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Artigo

Assessment of effects of a formula used in the traditional Chinese medicine (*Buzhong Yi Qi Wan*) on the morphologic and osmotic fragility of red blood cells

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RESUMO: "Determinação dos efeitos de uma fórmula usada na medicina tradicional chinesa (*Buzhong Yi Qi Wan*) na morfologia a fragilidade osmótica de hemácias". *Buzhong Yi Qi Wan* (BYQW) é uma combinação de algumas ervas medicinais amplamente usada na medicina tradicional chinesa para tratar o sangue, baço e desordens do estômago. A análise morfométrica e o ensaio de fragilidade osmótica têm sido usados para avaliar alterações na integridade da membrana de hemácias. O objetivo deste trabalho foi avaliar os efeitos de um extrato aquoso de BYQW na morfologia e na fragilidade osmótica de hemácias. Amostras sangüíneas foram tratadas com o extrato de BYQW, análise morfológica quantitativa/qualitativa e o ensaio de fragilidade osmótica foram realizados e comparados com grupo controle tratado com salina. Os dados obtidos indicaram ausência de modificações na morfologia, mas o ensaio de fragilidade osmótica sugeriu um aumento significativo ($p<0,05$) da hemólise em hemácias isoladas de sangue tratado com extrato aquoso de BYQW. Em conclusão, o extrato aquoso de BYQW poderia afetar a integridade da membrana diminuindo a resistência osmótica sem alterar a forma das hemácias.

Unitermos: *Buzhong Yi Qi Wan*, fragilidade osmótica, hemácias, morfologia.

ABSTRACT: *Buzhong Yi Qi Wan* (BYQW) is a combination of some medicinal herbs widely used in traditional Chinese medicine to treat blood, spleen and stomach disorders. Morphometric analysis and osmotic fragility assay have been used to evaluate changes on membrane integrity of red blood cells. The aim of this work was to evaluate the effect of an aqueous BYQW extract on the morphology and osmotic fragility of red blood cells. Blood samples were treated with BYQW extract, quantitative/qualitative morphological analysis and osmotic fragility assay were carried out against control groups treated with saline. The data obtained indicated no modification on morphology but osmotic fragility assay suggested a significant ($p<0.05$) increasing of hemolysis in red blood cells isolated from blood treated with aqueous BYQW extract. In conclusion, the aqueous BYQW extract could affect the membrane integrity decreasing the osmotic resistance but without altering the shape of red blood cells.

Keywords: *Buzhong Yi Qi Wan*, morphology, osmotic fragility, red blood cell.

INTRODUCTION

Buzhong Yi Qi Wan (BYQW) is a mixture of some medicinal herbs widely used in Traditional Chinese Medicine to treat blood (circulation), spleen (immunology) and stomach disorders, as peristalsis and digestive process (Wang et al., 2002). This Chinese formula is composed by the herbs: *Radix astragali* (27.8%), *Radix codonopsis* (8.3%), *Radix glycyrrhizae* (14%), *Rhizoma atractylodis macrocephalae* (8.3%), *Radix angelicae sinensis* (8.3%), *Rhizoma cimicifugae* (8.3%), *Radix bupleuri* (8.3%), *Pericarpium citri reticulatae* (8.3%), *Rizhoma zingiberis recens* (2.8%) and *Fructus jujubae* (5.6%) (Tu et al., 1994).

Results have demonstrated that the beneficial effects of BYQW in *miasthenia gravis* as anti-

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inflammatory action (Tu et al., 1994). Another study showed that BYQW is efficient to treat hepatitis-B and cancer (Ji et al., 1989). Du et al. (1993) showed that BYQW has marked effects on hepatic DNA, RNA and protein synthesis. It was considered that the mechanism of anti-hepatitis effects could be related to the enhancing protein synthesis in liver, promoting the repairs of the damaged hepatic tissue and improving the defense function of organism as a whole. Kuroiwa et al. (2004) obtained an increased immunological response against K562 target cells in old patients submitted to BYQW treatment.

