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Effects of the nonsteroidal anti-inflammatory drug naproxen on human erythrocytes and on cell membrane molecular models

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ABSTRACT

Naproxen, a nonsteroidal anti-inflammatory drug (NSAID), has been widely investigated in terms of its pharmacological action, but less is known about its effects on cell membranes and particularly those of human erythrocytes. In the present work, the structural effects on the human erythrocyte membrane and molecular models have been investigated. The latter consisted in bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of lipids found in the outer and inner moieties of the erythrocyte and most cell membranes, respectively. This report presents evidence that naproxen interacts with red cell membranes as follows: a) in scanning electron microscopy (SEM) studies on human erythrocytes it has been observed that the drug induced shape changes, forming echinocytes at a concentration as low as 10 μM; b) X-ray diffraction showed that naproxen strongly interacted with DMPC multilayers; in contrast, no perturbing effects on DMPE multilayers were detected; c) differential scanning calorimetry (DSC) data showed a decrease in the melting temperature (T_m) of DMPC liposomes, which was attributed to a destabilization of the gel phase, effect that was less pronounced for DMPE. These experimental results were observed at concentrations lower than those reported for plasma after therapeutic administration. This is the first time in which the structural effects of naproxen on the human erythrocyte membrane have been described.

1. Introduction

Naproxen (Fig. 1) is a member of the sodium arylacetic group of nonsteroidal anti-inflammatory drugs (NSAIDs). It is an effective analgesic used for the reduction of moderate to severe pain, fever and inflammation [1,2]. Advantage of sodium naproxen in comparison to the acidic form is its better absorption by the gastrointestinal tract, reaching maximum systemic concentrations in 1 h (0.4 mM) [3,4]; its long half-life in the body is approximately 12–15 h [5]. Naproxen most serious side effects are bleeding and ulcers in both stomach and intestine [6,7]. It has been associated with several potential interactions with the cell membrane, such as changes in the gastric mucosa from a hydrophobic to a more hydrophilic state; this effect is caused through a COX-independent mechanism that alters the physical properties of the gastric phospholipids [8]. In this paper the interaction of sodium naproxen with red blood cells (RBC) and molecular models of the erythrocyte membrane are described. Human erythrocytes were chosen because having only one membrane and no internal organelles are an ideal cell system for studying drug-membrane interactions. Although less specialized than many other cell membranes, they carry on as many functions in common with them to be considered representative of the plasma membrane in general. The erythrocyte membrane shows an asymmetric distribution in its phospholipid composition. The outer leaflet of the lipid bilayer is composed primarily of sphingomyelins (SM) and phosphatidylcholines (PC), whereas the inner leaflet contains mainly phosphatidylserines (PS) and phosphatidylethanolamines (PE) [9,10]. According to the bilayer couple hypothesis of Sheetz and Singer [11], the asymmetric composition of the two membrane bilayer leaflets leads to different responses to perturbations of exogenous molecules which would lead to diverse functional consequences, including shape changes of the intact cell. Given the molecular complexity of the erythrocyte membrane, phospholipid bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were used as molecular models for the erythrocyte membrane. The capacity of naproxen to perturb

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; SEM, scanning electron microscopy; DSC, differential scanning calorimetry; T_m, main transition temperature; PGs, Prostaglandines; COX, Cyclooxygenase; RBC, Red blood cells.

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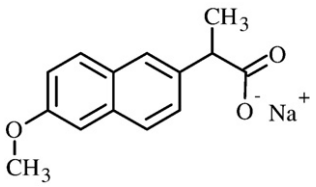

Fig. 1. Structural formula of sodium naproxen.

the bilayer structures of DMPC and DMPE was assessed by X-ray diffraction, while intact human erythrocytes were observed by scanning electron microscopy (SEM). These systems and techniques have been used in our laboratories to determine the interaction with and the membrane-perturbing effects of other drugs [12–14]. Additionally, differential scanning calorimetry (DSC) on DMPC and DMPE liposomes were performed to assess the phase properties of the membranes in the presence of the drug and to investigate lipid-naproxen interactions.

