Original Article

Validation of a mathematical model for determining the *Yin-Yang* nature of fruits

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A mathematical model for determining the *Yin Yang* nature of fruits was established in our previous study. The objective of this study was to validate the mathematical model using animal experiments. One hundred and twenty Wistar II female rats were randomly divided into five diet groups: A, B, C, D and E that were administered with saline solution, hot Traditional Chinese Medicine (TCM) tonic, cold TCM tonic, hot (*Yang*) formulated mineral solution and cold (*Yin*) formulated mineral solution, respectively. The consumption of drinking water of rats in each group was determined during a feeding period of 21 days. On the last day of the experiment, the lingual superficial structure of the rats was examined, which is a practice in traditional Chinese medicine to diagnose *Yin-yang* symptoms, and blood samples were collected from the rats to determine serum thrombocytin (5-HT) and thyrotrophic hormone (TSH) and plasma noradrenaline (NE). The diet D group, administered with the *Yang* mineral solutions demonstrated the same trend as the diet B group fed with hot TCM tonic (hot control), while the diet E group administered with the *Yin* mineral solutions has the similar trend as diet C group fed with cold TCM tonic (cold control). It was concluded that the diet D had *Yang* nature, whereas the diet E had *Yin* nature. The results from current study confirmed the findings from the previous study that the *Yin-Yang* nature of the fruits could be determined by the ratio of copper, iron and magnesium content using the mathematical model.

Key Words: validation, mathematical model, *Yin-Yang* nature, fruit, animal experiment

Introduction

In the Traditional Chinese Medicine (TCM), all foods are defined as *Yin* and *Yang*, or four natures: cold, cool, warm and hot in terms of their health functionality or health-related attributes.[1] The cold and cool nature belong to *Yin* while the warm and hot nature belong to *Yang* based on how the food interacts with health. 'Even'nature lies between the warm and the cool. The theory has become a ubiquitous guideline for food and health in China for centuries, contributing successfully to the general wellbeing of the Chinese nation.

In our efforts to elucidate a possible biochemical mechanism behind the theory, the correlation between composition and distinctive *Yin-Yang* nature of 25 fruits were analyzed and classified according to their respective natures. The data of eight components of each fruit including water, energy, carbohydrate, dietary fiber, fat, protein, vitamins and minerals, were collected from data base and analyzed by the Step Multivariate Discriminate Method, using a SPSS program (version 11.0). It was found that the mineral content could be used as the biochemical indicator for differentiating nature of the fruits with a 96% correct rate of back-substitution. The further analysis revealed that copper, iron and magnesium were the major factors for differentiating the *Yin-Yang* nature in fruits, which had 100% correct rates of back substitution. A mathematical model for determining the *Yin-Yang* nature of fruits was established. The equation was $Y=-10.19173X_{Cu}-1.42593X_{Fe}+0.14975X_{Mg}$ and its discriminate interval was (-0.6423171, 0.5275019), in which X_{Cu}, X_{Fe} and X_{Mg} represented the contents of copper, iron and magnesium; if the value was between its discriminate interval, it meant that the fruit belonged to the *Yang* group; if not, the fruit belonged to the *Yin* group.[2]

This animal experiment was carried out to validate the mathematical model for determining the *Yin-Yang* nature of fruits. According to TCM *Yin-Yang* theory, body temperature, lingual superficial structure, movement, face color, and so on are normally used as the signs to diagnose *Yin-yang* nature in human. Recent studies have revealed that similar *Yin-Yang* symptoms can also observed from animals. For example, an improvement in consumption of oxygen and drinking water by a TCM tonic with *Yang* nature was observed in rats just like in human beings. There are more reported biomarkers associated with the *Yin-Yang* symptoms. As the literatures[3,4] reported, the effects of TCM tonic with *Yang* nature was opposite to that with *Yin* nature. hormone (LH), but also suppress NE synthesis and elevate 5-HT level. A hot TCM tonic could activate the dopamine-β-hydroxyl (DβH) enzyme, induce synthesis noradrenaline (NE) synthesis, and increase catecholamine (CAs) content in nerve centre, visceras, even in urine, and decrease the thrombocytin (5-HT) level. While a TCM tonic with *Yin* nature not only could inactivate the DβH enzyme via certain chemicals formed in the centre

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nerve of rats suppressing the release of adrenocorticotropic hormone (ACTH), thyrotrophic hormone (TSH), and luteinizing hormone (LH), but also suppress NE synthesis and elevate 5-HT level.