Morphometric analysis has been used to evaluate morphological changes induced in different cellular systems as: (i) chronic ocular hypertensive effects on thickness of retinal nerve fiber layer and optic disc structure (Shimazawa et al., 2006), (ii) relationship between miocardiac infarction-related artery stenosis and capillary density (Prech et al., 2006) and (iii) effects of sexual hormones on mammal gland (Pompei et al., 2005). Red blood cells have been proposed as a prototypical cellular system regarding drug mediated plasma membrane effects (Li et al., 1999). Different techniques have demonstrated that therapeutic drugs can modify the structure and morphology of these cells (Nwafor; Coakley, 1986; Scheiman; Elta, 1990; Li et al., 1999; Shacter; Weitzman, 2002; Suwalsky et al., 2003; Hubner et al., 2005; Santos et al., 2005; Zhang et al., 2005). The morphometric analysis (area, shape and volume measurements) has been used to evaluate the alterations induced by natural products and synthetic drugs on membrane of red blood cells (Oliveira et al., 2002; Moreno et al., 2004).

The osmotic fragility assay can be used to verify the membrane integrity of red blood cells treated with drugs (Cinara et al., 2006; Spengler et al., 2007). The "osmotic fragility curve" reflects the structural and geometrical changes on red blood cells due to osmotic treatment. A hemolytic result from a structural disturbance of these cells and in their cytoskeleton was caused by high distribution of the partition coefficient in the membrane (Cavalcanti et al., 2003; Didelon et al., 2000).

As in humans the use of BYQW extract and several of its effects are not well understood yet, the aim of this work was to evaluate the effect of an aqueous extract of BYQW on the morphology and osmotic fragility of red blood cells.

MATERIAL AND METHODS

Animals

Adult male *Wistar* rats (n = 9, 3-4 months, 250-300 g) were maintained in an ambient with controlled light (12 h light/12 h dark) and exhaustion. Food and water were *ad libitum*. The experiments were followed the Ethical Guidelines of the *Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro* with the protocol number CEA/114/2006.

Preparation of BYQW extract

A commercial *Buzhong Yi Qi Wan* (Gansu Medicines & Health Products Import & Export Corporation, validity November 2008) was used in the assays. As indicated by this manufacturer, lyophilized Buzhong was used to prepare this dried powder. In the preparation of the extract, 128 mg of the material was put in a tube with 10 mL of saline solution (NaCl 0.9%) that was gently shaken. This suspension was centrifuged in a clinical centrifuge (3000 rpm, 5 min) and the supernatant was considered to be 12.8 mg/mL. Dilutions of this solution were performed with 0.9% NaCl solution to obtain diluted solutions.

Spectrophotometric measurements

Immediately after the preparation, the optic densities of aliquots of BYQW extract were measured in a spectrophotometer (Analyser 800M, Analyser, Brazil) to obtain the absorbance spectrum and the value at 480 nm was 0.33 ± 0.07. This procedure was carried out as the preparation standard of the extract used in the experimental assays.

Morphology evaluation

Histological preparations were carried out with blood samples *in vitro* treated with BYQW extract at different concentrations during 60 min at room temperature, or with saline solution as control group. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giemsa method (Junqueira; Carneiro, 2004). After that, the images of the red blood cells were acquired (Optronics, USA) from blood smears to qualitative morphology analysis under optical microscopy (x1000, Olympus, BX model, Japan). To morphometric analysis of red blood cells, the perimeter/area ratio was obtained from images by specific program (Image ProPlus Software, media Cibernetics, USA). Five fields per each slide were analyzed.

