especially some Chinese herbal medicines, such as ephedra, vitex, citrus, American ginseng, nanshan vine (Muthu et al., 2006), coptis (Moon et al., 2017), and andrographolide (Kim et al., 2014). In some animal experiments, it has been demonstrated that natural extracts such as magnolol and ginsenoside Rb1 (Liu et al., 2021) have a significant alleviating effect on heatstroke mediated damage. Therefore, from these findings resulting based on the targets of phytochemicals’ mechanism of action, we hypothesize dietary intervention can also provide evidence for improving certain disorders and stress.

Bioinformatics is widely used in the field of Chinese medicine to determine the relationship between certain components of Chinese medicine and potential benefits for disease. Based on these methods and investigations, we applied this method to the field of functional foods. Compared with the typical application of isolated studies involving a few functional factors) that play a crucial role. Therefore, some food compounds can be used as medicines and are under investigation for these beneficial effects. Many phytochemicals have been demonstrated to have beneficial effects for disease prevention and treatment of high-temperature environments. Functional factors such as tyrosine, glutamic acid, arginine can significantly reduce the body’s nitrogen loss under heat stress and improve the body’s thermal adaptability. mineral elements (mainly sodium, potassium, chromium, and selenium) can generally enhance the body heat stress ability. Likewise, natural phytochemicals can improve the body’s resistance to heat stress, especially some Chinese herbal medicines, such as ephedra, vitex, citrus, American ginseng, nanshan vine (Muthu et al., 2006), coptis (Moon et al., 2017), and andrographolide (Kim et al., 2014). In some animal experiments, it has been demonstrated that natural extracts such as magnolol and ginsenoside Rb1 (Liu et al., 2021) have a significant alleviating effect on heatstroke mediated damage. Therefore, from these findings resulting based on the targets of phytochemicals’ mechanism of action, we hypothesize dietary intervention can also provide evidence for improving certain disorders and stress.

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2. Methods and materials

2.1. Screening of potential food functional factors

Search the key words related to nerve damage caused by heat stress through GeneCards database (https://www.genecards.org/) and import the target into the UniProt website to correct the official name. Then aggregate, merge and screen these targets to complete the construction of the protein interaction network with String database (https://stridergdb.org/cgi/input.pl?sessionId=diDi0032&Rt0KInput_page_show_search=on) and Cytoscape 3.6.0 software. Based on this results, the score calculation of protein interaction is carried out. At the same time, GO (geneontology) and KEGG (Kyoto encyclopedia of genes and genomes) enrichment analysis are performed on the targets. The R software bioconductor package was used to convert key target names into IDs. The transformed IDs were then subjected to GO enrichment and KEGG enrichment analysis on the metascape online database. The filter conditions were P value < 0.05, Q value < 0.05. On the basis of this analysis, Drugbank (https://www.drugbank.ca/) and PubChem (http://pubchem.ncbi.nlm.nih.gov/) and Funfood (www.foodies.ac.cn) are further used to screen the known targets for compound matching. Screening conditions include target matching, oral availability (OB), intestinal system permeability (Caco-2), drug-like properties (DL) and blood-brain barrier permeability (BBB). The screened compounds are classified according to the compound structure, and the count of targets involved and the pathways of action. On the basis of screening, the compound composition, target, physical properties, bioavailability, and sensory characteristics in the food are integrated to select the nutrients for the formulation of nutritional supplements.

2.2. Reagents and instruments

Nutrients including L-tyrosine (CAS No.: 60-18-4), Vitamin B1 (CAS No.: 67-03-8), Vitamin B2 (CAS No.: 83-88-5), Vitamin B3 (CAS No.: 137-08-6), Vitamin B6 (CAS No.: 58-56-0), Vitamin B12 (CAS No.: 68-19-9), Quercetin (CAS No.: 117-39-5), Resveratrol (CAS No.: 501-36-0), Tea Polyphenols (CAS No.: 84650-60-2), Genistein (CAS No.: 446-72-0), Baicalein (CAS No.: 491-67-8), Luteolin (CAS No.: 491-70-3), Pyrrolo- cyanidin (CAS No.: 4852-22-6) and Naringin (CAS No.: 10236-47-2) are all purchased from Yuanye Biotechnology (Shanghai, China); CCK8 kit and ROS kit are purchased from Beyotime Biotechnology (Shanghai, China).

Instruments include Artificial climate box (ZRQ-400); Morris water maze (JLBehav-MWMG); Shuttle box (JLBehav-STG-4); Fluorescent dye 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, USA).