In our previous study investigating the influences of the hot and cold mineral formulations as deduced by the mathematic model, rat's anus temperature was established as an index to evaluate nature of the formulated mineral water. The hot and cold mineral formulations demonstrated effects very similar to the TCM tonic counterparts in the animal test. This indicated the feasibility of validating the mathematic model by animal test.

In this study, the validity of the mathematic model, for discriminating between fruits, was further evaluated by investigating the effects of the hot and cold mineral formulations on consumption of drinking water during the feeding time, the lingual superficial structure of rats and serum 5-HT and TSH level, as well plasma NE level at the end of experiment in comparison with the typical hot and cold TCM tonics.

Materials and methods

Experimental Solution Preparation

The *Yang* and *Yin* mineral formulations were prepared according to the mathematical model using magnesium sulfate ($MgSO_4$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and copper sulfate ($CuSO_4 \cdot 5H_2O$). By orthogonal experimental design with three factors and three levels (Table 1-4), nine formulations for both Yin and Yang formulated mineral solution were prepared. The hot TCM tonic solution was decocted from 50g *Rhizoma Zingiberis* and 50g *Rhizoma Typhonii* in 100ml distilled water. The cold TCM tonic solution was decocted from 57.15g *Gypsum Fibrosum* and 42.85g *Rhizoma Anemarrhenae* in 100ml distilled water.

Animal Test Design

One hundred and twenty WistarII female rats (Medical & Science Center of China, Bejing) with 'even' nature and a body weight of 180 to 200g were randomly divided into five diet groups: A, B, C, D and E. In a 21 days feeding trial, 4 ml experimental solutions were administered daily to the five groups in addition to normal feedings.[5] The experimental solutions for A, B, C, D and E groups were saline solution, hot TCM tonic solution, cold TCM tonic solution, *Yang* formulated mineral solution, and *Yin* formulated mineral solutions, respectively. In group B and C, 10 rats were with hot TCM tonic and cold TCM tonic, respectively, while in Diet group D and E, each of the 9 formulations of *Yang* or *Yin* formulated mineral solutions was administered to 5 rats, respectively. There were 45 rats in total in both diet D and E groups. On the last day of the experiments, after 6h fasting, the rat was put under light diethyl ether anesthesia before a blood sample was collected into a non clot activation tube from the abdominal aorta and centrifuged at 3000r.p.m. for 5 min within 30min to separate serum and plasma.

Consumption of drinking water

The consumption of drinking water of each rat was recorded daily by weight. Statistic analysis of drinking wa-

Table 1. Factors and levels of mineral formulated solutions with *Yang* nature

Levels	Factors (mg/100ml)		
	Mg	Fe	Cu
1	9.50	0.400	0.088
2	11.4	0.624	0.104
3	12.1	0.800	0.128

Table 2. Orthogonal table $L_9(3^3)$ of mineral formulated solutions with *Yang* nature

Experimental Numbers	Factors (mg/100ml)		
	Mg	Fe	Cu
1	9.50	0.400	0.128
2	11.4	0.400	0.088
3	12.1	0.400	0.104
4	9.50	0.624	0.104
5	11.4	0.624	0.128
6	12.1	0.624	0.088
7	9.50	0.800	0.088
8	11.4	0.800	0.104
9	12.1	0.800	0.128

Table 3. Factors and levels of mineral formulated solutions with *Yin* nature

Levels	Factors (mg/100ml)		
	Mg	Fe	Cu
1	13.0	0.20	0.032
2	14.1	0.40	0.064
3	19.0	0.60	0.080

Table 4. Orthogonal table $L_9(3^3)$ of mineral formulated solutions with *Yin* nature

Experimental numbers	Factors (mg/100ml)		
	Mg	Fe	Cu
1	13.0	0.20	0.080
2	14.1	0.20	0.032
3	19.0	0.20	0.064
4	13.0	0.40	0.080
5	14.1	0.40	0.032
6	19.0	0.40	0.064
7	13.0	0.60	0.080
8	14.1	0.60	0.032
9	19.0	0.60	0.064

ter data was performed using a T-test. Significance of differences was defined at a critical value of 2.021.