Osmotic fragility assay

The osmotic fragility evaluations of the red blood cells were performed with blood samples incubated with extract BYQW at higher concentration used (1.28 mg/mL) or with sodium chloride solution (0.9% NaCl) as a control for 60 minutes at room temperature. Briefly, blood samples (100 μL) were gently mixed with different NaCl concentrations (0.12 up to 0.9%) (Dacie; Lewis, 2001). After 30 minutes at room temperature these tubes

were centrifuged (1500 rpm, 15 min). The supernatants were isolated and the optical densities (OD) were determined in a spectrophotometer (Analyser 800M, Analyser, Brazil) at 540 nm. The optical density of each supernatant was compared with that corresponding to 100% lysis (NaCl 0.12%). The supernatant of the tube with NaCl 0.90% was considered the standard tube for the procedure. After, the hemolytic percentage, "fragility curves" were drawn by plotting the percentage of hemolytic (% hemolytic) for each tube (relative to 100% hemolytic tube) and the corresponding NaCl concentrations. According to Cavalcanti et al. (2003), three intervals were determinate: interval I (from 0.12 to 0.36%), interval II (from 0.36 to 0.60%), and interval III (from 0.60 to 0.9%) according the curve tendency.

The means and standard deviations of each interval were determinate and the statistical analysis was performed.

Statistical analysis

The data were presented as media ± standard deviation of perimeter/area ratio and hemolytic percentage. To perimeter/area ratio, the comparison between treated and control groups were performed by one-way ANOVA, followed by the Turkey-Kramer Multiple Comparisons Test. To hemolytic percentage, paired t-test was used to compare the intervals I, II and III between treated and control groups. A significance level at $p<0.05$ was adopted. InStat Graphpad software was used to perform statistical analysis (GraphPad InStat version 3.01 for Windows 95/NT, GraphPad Software, San Diego, USA).

RESULTS

The Figures 1 and 2 represent the photomicrographs of blood smears from samples of whole blood treated with saline (control) and treated BYQW extract at the highest concentration used (12.8 mg/mL). The comparison between these figures indicates that the extract was not capable to induce alterations on morphology of red blood cells.

To confirm the absence of effects on morphology of red blood cells by extract BYQW, morphometric evaluations were carried out. The data of perimeter/area ratio are showed in figure 3 and indicate that no modifications on this morphometric parameter confirming the qualitative evaluations of the blood smear.

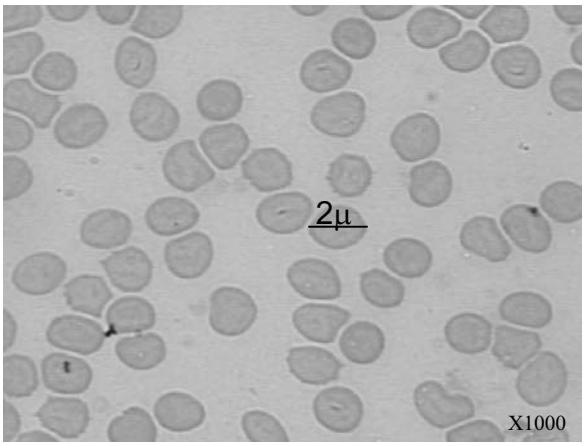
Osmotic fragility was used as another technique to study the effects of BYQW on membrane integrity. Our data suggest that the treatment with BYQW extract could induce significantly ($p<0.05$) the hemolytic at higher concentration used and therefore to increase osmotic fragility of red blood cells when compared to control group (Figure 4). The comparison of the means of hemolytic between blood treated with BYQW extract and control was significant ($p<0.05$) to the interval III (Figure 5).

DISCUSSION

BYQW is a traditional magistral formula composed by different herbs that has been used a long time in the Traditional Chinese Medicine due to its importance to health of human beings and several authors have reported biological effects associated with this formula (Du et al., 1993; Tu et al., 1994; Kuroiwa et al., 2004). The action mechanism related with the extract of BYQW is not fully understood (Cordeiro et al., 2005). The use of different assays could permit to evaluate and to obtain more information about the possible action mechanisms of this secular formula.