2.3. Animals and cells

The experimental animals were male 8-week-old C57BL/6 mice (Shanghai Jihui Experimental Animal Breeding Co., Ltd.), weighing 22–26 g. Six mice were kept in the standard-sized cages (size: 40 cm length, 25 cm width, and 18 cm height) for 5 days to adapt to the environment. They were free access to common rat chow and purified water. The rearing environment temperature is 23 ± 1 °C, the relative humidity is 60 ± 10% and the day-night cycle is 12 h. All experiments were performed in compliance with the Naval Medical University’s policy on animal use and ethics.

HT22 mouse hippocampal neurons were purchased from Shanghai Fuheng Technology Co., Ltd. (Shanghai, China) and cultured according to the provided guidelines (high glucose DMEM + 10% FBS, cultured at 37 °C and under 5% CO2).

2.4. Heat stress exposure and drug administration

2.4.1. Heat stress exposure and drug administration of animals

After the end of the adaptation period, the group entered the 14-days gavage stage. Animal were randomly divided into 12 groups with 6 mice in each group: Control, Heat stress, L-Tyrosine (300 mg/kg/day) (Lieberman et al., 2005), Quercetin (160 mg/kg/day) (Sullivan et al., 1951), Resveratrol (50 mg/kg/day) (Thomas et al., 2013; Gueguen et al., 2015), Tea Polyphenols (250 mg/kg/day), Genistein (230 mg/kg/day), Baicalein (300 mg/kg/day) (Rui et al., 2020), Luteolin (285 mg/kg/day) (Li et al., 2015), Pyrrolo-cyanidin (350 mg/kg/day) (Ray et al., 2001), Naringin (100 mg/kg/day) (Liu et al., 2016) and Multivitamin B (VB1:5.5 mg/kg/day; VB2:5.5 mg/kg/day; VB5:5.5 mg/kg/day; VB6:13.75 mg/kg/day; VB12:9.25 mg/kg/day). The oral dose of each kind of nutrition except Multivitamin B is calculated according to the Mecho formula, body surface area (mice/adult males) = 0.00253, so the equivalent doses (mice: adult males) = 0.00253 × (70/0.02): 1 ≈ 8.861.
The number 70 represents the weight of a 70 kg adult male and the number 0.02 represents the weight of a 20 g mouse. The oral intake of Multivitamin B is calculated based on 30 times except vitamin B₉ is calculated based on 20 times of the recommended intake for a 70 kg adult male.

The nutritional supplements are assembled on the basis of the determined concentration of the monomers. Formula of Plant Compounds(F1) is composed of Quercetin, Proanthocyanidin and Naringin. Formula of Nutritional fortifier (F2) is composed of L-Tyrosine and Multivitamin B. The gavage concentration of each nutrient is the same as above.

Each mouse in the control group and the heat stress group was gavaged with 0.2 ml of 0.9% NaCl daily. The other groups were given different nutrient dissolved in 0.9% NaCl. After seven days, heat exposure and behavioral experiments were added simultaneously with gavage. For heat exposure, animals were transferred from their home cage into an artificial climate box maintained at 42 ± 0.5 °C and 60 ± 10% humidity and exposed to heat stress for 1 h. To avoid the influence of diurnal cycling, mice were exposed to heat at approximately the same time between 8:30 a.m. and 10:30 a.m. each day.

2.4.2. Heat stress exposure and drug administration of cells

HT22 mouse hippocampal neurons were inoculated in 6-well plates (cell seeding densities: 3 × 10⁵) or 96-well plates (cell seeding densities: 5 × 10⁴). After 24 h of adherent growth, media were replaced with fresh media according to their respective treatments. Cells in control group and heat stress were cultured with high glucose DMEM (10% FBS). The formula of plant compounds used for intragastric administration of mice was diluted with the culture medium of the control group to required concentrations for cell intervention in different test. The thermal exposure method involved placing an orifice plate in an environment of 43 °C for 80 min and was carried out after 20 h of cell growth and medium replacement.

2.5. Behavioral tests

Animals were subjected to behavioral tests to assess cognitive performance simultaneously with heat stress exposure. The water maze positioning and navigation experiment was performed on days 1–6, and the water maze space exploration experiment was performed on the 7th day. In addition, the nutritional supplement group was subjected to the shuttle avoidance experiment on days 6–7.

2.5.1. Morris water maze test

The Morris water maze system consisted of a circular tank that was divided into four quadrants. The tank was filled with opaque water, and its level was at least 1 cm above the upper surface of the platform. The mice were trained in different quadrants for 6 days. Each mouse was given 1 min to find their way to the platform. If the mouse could not locate the platform within 1 min, the mouse would be gently guided to the platform for 10 s. After 6 days of training, the platform was removed. The ratio of the time and the distance near the original platform to the total time was recorded on the 7th day. The video tracking device also recorded the number of across times of the removed platform (Charles V, Vorhees and Michael T, Williams, 2006; Mark D Whiting and Olga N, Kokiko-Coehran, 2016; Daniel N Barry and Sean Commins, 2019).