Lingual superficial structure

General examination of lingual superficial structure was carried out by an experienced herbalist doctor during the 21 days.[6] At the end of the experiment, the lingual superficial structure of rats were examined by scanning electron microscope (SEM)[7]. The samples were washed and fixed in a 2.5% glutaraldehyde solution at 4°C, then dehydrated in a graded ethanol series. Critical point dried samples were placed on aluminum stub, gold sputtered, and examined at 25kV by SEM (JEM-35CF, JEOL Ltd.).

Determination of serum 5-HT

0.5ml serum was mixed with 4.5ml acidic butyl alcohol for 5 minutes and centrifugated at 3000r.p.m. for 10 minutes. The supernatant was collected and mixed with 3.0ml heptane and 1.0ml 0.1mol/L hydrochloric acid for 5 minutes, then centrifugated at 3000r.p.m. for 5 minutes. The lower aqueous phase was colleted and mixed with 5-HT standard solution, 0.1mol/L hydrochloric acid, 82.4mmol/L Cysteine solution and 447.3umol/L (60mg/L) o-phthalaldehyde (OPT) solution, then incubated at 100℃ for 10 minutes. The mixture was cooled to room temperature and measured for its intensity of fluorescence in excitation wavelength of 350nm and emission wavelength of 477nm by fluorescent spectrophotometer (MPF-4, Hitachi, Japan). Distilled water was used instead of the blood serum as a blank.[8, 9]

Measurement of plasma NE

Plasma NE was measured by the method of fluorescence analysis.[10]

Assay of serum TSH

Serum TSH was assayed by radioimmunoassay on γ-radiation immunity counter (GC-911, University of Chinese Science and Tech. Co.)[11]

Determination of serum copper, iron and magnesium

Serum copper, iron and magnesium were determined by the method of spectrophotometer.[12,13,14,15] Data were expressed as mean ± standard derivation (SD). Coefficient between mineral contents in formulated solution and serum of rats at the end of feeding trial was analyzed using Pearson correlation. Statistic analysis of the data was performed using software of SPSS Statistical Package version 7.0 (SPSS Inc., Chicago, IL, USA). p values <0.05 were considered as significant.

Results and discussion

As a control, the average drinking water consumption of the diet group A was 19.39mL/rat in 21days, while the diet group B, C, D, and E consumed 26.61mL/rat, 15.40mL/rat, 23.22mL/rat, and 16.95mL/rat, respectively. The diet group B, a hot TCM tonic group, and the group D fed with 'hot' formulated mineral solution, consumed more drinking water than the diet A group. The diet group C group, a cold TCM tonic group and the diet E group fed with 'cold' formulated mineral solution, consumed less water than the control group. The trend of the daily average water consumption of the group B, C, D and E could be expressed by the flowing equations $y = 22.528e^{0.0146x}$, $y = 16.407e^{-0.0064x}$, $y = 21.337e^{0.0074x}$, and $y = 17.952e^{-0.0053x}$ (Fig 1). As T test results in Table 5 indicate, the water consumption between either group B and D or group C and E is not significantly different, while the water consumption between the two 'hot' formula groups (diet group B and D) and two 'cold' formula groups (diet group C and E) are significantly different. It is apparent that the diet group B and D, the diet group C and E share similar properties. According to 'Ben Cao Gang Mu', one of the most important Chinese medical material books, *Rhizoma Zingiber* and *Rhizoma Typhonii* are of very hot nature while *Gypsum Fibrosum* and *Rhizoma Anemarrhenae* are of very cold nature. The combination of the two hot herbals constitutes the most representative 'hot' formulation and the combination of two cold herbals represents the 'cold' formulations in TCM. Based on the results of drinking water consumption, the nature of the 'hot' formulated mineral solution for group D should be *Yang*, and that of the group E should be *Yin*.

