Rhodiola Rosea as antioxidant in red blood cells: ultrastructural and hemolytic behaviour

M. Battistelli, R. De Sanctis,[1] R. De Bellis,[1] L. Cucchiarini,[1] M. Dachà,[1] P. Gobbi
Istituto di Scienze Morfologiche, [1]Istituto di Chimica Biologica "Giorgio Fornaini",
Università degli Studi di Urbino "Carlo Bo", Urbino (PU), Italy

Rhodiola rosea L. (Crassulaceae) is a plant that lives at high altitude in Europe and Asia, usually used for its high capacity to increase the organism resistance to different stress conditions. Although a few international literature supports these effects, today *R. rosea* has become a common component of many dietary supplements also in the Western world. The aim of the present study was to investigate the effect of the *R. rosea* roots aqueous extract on *in vitro* human erythrocytes exposed to hypochlorous acid (HOCl)-oxidative stress. Several damages occur in human erythrocytes exposed *in vitro* to HOCl, among these membrane protein and lipid modifications, shifting from the discocyte shape to the echinocyte one, and determining lysis ultimately. Therefore, in the present work, the evaluation of the antioxidant capacity of the *Rhodiola* extract has been carried out by means of scanning electron microscopy and of hemolytic behaviour on human erythrocytes exposed to HOCl in the presence of increasing doses of the aqueous extract in different experimental environments (co-incubation and subsequent incubations). The results obtained are consistent with a significative protection of the extract in presence of the oxidative agent, but a cautionary note emerges from the analysis of the data related to the cell exposition to the plant extract in the absence of any induced oxidative stress. In fact, the addition to erythrocyte of high doses of *R. rosea* extract always determines severe alterations of the cell shape.

Key words: rhodiola; erythrocytes; oxidative stress; echinocytes; stomatocytes; sem; hemolysis.

Correspondence: Prof. Pietro Gobbi
Istituto di Scienze Morfologiche,
Campus Scientifico, Loc. Crocicchia
61029 Urbino (PU), Italy
Tel/Fax: +39.0722.304244.
E-mail: pgobbi@uniurb.it

Paper accepted on April 18, 2005

European Journal of Histochemistry
2005; vol. 49 issue 3 (Jul-Sep): 243-254

Rhodiola rosea L. (Crassulaceae), also known as *arctic root* or *golden root,* is a plant living at high altitudes in Europe and Asia. Roots and rhizomes have long been used in the traditional medical system of these geographical areas to increase the organism resistance to different chemical, biological and physical stress (Kelly, 2001). It has also been demonstrated that the administration of *R. rosea* extracts to rats and mice treated with antitumoral drugs such as cisplatin and cyclophosphamide, makes these drugs more efficient and less cytotoxic (Udinstev and Schakhov, 1991; Rege *et al.,* 1999). For all these reasons, today this plant has become an important component of many dietary supplements.

Twenty-eight compounds have been isolated from the roots and above ground-parts of *R. rosea* such as p-tyrosol, organic acids (gallic acid, caffeic acid, and chlorogenic acid) and flavonoids (catechins and proantocyanidins) (Linh *et al.,* 2000). Salidrosides and cinammoyl-glycosides (rosin, rosavin and rosarin, distinctive of the *rosea* species) along with p-tyrosol are responsible for high therapeutic action (Linh *et al.,* 2000).

In the present study the effect of *R. rosea* aqueous extract has been investigated in an *in vitro* model of human erythrocytes exposed to oxidative stress. The oxidative stress condition, defined as a shift in the oxidant-antioxidant balance toward oxidants, is thought to arrange for the onset of many diseases such as atherosclerosis and cancer (Sies, 1985; McCall and Frei, 1999). If the cellular antioxidant defence could not limit the damage, a compromising of a range of cellular functions may occur, leading to pathological change and ultimately cell death (Rice-Evans and Diplock, 1993). Erythrocytes have been used extensively as a model system for investigating mechanisms of oxidative stress since these cells, lacking protein synthesis machinery, represent a simplified model (Vissers *et al.,* 1994; Mawatari and Murakami, 2001).

The erythrocyte has a characteristic biconcave shape really important for all its functions (i.e., deformability, O2 exchange) but also very susceptible to morphological changes with consequent lack of functionality. Among the factors causing morphological modifications, changes in osmolarity, pH conditions and presence of oxidants are described (Bobrowska-Hagerstrand *et al.,* 1998; Lim *et al.,* 2002; Cevc, 2003). Oxidants, in fact, produce alterations in erythrocyte membranes as manifested by a decreased cytoskeletal protein content and production of high-molecular-weight proteins, which can lead to abnormalities in erythrocyte shape and rheologic properties (Flynn et al, 1983; Snyder *et al.,* 1985; Somer and Meiselman, 1993). Furthermore, experiments with erythrocytes incubated with H_2O_2 and ascorbate/Fe^{+2} have shown morphological changes characterised by a dose-dependent increase in echinocyte formation, indicating the role of the oxidative damage in compromising the rheologic behaviour of the erythrocytes (Srour *et al.,* 2000).

In our model system we have utilised hypochlorous acid (HOCl), a powerful oxidant generated by activated phagocytes (neutrophils and monocytes), that is produced by the myeloperoxidase (MPO)-catalysed oxidation of chloride by hydrogen peroxide (Grisham *et al.,* 1984; Zavodnik *et al.,* 2001). Because of the high reactivity toward a large number of biological molecules, HOCl is considered to be a major cause of tissue injury in inflammation (Zavodnik *et al.,* 2001). Several studies have reported HOCl damages on bacteria, endothelial cells, tumour cells and erythrocytes (Weiss and Slivka, 1982; Rosen and Klebanoff, 1985; Tatsumi and Fliss, 1994; Zavodnik *et al.,* 2001). In erythrocytes, it is reported that oxidation of GSH and of -SH groups of membrane proteins occurs at low concentration of hypochlorous acid (Zavodnik *et al.,* 2001); the reaction between HOCl and protein lysine residues yields chloramine species which subsequently decompose giving nitrogen-centered protein radicals (Hawkins and Davies, 1998 a,b); cholesterol and fatty acids are modified by HOCl yielding α- e β-chlorohydrins (Carr *et al.,* 1997). Furthermore, some authors have demonstrated that in human erythrocytes HOCl causes increase of K^+-leak, alteration of membrane deformability leading to echinocyte shape, cross-linking of membrane proteins and extensive disruption of the membrane inducing lysis (Vissers *et al.,* 1998). In a recent work it was demonstrated that *R. rosea* aqueous extract is able to protect human erythrocytes from some of the damages induced by HOCl, i.e. GSH depletion, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inactivation and hemolysis more efficiently than ascorbic acid, a natural scavanger of HOCl (De Sanctis *et al.,* 2004).