2.5.2. Shuttle test

The shuttle box system consists of an opaque box divided into two parts, including a video acquisition and processing system. The testing lasted for two days, which were conducted on the 6th day of heat exposure training and the 7th day of the heat exposure test. The mice were allowed 5 min of free activities in the box, then a red light and buzzer stimulation was given for 15 s, and electrical stimulation was given within 5 s. If the mice fled to the safe box before the shock, this was recorded as an active escape response. If they fled to the safe box after the shock, this was recorded as a passive escape response. After escaping to the safe box, we stopped stimulating the mice and allowed them to enter the rest period of 5 s, after which the next cycle began. The number of cycles was set at 20 in the training phase and 30 in the test phase. The test phase was carried out 24 h after the end of the last training.

2.6. Evaluate formula 1 with hippocampal cells

2.6.1. Assays of cell viability and reactive oxygen species

Formula intervention was performed on control cells and heat-stressed cells, respectively. Undiluted formula 1 and dilute formula 1 (500 times, 1000 times, 2000 times, 4000 times and 8000 times) were used to intervene for 20 h. After nutritional formula intervention, the cells were exposed at 43 °C for 80 min and recovery at 37 °C for 1 h. The control group was placed in a 37 °C incubator waited for the next step to be performed simultaneously with the heat-treated cells. Reactive oxygen species (ROS) and cell viability assays of HT22 were performed according to the manufacturer’s protocol of Cell Counting Kit-8(CCK8) and ROS Assay Kit (Beyotime, Shanghai, China). Briefly, cells were treated with reagents to detect the fluorescence intensity or absorbance.

2.6.2. Real-time quantitative PCR

Total RNA from HT22 cells was extracted and reverse transcribed according to the manufacturer’s protocol of RNAiso Plus (Takara, Japan) and reverse transcription kit (Takara, Japan). Primer design was performed on primer-Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PR OGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and the primer sequences are shown in Table 1 β-Actin mRNA was used as an internal reference for normalization. qPCR was performed using SYBR Green Master Mix (with ROX) dye (Bimake, USA).

2.6.3. Western blot analysis

The HT22 cell lysates were separated by 10%, 12.5%, or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an NC membrane, and incubated in a blocking solution (Epi- zyme, Shanghai, China) for 15 min. Following blocking, primary antibodies (BDNF, PKA (phospho S99); Abcam, UK) were incubated with the membrane at 4 °C overnight. The next day, an infrared fluorescent dye-labeled secondary antibody (LICOR, USA) was incubated for 1 h and visualized using a two-color infrared fluorescence imaginar system (LICOR, USA). The band strength was normalized to β-actin or GAPDH protein band strength.

2.7. Statistical analyses

All data were statistically analyzed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Data were presented as mean ± standard deviation (SD) of at least three independent trials. All data were tested for normality and homogeneity of variance. Repeated measurement ANOVA was used for the data from the orientation navigation test. LSD-T test was used to compare between monomer groups and Dunnett’s multiple comparison method was used to compare between formulation groups.

3. Results

3.1. Obtaining of potential food functional factors

To identify functional food factors and genes that may be related to heat stress for our investigations, we searched keywords related to nerve damage caused by heat stress such as “Heat shock”, “Amnesia”, “Anxiety” through the database and scored the result of targets related to high temperature and cognition. We performed target interactive analysis and cross-comparison of different samples. Based on GeneCards database search, 253 targets related to amnesia (Amnesia), 4055 targets related to anxiety (Anxiety), and 4815 targets related to
heat stress (Heat stress) were identified. All targets were interactively analyzed, and targets of different samples were cross-referenced. Finally 103 co-expressed genes were determined (Fig. 1A). All target sources are human.

Taking the protein interaction relationship in the String database as the background, the plain text relationship and unconnected targets were screened and eliminated. There are 93 nodes, and 2213 edges were included in the network (Fig. 1B). The statistical significance of the number of connections between different proteins is shown in Fig. 1C. These results only show the protein whose connection number contains 10 and above. The top ten key targets APP, INS, KNG1, ALB, POMC, SST, BDNF, IL-6, MAPK1, and FOS. These ten proteins have the most interactions and the numbers were 40, 34, 29, 22, 22, 22, 21, 21, 21, 20.

