

## Acetylsalicylic Acid and Morphology of Red Blood Cells

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### ABSTRACT

*This work evaluated the effect of in vitro and in vivo treatment with ASA on the morphology of the red blood cells. Blood samples or Wistar rats were treated with ASA for one hour. Blood samples or animals treated with saline were used as control group. Blood smears were prepared, fixed, stained and the qualitative and quantitative morphology of red blood cells were evaluated under optical microscopy. Data showed that the in vitro treatment for one hour with ASA at higher dose used significantly ( $p < 0.05$ ) modified the perimeter/area ratio of the red blood cells. No morphological alterations were obtained with the in vivo treatment. ASA use at highest doses could interfere on shape of red blood cells.*

**Key words:** Acetylsalicylic Acid; Blood Cells; Morphology

### INTRODUCTION

Acetylsalicylic acid is a classical nonsteroidal antipyretic, analgesic and anti-inflammatory drug and in the United States alone, 35,000 kg are consumed daily (Jack, 1997). Its actions are based on irreversible inhibition of cyclooxygenases 1 and 2 that are responsible for the prostaglandin synthesis and some autacoids (Catella-Lawson, 2001; Amann and Peskar, 2002; Aude and Mehta, 2002). The anti-thrombotic action of acetylsalicylic acid is mainly due to its antiplatelet action (Grotta et al., 1985; Catella-Lawson, 2001; Insel, 2001) but it has been postulated that the inhibition of oxidative stress could be related to the ability of this drug to prevent cerebrovascular accidents (Sagone and Husney, 1987; Guerrero et

al., 2004). On the other hand, acetylsalicylic acid at therapeutic doses could induce the gastrointestinal adverse effects as gastric ulcer, erosive gastritis, gastrointestinal hemorrhage and exacerbation of peptic ulcer symptoms (Bollini et al., 1992), as well as ions imbalance associated to respiratory alkalosis with increased  $\text{Na}^+$ ,  $\text{K}^+$  and bicarbonate excretion (Lauwerys and Bernard, 1989; Nuyts et al., 1989).

Morphometric analysis has been used to evaluate the morphological changes induced in different cellular systems: (i) chronic ocular hypertensive effects on thickness of retinal nerve fiber layer and optic disc structure (Shimazawa et al., 2006), (ii) relationship between infarct-related artery stenosis and capillary density (Prech et al., 2006), and (iii) effects of sexual hormones on mamma gland

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(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 and Coakley, 1986; Scheiman and Elta, 1990; Li et al., 1999; Shacter and 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 aim of this work was to evaluate the effect of *in vitro* and *in vivo* treatment with acetylsalicylic acid on the morphology of the red blood cells.

## MATERIAL AND METHODS

### Animals

Adult male Wistar naive rats (3-4 month of age, body weight 250-350g) were housed, five per cage, in an environmental controlled room. Animals had free access to water and food and ambient temperature was kept at  $25 \pm 2^\circ\text{C}$ . Experiments were conducted in accordance with the Department Committee of Animal Care.

### Drugs

Commercial acetylsalicylic acid used in this study was purchased from Bayer (Aspirin®, Brazil).

### In vitro treatment with acetylsalicylic acid

Samples of heparinized whole blood were treated for one hour with acetylsalicylic acid at different doses (0.01, 0.10 and 1.00 mg/mL). Blood samples treated with 0.9% NaCl were used as control group. These concentrations were similar to the plasma levels in humans under antiinflammatory therapy with this drug (Insel, 2001).

### In vivo treatment with acetylsalicylic acid

Wistar rats (n=9) were treated for one hour with acetylsalicylic acid at different doses (1.5, 3.0 and 6.0 mg/kg, intragastric administration) and after heparinized, whole blood was withdrawn. Animals (n=10) treated with saline solution (0.9% NaCl, intragastric administration) were used as control group. These doses were similar to that used in

antiinflammatory therapy with this drug (Insel, 2001) and experimental investigations (Guerrero et al., 2004).

### Morphological evaluation of red blood cells

Histological preparations were carried out with blood samples *in vitro* or *in vivo* treated with acetylsalicylic acid for one hour at room temperature, or with NaCl (0.9%) as control group. Blood smears were prepared, dried, fixed and stained by May-Grünwald-Giensa method (Junqueira and Carneiro, 2002). After that, the images of red blood cells were acquired (Optronics, Japan) from blood smears under optical microscopy (x1000). For the morphometric analysis of red blood cells, the perimeter/area ratio was obtained from images by specific program (Image ProPlus Software). Morphological analyses were carried out by blind way by a specialist in histological analysis.