2. Materials and methods

2.1. X-ray diffraction studies of DMPC and DMPE multibilayers

Sodium naproxen (lot 1241487, MW 252.2) from Sigma (St. Louis, MO), synthetic DMPC (lot 140PC-241, MW 677.9) and DMPE (lot 140PE-58, MW 635.9) from Avanti (ALA, USA), were used without further purification. Ca. 2 mg of each phospholipid were suspended in 200 µl of (a) distilled water and (b) aqueous solutions of naproxen in a range of concentrations (0.01 mM to 2.0 mM). Samples were incubated for 30 min at 37 °C and 60 °C with DMPC and DMPE respectively, and centrifuged for 10 min at 2500 rpm. Samples were then transferred to 1.5 mm dia special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) and X-ray diffracted. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray generator was used. The relative reflection intensities were obtained in a MBraun PSD-50 M linear position-sensitive detector system (Garching, Germany) and no correction factors were applied. The experiments were performed at 18 °C ± 1 °C, which is below the main phase transition temperature of both phospholipids. Higher temperatures would have induced transitions to more fluid phases making the detection of structural changes more difficult. Each experiment was performed at least in triplicate. X-ray data was analyzed using the Origin software 7.0.

2.2. Differential Scanning Calorimetry (DSC) on DMPC and DMPE

DSC measurements were performed with a VP-DSC calorimeter (MicroCal, Inc., Northampton, MA, USA) at heating and cooling rates of 1 $K \cdot min^{-1}$. DSC samples were prepared by dispersing a known amount (5.6 mM) in 10 mM PBS buffer (pH 7.4). Lipid-naproxen mixtures were investigated up to a molar ratio of lipid:naproxen 1:2 corresponding to a naproxen concentration of ca. 11.2 mM. Samples were hydrated in the liquid crystalline phase by vortexing. Before measurements, samples were stored at 4 °C for a defined period. Measurements were performed in the temperature interval from 5 °C to 65 °C. In the figures the temperature range is shown only where phase transitions were observed. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample [15]. The accuracy was ± 0.1 °C for the main phase transition temperature and ± 1 $kJ \cdot mol^{-1}$ for the main phase transition enthalpy. The DSC data were analyzed using the Origin software. The phase transition enthalpy was obtained by integrating the area under the heat capacity curve [16].

2.3. Scanning Electron Microscopy (SEM) studies of human erythrocytes

Interaction of sodium naproxen with RBC was studied by means of the incubation of intact erythrocytes with different drug concentrations. Blood was obtained from healthy human male donors under no pharmacological treatment. Blood samples (0.1 ml) were obtained by puncturing the ear lobule and mixed with 10 µl of heparin (5000 UI/ml) in 0.9 ml of saline solution (NaCl 0.9%, pH 7.4). Sample was centrifuged (1000 rpm × 10 min) and the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. The sedimented erythrocytes were suspended in 0.9 ml of saline solution and 100 µl aliquots of those RBC were mixed with equal volumes of (a) saline solution (control), and (b) 100 µl of each naproxen stock solutions in saline solution. The final naproxen concentrations were in the range of 0.01 mM to 2 mM. Samples were then incubated for one hour at 37 °C. Following incubation, samples were centrifuged (1000 rpm × 10 min) and the supernatant was discarded. Fixation was performed by addition of 500 µl of 2.5% glutaraldehyde and overnight incubation at 4 °C. Fixed samples were washed with distilled water, placed over Al glass cover stubs, air dried at 37 °C for 30 min to 1 h, and gold-coated for 3 min at 10^{-1} Torr in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in a Jeol SEM (JSM 6380 LB, Japan).

