EFFECTS OF NANOCAPSULES CONTAINING ALL-TRANS-RETINOIC ACID UNDER HEMOLYTIC AND COAGULATION ACTIVITY

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Abstract: The chemotherapeutic all-trans retinoic acid (ATRA) used in the treatment of Acute Promyelocytic Leukemia has adverse effects on its oral administration, with which we incorporated a system of drugs, the nanocapsules, in order to have a possible improvement in solubility, photosensitivity, lower toxicity, generating pharmacological efficacy. The objective was to evaluate and compare the hemolytic and coagulation activity of the free drug (AL), nanoencapsulated (NA) and the white nanocapsules (NB) by analyzing the results of hemolysis, Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). We developed a prospective study of treatments at different concentrations of 0.25; 0.5; 1.0; 1.5; 2.0; 2.5 µg/mL. For the first test, all concentrations showed hemolytic activity, but when compared to NA with ATRA it is observed that these carriers induced lower hemolytic toxicity. In the PT test the nanoparticles at the two lowest concentrations remained in the physiological range (12 - 15 seconds). For the APTT test the three lowest concentrations remained within the control (25 - 35 seconds). Thus, we believe there is a promising benefit of using these nanoparticles developed and no doubt further studies will be performed to confirm the responses obtained here.
Keywords: Coagulation cascade. Hemolysis. Leukemia.

1 INTRODUCTION

Bleeding is a frequent and potentially lethal complication in cancer patients. It may be directly related to the disease, to the antineoplastic treatment or to factors not related to the neoplasia itself.[1]

The control of this alteration in the site of the endothelial lesion includes the action of subendothelium, endothelium, platelets and plasma proteins, a process which it is called hemostasis.[1]

One of the clotting disorders severely encountered in the clinical course of many pathologies, especially Acute Promyelocytic Leukemia (APL) is Disseminated Intravascular Coagulation (DIC). DIC is currently defined as an acquired syndrome, characterized by the diffuse activation of intravascular coagulation, leading to the formation and deposition of fibrin in the microvasculature.[2]

The clinical course of APL in recent years has been modified from a rapidly fatal acute leukemia to one of the more curable AML subtypes. This revolutionary progress in the prognosis of the disease is essentially associated with the remarkable advances in its treatment, more specifically with the introduction of new therapeutic agents that act directly in the molecular alteration, as is the case of *all-trans retinoic* acid derived from vitamin A (ATRA) and arsenic trioxide (ATO).

After following the administration, a rapid increase in leukocytes is observed, which is accompanied by Differentiation Syndrome[3] On the other hand, when administered orally, its bioavailability is reduced, and this can be attributed to its low solubility in water. Moreover, this type of treatment, when prolonged, provides a progressive reduction of its plasma concentration, which can be attributed to its increased metabolism and increased haemolytic toxicity.[4]

To reduce this disadvantage and adverse effect of free ATRA, this work aimed to incorporate the ATRA into a different drug delivery system, the nanocapsules, which when combined with the drug can improve the solubility, photosensitivity, toxicity, chemical stability, bioavailability and/or pharmacological efficacy.[5] In addition, the ATRA is a lipophilic

drug that is very poorly soluble in water, and its incorporation into lipid nanocarriers is an interesting alternative to allow its intravenous administration to replace oral use.[6]

Therefore, we used the determination of hemoglobin release in red blood cells as an *in vitro* model, as well as the possible effects on blood coagulation to study the toxicity of ATRA, ATRA nanocapsuled and blank nanocapsules.

2 MATERIALS AND METHODS

This work consisted of two stages: the first one to produce and characterize the nanocapsule suspensions, and a prospective *in vitro* study was carried out in which red blood cells and plasma were used as an experimental model to investigate the potential hemolytic and coagulation effects of the chemotherapeutic ATRA and its nanoencapsulated form.

The discard samples were obtained from healthy donors from peripheral blood, from the Laboratory of Clinical Analysis (LEAC) of the Universidad Franciscana under the Ethics Committee of the institution and CAAE registry: 31211214.4.0000.5306.

The samples were collected by venipuncture using Vacutainer tubes (BD Diagnostics, Plymouth, UK) containing EDTA (ethylenediaminetetraacetic acid) for hemolysis and sodium citrate for coagulation.

