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Cross-cytotoxicity study of antimalarial drugs and traditional medicinal plants on Renal Cells

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Abstract

Objective: The association of Artemisinin based combination therapy with herbal medicine seems to be a factor favouring the occurrence of serious adverse effects, hepatitis disorders and acute renal failure in particular. The aim of this study was to evaluate the cross- toxicity of these associations on renal cells.
Methods: African green monkey kidney cells (VERO) were incubated with anti-malarial drugs and plant preparations. The products used were: Artesunate 100mg / amodiaquine 270mg (ASAQ) and artemether 80mg / lumefantrine 480mg (AL), and *Sida acuta* and *Enantia polycarpa* at a concentration of 10 µg / ml. After 5 days of incubation, a cell count was performed using a haemocytometer.
Results: ASAQ resulted in significant cell destruction compared to the control between Day2 ($p < 0.05$) and Day5 ($p < 0.01$) with a peak at day 3 ($p < 0.001$). There was also a significant difference between *Sida acuta* and control on Day4 ($p < 0.05$) and Day5 ($p < 0.001$). Cell mortality was> 30% in the tubes treated with ASAQ and *Sida acuta*. Finally, cell destruction was statistically significant in tubes treated with the combination of antimalarial drugs and traditional plants compared to the control tube from Day2 ($p < 0.001$).
Conclusion: The renal toxicity observed with ASAQ, *Sida acuta* and combination of antimalarial drugs and herbs, appears dose-dependent or cumulative. The mechanism of this renal toxicity should be better analysed in further studies.
Keywords: Cytotoxicity, Artemisinine based Combination Therapy, Traditherapy, Malvaceae, Annonaceae.

1. Introduction

In developing countries, fighting malaria is a major public health concern. Malaria is responsible for higher morbidity/mortality rate, and represents 40 to 60 % of hospitalization in Cote-d'Ivoire [1]. In 2001, a World Health Organisation (WHO) expert panel recommended the use of Artemisinin-based Combination Therapy (ACT) for treatment of uncomplicated falciparum malaria in all endemic countries [2]. Since the introduction of ACT in Cote-d'Ivoire in 2003-2004, there has been a change in their safety profile and an increase of serious cases of side effects related to their use. In 2005, the incidence of serious side effects associated with antimalarials increased to 57% [3]. In July 2008, stagnancy of cases of Blackwater fever was noticed and an abnormal increase of cases of hepatonephritis. As a matter of fact this development prompted drawback in the uses of ACT for malaria treatment. This multi-visceral attack is associated with simultaneous severe hepatic and renal impairment. The circumstances responsible for this occurrence and factors inherent to this malfunction seem to be unknown. In this context, pharmacovigilance investigation was carried out, and ACT was found to be involved. The favoring factors of

hepatonephritis were self-medication, concomitant or successive intake of more than two antimalarials, associated with antibiotics of fluoroquinolone family or with herbal Medicinal recipes [4,5]. Renal impairment accounted for 80 to 90% of severe cases. Previous studies have reported possible toxic or immuno-allergic mechanisms that might have been responsible for functional organs (liver and kidney) and nervous system lesions. In West Africa, likewise across the continent, more than 80% of the population depend on traditional Medicine or plants for primary health care [6]. In addition, the association of modern treatment with traditional plants is a very common fact in this part of the world. However, this method of treatment could be disadvantageous. Therefore, the aim of this study was to evaluate the toxicity of the association of ACT with extracts of antimalarial plants on renal cells (Vero).

2. Materials and methods

Plant aqueous extracts and suspensions of ACT were put into contact with renal cells (Vero cell lines).

2.1. Cell Lines

Cell Lines are African green monkey kidney cells (Vero E6). Cells were thawed and seeded in T-75 cm^2 tissue culture flasks in Minimum Essential Medium (MEM) containing 10%, fetal bovine serum (FBS), mixture of penicillin 10 UI/ml 1%, streptomycin 10 mg/ml, L-Glutamine 1% (Sigma St Louis MO, USA).