3. Results

3.1. X-ray diffraction studies of DMPC and DMPE multilayers

Fig. 2A exhibits the results obtained by incubating DMPC with water and naproxen. As expected, water altered the structure of DMPC; its bilayer repeat distance (phospholipid bilayer width plus the layer of water) increased from about 55 Å in its crystalline form [17] to 64 Å when immersed in water, and its low-angle reflections (indicated as LA) were reduced to only the first two orders of the bilayer repeat. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region (indicated as WA), which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. These results were indicative of the gel state reached by DMPC bilayers. Fig. 2A discloses that after exposure to 0.1 mM naproxen, there was a considerable decrease of DMPC 4.2 Å reflection intensity, which became almost negligible with 0.5 mM. On the other hand, up to a concentration of 0.3 mM a monotonically decrease of the low angle reflection was induced. From these results, it can be concluded that naproxen produced a structural perturbation of DMPC bilayers and, as a consequence, a disruption of the in-plane structure and the bilayer stacking. Results from similar experiments with DMPE are presented in Fig. 2B, which shows only two reflections: one of 56.4 Å corresponding to the bilayer width, another of 4.2 Å similar to that observed in DMPC. Additional reflections were not detected with the used set-up. As reported elsewhere, water did not significantly affect the bilayer structure of DMPE [17]. As it can be observed, increasing naproxen concentrations did not significantly affect DMPE structure even at the maximum assayed concentration (2 mM).

3.2. Differential Scanning Calorimetry (DSC) of DMPC and DMPE

Using calorimetric analysis, information of the phase transition temperature changes and phase transition enthalpy changes were monitored as a function of added naproxen. These parameters gave insights into the lipid–drug interaction. Heat capacity versus temperature is plotted in Fig. 3. The analysis was performed for the pure phospholipids and for their mixtures with the drug at different molar ratios. Results from DMPC are shown in Fig. 3. For pure DMPC, two peaks, a small and a large endothermic one, were observed. The

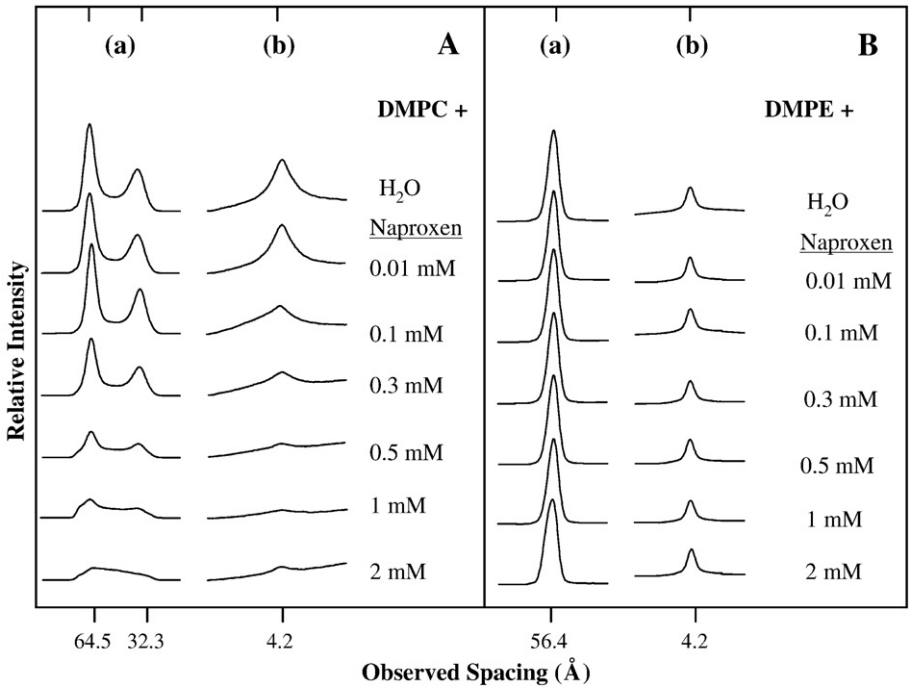

Fig. 2. Microdensitograms from X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous solutions of sodium naproxen; (a) and (b) correspond to low- and wide-angle reflections, respectively.