The nanoparticles loaded with ATRA (NA) and the blank nanocapsules (NB) - drug-free in the structure - were produced according to the formulation described by Ourique *et al.*,[6] with some adaptations. For the preparation of the nanocapsules, the interfacial deposition method of the polymer, described by Fessi, Puisieux, Devissaguet, Ammoury, and Benita,[7] was used.

The ATRA content found for the drug loaded nanocapsules was 98% at a concentration of 0.5 µg/mL. The treatment for both tests was performed with ATRA, nanocapsuled ATRA and blank nanocapsule, in the following concentrations of 0.25; 0.5; 1.0; 1.5; 2.0; 2.5 µg/mL.

2.1 HEMOLYTIC ACTIVITY

The methodology followed according to Dobrovolskaia *et al.*[8] Blood was added to 1x PBS at 1: 1 ratio and centrifuged 15 minutes at 1000 rpm, the supernatant was discarded, and this procedure was performed three times. Subsequently, in a new tube containing 1 mL of 1x PBS, 400 µL of already washed red blood cells and 80 µL of treatment were added. As negative control cells were used (400 µL) and PBS 1x (1mL) and as positive control cells (400 µL), PBS 1x (1mL) and H_2O_2 (80 µL). The tubes were incubated in a 37 °C at 5% CO_2 for 1 hour. It was then centrifuged for 5 minutes at 1000 rpm and 100 µL of the supernatant to a 96-well plate, which was read in ELISA reader at 405 nm.

2.2 COAGULATION TEST

This assay was performed according to protocol *Nanotechnology Characterization Laboratory* (Frederick *National Laboratory for Cancer Research Leidos Biomedical Research*, Inc. Frederick, MD 21702). Whole blood was collected and centrifuged 10 minutes at 2500 rpm. Then, 225 µL of plasma was separated into wells along with 25 µL of treatments and incubated at 37 °C for 30 minutes. Two independent experiments were performed in duplicate with different donors. It was later performed in reading coagulometer Quick Timer II (Drake) properly calibrated, according to the manufacturer's recommendations for TP Hemostasis tests (Labtest – Lot: 4008) and TTPA Hemostasis (Labtest – Lot: 4006). We used as reference for the TTPA normal value the interval between 25 and 35 seconds, while the baseline values for TP used the interval between 12 and 15 seconds according to the authors.[9,10]

2.3 ANALYZE STATISTICS

The hemolysis assay was performed in triplicate. The results were expressed as percentage of positive control (100%), mean ± standard deviation (S.D.). The analyzes were performed by variance (ANOVA) of 1-way, followed by Dunnett's post hoc test. Values with p <0.05 were considered statistically significant. Coagulation was performed in two independent

experiments, these in duplicate with different donors. The results were expressed as mean ± S.D. and compared to the corresponding physiological times of each test.

3 RESULTS

The suspensions of nanocapsules produced had smooth and milky, and the white tone exhibited NBs and NAs exhibited slight yellowish tint due to the presence of the drug, which in its free form has a yellow color.

The average pH of the formulations was 6.1 and 6.3 for the NB to NA. The results are in accordance with the results reported by Fachinetto, Ourique, Lubini, Tedesco, Silva, and Beck,[5] indicating that the formulations of nanocapsules produced according to pH have stability.

Regarding the size, the suspensions in this study showed an average diameter of 209 nm to 218 nm for NB and NA.

The PDI for the NB was 0.195 and for the NA of 0.158.