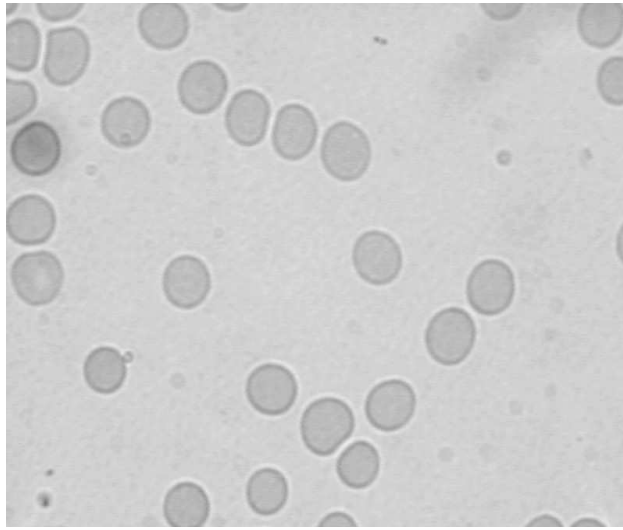
### Statistical analysis

Data are reported as means  $\pm$  SD of perimeter/area ratio. They were compared between the treated and control group by one way analysis of variance (ANOVA), followed by Bonferroni post test with a  $p < 0.05$  as significant level. InStat Graphpad software was used to perform the statistical analysis (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California, USA).

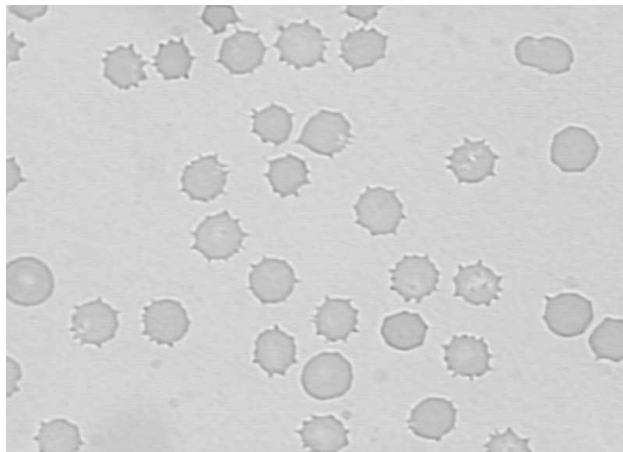
## RESULTS

Figs. 1 and 2 show the photomicrographs of the blood smears from blood *in vitro* treated with 0.9% NaCl solution (control) and with acetylsalicylic acid at the highest concentration used (1.0 mg/mL), respectively. The qualitative morphological analysis by the comparison between these figures showed that the acetylsalicylic acid induced major changes on the shape of the red blood cells.

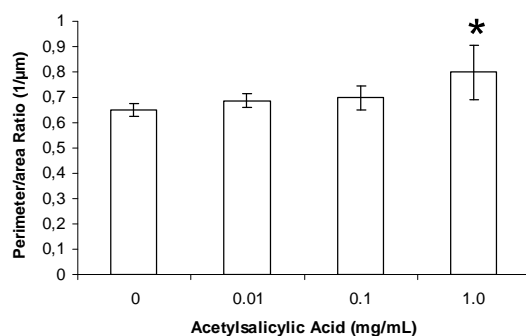
Fig. 3 shows the perimeter/area ratio for red blood cells from blood samples *in vitro* treated with acetylsalicylic acid at different concentrations. The analysis of these data indicated that acetylsalicylic acid significantly ( $p=0.014$ ) modified the perimeter/area ratio of red blood cells at the higher concentration used.



**Figure 1** - Photomicrography of blood smears from blood samples *in vitro* treated with 0.9% NaCl solution (control group). Samples of whole blood from *Wistar* rats were treated with 0.9% NaCl solution during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa 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 with acetylsalicylic acid. Samples of whole blood from *Wistar* rats were treated with acetylsalicylic acid (1.0 mg/mL) during 60 minutes. Blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image capture.

