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Original Article

Comparison of cytological parameters of exfoliated buccal mucosal cells in different temperament groups

Zahra Zendehboodi[1]*

1. Dept. of Biology, College of Sciences, Shiraz University, Shiraz, Iran.

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***Corresponding Author**:
Department of Biology, College of Sciences, Shiraz University, Shiraz, Iran.
Tel: +98 71 36137359
Fax: +98 71 32280916

Email:
zendehboodi@shirazu.ac.ir
zahrazendehboody@yahoo.com

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Abstract

Introduction: Temperament (Mizaj) forms the basic concept of Iranian traditional medicine (ITM), and greatly influences the diagnosis and treatment of diseases, as well as maintains the ideal healthy state of an individual. In particular, temperament is presumed to affect the morphological, physiological, and psychological features of a person; however, its influence on biological features remains unclear in practical ITM. This study aimed to evaluate the association between the temperament and the cytological features of buccal mucosa in healthy people.

Methods: The study sample included 75 healthy individuals from Fars province, southern Iran. The temperament was determined using a self-reported temperament identification scale. Based on the questionnaire, volunteers were classified in nine temperaments including one equilibrium, four simple temperaments (warm, cold, moist, and dry,) and four combined temperaments (warm–moist, warm–dry, cold–moist, cold–dry). Smears collected from the buccal mucosa of participants were analyzed for biomarkers of DNA damage, cytokinetic defects, proliferative potential, and cell death using micronucleus (MN) assay. Student's t-test or Mann–Whitney U test was applied to identify the differences between groups.

Results: DNA damage (nuclear buds) and cell death biomarkers (condensed chromatin, karyorrhexic, pyknotic, and karyolitic cells) reported significant differences between certain temperament groups.

Conclusions: The present study reported that the aforementioned cytological parameters could be affected by the temperament; however, more studies with greater sample sizes are warranted.

Introduction

Iranian traditional medicine (ITM), one of the oldest means of traditional medicine, strives to provide optimum paths for a healthy life with minimal illness (1, 2). Temperament forms the most essential concepts of ITM. Temperament is a result of the interaction of elements with different qualities (hot, cold, wet, dry) of the human body. Temperament is presumed to be a dominant quality and is essential in maintaining the ideal healthy state of an individual by affecting the general physical and emotional characteristics and also the physiological functions of the body. Based on the degrees of warmness and wetness, the experts of ITM categorize the temperament into nine major groups. These groups comprise one equilibrium temperament, which is in equilibrium for both warmness and wetness; four simple temperaments (warm, cold, moist, and dry), which are in equilibrium for one quality and out of that for another quality; and four combined temperaments (warm–moist, warm–dry, cold–moist, cold–dry), which are out of equilibrium for both qualities. According to ITM, a person with a balanced temperament is considered to be healthy, whereas the one with a imbalanced temperament is prone to diseases (3, 4).

Oral exfoliative cytology represents the microscopic examination and measurement of cells, which have been shed or removed from the buccal epithelial surface. The smear obtained by oral exfoliative cytology can be used for buccal micronucleus cytome test (MN assay). Based on the cytological and nuclear features, the MN assay has been used to measure DNA damage, proliferative potential, and cell death (5). Several parameters including certain diseases (7), radiation (9, 10), nutrition (15), smoking, sex and age (12), etc., are known to affect the MN assay parameters.

Despite its effectiveness, the existing knowledge on the functional mechanisms of ITM based on temperament is inadequate, and despite the progression of scientific prospects, the therapeutic methods of ITM are chiefly based on practical experiences and texts retrieved from ancient literature. We assume that further studies may help us in clarifying the biological mechanisms of practical traditional medicine, which in turn can be used in conjunction with and as an aid to the modern medicine. Considering the fact that morphological, physiological, and psychological features of a person are affected by the temperament (3), this study aimed to investigate whether MNs and other parameters of genome damage and cell death in the buccal mucosa of healthy people are associated with the temperament. Notably, identifying the biomarkers of these temperaments may help us better understand the mechanism of these differences.

Materials and methods

Study population

The study included 75 healthy students of both sexes, aged between 19–24 years. All participants belonged to the Fars province, southern Iran. Exclusion criteria for the subjects

were presence of a disease, alcohol or smoking habit, carrying restorative dental fillings, using a mouthwash, drug treatment, dental treatment, or recent exposure to facial or oral radiographs. This study was approved by the Ethics Committee of Biology Department of Shiraz University, Iran (ECBDE-SU-9-6177616), and informed consent was obtained from all participants.

