Fountain Journal of Natural and Applied Sciences: 2013; 2(1): 6-10

DOI: 10.53704/fujnas.v2i1.41

A publication of College of Natural and Applied Sciences, Fountain University, Osogbo, Nigeria
Journal homepage: www.fountainjournals.com

Assessment of Erythrocytes as a Model for *In Vitro* Drug Toxicity

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Abstract

Mitochondria and lysosome have been used to study drug-cell interactions both *in vitro* and *in vivo*. However, their preparations to free them from other sub-cellular organelles are tedious and cumbersome. Erythrocytes (being a simple type of cells without sub-cellular organelles and very easy to obtain in pure form) was assessed for *in-vitro* toxicity testing of selected chemical compounds by monitoring the release of its hemoglobin content as an index of damage to cell membrane. Washed erythrocytes from male wistar rats were prepared in sodium phosphate buffer (pH 7.5) incubated with and without acetylsalicylic acid (a membrane stabilizer) and chloroquine (a membrane labilizer). The light scattering properties of the suspensions and the hemoglobin released were determined using standard methods. Acetylsalicylic acid did not significantly alter the degree of hemolysis ($P > 0.05$) whereas the chloroquine alone as well as the mixture of acetylsalicylic acid and chloroquine significantly increased ($P < 0.05$) it. Acetylsalicylic acid stabilized the erythrocytes membrane while chloroquine destroys it causing lysis of the cell membrane. Results obtained in this study suggest that the drugs have interacted with the erythrocytes in a manner that corresponds to their mode of action. Erythrocytes can therefore be used to study interactions between drug molecules and cell membrane.

Keywords: Acetylsalicylic acid; Cell membrane; Chloroquine; Erythrocytes; Hemoglobin

Introduction

Drug can be defined as any chemical substance intended for use in medical diagnosis, cure, treatment, or prevention of disease. Many drugs need to pass through one or more cell membranes to reach their site of action. The molecular description of the interactions between drugs and membrane constituents is a powerful tool towards understanding their mechanism of activity or toxicity. Development of new drugs involves the assessment of the effect of such drugs on target cell membrane and part of quality of drugs that recommends it for usage is its ability to cross the membrane barrier and arrive at its intended site with minimum damage to the cellular integrity. Prior to this time, mitochondria and lysosome have been extensively used to study drug-cell interaction both *in vitro* and *in vivo* (Ngaha *et al.*, 1989; Akanji and Ngaha, 1995; Akanji *et al.*, 1999). However, their preparations are tedious in order to free them from other sub-cellular organelles since this involve isolation, homogenization of a tissue, differential and density-gradient centrifugation at varying speeds and other precautionary steps which are time and energy-

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consuming. The objective of this study is to assess if erythrocytes (being a simple type of cells without sub-cellular organelles and very easy to obtain in pure form) can serve as a cheaper model to carry out in-vitro toxicity testing of new drugs by monitoring its hemoglobin content to see if the membrane is damaged by the movement of drug molecules across it.

Materials and Methods

Materials

Animals

Four wistar rats (*Rattus novergicus*) weighing between 160 – 180 g were obtained from the Animal House, Department of Biochemistry, University of Ilorin, Nigeria.

Chemicals

Acetylsalicylic acid (aspirin) used was a product of Juhel Pharmaceuticals Limited, Lagos, Nigeria and chloroquine phosphate was a product of Jopan Pharmaceuticals Nigeria Limited, Lagos.

Methods

Collection and preparation of erythrocytes

The rats were anaesthetized with chloroform. When they became unconscious, blood samples were collected into bottles containing EDTA (Akanji and Ngaha, 1989). The erythrocytes suspension was prepared by washing the collected blood free of plasma and buffy coat layer four times in cold 0.95 % (w/v) sodium chloride in 5 mM sodium phosphate buffer (pH7.5) (Ralston, 1976). After each wash, red cells were centrifuged at 2000 g for 5 mins. The supernatant and buffy coat was carefully removed by aspiration.