The aim of this work is, therefore, to define better the antioxidant activity of the aqueous extract of Rhodiola *rosea* from a morphological point of view, evaluating the ability of the extract to counteract HOCl induced-echinocyte formation.

Materials and Methods

Reagents

Sodium hypochlorite (NaOCl) was purchased by Sigma-Aldrich (Italy). At pH 7.4 this reagent contains approximately 1:1 ratio of HOCl and OCl- and is subsequently referred to as HOCl (Vissers and Winterbourn, 1995). All reagents used for biochemical determinations were purchased by Sigma-Aldrich (Italy) and ICN (Italy). Composition of phosphate buffered saline (PBS) was 0.15 M NaCl, 1.9 mM NaH_2PO4, 8.1 mM Na_2HPO_4, pH 7.4.

Rhodiola rosea aqueous extract

Population, in ancient habits, have always assumed Rhodiola *rosea* as tea of root and rhizomes; for this reason it has been decided to use an aqueous extract, which moreover is more compatible than organic solvents with our experimental cellular context. The *R. rosea* roots and rhizomes were obtained by Botanic Garden *Chanousia* (Aosta, Italy). A threefold extraction of 100 mg of dry powder of *R. rosea* rhizomes with 2.4 ml final volume of distilled water at room temperature was done, obtaining a final proportion of 41.6 mg/mL. The obtained aqueous phase was evaporated under vacuum, dissolved in the same volume of PBS and incubated with erythrocytes in the presence or absence of HOCl. All experiments were performed using the same batch.

Blood samples

Erythrocytes were obtained from healthy consenting donors. Heparinazed blood was centrifuged at 3,000 rpm for 10 min. at 4°C. After removal of plasma and buffy coat, the erythrocytes were washed three times with cold PBS and processed for subsequent analyses.

Incubation experiments

All incubation experiments were performed in a shaking bath for 15 min. at 37°C with erythrocytes 10% v/v in PBS. After 15 min. of each incubation, an aliquot of erythrocyte suspension was centrifuged at 3,000 rpm for 10 min. at 4°C, washed one time with PBS, and treated as reported below for the evaluation of hemoglobin and for the morphological observation.

1- Incubation of erythrocytes with HOCl

Erythrocytes were incubated with 0.5 mM HOCl added as a single bolus of a diluted solution in PBS, which concentration was determined spectrophotometrically at 292 nm (ε=350 M^{-1} cm^{-1}) (Morris, 1966).

2- Co-incubation of erythrocytes with R. rosea extract and HOCl

Erythrocytes were incubated with increasing doses of *R. rosea* extract (10-25-50-100-150-200 μL) and with 0.5 mM HOCl at the same time. The incubation was carried on as previously described.

3- Pre-incubation of erythrocytes with R.rosea extract and subsequent HOCl treatment

Erythrocytes were pre-incubated with different aliquots of *R. rosea* extract (10-25-50-100-150-200 μL) for 10 min. in a shaking bath at 37°C. After centrifugation at 3,000 rpm for 10 min. at 4°C, the supernatant was discarded and packed erythrocytes were resuspended in PBS in the presence of 0.5 mM HOCl. The incubation was carried on as previously described.

4- Incubation of erythrocytes with pure R. rosea extract

Erythrocytes were incubated with increasing doses of *R. rosea* extract (10-25-50-100-150-200 μL). The incubation was carried on as previously described.

Determination of osmolarity and pH condition

The osmolarity of the incubation medium was measured with an automatic osmometer (Roebling). In all the experimental conditions osmolarity and pH similar to control sample were obtained.

Evaluation of hemolysis

After incubation experiments, erythrocyte suspension was centrifuged at 3,000 rpm for 10 min. and the supernatant used for haemoglobin determination. Hb was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler (1975) The hemolysis rate was determined by the evaluation of the haemoglobin (Hb) released by the erythrocytes in the supernatant in respect to the total Hb amount. Erythrocyte hemolysis in control sample was evaluated as 0.6±0.14%.

Statistical analyses and SEM morphological evaluation

Statistical analyses were performed using Kruskal-Wallis test. All data are expressed as mean ± S.D. of at least five different determinations.

The morphological alterations were evaluated by three different observers, in a separate observation session, on at least 10 frames at the same magnification for each field. It has been selected central area of the specimens on which erythrocytes appeared homogeneously distributed in the absence of evident artificial aggregates induced by poly-L-lysine.

Scanning Electron Microscopy (SEM)

For SEM analyses, cells were washed and fixed in suspension with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 1hr. They were quickly washed in 0.15 M phosphate buffer pH 7.3 and drops of the suspension were deposited on poly-L-lysine-coated coverslips as previously described (Falcieri *et al.,* 1988). The adhesion was carried out overnight in a moist and sealed chamber at 4°C. The slides were then washed and postfixed with 1% OsO_4 in the same buffer for 1hr. A gentle progressive alcohol dehydration was performed and specimens were critical point-dried. After mounting on conventional SEM stubs by means of silver glue, slides were gold-coated by a sputtering device. Observations were carried out with a Philips 515 scanning electron microscope.

Results

1- HOCl treated samples and control

Control and treated samples are observed at SEM level in order to evaluate the erythrocyte morphological aspects in the different experimental conditions. Untreated normal erythrocytes show (Figure 1A; Table I.1) the typical biconcave discoid shape (discocyte) with a smooth membrane outlined by a round profile. Not particularly evident is