2.2. Drugs used

Antimalarial drugs used made up of artesunate 100 mg/amodiaquine 270 mg (ASAQ) and artemether 80 mg/lumefantrine 480 mg (AL). Medicinal plant extracts tested involved *Sida Acuta* (PSA) from the *Malvaceae* family and *Enantia polycarpa* (PEP) from *Annonaceae* group. These plants showed strong antimalarial activity in previous studies [7-9].

2.3. Cell culture

Cells were trypsinized and a suspension of 0.5 x 10^5 Cell/ml was seeded in 51 tubes T-25 cm^2 of 2 ml MEM 10% respectively for 48 h at 37ºC in humidified air and 5% CO_2 . Then the growth medium was changed appropriately.

2.4. Preparation of Plants

2.4.1 Enantia Polycarpa

Step 1: crushing the bark of *Enantia polycarpa*

The bark of *Enantia polycarpa* was thoroughly washed and then dried. They are then broken into smaller pieces and then crushed using a suitable grinder to obtain a very fine powder.

Step 2: preparation of the extract

Two hundred grams (200 g) of powder of *Enantia polycarpa* are placed in two liters (2L) of distilled water. The mixture was stirred for 24 hours using a magnetic stirrer. The solution obtained was filtered three times on hydrophilic cotton and on Watman paper and then dried in an oven at a temperature of 40 degree. The powder obtained is the aqueous extract of the powder of the bark of *Enantia polycarpa*.

2.4.2 Sida acuta

A preparation of the aqueous extract of the leafy stems of *Sida acuta* was performed. Two hundred grams (200 g) of leafy stems of *Sida acuta* was washed and then placed in two liters (2L) of distilled water. The mixture was boiled for 15 minutes. The solution obtained was cooled and filtered three times on hydrophilic cotton and on Watman paper and then dried in an oven at a temperature of 40 degree. The powder obtained is the aqueous extract of leaves of *Sida acuta.*

2.5. Drug Preparation

Artesunate tablets 100mg / amodiaquine 270mg (ASAQ) and artemether 80mg / lumefantrine 480mg (AL) were crushed. The powder obtained was suspended in 2% MEM.

2.6 Study of Cell Toxicity

A concentration of 1mg / ml was prepared for all our products. Two dilutions of ½ made it possible to reduce this concentration to 10 µg / ml. Thus, 5 ml of the plant and drug preparations were brought in contact with the VERO cells in the T-25 cm^2 culture tubes. We first carried out separate tests with artemether / lumefantrine and artesunate / amodiaquine and *Sida acuta* and *Enantia Polycarpa*. Then we carried out combinations tests by combining antimalarials with traditional plants 2 by 2. Thus, for the separate tests, ASAQ was put in 5 tubes, AL in 5 tubes, PSA in 5 other tubes and PEP in 5 tubes. In the last 5 tubes, no products were added; they are to serve as controls. For the combined tests, ASAQ + PSA was put in 5 tubes, ASAQ + PEP in 5 others, AL + PSA in 5tubes, AL + PEP in 5 tubes. The last five tubes to serve as control. After inoculation, the tubes were placed in an oven at 36 °C to 37 °C under 5% CO_2. Cells were observed daily for 5 days under the inverted fluorescence microscope for carpet confluence. Measurement of the effect of antimalarial drugs and traditional plants on renal cells (Vero) was achieved by daily counting of the number of living cells using the haemocytometer. Indeed according to Coulerie [10], a product is considered to be cytotoxic when it causes a cell mortality greater than 30% at a concentration of 10 µg / ml. Finally, each assay was carried out in triplicate.