After the fourth wash, the erythrocytes were reconstituted with the 0.95% (w/v) sodium chloride in 5 mM sodium phosphate buffer pH (7.5) and used immediately for light scattering experiment and the hemoglobin release assay.

Preparation of chemical agents

Solutions of various concentrations of acetylsalicylic acid and chloroquine were prepared using 0.95% (w/v) sodium chloride in 5 mM sodium phosphate buffer pH (7.5) as the solvent.

Another solution containing the two chemical compounds together (acetylsalicylic acid and chloroquine) was also prepared. The solution contains 10^{-3} M acetylsalicylic acid and 10^{-2} M chloroquine.

Light Scattering Experiment

The light scattering experiment was done using the procedure described by Fry *et al.* (1979); Ngaha *et al.* 1979. Briefly, erythrocyte suspension was carefully diluted using sodium phosphate buffer (pH 7.5) containing 0.95% sodium chloride to give approximately 0.4 units. The change in the absorbance of the appropriately diluted erythrocyte suspension with or without varying concentrations (10^{-3} M - 10^{-2}M) of acetylsalicylic acid and chloroquine was read at 450 nm at 5 mins interval for 20 mins.

In the first set of experiments, 1 mL of different concentrations (10^{-3} M - 10^{-2} M) of acetylsalicylic acid and chloroquine were added separately to 20 μL of erythrocyte suspension in 1.5 mL sodium phosphate buffer (pH 7.5) containing 0.95 % sodium chloride to make a total volume of 2.5 mL. The absorbance of the mixtures was read at 5 mins interval over a period of 20 mins.

In another set of experiments, a mixture of 0.5 mL each of 10^{-3} M acetylsalicylic acid and 10^{-2} M chloroquine was added to 20 μL of erythrocyte suspension in 1.5 mL sodium phosphate buffer (pH 7.5) containing 0.95 % sodium chloride to make a total volume of 2.5 mL and the absorbance was read over a period of 20 mins as previously described.

Hemoglobin release assay

The method of Sadique *et al.* (1989) as modified by Oyedapo and Famurewa (1995) and Oyedapo *et al.* (2004) was employed to study the stabilization of erythrocyte membrane following incubation with the two chemical compounds. This assay mixture consisted of 2 mL of hyposaline, 1 mL of 5 mM sodium phosphate buffer at pH (7.5),

0.5 mL of 2% (v/v) erythrocyte suspension in isosaline and 1 mL of the drug solution to give a total assay volume of 4.5 mL. The reaction mixtures were incubated at 56°C for 30 mins. The tubes were cooled under running water followed by centrifugation at 5000 rpm for 10 mins. The supernatant was collected and absorbance of the released hemoglobin was read at 560 nm. The percentage membrane stability was determined using the expression:

$$\text{Membrane stability} = 100 - [100 \times \frac{(\text{Drug test} - \text{Drug control})}{\text{Control value}}]$$

Where:
Drug test = absorbance of hemoglobin released when the chemical compounds where added.

Drug control = absorbance of hemoglobin released when the erythrocyte suspension was omitted.

Control = absorbance of hemoglobin released when the chemical compounds were omitted.

Statistical analysis

Results were expressed as the mean ± SEM of five determinations. The data were analyzed using Duncan Multiple Range Test and complemented with Student's t-test. The differences were considered statistically significant at $P < 0.05$.

Results

Incubation of the erythrocyte suspension with different concentrations of acetylsalicylic acid gave an increase in optical density than when erythrocyte suspension was incubated alone (Figure 1) while incubation of erythrocyte suspension with different concentrations of chloroquine gave a decreased optical density than what was observed when erythrocyte suspension was incubated alone and when it was incubated with acetylsalicylic acid (Figure 2). Also, a decreased optical density was observed in the erythrocyte suspension to which a mixture of 10^{-3}M acetylsalicylic acid and 10^{-2}M chloroquine was added which is much lower than the optical density observed when erythrocyte suspension was incubated alone and when it was incubated with acetylsalicylic acid (Figure 3). In the membrane stability (hemoglobin release) assay, there was about 95% stability when the erythrocyte was incubated with acetylsalicylic acid, 87 % when it was incubated with chloroquine and about 41 % when it was incubated with the mixture of the drug solutions (Figure 4).