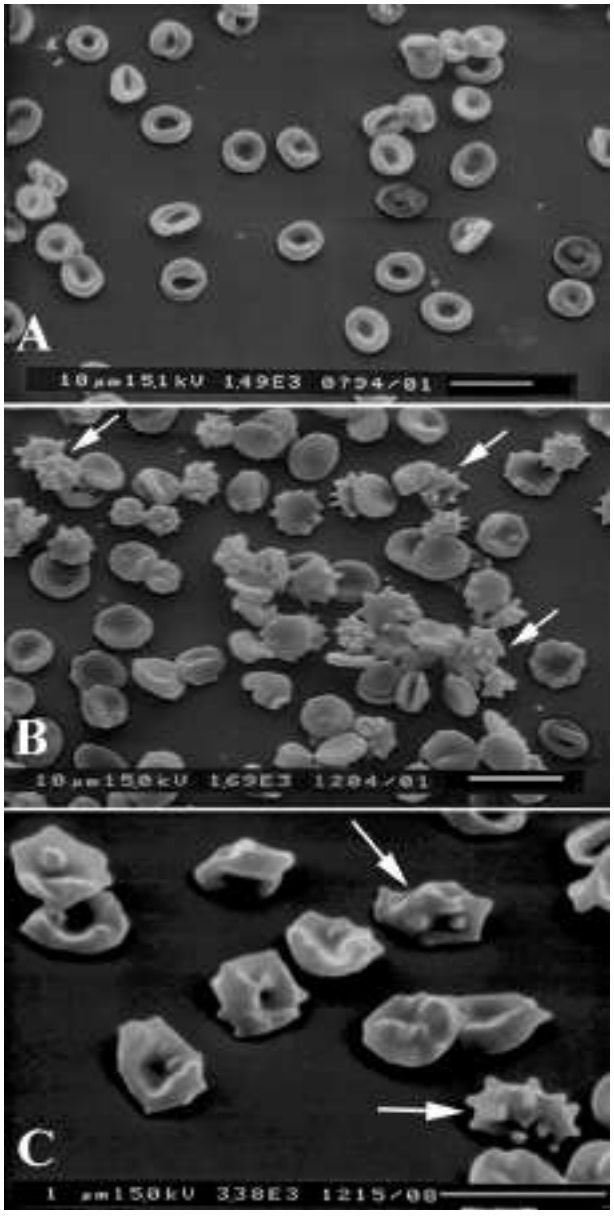

Figure 1. SEM images of control and HOCl treated samples. 1A: control erythrocytes (x 1,490). 1B: erythrocytes incubated for 15 min. with 0.5 mM HOCl (x 1,690). Arrows indicate some altered erythrocytes. 1C: higher magnification (x 3,380) with arrows indicating echinocytes.

the cell aggregation and a spontaneous hemolysis corresponds to 0.6±0.14 % (Figure 2:Δ).

In the first experiment (oxidant only), samples were exposed to 0.5 mM HOCl (Figure 1B; Table 1.1). 75±5% of total erythrocyte population shows morphological alterations. Most of the altered cells presents a deeply crenated shape (echinocyte) (Figure 1C), on which diffuse spoke-like membrane protrusions or convex rounded protuberances are regularly present. 7.1±0.85% hemolysis is observed in this experimental condition confirming pre-existing data (Vissers *et al.,* 1998; De Sanctis *et al.,* 2004) (Figure 2:▲).

2- Co-incubation

In the second experiment (co-incubation), in which the oxidant was simultaneously incubated with increasing doses of *R. rosea* aqueous extract, different ultrastructural features of erythrocytes coming from *R. rosea* addition can be observed. In the samples incubated with 0.5 mM HOCl together with 10 and 25 μL of the extract, about 90% of erythrocytes with a crenated shape, similar to the samples of the previous experiment, is shown (Figure 3A-B; Table I.2). In the sample incubated with 50 μL of extract (Figure 3C; Table I.2) a reduction of altered cells up to a 55±5% of the whole population can be detected. The altered shapes are now represented not only by echinocytes but also by stomatocytes, cells with deep concave invaginations of the surface; at this experimental step, the ratio between echinocytes and stomatocytes is about 3 to 1 (Table I.2).

After incubation with 0.5 mM HOCl and 100 μL of extract (Figure 3D; Table I.2), an inversion of the echinocyte-stomatocyte ratio (1 to 3, Table I.2) can be described, as well as a decrease of the total amount of erythrocytes on the specimen stand. Few cnizocytes, cells with multiple deep concave invaginations of the surface, are also visible.

Further reductions in cell shape alteration can be observed after incubation with 0.5 mM HOCl and 150 μL of the extract (Figure 3E; Table I.2). In fact, at this condition about 30% of erythrocytes is mainly in the stomatocyte form. Small cell clusters are easily shown and the amount of cells on the stand seems to be reduced in respect to the control.

A remarkable effect is evident after incubation of 0.5 mM HOCl with 200 μL of the aqueous extract (Figure 3F; Table I.2). At this stage less than 20% of erythrocytes appears with the stomatocyte shape. During observation of this sample, it is difficult to find homogeneously distributed erythrocytes: in fact they appear in minor amount and aggregated in small clusters.

In co-incubation experiments a dose-dependent effect of Rhodiola extract is observed vs. HOCl-induced hemolysis. In fact, as shown in Figure 2:-•- the hemolysis ratio progressively decreases increasing the extract dose. A significant protection was already evident with 25 μL of the extract, while the

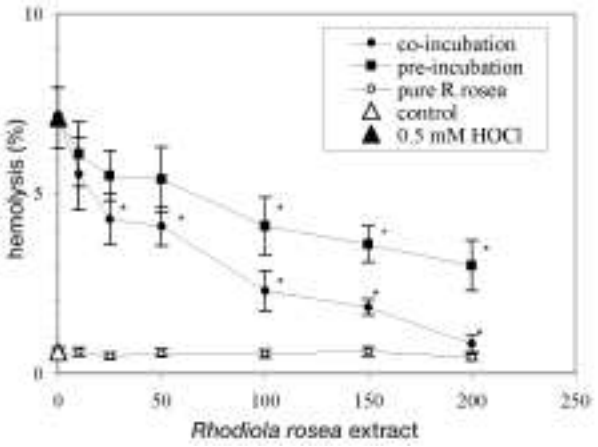

Figure 2. Hemolysis data in the different experimental environments: Data are the mean +/- SD of five different determinations. *p<0.05 vs. HOCl.

addition of 200 μL results in the minimal hemolysis (0.83±0.24%), similar to the control sample (0.6±0.14%).

3- Pre-incubation

In the third experiment (pre-incubation) the samples are preliminarily treated with rising doses of *R. rosea* extract and afterwards, subsequent to extract washing, with the oxidant.

The samples exposed to 0.5 mM HOCl after the pre-incubation with 10 μL and 25 μL of the antioxidant, reproduce morphological effects similar to the previous experiment of co-incubation with the same amount of the extract, presenting about 90 % of whole erythrocytes the echinocyte shape alteration (Figure 4A-B; Table I.3). It has to be noted that, starting from the addition of 25 μL of the extract, the total amount of cells present on the specimen holder, still remains abundant, but erythrocytes appear to be widely scattered throughout the stand, with a reduced tendency to the aggregation. Similar features are shown In the sample exposed to 0.5 mM HOCl after pre-incubation with 50 μL of the extract (Figure 4C): a slight reduction, of about 10 %, of altered echinocyte shape has to be mentioned (Table I.3). In the sample exposed to 0.5 mM HOCl after pre-incubation with 100 μL of extract, a smooth crenated outline is evident on 65±5% of erythrocytes (Figure 4D; Table I.3) similar to echinocytes in a precocious step of alteration. Just few stomatocytes are present (about 2%). A similar amount of affected erythrocytes is observable in the sample exposed to 0.5 mM HOCl after pre-incubation with 150 μL of