Figure 1 below shows the results regarding the hemolytic activity of the chemotherapeutic ATRA in its free form (ATRA), ATRA nanoencapsulated (NA) and blank nanocapsule (NB).
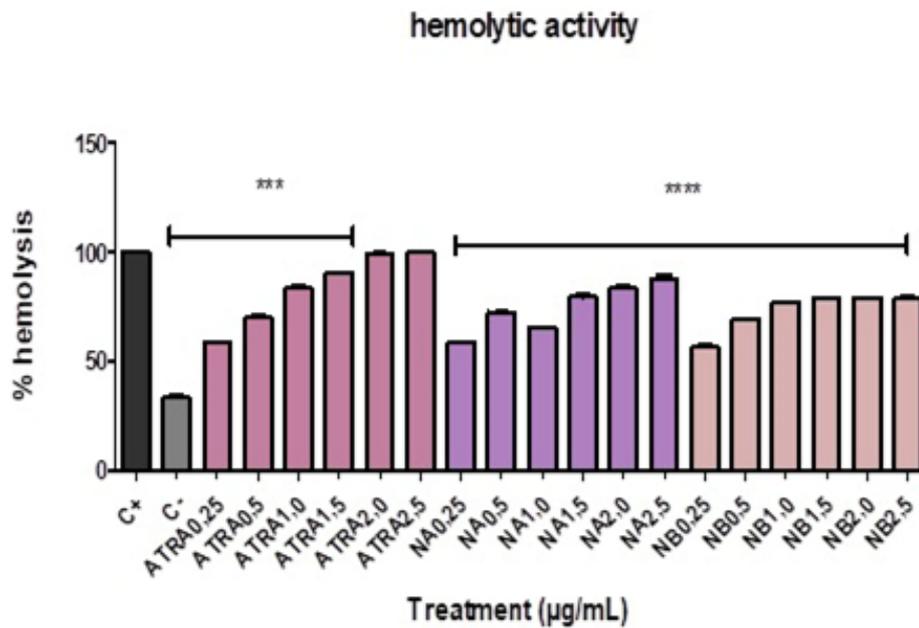
Figure 1 – Hemolysis assay with different treatments
Notes: Results expressed as percentage of positive control (100%). Data were expressed as mean ±
standard deviation (SD). Analyzes were performed by 1-way ANOVA, followed by Dunnett's post hoc
test. Values with p <0.05 were considered statistically significant.

The results found in the hemolytic activity of ATRA, evidenced the increase of
the same, dependent concentration.

Performed a new statistical analysis and comparing free and nanocapsules
concentrations for the presence of significant differences, as shown in Table 1

Concentration	ATRA	NA	"p" value
1,0 µg/mL	83,49%	65,1%	<0,001
1,5 µg/mL	90%	79%	<0,001
2,0 µg/mL	100%	83%	<0,001
2,5 µg/mL	100%	86%	<0,001

Table 1 – Comparative analysis between treatments with AL
         and NA through analysis of variance (ANOVA) of 1
         way, followed by Tukey post hoc test

These results demonstrate that although the activity of the ATRA-loaded
nanocapsules showed haemolytic activity, still these carriers induced lower haemolytic
toxicity compared to ATRA.

The prothrombin time (PT) and Activated Partial Thromboplastin Time (APTT) were measured by measuring the coagulant activity. As previously mentioned, blood from healthy patients, specifically isolated plasma, was used for this study. The results of coagulation tests are related to the type and concentration of treatments as can be observed in the figures below, being TP (Figure 2) and APTT (Figure 3). Where for the first test, only the treatments with the nanoparticles in the concentration of 1,0 µg/mL remained above the physiological range. In the second test, all are within the range. Results greater than 1.5 µg/mL are not represented graphically by producing an anticoagulant effect, values greater than 120 seconds and 240 seconds, respectively.
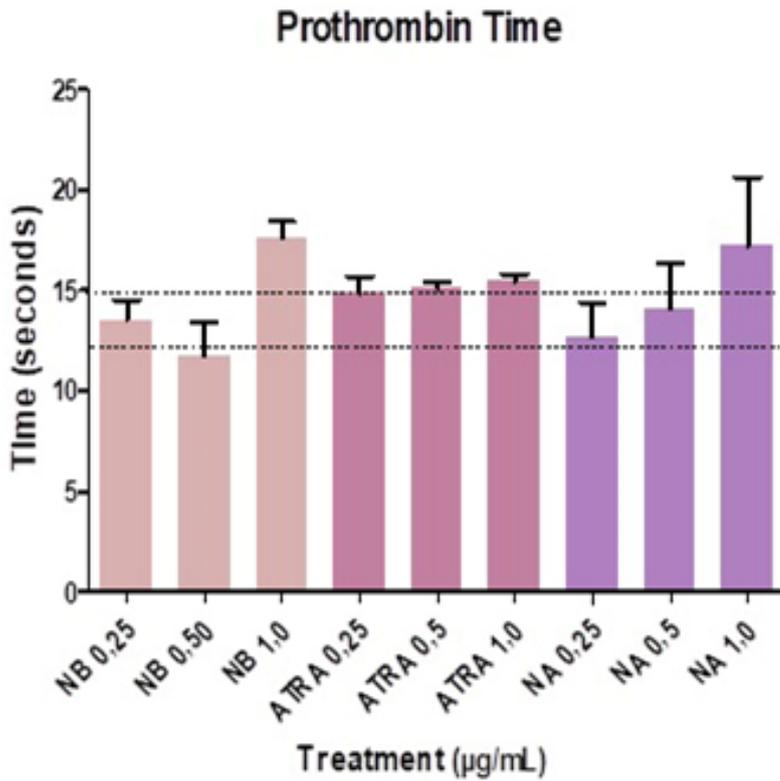