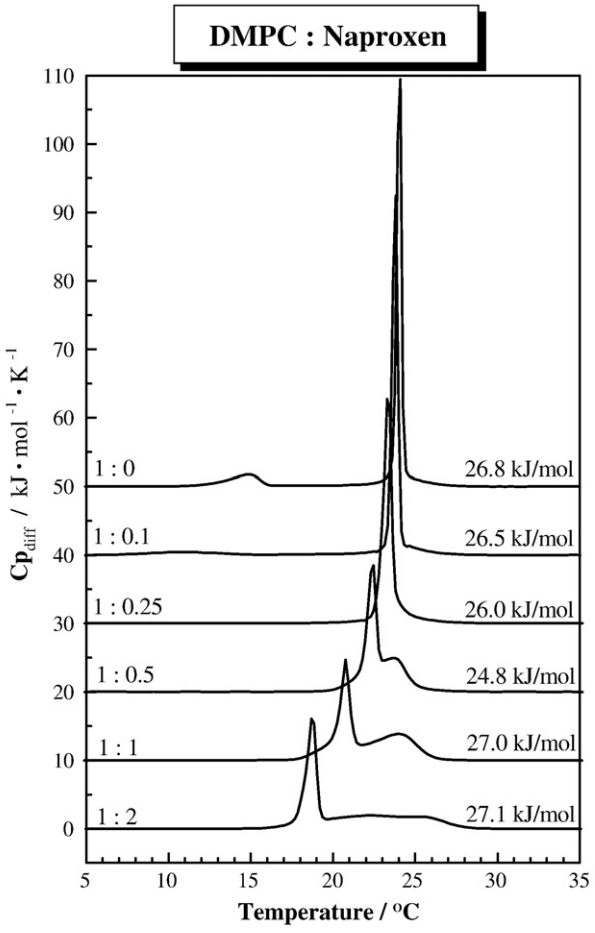

Fig. 3. DSC thermograms of DMPC in the presence of various lipid to naproxen molar ratios enthalpy is obtained by peak integration. The Naproxen concentrations correspond to 0 to 11.2 mM (for the 1:2 molar ratio). Adapted according to Manrique-Moreno et al. [26].

small peak at lower temperature (ca. 15 °C, pretransition) corresponds to the transition from the L_β phase into the P_β ripple phase, whereas the large peak at ca. 24 °C corresponds to the main transition [18]. The P_β gel phase is induced by structural constraints between the packing characteristics of the two acyl chains and the headgroup [19]. At a DMPC:naproxen 1:0.1 molar ratio the gel to liquid crystalline main phase transition was only slightly affected by the presence of the drug. A broadening and shifting effect to a lower temperature was observed in thermograms. However, the low amount of 0.56 mM naproxen (molar ratio of 1:0.1) was sufficient to induce the disappearance of the ripple phase (P_β). This is in accordance to Lygre et al. [20] and Du et al. [21]. Naproxen was able to alter the cooperativity of the phase transition of DMPC as a function of the lipid:drug molar ratio. Phase separation was induced at a DMPC: naproxen molar ratio of 1:0.5. By an increase of the naproxen concentration to 11.2 mM corresponding to a DMPC: naproxen molar ratio of 1:2, the main phase transition temperature (T_m) was shifted below 18.5 °C. This result indicated that a DMPC: naproxen interaction was induced. Above a naproxen concentration of 1.4 mM (DMPC: naproxen 1:0.25), a phase separation was induced, showing the presence of two transitions. By increasing the naproxen ratio the phase transition enthalpy of the higher melting phase (phase composed mainly of pure lipid) decreases, whereas the phase transition enthalpy of the lower melting phase (phase containing higher amounts of DMPC-naproxen "complexes") increases. In contrast to the evident influence on the phase transition temperature the overall molar enthalpy of the transition remains constant at all molar ratios of the DMPC:naproxen mixtures (see phase transition enthalpy data included in Fig. 3). However, it has to be considered that the overall phase transition enthalpy is the addition of the phase transition of the different phases formed.