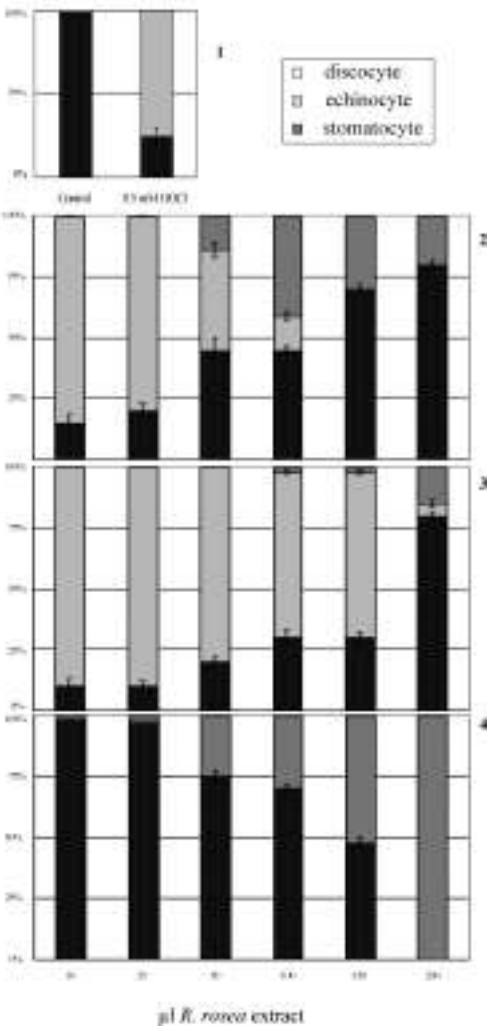

Table I. Erythrocyte shape behaviour in the different experimental environments: I.1.: control and 0.5 mM HOCl; I.2: co-incubation experiment; I.3: pre-incubation experiment ; I.4: pure R. rosea extract.

Rhodiola (Figure 4E; Table I.3). The altered cells are mainly echynocytes / smooth crenated cells. The stomatocyte shape is about 2%. In the sample exposed to 0.5 mM HOCl after pre-incubation with 200 μL of extract only 20 % of erythrocytes shows alterations in cellular profile (Figure 4F; Table I.3); 15 % of modified cells presents a stomatocyte shape, while 5% assumes an undulated and crenated outline. As mentioned above, at this experimental point erythrocyte aggregation is not widely detectable. Hemolysis data of the pre-incubation experiments confirm the *R. rosea* dose-dependent protective effect observed in the previous co-incubation experiment (Figure 2: -■-), but minor efficacy is noted at various doses of the extract, in fact a significant protection against HOCl hemolytic effect is evident starting from the addition of 100 μL of *R. rosea* extract.

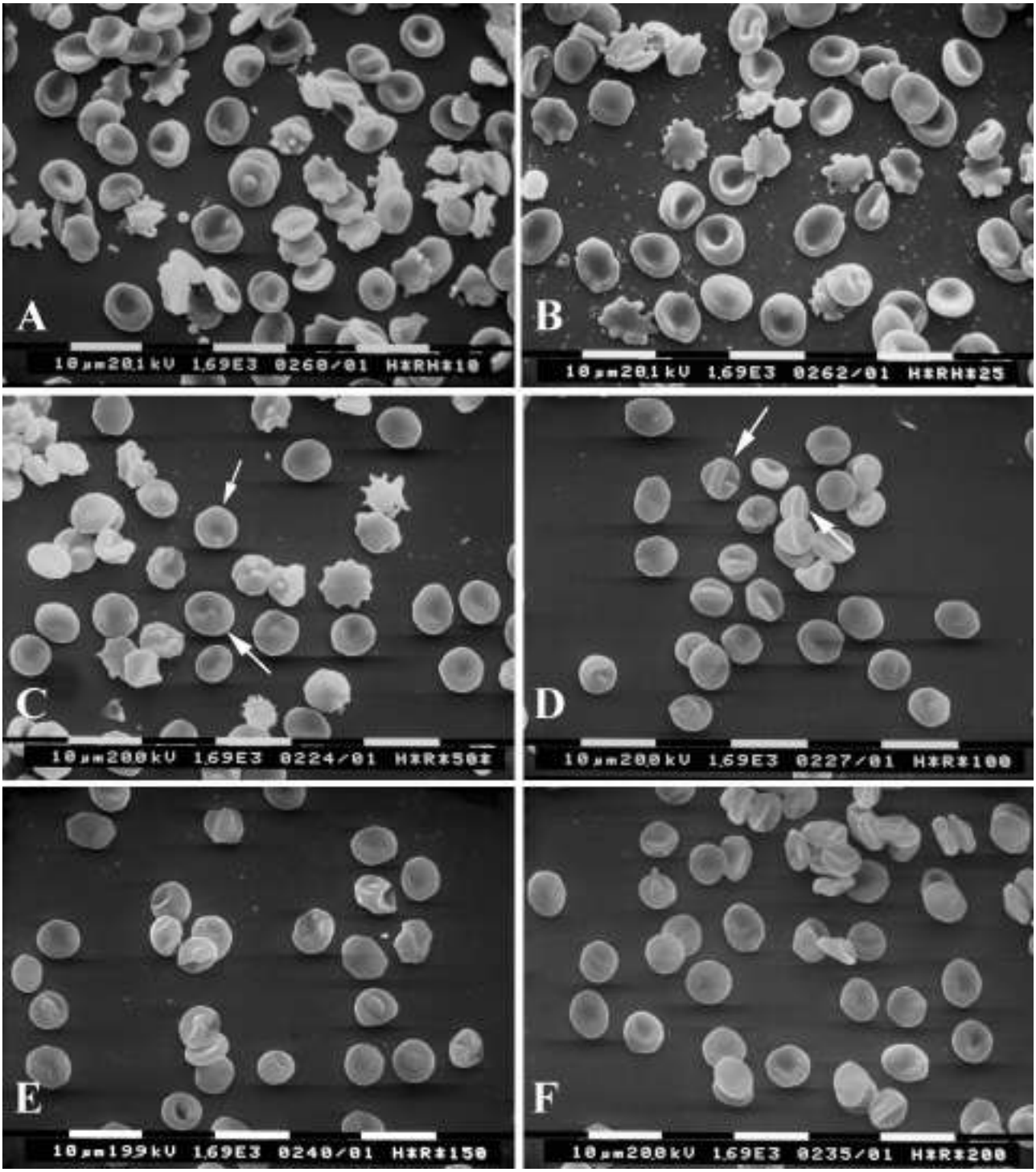

Figure 3. Erythrocytes co-incubated with 0.5 mM HOCl and increasing doses of R. rosea aqueous extract at the SEM analysis (x 1,690). 2A: 10 µL R. rosea extract; 2B: 25 µL R. rosea extract; 2C: 50 µL R. rosea extract. Arrows indicate stomatocytes; 2D: 100 µL R. rosea extract. Few cnizocytes are evident (arrows); 2E: 150 Ìl R. rosea extract; 2F: 200 µL R. rosea extract; small cell clusters are evident.

4- Pure *R. rosea* treated samples.

In the fourth experiment (pure extract), samples have been incubated with the aqueous extract of *R. rosea* only, and a different morphological behaviour, increasing the doses of the extract, has been noted.