Participants' temperaments were determined using a self-reported temperament identification scale, constructed by Mojahedi, et al. (16). Based on the questionnaire, volunteers were classified in 9 temperaments including one equilibrium, four simple temperaments (warm, cold, moist, and dry,) and four combined temperaments (warm–moist, warm–dry, cold–moist, cold–dry).

Cell sampling and preparation

Buccal cells (BCs) were collected from volunteers. Prior to BC collection, their mouth was rinsed thoroughly with water to remove any unwanted debris. Small-headed toothbrushes were rotated 30 times in a circular motion against the inner part of the left cheek. The bristles of the toothbrush were placed into 14-ml falcon containing BC buffer [0.01 M Tris–HCl, 0.13 M EDTA, 0.02 M sodium chloride] at pH 7.0 and were agitated to dislodge the cells. The cells were centrifuged for 10 min at 500 g. Supernatant was extracted and replaced with 10 ml of fresh BC buffer. Cells were spun and washed again twice. Furthermore, the supernatant was extracted and cells were resuspended in 3 ml of fixative (ethanol–acetic acid (3:1)). After 5 min the cells were centrifuged, resuspended in 200 µl of fixative and were then placed on slides and allowed to air-dry for 10 min. Slides were treated with 5 M HCL for 30 min and were then stained with the Schiff's reagent for 60 min. The cells were counterstained with 0.2% light green for 30 s.

Microscopic observation

Using MN assay, biomarkers of DNA damage (micronuclei and nuclear buds), cytokinetic defects (binucleated cells), proliferative potential (basal cell frequency), and cell death (condensed chromatin, ***karyorrhexic***, pyknotic, and karyolitic cells) could be evaluated (5). The distinct cell populations scored in this assay (Fig. 1), were identified based on the criteria outlined by Thomas et al. (17). According to these criteria, *basal cells* have a uniformly stained nucleus. They have smaller size and larger nuclear–cytoplasmic ratio compared to differentiated cells. *Normal differentiated cells* have a uniformly stained nucleus. They have larger size and smaller nuclear–cytoplasmic ratio relative to basal cells. The *micronucleated cells* comprise both the main nucleus and one or more smaller nuclei called micronuclei (MNs). MNs are round or oval with similar stain intensity as the main nucleus and are 1/3–1/16 diameter of the main nucleus. The *cells with nuclear bud* possess nuclei attached to the main nucleus, suggestive of a budding process. The nuclear bud reveals a similar morphology and staining properties as the nucleus and its diameter may range from a quarter to half of the nucleus. *Binucleated cells* have two nuclei with the same morphology. The nuclei are in close proximity or may contact each other. *Condensed chromatin cells* have nucleus with striated pattern of parallel tracts of aggregated chromatin. The nucleus is intensively stained in distinct areas of chromatin condensation. The karyorrhexic *cells* have nucleus with extensive chromatin aggregation, leading to fragmentation and disintegration of the nucleus. The *pyknotic cells* have small and shrunken nucleus, which is uniformly and densely stained. The nuclear diameter is approximately 1/3–2/3rd of the normal nucleus. The *karyolytic cells* are cells with DNA depleted nucleus. The nucleus has no Feulgen staining and appears as a ghost-like image. The frequency of each cell type was determined in 1000 cells for each person. The cells were analyzed under a total magnification of 400× using a Nikon microscope.

Statistical analysis

The normality of the variables was evaluated using the Shapiro–Wilk test. Student's t-test was used to determine the significance of the cellular parameters measured between the study groups, in the case of normal distributed variables; at other instances, Mann–Whitney U test was applied. The chi-square and Kruskal–Wallis tests were used to compare the sex ratio and mean age of the study groups, respectively. $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS version 22.

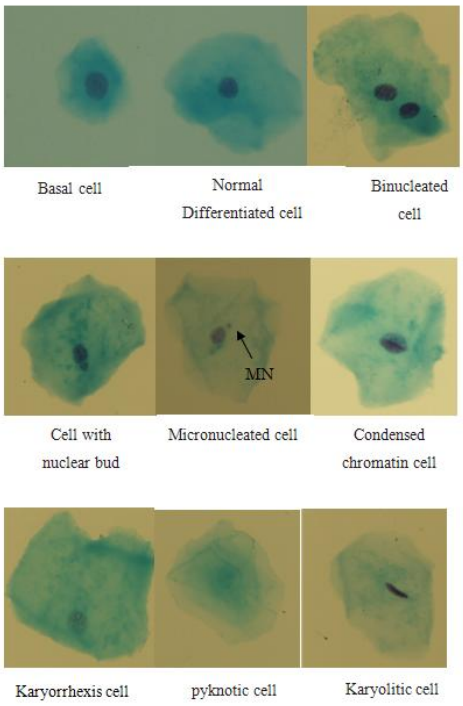

Figure 1. Images showing distinct buccal cell types as scored in the buccal cytome test

Results

As revealed by the data of the gender and age of the study population, no significant difference was observed between the means of age in different temperament groups, and the sex ratio was nearly similar in all groups (Table 1). Since no statistical association was observed between sex and temperament ($\chi 2 = 7.68$, df = 8, $P = 0.465$), the sex groups were pooled. According to Table 1, the study population contains all nine temperament groups and the equilibrium group includes the maximum participants.