Fig. 4 shows the DSC results obtained with pure DMPE and with its mixtures with naproxen. An increase of the naproxen concentration from 0.56 to 11.2 mM corresponding to DMPE: naproxen molar ratio from 1:0.1 to 1:2, a broadening of the phase transition was observed accompanied of a slight destabilization of the gel phase. A high naproxen concentration of 11.2 mM even induced a phase separation, which was clearly seen as a double peak for the phase transition. The

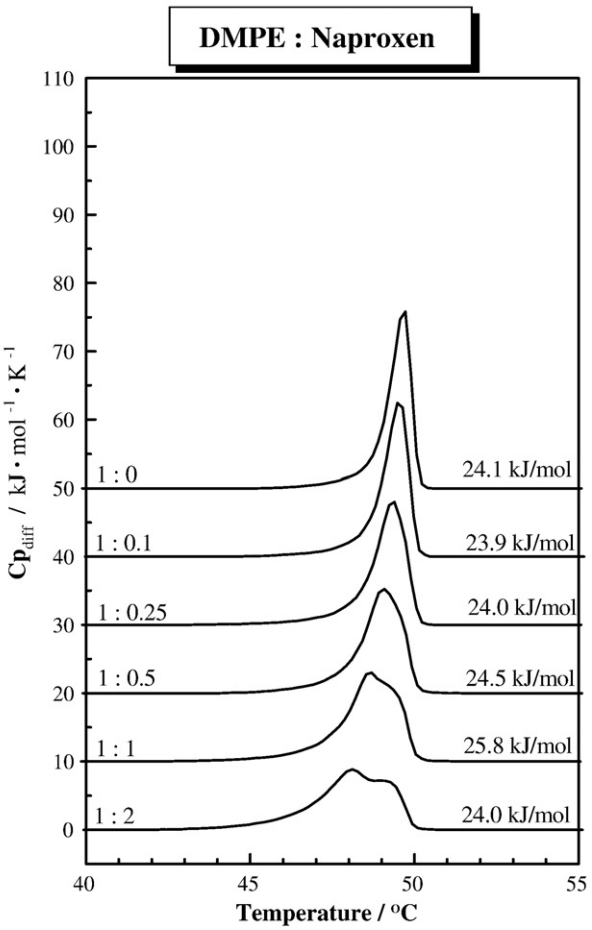

Fig. 4. DSC thermograms of DMPE in the presence of various lipid to naproxen molar ratios enthalpy is obtained by peak integration. The Naproxen concentrations correspond to 0 to 11.2 mM (for the 1:2 molar ratio). Adapted according to Manrique-Moreno et al. [26].

higher melting peak located at 49.5 °C corresponded to more or less pure DMPE, whereas the peak at 48 °C corresponded to DMPE-naproxen mixtures. The overall phase transition enthalpy was quite unaffected by the presence of naproxen (Fig. 4).

3.3. Scanning Electron Microscopy (SEM) studies of human erythrocytes

The effects of the *in vitro* interaction of naproxen with human erythrocytes were followed by SEM. Under physiological conditions, normal human red blood cells assume a flattened biconcave disc shape (discocyte) ~ 8 μm in diameter. The control experiment consisted in erythrocytes incubated with saline solution (Fig. 5A). The observation of human erythrocytes incubated with naproxen in the range of 0.01–2 mM revealed that the drug induced changes in the normal biconcave shape of the erythrocytes. A morphological analysis revealed that a few of the cells treated with 0.01 mM presented echinocytosis, an altered condition in which the erythrocytes show a spiny configuration, exhibiting blebs or protuberances in their surfaces (Fig. 5B); 0.3 mM induced to some red blood a knizocytic type of deformation (cells with two or three concavities due to indentations in the red cell membrane, Fig. 5C), 0.5 mM produced echinocytosis in some cells and in a number of the rest stomatocytosis (a cup-shaped form with evagination of one surface and a deep invagination of the opposite face, Fig. 5D); several of the cells treated with 1.0 mM concentration showed echinocytes and a few stomatocytes (Fig. 5E), while the majority of the cells incubated with 2.0 mM sodium naproxen showed echinocytes, and a few stomatocytes, knizocytes and elliptocytes (elliptical cells also called ovalocytes, Fig. 5F). (The nomenclature of red cell shapes and their corresponding micrographs are described in ref. [22]).