Examination of tongue coating is an important means for TCM doctors to diagnose a person's health status, especially in terms of Yin and Yang balance. Generally when one is under *Yang* status, his or her tongue fur would be thick, yellow and dry, when one's nature is *Yin*, his or her tongue fur would appear thin, white and moisturized. The tongue examination by an experienced herbalist showed that the lingual superficial structure of the rats in group B and D were slightly yellow and dry, while

Table 5. T-test of the average consumption of drinking water of each group

T	Degrees of freedom
T_{AB}=3.55	40
T_{AC}=3.07	40
T_{AD}=3.07	40
T_{AE}=4.10	40
T_{BC}=4.66	40
T_{BD}=1.43**	40
T_{BE}=4.58	40
T_{CD}=4.37	40
T_{CE}=1.10**	40
T_{DE}=4.59	40

Table 6. Contents of copper, iron and magnesium in the blood serum of rats

	Mg（μmol/L）	Fe（μmol/L）	Cu（μmol/L）
Blood Serum of Diet D group	0.524±0.0230	64.3±6.629	35.8±4.42
Blood Serum of Diet E group	0.633±0.0529	57.5±5.47	24.3±6.17

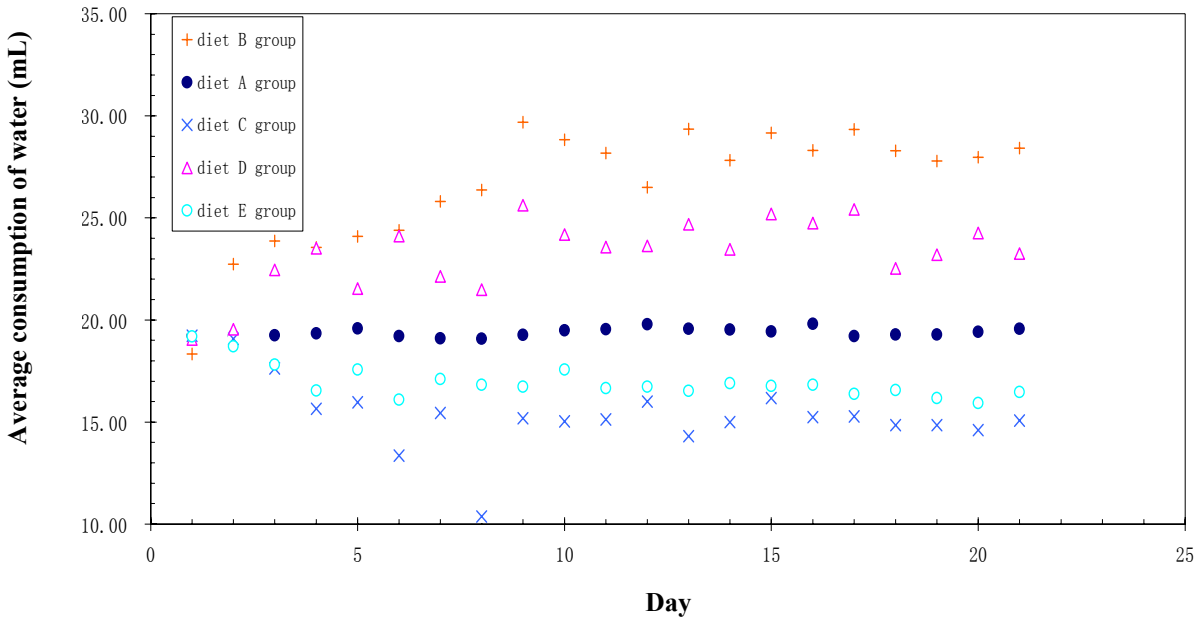


Figure 1. Daily average water consumption of each group of rats.

the rats in group C and E had slightly white and wet tongues. The results indicated that the rats in group D had *Yang* nature while those in group E had *Yin* nature after feeding with the *Yang* and *Yin* formulate mineral solutions, respectively. It can be concluded that the diet D had similar nature to diet B as the diet E to the diet C.