Different techniques have been used to evaluate the effects of the interaction between drugs and plasma membrane (Li et al., 1999, Pompei et al., 2005). Qualitative and quantitative morphological analysis have permitted to verify the effects of natural products on membrane of red blood cells (Oliveira et al., 2002; Moreno et al., 2002; Costa et al., 2002; Oliveira et al., 2003; Fernandes et al., 2005; Aquino et al., 2007). The morphological analysis of blood smears suggested no alteration on shape red blood cells from blood treated with BYQW extract in quantitative and qualitative assays (Figures 1, 2 and 3). However, the data obtained from osmotic fragility experimental study have indicated that BYQW extract could alter the membrane integrity at NaCl concentrations near to physiologic level (0.9%). The data from morphological analysis could be useful to understand the use do this magistral formula to treat several disorders (Kiyohara et al., 2006; Shinozuka et al., 2007; Yamaya et al., 2007; Onogi et al., 2006; Tajima et al., 2006; Kanehara et al., 2006). Furthermore, considering the concepts of the Traditional Chinese Medicine, the BYQW would be used to treat blood and spleen disorders (Wang et al., 2002), due to these applications could be associated with the direct production of blood or related with the transformation action of the food by the spleen to aid in the production of blood (Maciocia, 2007). These uses in circulatory, immunology and digestive systems have been studied by various occidental and oriental researches. By the way, the distribution mechanism of energy and compounds food, as well as the blood circulations, probably can be altered by BYQW. The major compound of BYQW is the radix *Astragalus* (radiech) wealthy in saponins and flavonoids, which promote tissue repairs (Du et al., 1993), anothers health effects.

The result obtained by osmotic fragility assay is in according with the antibacterial effects (Yamaoka et al., 1998; Yamaoka et al., 2001; Yan et al., 2002) suggesting that BYQW could act on bacteria cells modifying their membrane integrity.



Figures 1. Photomicrography of blood smears from blood samples *in vitro* treated with NaCl 0.9% solution (control group). Blood smears were stained with May-Grünwald-Giemsa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image capture.

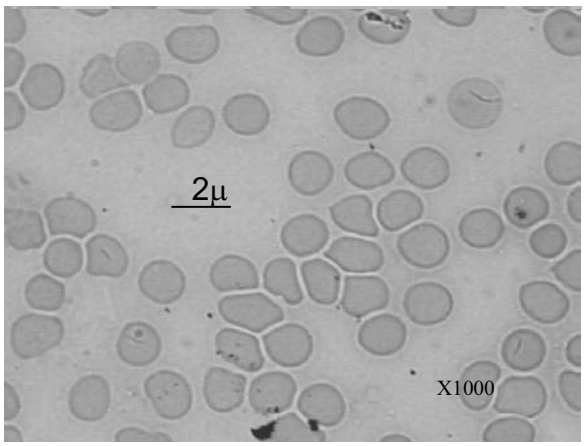


Figure 2. Photomicrography of blood smears from blood samples *in vitro* treated (100%) with BYQW extract. Samples of whole blood from *Wistar* rats were treated with BYQW extract (1.28 mg/mL) during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giemsa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image capture.

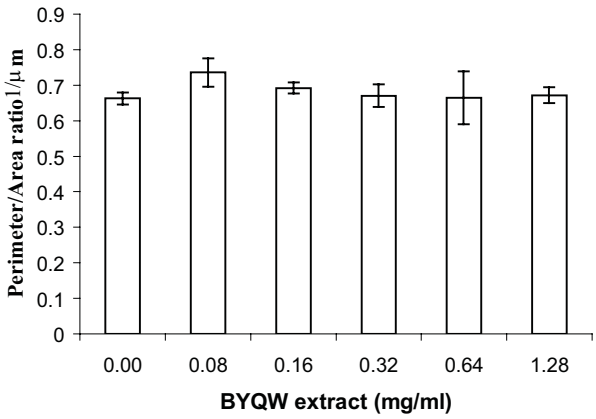


Figure 3. Effects of BYQW extract on the perimeter/area ratio of red blood cells from blood *in vitro*. Samples of whole blood from *Wistar* rats were treated with BYQW extract at different concentrations during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giemsa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after the capture of images in five fields for each smear and five smears for each BYQW extract concentration. After that, morphometric measurements (perimeter and area) were carried out and perimeter/area calculated.