The addition of 10 µL of *R. rosea* extract to erythrocytes (Figure 5A; Table I.4) shows an ultrastructure completely superimposable to the control

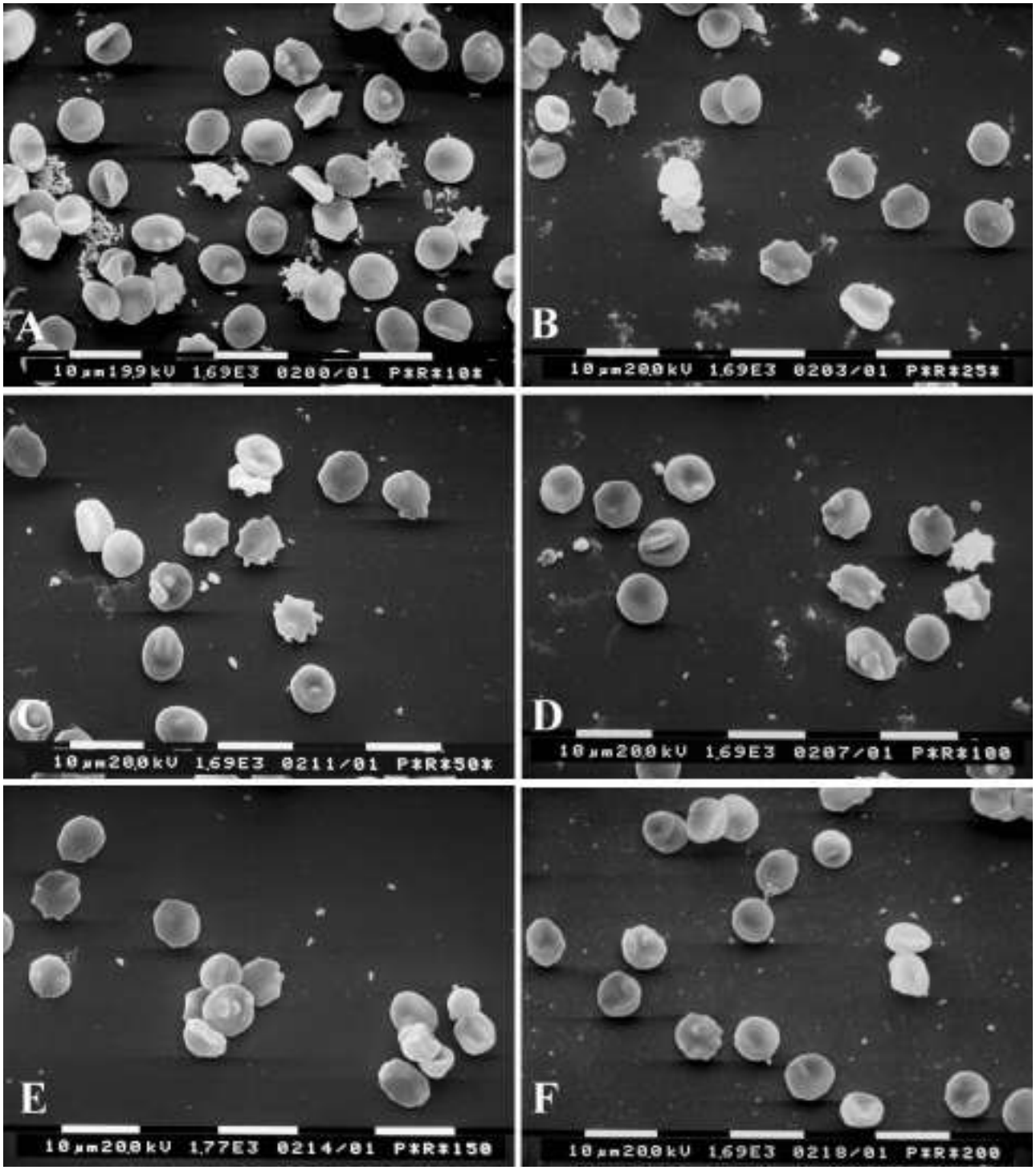

Figure 4. SEM pictures of erythrocytes preliminarily treated with increasing doses of *R. rosea* extract and afterwards, subsequent to extract washing, with 0.5 mM HOCl (pre-incubation). 3A: 10 µL *R. rosea* extract (x 1,690); 3B: 25 µL *R. rosea* extract (x 1,690); 3C: 50 µL *R. rosea* extract (x 1,690); 3D: 100 µL *R. rosea* extract (x 1,690); 3E: 150 µL *R. rosea* extract (x 1,770); 3F: 200 µL *R. rosea* extract (x 1,690).

samples, both in terms of morphology and of distribution on the stand. After incubation with 25 µL of the extract, a small number of erythrocytes (about 3 %) shows a smooth undulated outline and, at the same time, the majority of erythrocytes appears similar to control cells (Figure 5B; Table I.4). After 50 µL of extract addition, 25% of erythrocytes appears as stomatocytes (Figure 5C; Table I.4). An