Based on the targets obtained by PPI network analysis, routine GO enrichment analysis was performed using the R software bioconductor package. The results showed that these targets were enriched to obtain signal receptor activator activity, receptor ligand activity, hormone activity and other functions in brain (Fig. 1D). In order to determine more detailed information, the metascape online database was used to conduct a classification of GO enrichment analysis, including cellular component (CC), molecular function (MF), and biological process (BP) (Fig. 1E). KEGG analysis can show the pathway of the target through its upstream and downstream analysis. In this study, 46 pathways were enriched and the results of the top 20 ranked by correlation are shown in Fig. 1F. It mainly includes Neuroactive ligand-receptor interaction, cAMP signaling pathway, serotonergic synapse, Alzheimer’s disease and Leishmaniasis. The amplified version of Fig.(1B,1D,1E,1F) are in the appendix.

Following identification, we then matched the screened targets with compounds through the Drugbank, Pubchem, and Funfood databases. Subsequently, we ranked the targets according to importance in the protein PPI network. If equally important, the targets were then sorted

| Gene   | Forward          | Reverse          |
|--------|------------------|------------------|
| TNF-α  | ATGTCTCAGCCTCTTCTCATTC | GCTTGTCACTGGAATTTTGAGA |
| IL-1β  | CACTACAGGCTCGAGAATGAAACAC | TGTGTGCTGGTGTCCTCCTTGAC |
| IL-10  | TCTCTTTCAAAACAAAGGACCCGC | GCAAACCAAGTAACCTTAAAG |
| IL-6   | TGGCAAGACCTGGCTATAC | CCATTGCAAACCTCTGTTCAG |
| INOS   | GGAGATGGCTGCGAAGAGAG | GTCCCAGAGTAGACCTTGAGG |
| COX-2  | CAGGCTGAACTCTGAAACA | GTCGAAGGGCCACTGATACCCTA |

Fig. 1. Bioinformatics of the target protein analysis (A) Intersection display of target interaction (B) PPI protein network construction (C) Barplot of target protein (D) Bubble chart of biological process entry for GO enrichment analysis (E) Cellular component (CC), molecular function (MF) and biological process (BP) of GO enrichment analysis (F) KEGG pathway enrichment histogram.
by quantity. We obtained 129 food function factors and the results of screening compounds are ranked according to the number of intervention targets. The top ten results are: Vitamin A, Vitamin B₁, Vitamin B₂, L-Tyrosine, Caffeine, Capsaicin, Vitamin B₆, Taurine, Resveratrol and Quercetin (Table 2). These compounds’ quantity of intervention targets is more than 30, showing that these are related to multiple signaling pathways.

Taking into account their classification, oral administration, literature reports, familiarity in life and other factors, we individually selected 14 species from 129 food function factors and carried out animal behavior experiments to verify the effect. These selected factors are L-Tyrosine, Vitamin B₁, Vitamin B₂, Vitamin B₆, Vitamin B₁₂, and Vitamin B₉; Quercetin, Naringin, Genistein, Baicalein and Luteolin, Resveratrol, Tea polyphenol and Proanthocyanidins. Considering that Vitamin B₁, Vitamin B₂, Vitamin B₆, Vitamin B₉ and Vitamin B₁₂ are all Vitamin B compounds, we mixed them to test the comprehensive effect of multiple vitamin B(MVB) in further animal experiments.

3.2. Behavior test

3.2.1. Potential food functional factors

The orientation navigation test can detect mice’s learning and memory acquisition ability. With the progress of training, the time required for boarding the platform (latency period) will gradually decrease because mice gradually acquire the memory of platform position through learning during training. The results showed that the latency time of mice in all groups decreased with the increase of training days. Compared with the control group, the latency time of the mice in the heat stress group was significantly longer (P < 0.01), which indicated that the learning and memory ability of the mice was impaired. After the intervention of food functional factors in other nutrient groups, the incubation period was shortened, and the naringin group showed the most obvious performance (P < 0.01) (Fig. 2A). The ratio between day 1 and day 6 of latency period is shown in Fig. 2B for clearer display. The smaller the ratio, the better the learning and memory effect of mice. The L-tyrosine group also showed significant differences (P < 0.01) except for naringin and the control group.

In the space probe test, the platform was removed. Mice would keep swimming around the platform and repeatedly crossing the original position to try to board the platform and the behavior of mice can reflect spatial memory ability. The results showed that compared with the control group, the mice in the heat stress group spent less time (Fig. 2C), less distance (Fig. 2D) swimming in the quadrant of the platform and less frequently (Fig. 2E) crossed the platform. Compared with the heat stress group, tyrosine, multivitamin B, quercetin, baicalein, proanthocyanidin and naringin group increased the percentage of time swimming (Fig. 2C) and the percentage of distance (Fig. 2D) in the quadrant where the platform was located; The number of platform crossings was increased in mice in the tyrosine, B multivitamin, quercetin, resveratrol, baicalein, proanthocyanidin, and naringin groups (Fig. 2E).