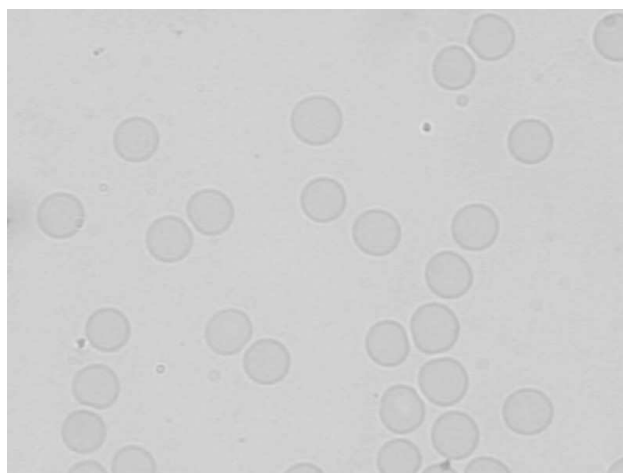


**Figure 3** - Effects of acetylsalicylic acid on the perimeter/area ratio of red blood cells from blood *in vitro* treated. Samples of whole blood from Wistar rats were treated with acetylsalicylic acid at different concentrations during 60 minutes. Blood smears were prepared, dried, fixed and staining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

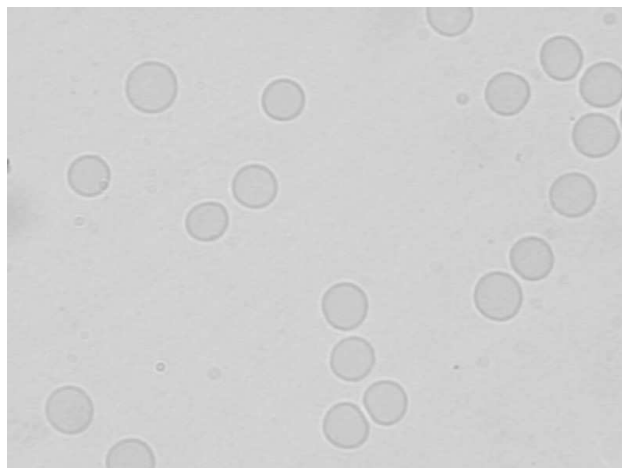
Figs. 4 and 5 show the photomicrographs of the blood smears from blood *in vivo* treated with 0.9% NaCl solution (control) and with acetylsalicylic acid at the highest dose used (6 mg/kg), respectively. The qualitative morphological analysis by the comparison between these figures shown that the *in vivo* acetylsalicylic acid did not

induce any significant modifications in the shape of the red blood cells.

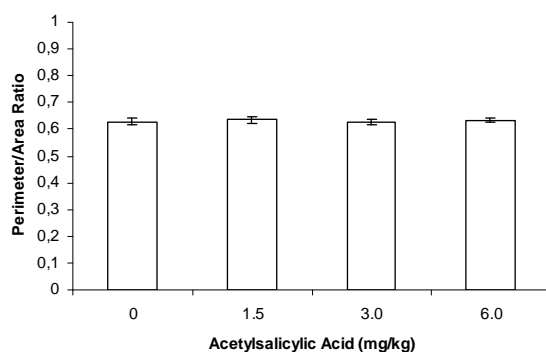
Fig. 6 shows the perimeter/area ratio for red blood cells from blood samples *in vivo* treated with acetylsalicylic acid at different doses. The analysis of these data indicated that *in vivo* acetylsalicylic acid did not alter significantly ( $p=0.749$ ) the perimeter/area ratio of red blood cells.



**Figure 4** - Photomicrography of blood smears from blood samples *in vivo* treated with 0.9% NaCl solution (control group). Wistar rats were treated with NaCl 0.9% solution during 60 minutes. After that, blood samples were withdraw, blood smears were prepared, dried, fixed and staining by May-Grünwald-Giensa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image capture.



**Figure 5** - Photomicrography of blood smears from blood samples *in vivo* treated with acetylsalicylic acid. Wistar rats were treated with acetylsalicylic acid (6.0 mg/kg) during 60 minutes. After that, blood samples were withdrawn, blood smears were prepared, dried, fixed and stained by May-Grünwald-Giemsa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image capture.



**Figure 6** - Effects of acetylsalicylic acid on the perimeter/area ratio of red blood cells from blood *in vivo* treated. Wistar rats were treated with acetylsalicylic acid at different concentrations during 60 minutes. After that, blood samples were withdrawn, blood smears were prepared, dried, fixed and stained by May-Grünwald-Giemsa method. The morphology of red blood cells was evaluated under optical microscopy (x1000) after image captures of five fields for each smear and five smears for each acetylsalicylic acid concentration. After that, morphometric measurements (perimeter and area) were carried out and perimeter/area calculated.