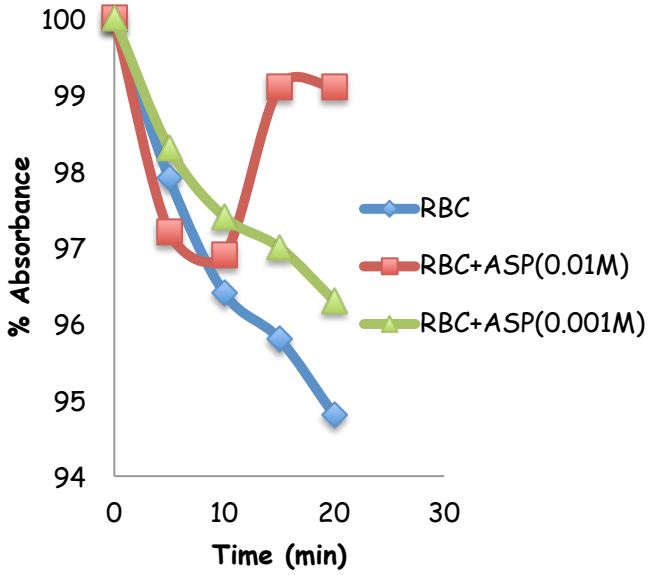

Figure 1: Effect of different concentrations of aspirin on light scattering properties of erythrocyte membrane.

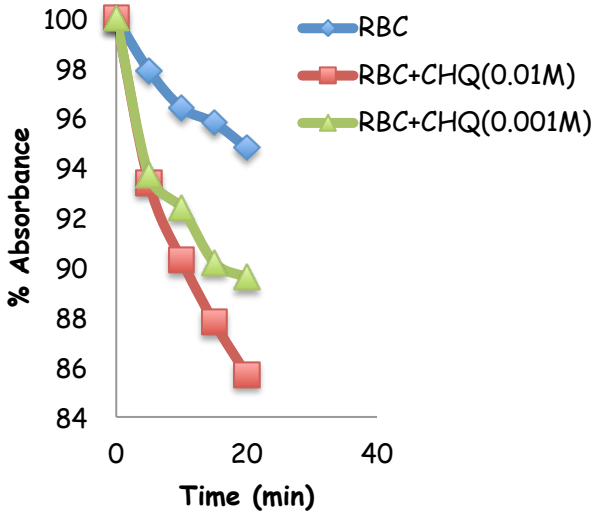

Figure 2: Effect of different concentrations of chloroquine on light scattering properties of erythrocyte membrane.

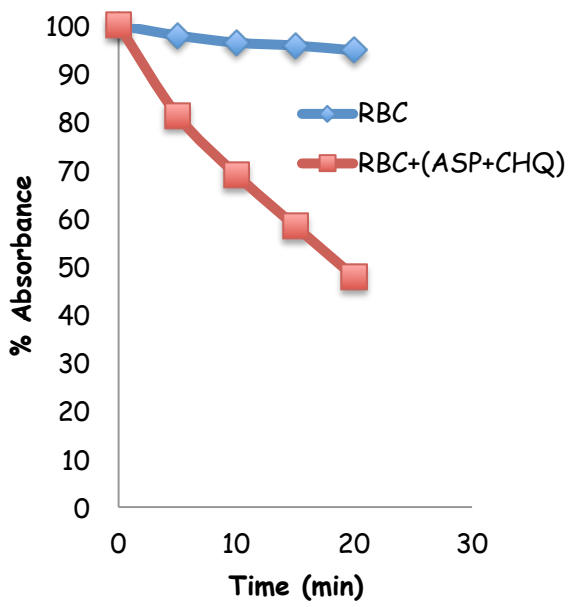

Figure 3: Effect of addition of a mixture of 0.001 M aspirin and 0.01 M chloroquine to erythrocyte suspension.