Although some nutrient differences were not significant, the results of the water maze experiment showed that the 10 nutrients that were screened improved the cognitive decline of mice in a heat-stress environment.

3.2.2. Formula 1: Formula of plant compounds

We selected three plant compounds, naringin, quercetin, and proanthocyanidin to formulate a nutritional supplement according to our findings of nutrients’ effect on learning and memory in heat-stress-exposed rats. The water maze navigation results showed that the time for the mice to board the platform gradually shortened with an increase in training days (Fig. 3A). On the last day of the experiment, our findings demonstrated that the naringin and formula group were better than the control group (P < 0.01). The ratio between day 1 and day 6 of latency period is shown in Fig. 3B for clearer display. The smaller the ratio, the better the learning and memory effect of mice.

The water maze space exploration experiment showed that the time (Fig. 3C), the distance (Fig. 3D) spent in the quadrant of the original platform, and the number of times (Fig. 3E) crossing the platform position of the mice in the high-temperature treatment group were significantly less than those in the control group. These three indicators were significantly improved after the nutritional intervention. Except for the time (Fig. 3C) and the distance (Fig. 3D) spent of naringin, the overall performance of the nutritional supplement group was better than that of the single nutrient group.

In the shuttle experiment, the mice learned the relationship between sound and light and electric shock through training, so as to judge the timing of the shuttle, and still keep the memory of this in the final experiment, so the shuttle experiment can reflect the learning, memory and decision-making ability of the mice. In the shuttle experiment, the heat-stressed mice compared with the control group actively shuttled during the acousto-optic stimulation phase to avoid electric shocks less frequently and the nutritional intervention group took more active escapes than the heat stress group (Fig. 3F). Notably, the proanthocyanidin group had the best effect (P < 0.01), followed by the nutritional supplement group (P < 0.01).

### Table 2
Top 20 potential food function factors.

| Compound Name | Total number of targets | Molecular Weight | OB (%) | Caco-2 | BBB | DL |
|---------------|-------------------------|------------------|--------|--------|-----|----|
| Vitamin A     | 85                      | 286.50           | 19.53  | 1.38   | 0.86 | 0.16 |
| Vitamin B₁    | 74                      | 265.35           | 20.54  | 0.8867 | 0.9333 |    |
| Vitamin B₂    | 69                      | 376.36           | 34.21  | 0.7122 | 0.8495 |    |
| L-Tyrosine    | 65                      | 181.18           |        |        |      |    |
| Caffeine      | 54                      | 194.22           | 89.46  | 0.58   | –0.01 | 0.08 |
| Capsaicin     | 35                      | 305.46           | 10.31  | 0.93   | 0.43  | 0.20 |
| Vitamin B₆    | 34                      | 231.14           | 45.63  | 0.8958 | 0.6889 |    |
| Taurine       | 33                      | 125.17           | 24.37  | –0.78  | –2.75 | 0.01 |
| Resveratrol   | 32                      | 228.26           | 19.07  | 0.80   | –0.01 | 0.11 |
| Quercetin     | 32                      | 304.27           | 66.44  | –0.34  | –1.11 | 0.27 |
| Vitamin B₁₂  | 31                      | 1355.56          | 80.74  | 0.6455 | 0.7477 |    |
| Genistein     | 28                      | 270.25           | 17.93  | 0.43   | –0.40 | 0.21 |
| Vitamin B₆    | 24                      | 219.24           | 61.8   | 0.6887 | 0.7005 |    |
| Linolenic acid| 23                      | 278.48           | 45.01  | 1.21   | 0.84  | 0.15 |
| Beta-carotene | 18                      | 536.96           | 37.18  | 2.25   | 1.52  | 0.58 |
| Luteolin      | 16                      | 286.25           | 36.16  | 0.19   | –0.84 | 0.25 |
| Tea polyphenol| 16                      | 458.40           | 55.09  | –0.57  | –1.70 | 0.77 |
| Baicalein     | 13                      | 270.25           | 33.52  | 0.63   | –0.05 | 0.21 |
| Piperine      | 13                      | 285.37           | 42.52  | 1.12   | 0.62  | 0.23 |
| Hmo           | 10                      | 268.28           | 38.37  | 0.79   | 0.25  | 0.21 |
3.2.3. Formula 2: Formula of nutritional fortifier

According to our findings of different nutrients’ effect on learning and memory in heat stress-exposed rats, we selected the tyrosine and multivitamin B to formulate the nutrition supplement. The water maze navigation results showed that after six consecutive days of heat stress intervention, the time for the mice to board the platform gradually shortened with the increase of training days. Compared with the control group, the latency of the heat stress group was significantly increased ($P < 0.05$). The last day results showed that the nutritional supplement group performed much better than the heat-stress group ($P < 0.05$) and the single nutrition group. (Fig. 4A). The ratio between day 1 and day 6 of latency period is shown in Fig. 4B for clearer display. The smaller the ratio, the better the learning and memory effect of mice.