Interesting results were obtained by the microscopic study of lingual superficial structure of rats fed with the 'hot'and 'cold' formulated solutions. As shown in Figure 2a of the scanning electron microscope (SEM) pictures of tongue filiform papillae and the fungiform papillae of a rat with 'even' nature, the filiform papillae appears thin and long while the fungiform papillae a mushroom shaped. The SEM pictures of filiform papillae of the rats from group D and E were shown in Fig. 2b and 2c, respectively. It can be observed that more cracks on the filiform papillae in D group rats than those of group E rats. The cracks on filiform papillae of group D rats could be seen more clearly in Fig 2c. Comparing image pattern of the fungiform papilla of group D rats (Fig 2f) with those of group E rats (Fig 2e), a large deep hole can be seen in the center of the fungiform papilla of group D rats, but no visible hole from that of group E rats. As reported in a study of human tongue[16], cracks on the filiform papillae and deep holes on the fungiform papilla forma coarser papilla surface as a result of keratinization of epithelial cells. The characteristic thick, yellowish and dried tongue fur, a typical lingual superficial structure for a person under *Yang* status in TCM, corresponds well with the coarser papilla surface caused by high degree of keratinization of tongue epithelial cells. It is evidenced by the rougher surfaces of both filiform and fungiform paplilla of the rats administered with 'hot' formulated mineral solution.

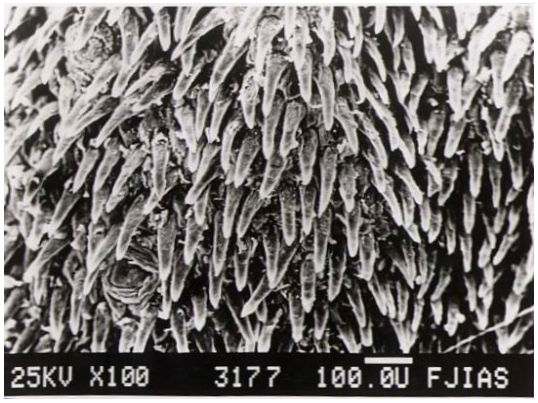
Examination of lingual superficial structure by an experienced TCM doctor can be amazingly accurate, nevertheless, still remains a subjective rather than objective process. Some efforts have been made to improve the lingual superficial structure analysis more scientific through graph analysis[17], but no work has been reported using ultrastructural means to investigate the TCM lingual superficial structure. Though much more work is necessary in order to define the ultrastructural patterns of tongues of *Yin* and *Yang* symptoms, our preliminary results of microscopic analysis of lingual superficial structure of *Yin* and *Yang* status clearly indicate the methodology can be extremely useful in modernizing the ancient and critical practice of tongue examination in TCM. There is no any available explanation to how a person under '*Yang*' status would have a higher degree of keratinization of tongue epithelial cells. It may become a meaningful breakthrough point to elucidate the scientific principles behind the most important practice of diagnosis in TCM.

Table 7. Pearson Correlation coefficient between mineral contents in formulated solutions and in the blood serum of rats after feeding the corresponding diets for 21 days

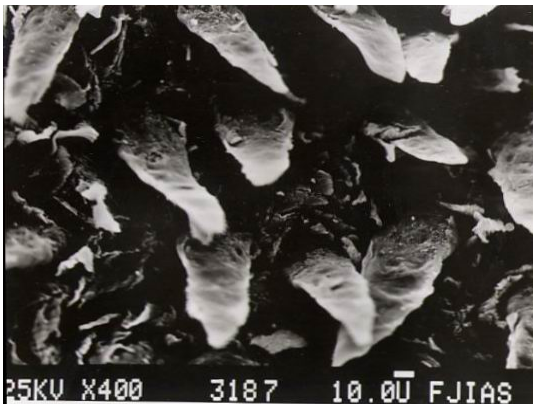
Minerals	Pearson Correlation coefficient
Magnesium content in diet group D	0.829(**)
Iron content in diet group D	0.988(**)
Copper content in diet group D	0.983(**)
Magnesium content in diet group Ep	0.978(**)
Iron content in diet group E	0.976(**)
Copper content in diet group E	0.985(**)