## DISCUSSION

The data obtained in this work showed that *in vitro* or *in vivo* acetylsalicylic acid did not affect the morphology of red blood cells at doses similar to used in anti-thrombotic, antipyretic or anti-

inflammatory therapy (Figs. 3, 5 and 6). However, *in vitro* acetylsalicylic acid at the higher dose (1.0 mg/mL) could alter the morphology of red blood cells as observed by the qualitative analysis and confirmed by the morphometric measurement of perimeter/area ratio (Figs. 2 and 3).

Different techniques have been used to evaluate the effects of the interaction between the drugs and plasma membrane. Using a photometric method, it was demonstrated that derivatives but not acetylsalicylic acid were capable to induce the changes in red blood cell shape (Li et al., 1999). However, an *in vitro* study showed that acetylsalicylic acid at highest doses could increase the deformability and osmotic fragility of the membrane of red blood cells (Bilto, 1999). By electron spin resonance spectroscopy, it was demonstrated that humans submitted to highest doses of acetylsalicylic acid presented structural changes in the membrane of red blood cells (Mazorow et al., 1985).

It was demonstrated that 30 minutes after a single dose of 0.65g, only 27% of acetylsalicylic was in acetylated form due its metabolism in plasma, liver and erythrocytes (Amann and Peskar, 2002). About 50% of acetylsalicylic acid are de-acetylated to salicylate already during and immediately after its absorption (Amann and Peskar, 2002). In this condition, acetylsalicylic acid reaches the detectable plasma level in 30 minutes but the higher plasma concentration occurs only after one hour (Insel, 2001). However, the present data showed that the acetylsalicylic acid concentration reached one hour after *in vivo* treatment could be low and no alterations on membrane shape was observed. This hypothesis was confirmed by *in vitro* treatment at high dose (1.0 mg/mL) where modifications on membrane shape were observed (Fig. 2 and 3).

Acetylsalicylic acid at normal dose (10mg/kg/day) can cause peroxidation in human erythrocytes, increasing glutathione peroxidase and catalase activities but without changing the susceptibility to oxidation (Durak et al., 2001). Other authors reported decrease in glutathione levels in *Wistar* rats after acetylsalicylic acid treatment at 10mg/kg with plasma levels about 10µg/mL (Guerrero et al., 2004). In fact, it was hypothesized that gastric damage induced by acetylsalicylic acid could be connected with the degradation of the lipid components of the cellular membranes (Javor et al., 1986). Salicylates may cause direct cellular toxicity via inhibition of membrane transport properties (Scheiman and Elta, 1990). Other data have demonstrated that acetylsalicylic acid can alter the inward calcium currents by voltage-gated  $Ca^{+2}$  channels (Greffrath et al., 2002; Kim et al., 2001). Indeed, salicylates uncouple the oxidative phosphorylation, leading diminished cellular ATP

concentrations at pharmacological relevant doses (Cronstein et al., 1994). This effect may alter the ions balance ATP-dependent and induce alterations on membrane. These mechanisms could be involved in effects of *in vitro* acetylsalicylic acid on membrane of red blood cells as observed, in the present work.

In conclusion, acetylsalicylic acid at doses similar to anti-thrombotic, antipyretic, analgesic or anti-inflammatory therapy could be not capable but at toxic doses, alterations on membrane of red blood cells could be induced.

## RESUMO

Este trabalho avaliou o efeito do tratamento *in vitro* e *in vivo* com AAS na morfologia dos eritrócitos. Amostras de sangue ou ratos *Wistar* foram tratadas com AAS por uma hora. Amostras sanguíneas ou animais tratados com salina foram utilizados como grupos controle. Distensões de sangue foram preparadas, fixadas, coradas e a análise morfológica qualitativa e quantitativa dos eritrócitos foi realizada em microscópio óptico. Os dados mostraram que o tratamento *in vitro* por uma hora com AAS na maior dose utilizada modificou significativamente ( $p < 0.05$ ) a relação perímetro/área dos eritrócitos. Não foram obtidas alterações morfológicas com o tratamento *in vivo*. O uso do AAS em doses altas poderia interferir na forma dos eritrócitos.

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