Figure 2 – Results of PT
Notes: The dotted boundaries correspond to expected physiological times for healthy donors (between 12 and 15 seconds). Mean ± S.D. values from two independentexperiments and from different donors.
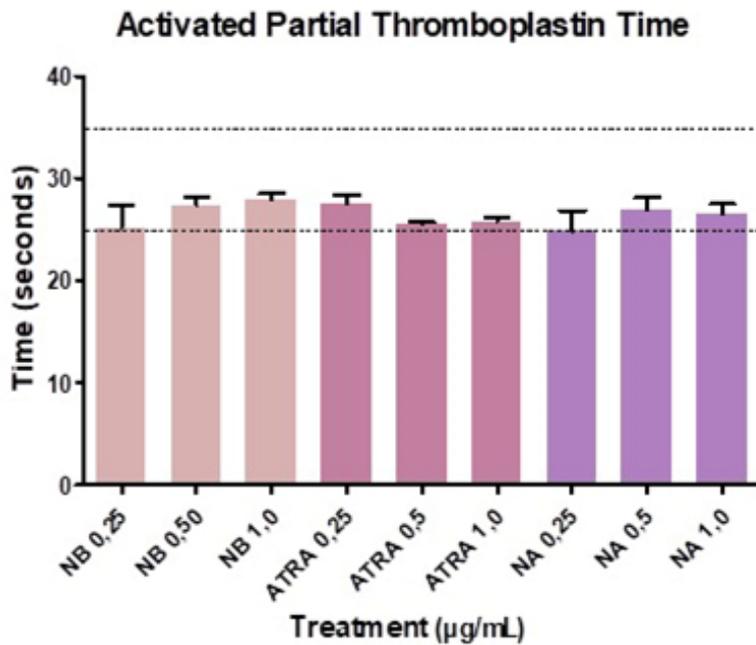
Figure 3 – APTT results
Notes: Dotted borders correspond to expected physiological times for healthy donors (between 25 and 35 seconds). Mean ± S.D. values from two independent experiments and from different donors.

4 DISCUSSION

Nanomedicine has evolved in the last years with the increase of the studies in this area. Nanoparticles increasingly play an important role in drug delivery, therapy and diagnosis. Because they have unique properties such as reduced size, large surface area and high reactivity. These characteristics make these nanoparticles have different behaviors according to the interaction medium.

For a particle to be considered nanometric, it is necessary that at least one of its dimensions is smaller than 1000 nanometers and that this causes changes in its natural properties. Thus, the results found for the sizes of the nanocapsules produced agree with the criteria established in the literature.[11]

The PDI measures the particle size distribution, which must be homogeneously distributed[12] suggests that in the case of manometric particles, the formulations should be monodispersed, in other words, with PDI values of less than about 0.25.