The results of water maze space exploration showed the time (Fig. 4C), the distance (Fig. 4D) spent in the quadrant of the original platform, and the number of times (Fig. 4E) crossing the platform position of the mice in the high-temperature treatment group were significantly less than those in the control group. The tyrosine and multivitamin B and the formula 2 performed better than the heat stress group in all three indicators, and formula 2 group was the best ($P < 0.001$).

The index of the number of active escapes in the shuttle experiment (Fig. 4F) showed that the performance of the heat-stress group was significantly less than the control group ($P < 0.001$). Compared with the heat stress group, the formula 2 group was found to have a significant improvement effect ($P < 0.05$).

3.3. Assessment of formula 1 (F1) with hippocampal cells

3.3.1. Cell viability and reactive oxygen species assessment

Based on the verification result of the animal experiments, we
diluted formula 1 to verify the activity of the hippocampal cells (Fig. 5). Without heat stress, the result shows gavage concentration was too high for the cells and the cell activity was 0. After diluting 500 times and 1000 times, cell activity dropped to 16.5% and 72.8% of control group. Dilution of the formula by more than 2000 times is nontoxic to cells and the cell viability was even stronger than the control group. The cell viability increases with the decrease of the concentration. The cell viability significantly dropped to 34.5% of control group after heat stress. On this basis, the cell viability was only 2.3% with the formula diluted 500 times. It is shown that the intervention of formula which diluted by more than 2000 times can largely offset the effect of heat stress on cell activity. Among them, the 4000 times dilution has the best effect, which cell viability is more than twice of heat stress group.

3.3.2. Real-time quantitative PCR

The results showed that the mRNA expressions of iNOS, COX-2, IL-1β, IL-6, TNF-α and IL-10 were significantly increased after heat stress, and IL-6 was the most obvious. After the intervention of formula 1, the results showed that the expression of the above genes caused by heat stress could be significantly inhibited, and the ratios of formula group and heat stress group were 25.3% (Fig. 7A), 65.3% (Figs. 7B), 28.2% (Fig. 7C), 20.2% (Figs. 7D), 33.4% (Fig. 7E), 22.6% (Fig. 7F).

3.3.3. Western blot analysis

To investigate the mechanisms of formula 1 to prevent cognitive impairment in high temperature, we measured phospho-PKA and BDNF expressions in the HT22. Compared with the control group, the contents of phospho-PKA and BDNF were significantly decreased in the heat stress group, and the contents of these proteins in the Formula group were significantly increased compared with the heat stress group (Fig. 8).
Fig. 4. Effect of Formula of Nutritional fortifier (F2) on learning and memory in heat stress-exposed rats. (A) Latency time (B) The change of day 1 and 6 of the latency time (C) Platform quadrant time (D) Platform quadrant distance (E) Number of across the platform of water maze; (F) Number of active avoidances of shuttle experiment; Each bar represents the mean ± SD (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. Heat Stress group; Statistical description: Dunnett’s multiple comparisons was used in Fig. 4 A, C, D, E, F, Uncorrected Fisher’s LSD test was used in Fig. 4B.

Fig. 5. Effects of nutritional supplements on cell activity after heat stress, #P < 0.05, ##P < 0.01, ###P < 0.01 vs. Control group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. Heat stress group; Statistical description: Uncorrected Fisher’s LSD was used in Fig. 5.
4. Discussion

This study aims not to treat diseases but to help the brain optimally adapt to a thermal environment before the potential onset of disease. This study will provide creative solutions for developing nutritional supplements for high-temperature workers and even special nutritional groups. We screened 103 targets related to heat stress and brain injury and 129 potentially beneficial food function factors using bioinformatics. Food functional factors such as naringin, quercetin, proanthocyanidin, multivitamin B, and tyrosine were screened and identified for beneficial effects verified by animal behavioral experiments. We made two efficient nutritional formulas to improve learning and memory ability in high-temperature environments from these findings.

The human nervous system is susceptible to high-temperature environments compared with other systems. This leads to a decline in learning and memory ability and decreased work efficiency in environments that may have higher temperatures. However, when we searched the literature for compounds that can benefit mental

![Figure 6](image1.png)

**Fig. 6.** ROS assessment of hippocampal cells after heat stress *P < 0.05, ***P < 0.001 vs. Heat Stress group; Statistical description: Uncorrected Fisher’s LSD was used in Fig. 6.