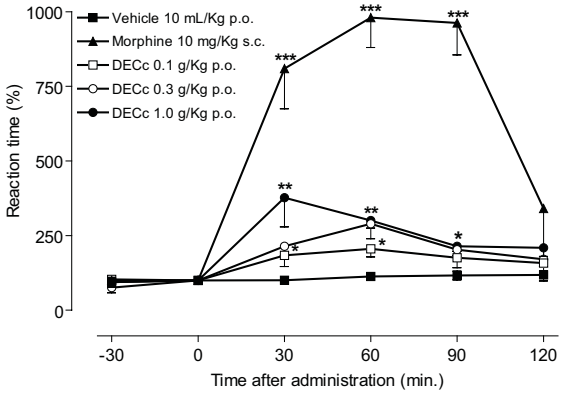


Figure 4. Osmotic fragility of blood samples treated or not treated with BYQW extract) or with sodium chloride solution (0.9% NaCl), as control, during 60 minutes at room temperature. Aliquots of blood (100 μL) were gently mixed with NaCl at different concentrations. After 30 minutes (room temperature) and centrifugation (1500 rpm, 15 min), the supernatants were isolated and the optical densities (OD) were determined in a spectrophotometer at 480 nm. The optical density of each supernatant was compared with that corresponding to 100% lysis (NaCl 0.12%). The supernatant at NaCl 0.9% was considered the standard to the procedure. The hemolytic percentage was calculated and "fragility curves" were drawn plotting the percentage of hemolytic (% hemolytic) for each NaCl concentration (relative to 100% hemolytic tube). (●) control, (■) treated with BYWQ extract.

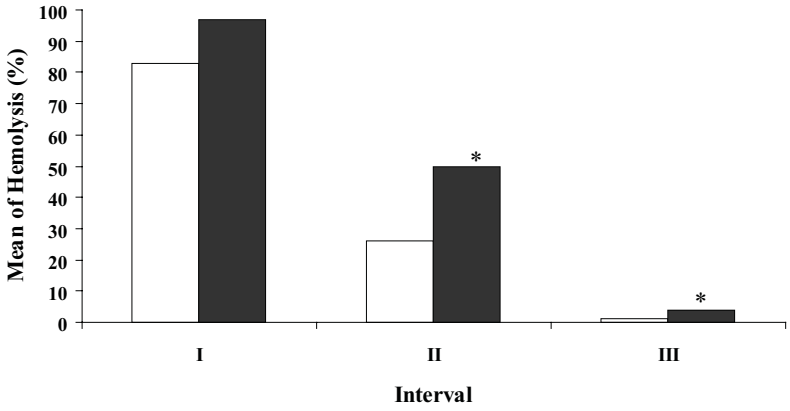


Figure 5. Means of hemolytic grade of the blood samples treated or not treated with BYQW extract. Three intervals were determinate: interval I (from 0.12 to 0.24%), interval II (from 0.24 to 0.48%), and interval III (from 0.48 to 0.9%) according to the curve tendency. The means and standard deviations of each interval were determined and the statistical analysis performed. (□) control bars, (■) treated bars with BYQW extract were statistically compared. (*) $p<0.05$.

In conclusion, probably substances present in aqueous BYQW extract could affect the membrane integrity decreasing the osmotic resistance but without altering the shape of red blood cells indicating that osmotic fragility assay could be more sensitive than the morphology analysis to evaluate effects of BYQW extract on membrane integrity of red blood cells.

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