4. Discussion

It is accepted that, among other side effects, the chronic use of NSAIDs may induce gastrointestinal bleeding [23]. The gastric mucosal barrier against acid back diffusion is a complex and dynamic defense system; its hydrophobicity is due to surface-active phospholipids, being the main phosphatidylcholines and phosphatidylethanolamines [24]. On the other hand, there are reports that clearly show that erythrocytes are one of the critical targets for NSAIDs [4]. The current study presents the following evidences that naproxen affects human erythrocytes and molecular models of its cell membrane. SEM observations showed that it induced the preferential formation of echinocytes, even at a concentration as low as 10 μM. According to the bilayer couple hypothesis of Sheetz and Singer [11], selective intercalation of the agents into the outer or inner monolayer of the membrane results in expansion of one monolayer relative to the other, inducing the observed shape changes. Thus, anionic amphipathic compounds produce echinocytes by preferential association with the outer monolayer. This morphological change is probably a result of the inability of the amphiphats to cross the bilayer, or can be due to charge repulsions by negatively charged inner monolayer lipids [11]. Among other equinocitic agents arsenate and valiomycin are recognized [25]. Under our experimental conditions (pH 7.4), naproxen ion has a negatively charged carboxylic moiety.

The X-ray diffraction results confirmed the perturbation effect exert by naproxen on bilayers made up of DMPC and DMPE, classes of the major phospholipids present in the outer and inner erythrocyte membrane, respectively. Results showed that naproxen interacted practically only with DMPC. DMPC and DMPE differ only in their terminal amino groups, being these $^{+}N(CH_3)_3$ in DMPC and $^{+}NH_3$ in DMPE. Moreover, both molecular conformations are very similar in their crystalline phases, with the hydrocarbon chains mostly parallel and extended and the polar head groups lying perpendicularly to them [17]. However, DMPE molecules pack tighter than those of DMPC. This effect, due to DMPE smaller polar group and higher effective charge, stands for a very stable multilayer arrangement that is not significantly perturbed by the presence of water. The strong hydrogen bond network of DMPE bilayers is certainly a reason for the reduced penetration of the drug into its interfacial head group region. On the other hand, the gradual hydration of DMPC results in water filling the highly polar interbilayer spaces with the resulting increase of the bilayer repeat (bilayer width plus the water layer). This phenomenon allows the incorporation of naproxen anions into DMPC intercalated water layers and then into the lipid bilayers. Given the amphipatic nature of naproxen ions one would expect that they locate in such a way that their negatively charged carboxyl groups electrostatically interact with the positively charged terminal $^{+}N(CH_3)_3$ groups modifying the electrostatic interactions between DMPC phosphate and amino groups disrupting its bilayer structure. However, FTIR analyses performed by Manrique-Moreno et al. [26] on DMPC membranes were unable to detect any relevant interaction of naproxen and the DMPC amino group.

DSC data show that the gel phase was actually destabilized by the presence of naproxen; the main phase transition was shifted to lower temperature. This effect was more pronounced for DMPC compared to DMPE. No changes in the overall enthalpy were detected for any phospholipid. A similar conclusion was obtained by Castelli et al. by means of differential scanning calorimetry on DMPC large unilamellar vesicles [27]. Their results showed that naproxen was able to depress the DMPC transitional temperature without inducing changes in the enthalpy (ΔH). The interaction between the drug and the DMPC liposomes was explained as a fluidifying effect, and the constant ΔH was described by surface interaction between amphipatic molecules