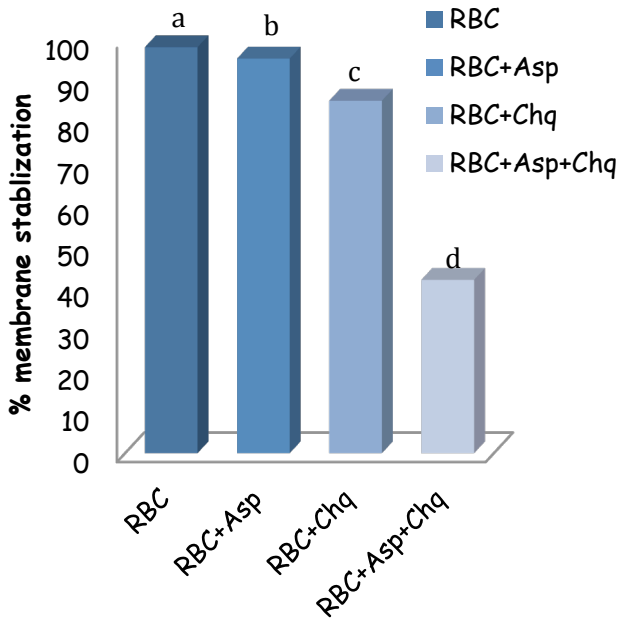

Figure 4: Effect of aspirin and chloroquine on erythrocyte membrane stability.

Discussion

Light scattering measurement has provided a convenient means of detecting changes in the integrity of membrane (Fry *et al.*, 1975). It has also been used previously to investigate the effect of pH and temperature on lysosome (Sawant *et al.*, 1964; Shibko and Tappel, 1965).

The significant increase in optical density following treatment of erythrocyte suspension with acetylsalicylic acid indicates that there was little or no hemolysis, which may be as a result of its membrane stabilizing property. Acetylsalicylic acid seems to play a stabilizing role, and thus maintained the integrity of the cell membranes. This accounted for the increased optical density throughout the period of the experiment. This may be attributable to the ability of acetylsalicylic acid to lodge itself in spaces created between molecules on the membrane structure. However, the significant decreased optical density observed after incubation of the erythrocyte suspension with chloroquine is an indication of hemolysis of the erythrocytes which may be as a result of the labilizing potential of chloroquine causing the cells to swell and hemolyse thus releasing their hemoglobin content. In the same vein, the significant decreased optical density observed after incubation of the erythrocyte suspension with a mixture of acetylsalicylic acid and chloroquine indicates there was a high degree of hemolysis. This finding is surprising as it contradicts previous finding that a mixture of a membrane stabilizer and labilizer will produce a synergistic effect on the membrane and leave it intact.

It has previously been shown that chemical compounds interact with cell membrane in order to gain entrance into the cell; such interaction could subsequently lead to either lysis of the cell or its stabilization. The implication of this result is that chloroquine, a well known lysosome membrane labilizer (Ngaha and Akanji, 1982) was able to labilize erythrocyte membrane as demonstrated by decrease in percentage of light absorbed.

In the same vein, acetylsalicylic acid was demonstrated to be able to stabilize erythrocyte membrane. The findings in the experiments showed that incubation of erythrocyte with chloroquine led to release of higher concentration

It is however surprising that when erythrocyte was incubated with the mixture of the two chemical agents, it increased the extent of labilization of erythrocyte membrane as shown in both the light scattering experiments and hemoglobin release studies as contrary to the views of previous workers who had shown that when a membrane labilizer and stabilizer are used together, their effects neutralize each other and the membrane is intact (Akanji, 1989, Ngaha *et al.,* 1989). It will therefore be necessary to investigate in future why this should be so.

Conclusion

From the results, erythrocytes have been able to show how acetylsalicylic acid and chloroquine interact with the cell membrane; therefore they can be used as an approach to predict organ toxicity. This *in- vitro* test will lead to reduction in cost for testing toxicity of newly developed chemical compounds and also mechanism of action of drugs.

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