![Figure 7](image2.png)

**Fig. 7.** The effect of formula 1 with hippocampal cells on iNOS, COX-2, IL-1β, IL-6, TNF-α and IL-10 mRNA expression by heat stress. Each bar represents the mean ± SD (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. Heat stress group; Statistical description: Dunnett’s multiple comparisons test was used in Fig. 7. expression of heat stress (ΔΔct relative quantitative), *P < 0.05, **P < 0.01, ***P < 0.001 vs heat stress group.
performance under high-temperature conditions, we found that there are few pieces of literature on improving mental performance skills such as cognition, memory, judgment, emotion, and consciousness. The lack of literature in this field demonstrates that this research field is limited. With very few relevant studies, we mainly identified studies that focused on basic research, and few focused on discovering new functional nutrients. In this study, we directly identified 129 potentially effective food function factors which can prevent cognitive impairment in a high-temperature environment by a disease-target-compound matching using bioinformatics. Having identified 129 potential factors, these findings may benefit added research to provide sufficient reference and evidence for related fields.

Fig. 8. Influence of formula 1 on the cAMP pathway. (A–B) The effect of Formula 1 on the expression of phospho-PKA in HT22. (C–D) The effect of Formula 1 on the expression of BDNF in HT22. Values are expressed as means ± SD. *P < 0.05 as compared with the heat-stress group; Statistical description: Dunnett’s multiple comparisons test was used in Fig. 8.

Considering the solubility, operability, food taste, animal behavioral experiments were utilized to further screen and validate the top-ranked related food function factors. Based on the classification of the chemical structure and the practical application, We selected factors that are Naringin (terpenoids), L-tyrosine (amino acid), Vitamin B1, Vitamin B2, Vitamin B5, Vitamin B6, and Vitamin B12, Quercetin (flavonoids), Genistein, Proanthocyanidins, Baicalein and Luteolin (flavonoids), Resveratrol (non-flavonoid polyphenols), Tea polyphenol. We don’t choose Vitamin A because of its solubility. Although capsaicin, piperine and some other substance were found to interact with many targets, irritation resulting from the pepper-like taste/odor may restrict their application. Considering that Vitamin B1, Vitamin B2, Vitamin B5, Vitamin B6, and Vitamin B12 are all vitamin B compounds, we mixed them to test the comprehensive effect of multiple vitamin B(MVB) in further animal experiments.

On the basis of effective of animal behavioral experiments, food function factors with optimal effects were designed to make two unique nutritional formulas. The results showed that the effect of combined nutrients was significantly better than that of a single nutrient. When formulating the nutritional supplements, we did not only consider the ranking of their combined effects but also classified these nutrients. We provide two perspectives; one is the formula of plant compounds. The ingredients in this formula are naringin, quercetin, and proanthocyanidin. The reason for choosing these three nutrients to form a formula is that they ranked in the top in all outcome indicators in animal behavioral experiments. Therefore, the effect of this formula may be relatively fast and more suitable for high-temperature work or high-intensity mental work. Naringin and quercetin are found in many foods such as grapefruit, oranges and onions, asparagus. Proanthocyanidins widely found in blueberries, black wolfberry, eggplant. Although baicalein is also more effective than other compounds, it is rarely found in daily food and mainly comes from Scutellaria baicalensis. So the sources of formula 1 are widely accessible and easy to obtain in daily life, which can not only reduce people’s concerns about their safety, but also reduce the difficulty of formula production and cost. The other is a nutritional fortifier, which uses multivitamin B and tyrosine. The nutrients in this formula are available in most foods consumed in everyday life. They are essential nutrients for the human body, and if they are deficient, certain diseases may occur. Therefore, based on not exceeding the maximum tolerance limit, this nutritional formula is of great help to prevent cognitive impairment and the normal metabolism and growth and development of humans and animals. However, relying on obtaining these nutritional factors from food is far from achieving a significant effect of preventing cognitive impairment and people working in high temperature areas need additional nutritional supplements. The oral intake of Multivitamin B is calculated based on 30 times except vitamin B5 is calculated based on 20 times of the recommended intake for a 70 kg adult male. This dose is based on the concentrations of nutritional preparations known on the market such as Red Bull (vitamin drink) on the market and we ensured that their daily intake did not exceed tolerable upper intake levels (UL) in this formula. With our focus on application, we choose that MVB, L-tyrosine, naringin, quercetin and proanthocyanidins which are involved in a wide range of targets and they are also convenient to obtain in the daily diet. These two nutritional formulas are our initial and proof-of-concept attempts to provide added ideas for food product development. This can provide a sufficient basis for the research and development of related functional food products to prevent cognitive impairment under a high-temperature environment.