The results for plasma NE, and serum 5-HT and TSH are summarized in Figure 3. NE and TSH levels from group B and D were elevated, in comparison with those of group A, while those of group C and E demonstrated lower values. On the other hand, the average 5-HT levels of group B and D were lower than that of group A, while group C and E had higher 5-HT levels. As previously reported[3,4], the hot TCM tonic could activate the synthesis of NE, elevate TSH, but decrease 5-HT, and the results from group B showed exactly the same tendency. In this study, group D, administered with the *Yang* mineral

2a



2b



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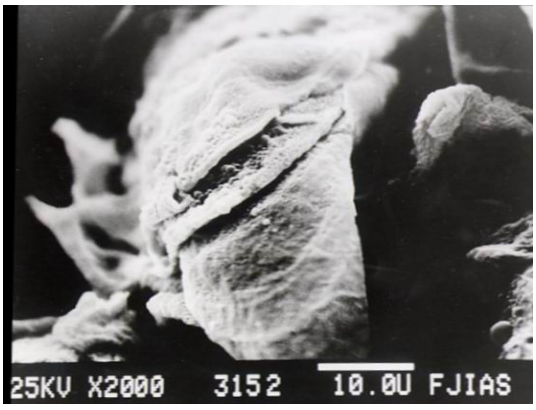
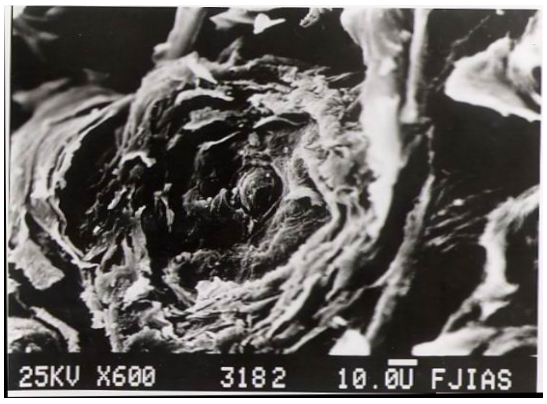


2e



2f

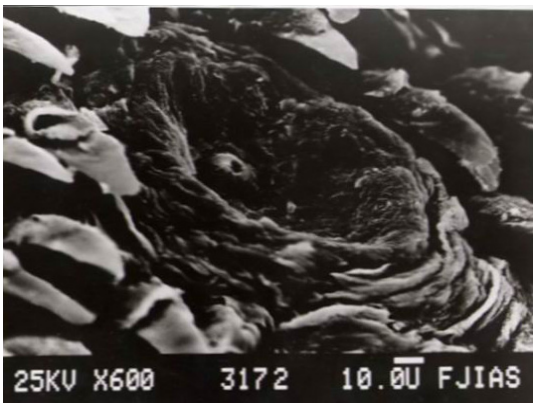


Figure 2. Scanning electron photographs of Filiform papillae and fungiform papilla. (a) A rat with 'even' nature, (b) Diet group E, (c) Diet group D, (d) Amplificatory photograph of filiform papillae of the diet group D, (e) Fugiform papilla of the diet group E (f) Fugiform papilla of the diet group D