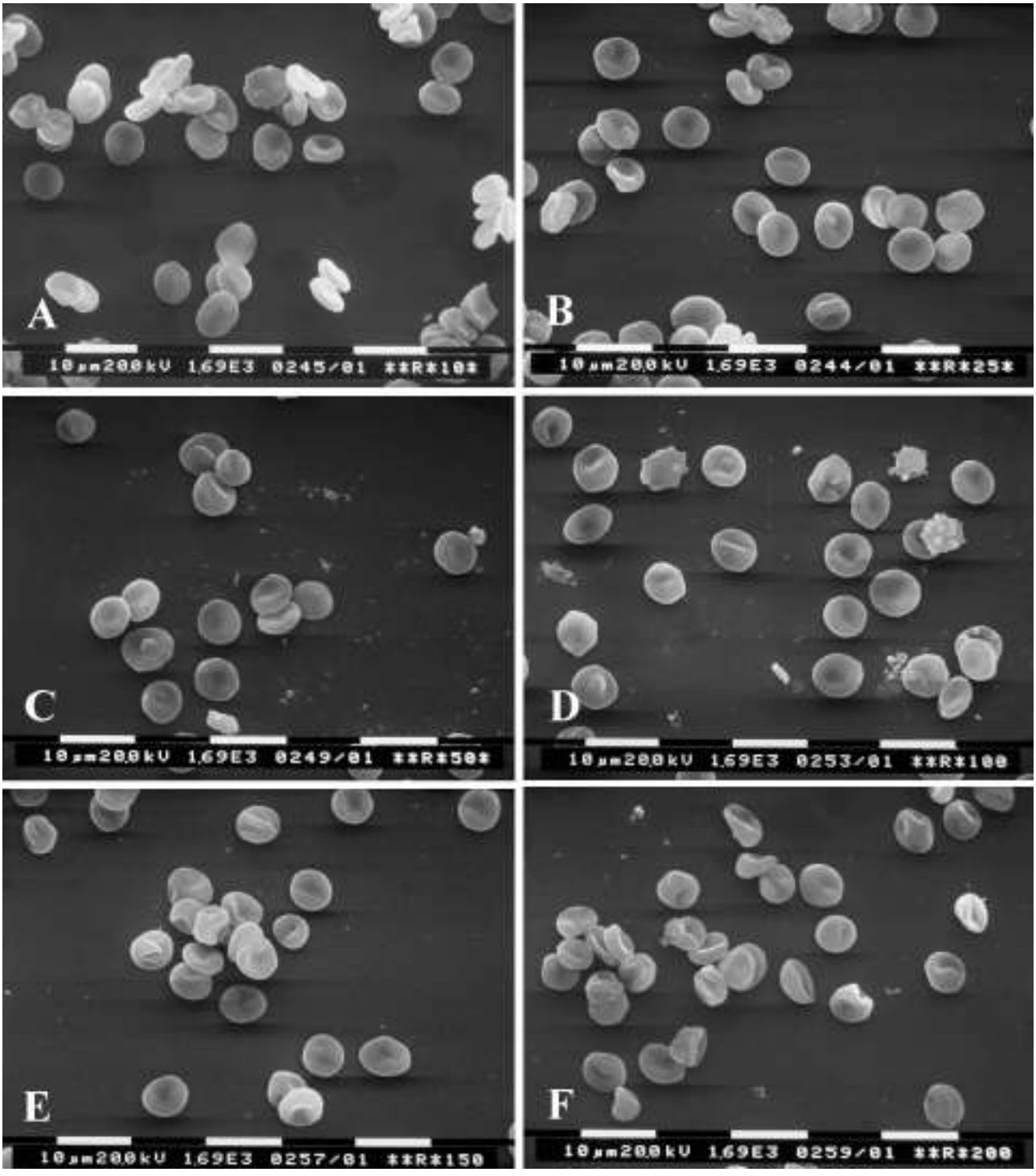

Figure 5. Erythrocyte incubation with increasing doses of pure R. rosea aqueous extract at the ultrastructural analysis (x 1,690). 4A: 10 µLv R. rosea extract; 4B: 25 µL R. rosea extract; 4C: 50 µL R. rosea extract; 4D: 100 µL R. rosea extract; 4E: 150 µL R. rosea extract; 4F: 200 µL R. rosea extract.

erythrocyte rarefaction seems to occur. Incubation with 100 µL of Rhodiola *rosea* extract induces alterations in about 30 % of erythrocytes, most of which shows a stomatocyte-cnizocyte form, while the remaining are represented by cells with crenated profile. Only few echinocytes are occasionaly present (Figure 5D; Table I.4). It has been detected an increasing of altered shapes in sample incubated with 150 µL extract. In fact, more than 50% of erythrocytes presents a stomatocyte-cnizocyte

profile, whereas echinocytes are not present (Figure 5E; Table I.4). As previously described, even at this condition a remarkable increasing in cell mutual adhesion and a decreasing in total amount of cells on the specimen has to be reported. With the highest addition of the extract (200 μL) the 100% of erythrocytes loses any discocyte profile, becoming stomatocytes or cnizocytes, often aggregates. (Figure 5F; Table I.4). Hemolysis data, at increasing doses of the extract, have a mean value of 0.5±0.12 (Figure 2: -∘-), very similar to control sample.

Discussion

In the present study, the antioxidant effects of the aqueous extract of Rhodiola *rosea* roots and rhizomes have been evaluated on human erythrocytes exposed to hypochlorous acid-induced oxidative stress utilising four different environments at different oxidative strength in presence or absence of the extract; nevertheless the electron microscopy has demonstrated morphological alterations induced by the extract. Human erythrocytes are a reliable and easily obtainable model to detect oxidative stress; their own very simple internal structure, depleted of nucleus and organelles, offers an ideal environment not affected by complex and renewable buffer system in which any cause-effect relationship can be clearly shown. For these reasons, several papers report the use of erythrocytes to study oxidative stress and, in particular, the effects of hypochlorous acid, even in the presence of different kind of antioxidant (Vissers *et al.,* 1994; Vissers and Winterbourn, 1995; Vissers *et al.,* 1998; Zavodnik *et al.,* 2001; De Sanctis *et al.,* 2004). Hypochlorous acid is reported as capable to oxidize GSH and SH groups of membrane proteins (Zavodnik *et al.,* 2001), to indirectly form nitrogen-centered protein radicals from chloramine species (Hawkins and Davies, 1998), to interfere with cholesterol and fatty acids, yielding chlorohydrins (Carr *et al.,* 1997) and to cause potassium leak and cell lysis due to alteration of membrane elasticity and cross-linking of membrane proteins (Vissers *et al.,* 1994; Visser *et al.,* 1998). Even the inactivation of glyceraldehyde-3-phosphate dehydrogenase, Na^+/K^+-, Ca^+/Mg^+-ATPase are reported as effect of the interaction between even low doses of HOCl and erythrocytes (Schraufstatter *et al.,* 1990; Zavodnik *et al.,* 2001, De Sanctis *et al.,* 2004). 0.5 mM HOCl has been used on erythrocytes in the first trial; the same oxidant but with contemporary addition of increasing doses of aqueous extract of Rhodiola *rosea* (from 10 to 200 μL) has been utilised in the second trial (co-incubation). A pre-incubation with increasing doses of the extract and a following oxidative stress has been carried out on the third experiment and, finally, only the aqueous extract at different doses in the fourth experiment (pure extract). The choice of the amounts of extract and HOCl applied in the experiments was due to preliminary studies (Vissers et al 1995, De Sanctis *et al.,* 2004): 0.5 mM HOCl is in fact consistent with an oxidative stress which impairs some cellular functions without being too harmful to loose any possible recovery of these damaged functions.

Therefore, SEM analysis, in association with hemolysis evaluation of the different environments, underlines interesting and stimulating behaviours of erythrocytes. In fact, the first experiment confirmed the role of HOCl as strong oxidant agent: the echinocyte, pathological erythrocyte shape with its characteristic spoke-like membrane protrusions, represents an useful, repetitive and reproducible morphological precocious sign of oxidative stress (Lim *et al.,* 2002) that often, together with the membrane protein clustering and spectrin alterations, may precede the cell lysis (Vissers *et al.,* 1998).

The difference between the percentage of modified cells detected at the ultrastructural level (about 70%) and hemolysis data (7.1%) after 15 min. of HOCl incubation only, underlines two important aspects of our technical approach. The first is the appropriateness of the oxidative stimulus, strong enough to alter most of the cells but not enough to disrupt all of them. This aspect can be explained with a different cell resistance to the stress, due to the different age of the erythrocytes and consequently, due to a progressive missing of intracellular buffer systems. The second one is that SEM analysis may represent a sensitive tool to detect precocious damages even in cells where, ceasing the stimulus and thanks to more abundant endogenous antioxidant capabilities, a reversion to a physiological status, can be supposed.