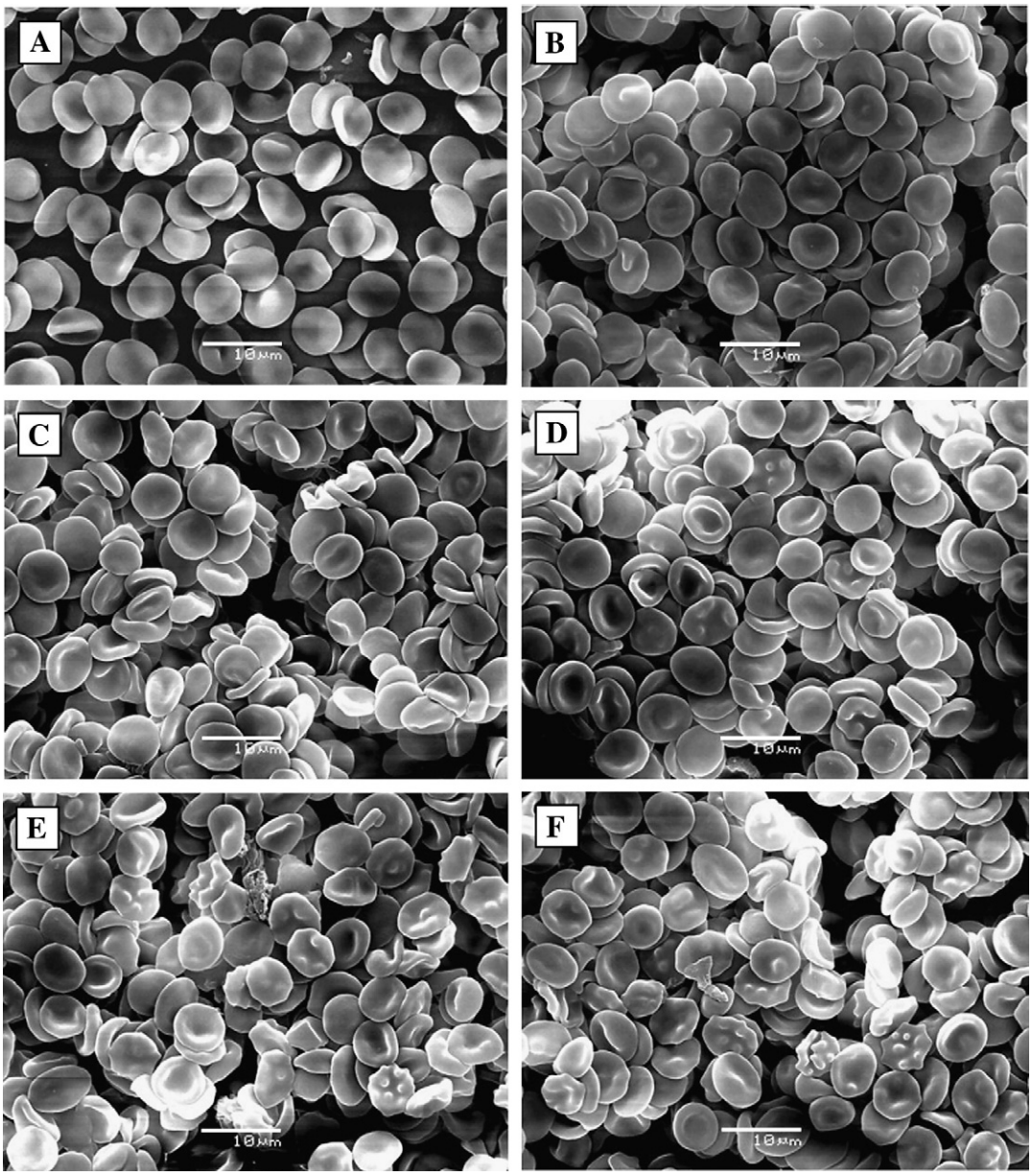

Fig. 5. Effect of naproxen on the morphology of human erythrocytes. Images obtained by scanning electron microscopy (SEM) of (A) control; (B) 0.01 mM; (C) 0.3 mM; (D) 0.5 mM; (E) 1 mM; (F) 2 mM sodium naproxen.

and DMPC polar heads, which occur only at the surface of lipid layers without strong interaction with the acyl chains. These results are also aligned with the infrared and FRET data presented by Manrique-Moreno et al. [26], which showed a phase transition temperature shifted to lower temperatures, a fluidization effect induced by the drug and no intercalation of naproxen into liposomes.