Nanoparticles may have longer systemic circulation times. These prolonged circulation time in the bloodstream increase the duration of contact with these blood components, including the coagulation system, potentially amplifying the activation of the coagulation cascade.[13]

In APL, malignant promyelocytes reveal procoagulant substances that activate the coagulation cascade, usually thrombin, release fibrinogen, coagulation factors, and platelets. Because of this reaction, patients with APL show DIC, fibrinolysis and proteolysis.[14]

The fibrinolytic system is generally largely inactivated during the phase of increased activation of coagulation, which contributes to the deposition of fibrin. However, in some situations (for example, in APL) fibrinolysis may be accelerated, thus contributing to severe bleeding.[15]

Fibrin deposition may lead to occlusion of the vessels and consequent impairment of blood supply to various organs, which, together with metabolic and hemodynamic changes, contribute to the failure of multiple organs. Consumption and consequent depletion of coagulation factors and platelets resulting from continuous procoagulant activity may lead to diffuse bleeding, which is often the first manifestation noted. It is important to emphasize that the DIC is always secondary to a basic disease and the identification and treatment of the predisposing condition are fundamental for the resolution of the syndrome.[16]

Morbidity and mortality in APL are strongly related to coagulopathy.[17] Clinically, APL differs from other Acute Myeloid Leukemias (AML) because it is associated with this alteration in about 60% to 90% of patients, the main cause of high mortality rates.[12] Hemostasis alterations were studied in 20 patients with APL prior to treatment, during induction of remission and after complete remission. The DIC was detected in 14 cases, 71% of them at the time of diagnosis.[17]

The results found in the hemolytic activity of free ATRA, evidenced the increase of the same, dependent concentration, corroborating with the findings Lim, Lee and Kim.[18] ATRA in free form has poor solubility, is chemically unstable, and when exposed to different factors, such as light, heat and oxidants, rapidly turns into isomerized products, such as isotretinoin or oxidized products such as *all-trans*-4-oxo. ATRA is highly lipophilic and has

this ability to permeate phospholipid bilayers, resulting in destabilization of erythrocyte cell membranes.[19]

With these findings and reporting to the clinic of patients with APL who acquire the syndrome of Differentiation to the ATRA, they are found to have symptoms of medullary relapse and severe anemia due to the use of high doses of this chemotherapy by the vein.[19]

Because ATRA is a hydrophobic drug, it has low aqueous solubility, it is a major disadvantage to administer it intravenously. But numerous efforts have been made to allow its administration by this route using nanocarriers, especially lipid systems such as liposomes, nanoemulsions (NE) and solid lipid nanoparticles (SLN) as a drug delivery system.[18]

No study was found, although the activity of the nanoparticles loaded with ATRA had the development team, yet they induced lower haemolytic toxicity. Moreover, these systems present potential for accumulation of the drug through a passive targeting called permeability and increased retention (EPR).[20]

Which is a complex process, activated by fibrinogen and involving other proteins. It is divided into two pathways: the intrinsic (antibody-dependent) and extrinsic (non-antibody-dependent) pathway, both ending in a common pathway.

However, the trends observed in studies of polymer-based nanoparticles, similar to that studied by us, are related to charge dependence as well as to hemolytic activity. Koziara, Oh, Akers, Ferraris, and Mumper13 showed that the reduction of the particle surface charge decreases platelet aggregation and activation. Previous studies have shown that polymeric nanoparticles injected intravenously could modify coagulation time by both pathways.[13]

Thus, the extrinsic coagulation pathway was measured using the PT test followed by the evaluation of the intrinsic coagulation pathway with the APTT. As previously mentioned, all treatments showed anticoagulants for concentrations higher than 1.5 µg/mL for the TTPA test, in the TP test the ATRA treatment maintained the results standard as the previous test, but in the nanoparticle treatments the anticoagulant activity was observed from the concentration of 1.0 µg/mL. As the main alterations occurred in the PT test, it is believed that a greater interaction with the factors VII, X, V, II of coagulation and fibrinogen could have occurred, however, as alterations were observed in the second test

(APTT), we did not rule out interactions with the intrinsic factors of coagulation, among them, factor VIII, IX, XI, XII and protein Precalicrine.[21] Other methods of analysis should be performed to confirm and better understand the results obtained here.

5 CONCLUSION

In conclusion of the interaction of the nanoparticles with the hemolysis tests and the coagulation cascade, we observed the occurrence of hemolysis with the nanoparticles with ATRA, but with a decrease in its toxicity compared to the free ATRA. The results for coagulation cascade showed that the two lowest concentrations present a safe profile, without intrinsic and extrinsic pathways alteration. Thus, we believe that there is a promising benefit to the use of these developed nanoparticles, but *in vitro* and in vivo cytogenotoxicity assays are necessary to ensure the use of these nanocarreadores aiming pharmacological safety of them.

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