According to similar assumptions, the second experiment (co-incubation) clearly indicates a positive dose-dependent antioxidant activity of the aqueous extract of *R. rosea*. It has to be considered

the mainly morphological approach of the present study in which protective effects have been evaluated as cell shape preservation. A constant confirmation of the ultrastructures from echinocytes to the normal shape, via stomatocytes, comes from the data of decreasing in hemolysis ratio (from 5.5 to 0.8%) while increasing the extract addition from 10 to 200 µl to the medium.

A similar result is detectable in the third experiment (pre-incubation). The positive dose-dependent effect of the aqueous extract is evident, but two main differences have to be pointed out: i) a preponderance of echinocytes in respect to stomatocytes, mainly presented in the co-incubation experiment; ii) the hemolysis curve presents higher values of damage at the same doses of the co-incubation experiment. Both the evidences of a minor antioxidant efficacy have to be correlated to the procedural differences between the two experiments. In fact, in the co-incubation experiment a protective effect of *R. rosea* extract can be supposed both in the intracellular as well as in the extracellular environment. On the contrary, in the pre-incubation experiment, being absent the extract during the HOCl exposition, a mere intracellular protection of the internalized antioxidant has to be hypothesize. In spite of that, the hemolysis rate, significatively decreased by extract addition, underlines an important intracellular activity. This demonstration and the presence of pathological stomatocytes after high *R. rosea* doses addition suggest a possible direct role of *R. rosea* extract on erythrocyte alteration. This hypothesis is confirmed by the fourth experiment (pure extract). In fact, in this last experiment it has to be noted that at the higher doses, 100% of erythrocytes showed morphological changes in the absence of the oxidant. Stated that echinocytes are the morphological sign of oxidative stress (Lim *et al.,* 2002), in our opinion, stomatocytes may be considered the sign of an "anti-oxidative" stress. While echinocytes can be interpreted as a premonitory sign of a hemolytic event, our hemolysis data suggest that not the same can be said about the stomatocyte shape, being 0.6 % only the hemolysis of the extract even at the maximal dose. The cnizocyte shape, detectable in the presence of the extract highest amount may represent a deeper alteration of the stomatocyte, due to the same causes and with the same hemolytic behaviour (Rosati and Colombo, 2003).

These findings agree with the fact that several antioxidants can express a prooxidant activity. For example, while antioxidants from various herbal medicines support a positive role in human nutrition and disease prevention, it is of note that an *in vitro* prooxidant activity was also observed for cytotoxic and proapoptotic effects (Ismail and Alan, 2001; Ueda *et al.,* 2002; Fedeli *et al.,* 2004). Moreover, a mutagenic effect related to flavonoid-mediated oxidative damage was also described (Yamashita *et al.,* 1999; Yoshino *et al.,* 1999) and it is suggested that the same structural features determining an antioxidant activity, i.e. a high number of hydroxyl groups, could be responsible for the prooxidant behaviour (Cao *et al.,* 1997).

A wide family of agents, drugs and substances has been described as *stomatogenics* (Hagerstrand *et al.,* 2000, Van der Heide and Poolman, 2000; Lim *et al.,* 2002), including chlorpromazine, cationic amphipaths, and also conditions of low salt, low pH and cholesterol depletion (Bobrowska-Hagerstrand, 1998; Lim *et al.,* 2002) and one or more agents could be present in the extract in order to induce stomatogenesis. Low salt or pH and the presence of chlorpromazine can be excluded because of the controlled experimental conditions. Even if the cholesterol depletion can not be completely excluded, it has to be considered that cholesterol depletion represents a preliminary way to cell lysis (Lim *et al.,* 2002) and this aspect does not agree with our hemolysis data. A more probable hypothesysis is the presence of cationic amphipats that could be implicated in stomatogenesis of our model. In fact, this category of molecules, including surfactants, (co)solvents, such as certain alcohols etc. (Cevc, 2003), could explain also other evidences of our experiments. In fact, in the presence of high amounts of *R. rosea* (co-incubation and pure extract trials) a relevant tendency to the reciprocal cell adhesion has been noted and it is known that cationic reagents can interfere with membrane polysaccharides and proteins causing erythrocyte adhesion (Nishiguchi *et al.,* 1998). In our opinion, this could explain the tight relationship between the dose-dependent shape alteration and the dose-dependent cell bond as well as the behaviour of erythrocytes undergoing to poly-L-lysine adhesion during SEM preparation. The data of low hemolysis suggest a constant erythrocyte dilution in the buffer. Therefore, a lowest amount of cells detectable at the SEM level in samples treated with high doses of the extract, has to be related to an

alteration of cell adhesion to the specimen stand. It is well known that the poly-L-lysine cover glass cell adhesion represents a purely electrostatic interaction between the poly cationic on the glass surface and the cell to bind (Canesi *et al.,* 2004); a disturbance in such process can be therefore obtained adding to the cell surface a relevant amount of cations, as probably in our experiment occur.

Studies are in progress in our labs in order to precisely identify the compound(s) responsible for the described effects and to better understand the modifications of the erythrocyte membrane at the cytoskeletal level.

In conclusion, considering that concentrated extracts of antioxidant-rich plants such as propolis, pine bark, green tea leaves, soy and grape seed are widely and superficially marketed as uncontrolled dietary supplements, caution should be taken in relation to the dose administered. Is our opinion that, such nutraceuticals, whose active compounds should be considered properly as drugs, in absence of certain scientific evidences, have to be care

fully administered.

Acknowledgements

We would like to thank Umberto Mancini for the technical skillful assistance and the staff of the *Centro Trasfusionale* of the Urbino Hospital for kindly providing blood samples from human volunteer healthy donors.

References

Beutler E. "Red Cell Metabolism. A manual of Biochemical Methods." Ed. Grune & Straton: New York; 1975.

Bobrowska-Hagerstrand M, Hagerstrand H, Iglic A. Membrane skeleton and red blood cell vesciculation at low pH. Biochem Biophys Acta 1998; 123-8.

Canesi L, Ciacci C, Betti M, Lorusso LC, Marchi B, Burattini S, Falcieri E, Gallo G. Rapid effects of 17beta-estradiol on cell signaling and function of Mytilus hemocytes. Gen Comp Endocrinol 2004; 136:58-71.

Cao G, Sofie E, Prior RL. Antioxidant and prooxidant behaviour of flavonoids: structure-activity relationships. Free Rad Biol Med 1997; 22:749-60.

Carr AC, Van der Berg JJ, Winterbourn CC. Chlorination of cholesterol in cell membrane by hypochlorous acid. Archives of Biochemistry and Biophysics 1996; 332:63-9.