The data of MN assay including biomarkers of DNA damage, cytokinetic defects, proliferative potential, and cell death were summarized and each group was compared with other groups for each parameter using Student's t-test or Mann–Whitney U test (Table 2). Significant differences in the mean values of nuclear buds, condensed chromatin, karyorrhexic, pyknotic, and karyolitic cells were observed between certain groups.

According to Table 2, the mean of karyolitic cells in warm–moist temperament was significantly higher than that in other groups; however, no significant difference was observed when these values were compared with those of the warm group ($P = 0.073$). The mean of pyknotic cells in warm–moist temperament was lower compared to that in other groups; as depicted by the data, these differences were significant compared with all groups except for the cold–dry group. Moreover, the warm–moist group reported significant decrease in the mean of condensed chromatin and nuclear buds compared to the moist group; however, notably, the frequency of these two parameters in the warm–moist group was lower compared to all other groups, but no statistically significant difference was seen. The mean of karyorrhexic cells in the cold group was lower compared to that in other groups; as depicted by the data, these differences were significant with the moist, equilibrium, and cold–dry groups. Comparison between other groups showed no difference for these parameters. Moreover, no difference was observed for the mean values of basal, binucleated, and micronucleated cells between any two groups.

Table 1. Age and gender ratio in study groups

Temperament Groups	N	Age (Mean ±SD)	Gender male/female
Equilibrium	18	20.8±1.4	5/13
Warm	5	20.2±0.5	0/5
Cold	7	20.6±1.8	0/7
Moist	12	21.1±1.2	1/11
Dry	13	20.6±1.0	4/9
Warm-Moist	5	22.0±1.6	1/4
Warm-Dry	5	21.4±2.0	1/4
Cold-Moist	5	20.8±1.5	2/3
Cold-Dry	5	20.4±1.1	2/3
P values		0.611	0.465

Table 2. Comparison of cytological biomarker in buccal epithelial cells between different temperament groups

Temperament	Basal cell	BN cell	CC cell	KH cell	PK cell	KL cell	MN	NBUD
Equilibrium	27.0±11.9	5.8±2.9	61.9±36.3	101.4±57.5ǂ	6.1±7.8*	100.9±84.2*	0.3±1.4	12.3±6.1
Warm	32.2±20.0	7.8±3.3	58.2±28.7	95.8±57.8	3.4±3.1*	110.2±88.8	0.8±1.8	13.2±8.5
Cold	24.9±6.2	7.0±4.7	60.3±27.4	66.0±15.3	4.0±3.5*	111.9±81.6*	0.0±0.0	12.9±5.6
Moist	26.3±9.6	5.3±3.1	75.3±22.3*	120.8±49.3ǂ	5.3±4.1*	78.1±39.6*	0.4±0.9	14.1±6.0*
Dry	28.7±14.5	6.9±3.9	62.9±45.0	90.7±37.1	3.9±3.9*	64.9±37.7*	0.9±2.2	13.5±6.0
Warm-Moist	18.8±12.3	6.8±3.6	40.0±19.6	106.4±86.7	0.2±0.5	233.2±99.5	0.2±0.5	8.6±5.5
Warm-Dry	27.8±12.4	8.6±6.0	76.6±58.0	76.2±68.4	6.4±5.6*	63.8±54.1*	0.0±0.0	15.8±12.1
Cold-Moist	34.6±16.5	8.6±4.7	59.8±34.3	110.6±98.1	5.8±6.4*	98.4±45.3*	0.0±0.0	12.2±4.8
Cold-Dry	31.2±12.3	2.4±1.3	65.6±29.8	122.8±43.4ǂ	3.0±2.8	101.0±39.8*	0.0±0.0	14.4±8.0

All values are given as mean±SD.

*, ǂ, $P < 0.05$ indicates significance level.

*, Significant difference between warm–moist and each of other groups.

ǂ, significant difference between ***cold*** and each of other groups.