This study did not verify all the nutrients in the list. It only selected some common daily nutrients for verification based on the ranking of the sorted targets. The ranking of the targets may be affected by current research articles, leading to limitations. Regardless, to improve the efficacy and response, we administered the highest possible concentration within the allowable range for 7 days. Long-term low-dose use of nutrient supplements on improving mental work ability under high-temperature intervention also needs to be verified.

According to the current research, the cognitive impairment caused by heat stress may be related to acute inflammation in the brain. Therefore, this study explored the effect of plant compound formula on the pro-inflammatory factor IL-1β/IL-6/TNF-α and anti-inflammatory
factor IL-10 in HT22 after heat stress. The results showed that the formula could significantly inhibit the expression of high temperature-induced inflammatory genes IL-1β, IL-6, and TNF-α. At the same time, the expression of anti-inflammatory factor IL-10 was also inhibited, which may be because the formula can reduce the level of inflammation, so the expression of anti-inflammatory factor is down-regulated. On this basis, we further determined the expression of Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2). iNOS and COX-2 are important mediators of neuroinflammation. They are involved in the early inflammatory response of brain tissue under the action of injury factors. COX-2 is an inducible enzyme that is less expressed in normal tissues, but is highly expressed when cells are stimulated by inflammation. Because of its rapid response to pro-inflammatory mediators and cytokines, it has been considered to play an important role in the pathological process of inflammation. Similarly, when the nervous system is injured, Inducible Nitric Oxide Synthase iNOS is induced and abundantly expressed. RT-PCR results showed that the expression of these two genes was up-regulated after heat stress and down-regulated after formula intervention. Based on this, this study believes that the formula can alleviate the inflammation of hippocampal cells caused by heat stress, and the mechanism may be related to the inhibition of the expression of inflammatory factors. This provides evidence that the formula can prevent cognitive impairment caused by high temperature environment. Although the experimental results illustrate the potential value of the formulation in applications, its mechanism still needs to be further studied.

Besides finding the food function factors, 103 targets related to heat stress and brain function were predicted in detail by using the PPI core network. APP, INS, KNG1, ALB, POMC, SST, BDNF, IL-6, MAPK1, and FOS are the top ten key targets. These ten proteins have the most interactions with other proteins. The GO enrichment and KEGG analysis show the pathways associated with the identified 103 targets, including neuroactive ligand-receptor interaction, cAMP signaling pathway, serotonergic synaptic signaling pathway, Alzheimer’s disease signaling pathway, and inflammation signaling pathway. This will provide a reference for us to study further the molecular mechanism of each functional food factor or its formulation in the future. To confirm the bioinformatic findings with regards to these compounds and heat-stress related pathways, we assess activation of cAMP pathways by looking at the expression of the phosphorylation of key pathway proteins PKA via immunoblot. We also examined the expression of BDNF, as it is one of the predicted key proteins and is a downstream protein of the cAMP pathway. The results showed that the expression of BDNF and phospho-PKA in the cells of HT22 was significantly decreased after heat stress treatment, and the expression levels were significantly increased after the intervention of formula 1 compared with the heat stress group. This indicates that formula 1 activates the cAMP pathway and affects the expression of the key protein BDNF downstream of the pathway.

This study confirmed the bioinformatic findings with regards to these compounds and pathways which would greatly benefit from in vitro studies. At the same time, by sorting targets and matching the food and function database, we obtained the foods corresponding to these targets. We then marked the molecular mass, oral administration, and intestinal penetration rate of the active substances screened to construct a high-temperature cognitive functions food database. These findings will help the daily dietary guidance of people working in high-temperature environments.

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**Author declarations**

There are no conflicts of interest to declare.

**CRediT authorship contribution statement**

Mengyu Cai: Writing – original draft. Yicui Qu: Data curation. Zifu Ren: Data curation. Xin Xu: Data curation. Chuyang Ye: Data curation. Hongtao Lu: Data curation. Yinyin Zhang: Data curation. Wenlan Pan: Data curation. Hui Shen: Funding acquisition. Hongxia Li: Writing – review & editing.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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Appendix

A. Chemical structures of each compounds

L-Tyrosine

Naringin

Proanthocyanidins

Quercetin

Resveratro

Tea polyphenol

Genistein

Baicalei

Luteolin

Vitamin B₁

Vitamin B₂

Vitamin B₅

Vitamin B₆

Vitamin B₁₂
B. PPI protein network construction

![PPI protein network construction diagram](image)

C. Bubble chart of biological process entry for GO enrichment analysis

![Bubble chart of biological process entry for GO enrichment analysis](image)
D. Cellular component (CC), molecular function (MF) and biological process (BP) of GO enrichment analysis

E. KEGG pathway enrichment histogram

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