Carr AC, Vissers MCM, Domigan NM, Witerbourn CC. Modification of red cell menbrane lipids by hypochlorous acid and hemolysis by preformed lipid chlorohydrins. Redox Reports 1997; 3:263-71.

Cevc G. Lipid vescicles and other colloids as drug carriers on the skin. Advanc Drug Deliv Rev 2004; 56:675-711.

De Sanctis R, De Bellis R, Scesa C, Mancini U, Cucchiarini L, Dachà M. *in vitro* protective effect of Rhodiola *rosea* extract against hypochlorous acid-induced oxidative damage in human erythrocytes. BioFactors 2004; 20: 147-159.

Falcieri E, Mariani AR, Del Coco R, Facchini A,. Maraldi NM. A high yield technique for freeze-fracturing of small fractions of isolated cells. J Submicrosc Cytol Pathol 1988; 20:623-7.

Fedeli D, Berrettini M, Gabryelak T, Falcioni G,. The effect of some tannins on trout erythrocytes exposed to oxidative stress. Mutata Res 2004; 563, 89-96.

Flynn TP, Allen DW, Johnson GJ, White JG. Oxidant damage of the lipids and proteins of the erythrocyte membranes in unstable hemoglobin desease. J Clin Invest 1983; 71:1215-23.

Grisham MB, Jefferson MM, Thomas EL. Role of monochloramine in the oxidation of erythrocyte haemoglobin by stimulated neutrophils. J Biol Chem 1984; 259:6757-65.

Hagerstrand H, Danieluk M, Bobrowska-Hagerstrand M, Iglic A, Wrobel A, Isomaa B, Nikinmaa M. Influence of band 3 protein absence and skeletal structures on amphiphile-and Ca2+-induced shape alterations in erythrocytes: a study with lamprey (Lampetra fluviatilis), trout (Onchorhynchus mykiss) and human erythrocytes. Biochem Biophys Acta 2000; 1466:125-38.

Hawkins CL, Davies MJ. Hypochlorite-induced damage to proteins: formation of nitrogen-centered radicals from lysine residues and their role in protein fragmentation. J Biochem 1998a 332:617-25.

Hawkins CL, Davies MJ. Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmantation reactions of nitrogen-centered radicals. Journal of Chemical Society 1998b; 2:1937-45.

Ismail N, Alani M. A novel cytotoxic flavonoid glycoside from Physalis angulata. Fitoterapia 2001; 72:676-9.

Kelly GS. Rhodiola *rosea*: a possible plant adaptogen. Altern Med Rev 2001; 6:293-302.

Lim GHW, Wortis M, Mukhopadhyay R. Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: Evidence for the bilayer-couple hypotesis from membrane mechanics. PNAS 2002; vol. 99, 26:16766-9.

Linh PT, Kim YH, Hong SP. Quantitative determination of salidroside and tyrosol from the underground part of Rhodiola *rosea* by high performance liquid chromatography. Arch Pharm Res 2000; 23:349-52.

Mawatari S, Murakami K. Effects of ascorbate on membrane phospholipids and tocopherols of intact erythrocytes during peroxidation by t-butylhydroperoxide: comparison with effects of dithiothreitol. Lipids 2001; 36:57-65.

McCall MR, Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans? Free Rad Biol Med 1999; 26:1034-53.

Morris JC. The acid ionisation of HOCl from 5 degree to 35 degree. J Phys Chem 1966; 70: 3798-805.

Nishiguchi E, Okubo K, Nakamura S. Adhesion of human red blood cells and surface charge of membrane. Cell Struct Funct 1998; 23:143-52.

Rege NN, Thatte UM, Dahanukar SA. Adaptogenic properties of six rasayana herbs used in Ayurvedic medicine. Phytother Res 1999; 13:275-91.

Rice-Evans CA, Diplock AT. Current status of antioxidant therapy. Free Rad Biol Med 1993; 15:77-96.

Rosati P, Colombo R. La Cellula. Edi-ermes, Milano, 2003 pp.139-42.

Rosen H, Klebanoff S. Oxidation of microbial iron-sulfur centers by the myeloperoxidase-H2O2-halide antimicrobial system. J Infect Immun 1985; 47:613-8.

Sies H. Oxidative stress: introductory remarks. In: Sies H, ed. Oxidative stress. Academic Press: New York, 1985, pp. 1-8.

Snyder LM, Fortier NL, Trainor J, Jacobs J, Leb L, Lubin B, et al. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglin cross-linking. J Clin Invest 1985; 76:1971-7.

Somer T, Meiselman HJ, Disorders of blood viscosity. Ann Mad 1993; 25:31-9.

Srour T, Bilto YY, Juma M, Irhimeh MR. Exposure of human erythrocytes to oxygen radicals causes loss of deformability, increased osmotic fragility, lipid peroxidation and protein degradation. 2000; 23:13-21.

Tatsumi T, Fliss H. Hypochlorous acid and chloramines increase endothelial permeability: possible involvement of cellular zinc. Am J Physiol 1994; 267:1597-607.

Ueda S, Nakamura H, Masutani H, Sasada A, Takabayashi Y, Yamaoka Y, et al. Baicalin induces apoptosis via mitochondrial pathway as

prooxidant. Mol Immunol 2002; 38:781-91.

Van der Heide T, Poolman B. Osmoregulated ABC-trasport system of Lactococcus lactis senses water stress via changes in the physical state of the membrane. PNAS 2000; vol.97, 13:7102-6.

Vissers MCM, Stern A, Kuypers F, Van den Berg CC. Membrane changes associated with lysis of red blood cells by hypochlorous acid. Free Rad Biol Med 1994; 16:703-12.

Vissers MCM, Winterbourn CC. Oxidation of intracellular glutathione after exposure of human red blood cells to hypochlorous acid. J Biochem 1995; 307:57-62.

Vissers MCM, Carr AC, Chapman ALP. Comparison of human red cell lysis by hypochlorous and hypobromous acids: insights into the mechanism of lysis. Biochem J 1998; 330:131-8.

Weiss SJ, Slivka A. Monocyte and granulocyte-mediated tumour cell destruction. A role for the hydrogen peroxide-myeloperoxidase-chloride system. J Clin Invest 1982; 69:255-62.

Yamashita N, Tanemura H, Kawanishi S. Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II). Mutat Res 1999; 425:107-15.

Yoshino M, Haneda M, Naruse M, Murakami K. Prooxidant activity of flavonoids: copper-dependent strand breaks and the formation of 8-hydroxy-2'-guanosine in DNA. Mol Genet Metab 1999; 68:468-72.

Zavodnik IB, Laphina EA, Zavodnik LB, Bartosz G, Soszynski M, Bryszewska M, Hypochlorous acid damages erythrocytes membrane proteins and alters lipid bilayer structure and fluidity. Free Rad Biol Med 2001; 30:363-9.