BN, binucleated; CC, condensed chromatin; KH, ***karyorrhexis***; PK, pyknosis; KL, karyolysis; MN, micronuclei; NBUD, nuclear bud.

The groups were compared with each other using t-test or Mann–Whitney U test.

Discussion

Temperament, classified into nine distinct patterns with specified signs and symptoms, forms the key feature of ITM theory. Many physiological and pathological events could be categorized based on temperament, and consequently a distinct therapeutic strategy could be identified. Thus, temperament is vital in diagnosis and treatment of diseases (3, 4). Previous studies have investigated the molecular and hormonal factors correlating with temperaments in human. Zhao et al. evaluated the diagnostic value of clinical inflammatory and immune indexes on heat or cold syndrome in female patients with rheumatoid arthritis and confirmed that patients with heat syndrome have higher expression of C-reactive protein than those with cold syndrome (11). Investigating certain parameters of neuroendocrine and immune systems in people with a hot or cold nature revealed that norepinephrine/epinephrine and norepinephrine/cortisol ratios were significantly higher in the hot nature group compared with those in the cold nature group; moreover, a significant association was observed between the IL-4/IFN-γ and warmth/coldness ratios (20). Tao Ma's research group examined the gene expression information of cold syndrome using the microarray and systems biology methods and indicated that the genes related to cold syndrome are involved in energy metabolism, which correlate with the genes of neurotransmitters, hormones, and cytokines in the NEI

interaction network (8). Using proteomic tools and network analysis of the mitochondria, a difference has been observed in the expression of certain proteins and also in protein–protein interaction networks of cold–dry temperament compared to that of hot–wet ones (6). Examining the gene expression profile in $CD4^+$ T cells of rheumatoid arthritis patients with cold or hot patterns revealed that the genes involved in small G protein signaling pathways, oxidation–reduction in fatty acid metabolism, and T cell proliferation were differentially expressed in these two temperaments (18). In agreement with the aforementioned evidence, studies correlating temperaments to distinct biological factors, as the association of temperament with blood groups, enzymatic variation, and body composition, have been reported (19, 21 , 22).

The results of this study revealed that the equilibrium group gathered the maximum participants, which is consistent with the best of our knowledge from "The Canon of Medicine"(23). In this study, exfoliative cytology was assessed for the cheeks of clinically healthy people with defined temperament, followed by comparing the frequency of DNA damage, cell death, and regenerative capacity biomarkers in each temperament groups with the other groups. According to the data, the maximum variation for the examined parameters was observed in the warm–moist group, that is, when compare to the most other groups, the frequency of karyolitic and pyknotic cells significantly increased and decreased, respectively. This temperament group also reported minimum frequency of condensed chromatin. The cells with condensed chromatin as well as karyolitic and pyknotic cells are markers of cell death since they indicate one of the stages leading to cell death (17). This result indicated that the mechanism of cell death in warm–moist temperament may differ from other groups. This temperament group also reported minimum frequency of nuclear buds and its difference with the moist group was significant. This finding indicated that wetness alone may increase the nuclear buds; however, when combined with warmness, its effect could be modulated. Another variation was observed for cold group, which reported the minimum frequency of karyorrhexic cells and its difference with moist, equilibrium, and cold–dry groups was significant. As karyorrhexic cells are a marker of cell death, this result may imply that the cell death in the cold group was different than that of other groups. The result of this study is in accordance with other evidences specifying temperaments by distinct biological phenomena as it identified a relationship between the cellular biomarkers and temperaments.

Notably, the present study has two limitations. The first limitation was the small sample size as a result of restricted exclusion criteria, especially dental filling and smoking. Since genotoxic damage increases in the oral mucosa cells of subjects with dental fillings (14), only the subjects with no dental fillings were included in the study; however a majority of people, even at young ages, have dental filling at least for one tooth. Another exclusion criterion was smoking, which reduced the male count in the study. Similar to dental filling, smoking also leads to increased genotoxic damage (12). The second limitation is the number of temperament groups. Since the sample size was small and about 24% participants gathered in the equilibrium group, only few subjects were placed in the other groups. Even though some studies have categorized the temperament into four major groups, which included just combined temperaments (19, 20, 13), this division pattern is inconsistent with ITM (3).

In conclusion, this is the first study to support the view that the cellular properties of individuals are affected by the temperament. Identification of cellular biomarkers of different temperaments may provide a basis to improve our understanding of the biological mechanisms leading to the development of different patterns of temperament; however, this is a preliminary study, and therefore, further research is needed to understand the possible effect of temperament on the cellular properties.

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Conflict of interest

The author declares no conflict of interest.

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