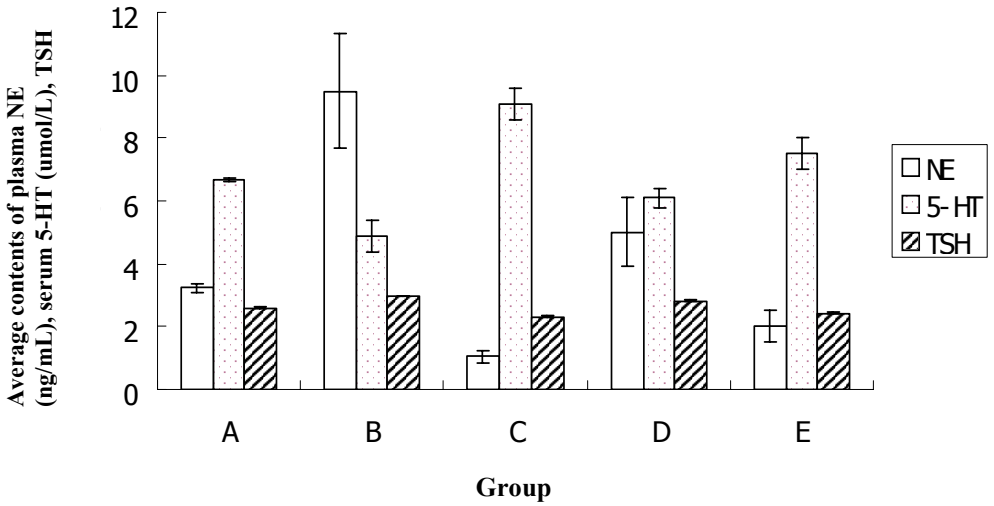


Figure 3. Average contents of plasma NE, serum 5-HT and TSH of each group of rats.

solution, demonstrated the same impact as group B which served as a control for hot TCM tonic, while group E administered with the *Yin* mineral solution was similar to group C. The results confirmed that group D shared the same nature as diet B while group E as group C.

Serum copper, iron and magnesium levels in the rats at the end of the feeding were also determined. Data analysis was carried out by Pearson Correlation coefficient. As shown in Table.2, the average contents of copper, iron and magnesium in the formulated minimal solutions correlates well with the average contents of copper, iron and magnesium in serum of the rats, implying that the minerals by feeding could be well assimilated thus caused the change in the *Yin*-*Yang* status of the rats.

It was previously reported that the hot-cold nature of TCM could be correlated to the contents of Fe, Mn, Cu and Zn based on an analysis of 176 TCM ingredients[18], and Fe content positively correlated to the nature of TCM ingredient from hot to cold (γ=0.547, $p<0.01$), while Mn content positively correlated (γ=-0.570 7, $p<0.02$). The greatest difference between those findings and our mathematical model is that the latter has a much higher correct rate of 100%, and that it is the proportions of Cu, Fe, and Mg rather than the content of individual element that determines the hot-cold nature. The remarkably lower correlation rate in TCM nature prediction may be due to the multiple process of preparation of TCM ingredients such as sun-drying, heating, extraction, which may result in complexity in the existing status of minerals as well as different availability of different minerals. On the contrary, it is relatively simple in the case of fresh fruit. Those negligence may well be the reason why the conclusion about influence of Fe on TCM nature in the reported work was the opposite to our conclusion about effects of Fe on fruit nature. It is likely that the correlation between the mineral contents to the TCM ingredient hot-cold nature would be dramatically improved if the influence of preparation processes, and mineral status were reconsidered.

One of the most interesting findings in this work is, quite certainly the interaction of minerals can be completely different from the effect of individual element, especially in the case of fruits. It is the relative proportion of different elements rather than the absolute level of individual element that dictates effects on the physiological status. Biochemical functions of individual elements of Cu, Fe, and Mg have been extensively investigated and elucidated, but the effects of different combination of different elements relatively unknown. Understanding the impact of a group of elements on health is important in elucidating fruits health function since each fruit is a package of minerals with a certain ratio.

Conclusion

Our previous work demonstrated that the hot and cold mineral formula deduced from our mathematical model exerted exactly the same influences of the rat anus temperature as the typical hot and cold TCM. This study has found that the hot and cold formulated mineral solutions based the mathematical model demonstrate exactly the same functions as the TCM *Yin* and *Yang* controls on rat biomarkers such as drinking water consumption, tongue phenomena, plasma NE level, serum TSH and 5-HT level, The results from current study have further proved that contents of copper, iron and magnesium and their relative ratio are the determining factor for fruit's *Yin*-*yang* nature, and that the mathematical model proposed in previous study can be used to differentiate the hot and cold nature. It has also been revealed that the cornification of the filiform papillae and the fungiform papillae is more serious in hot syndrome rats than that of in cold syndrome rats. Microscopic analysis of the tongue ultrastructure can be a useful method in diagnosing the *Yin* and *Yang* status. Finally, the importance of understanding of minerals as a group rather than individually is indicated in this study.

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