The hypothesis that naproxen binds on the membrane surface is confirmed by the results obtained by Giraud et al. [8] using fluorescence resonance energy transfer experiments and fluorescence anisotropy measurements on dipalmitoylphosphatydylcholine (DPPC) liposomes. They evaluated the binding mechanism behind the topical gastric irritancy of indomethacin and naproxen when administred intragastrically to rats. Their results showed that indomethacin was bound to the membrane surface in experiments carried out at pH 7. This behavior was explained by a 90% ionization of indomethacin under physiological conditions. Therefore, electrostatic interactions between the negatively charged carboxylic group of indomethacin and positively charged quaternary ammonium of the PC were not discarded. The studies performed by Giraud et al. [8] with naproxen in its acidic and highly liposoluble form showed that the drug located within the bilayer, at a level closer to the polar head groups than the fatty acid tail.

Our results with sodium naproxen, a very soluble form of the drug, can be probable explained by entropic effects due to the changes in the hydration shell by the presence of the drug when it is located at the surface of the polar head region of liposomes [26,28]. Lichtenberger et al. [29] studied a group of five NSAIDs and the possible association with zwitterionic phospholipids in order to form NSAID/lipid complexes. The existence of such DMPC-naproxen complexes can be deduced from the induction of a phase separation in the presence of the drug; a DMPC: naproxen enriched phase and a more or less pure DMPC phase are formed. This was not observed to that extent for DMPE. These complexes retarded the ability of the NSAIDs to interact with the extracellular phospholipid lining on the mucus gel layer. They found that the drugs were able to interact with the zwitterionic phospholipid DPPC possibly due to hydrophobic and electrostatic interactions [29].

Our experimental findings are certainly of interest as they indicate that a naproxen concentration as low as 10 μM affects the human

erythrocyte shape. The effects of this drug detected in the present work, particularly on human erythrocytes, were observed at concentrations lower than those reported in plasma after therapeutic administration (0.15–0.4 mM) [30]. An important fact to be analyzed is the increase of negative charges on the bilayer surface due to the location of the naproxen molecules in the head group region. This location would modify the electrostatic properties of the phospholipid molecules. The polar head groups of the lipids are oriented at the water membrane interface. Carbonyls, phosphates, carboxylic moieties and positively charged amino groups are the main zones of hydration in a phospholipid membrane [31]. The orientation of these groups and the organization of the water dipoles in its respective hydration spheres determine the correct function of the membrane. The location of naproxen in the polar head groups generates a reorganization of water molecules affecting the packing and cooperativity of the phospholipids explaining the phase separation effect observed by DSC. The formation of strong DMPC:naproxen complexes and the phase separation effect changes could induced also the alteration of the normal biconcave shape of red blood cells inducing preferentially echinocytes. This effect in vivo could increases the resistance of the erythrocytes to entry into capillaries [32], which could contribute to decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion [33].

In conclusion, our experimental results evidence that naproxen interacts with red cell membranes as follows: a) in scanning electron microscopy (SEM) studies on intact human erythrocytes it was observed that the drug induced the preferential formation of echinocytes at a concentration as low as 10 μM. The appearance of this shape is indicative of the insertion of naproxen molecules into the outer moiety of the erythrocyte lipid bilayer ; b) X-ray diffraction showed that naproxen strongly interacted with DMPC bilayers, class of lipid preferentially located in the outer monolayer of the erythrocyte membrane; c) differential scanning calorimetry (DSC) data showed a decrease in the melting temperature (T_m) of DMPC liposomes, which was attributed to a destabilization of the gel phase, effect that was less pronounced for DMPE. These experimental results were observed at concentrations lower than those reported in plasma after therapeutic administration. This is the first time that the structural effects of naproxen on the human erythrocyte membrane have been described.

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