2.7. Statistical Analysis

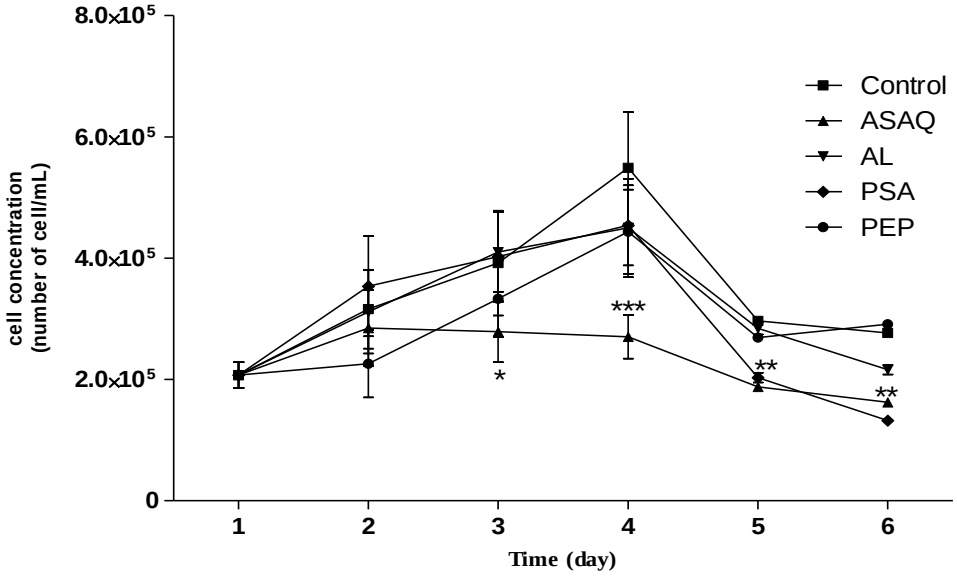
The results were expressed as mean ± standard error of mean (S.E.M.). Statistical analysis was performed using Graph Pad Prism 5.0® software. The difference between groups was assessed by analysis of variance (ANOVA), followed, when necessary, by the Turkey's test

or Bonferroni post-tests. Differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. Effect of monotherapies on Vero Cells.

The activity of antimalarial drugs and plant extracts on Vero Cells was compared to the control (Figure 1). From this figure, it is noticed that artesunate/amodiaquine brought about a significant cell mortality compared to the control, between Day 2 ($p<0.05$) and Day 5 ($p<0.01$) with a peak on Day 3 ($p<0.001$). There is also a significant difference in impact between the control and the *Sida Acuta* plant on Day 4 ($p<0.05$) and Day 5 ($p<0.001$).



*p<0.05 **p<0.01 ***p<0.001 compared to control

Figure 1: Effect of monotherapies on Vero cells

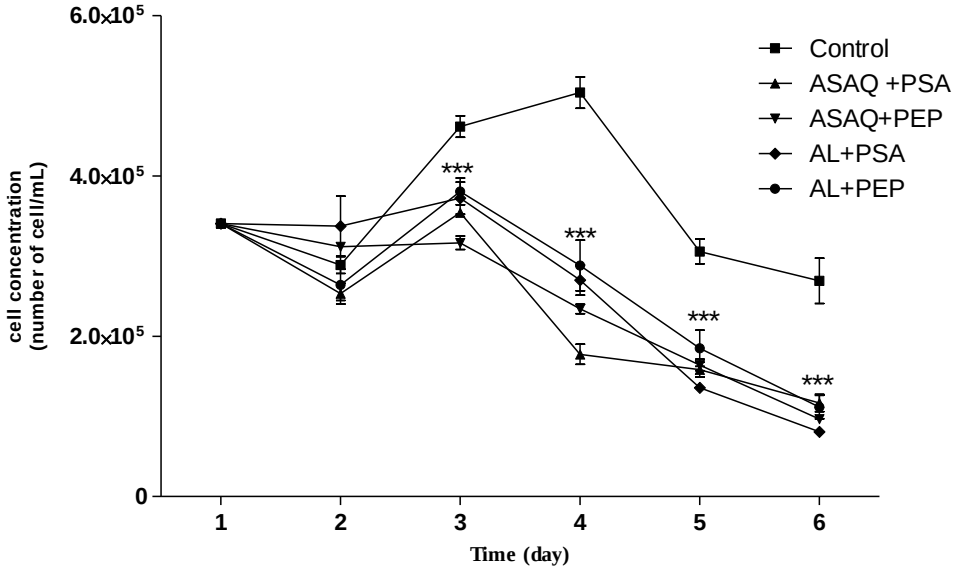
The mean percentage mortality of cells in treated sample tubes is given in Table 1. From this table, the cell mortality in samples treated with artesunate/amodiaquine is greater than 30% from Day 2 to Day 5. This indicates, according to Coulerie, that the test compound is toxic. Likewise, for *Sida Acuta* extract, Cell mortality was greater than 30% at Day 4 and Day 5. This also shows that the plant is toxic to Vero Cells.

Table 1: Effect of monotherapies on cell mortality (%)

Products	Days of incubation (D)					
	0	1	2	3	4	5
MEM 2%	0.85	1.3	0.27	0.6	1.24	1.15
ASAQ	0.85	12.48	37.39	48.55	57.41	57.64
AL	0.85	5.36	4.16	19.96	6.72	19.56
PSA	0.85	3.57	2.34	29.79	36.38	50.69
PEP	0.85	38.76	10.24	18.08	9.26	0

3.2. Effect of combination therapy on Vero Cells.

The combination of antimalarial drugs with traditional plants in Cell treatment is shown in Figure 2. The graphs displayed Cell apoptosis due to this combination therapy as compared to control is noticed from Day 2 ($p<0.001$).



***p<0.001 compared to control

Figure 2: Effect of combination therapy on Vero cells

The mean percentage Cell mortality in treated sample test-tubes is displayed in Table 2. From this table, in every combination therapy considered, Cell mortality was greater than 30% from Day 3. Therefore, the combination of antimalarial drugs with plant extract could be said to be toxic from Day 3.

Table 2: Effect of Combination Therapy on Cell Mortality (%)

Products	Days of incubation (D)					
	0	1	2	3	4	5
MEM 2%	0.17	0.19	0.3	0	0.10	0.28
ASAQ+PSA	0.17	18.75	25	63.72	47.85	57.14
ASAQ+PEP	0.17	7.16	31.57	54.18	46.69	66.81
AL+PSA	0.17	0	17.16	44.86	57.98	72.68
AL+PEP	0.17	0	16.31	38.90	35.40	57.56

4. Discussion

4.1. ACT Cytotoxicity

The evaluation of the toxicity mechanisms of ACT is the first of its kind in a combination therapy involving ACT with traditional plants extract. At a concentration of 10 µg/ml, artesunate/amodiaquine and extract of *Sida Acuta* seem to be more nephrotoxic than artemether/lumefantrine and *Enantia Polycarpa*. These results confirm a study conducted by Die Kacou *et al* [4], which showed kidney impairments in 80 to 90% of severe cases due to ACT treatment. Renal failure is acute, showing higher mean values of biological indicators. Thus, mean values of urea and creatinine, respectively reached 2.20 g/l and 109 mg/l. Renal failure caused by treatment with antimalarial drugs have been previously reported [11-14]. A study carried out by Daubrey [15] on Blackwater fever caused by antimalarial drugs showed renal failure with urea and creatinine values of 1.74 g/l and 62.74 mg/l respectively. According to the results of this study, renal dialysis was recommended to 61% of the patients. According to previous studies [12-14] from 22 to 47.6% of Ivorian patients had dialysis. A study conducted by Kamagate *et al* [16] also revealed renal failure after taking antimalarial drugs composed of quinine 36%, artemether/lumefantrine 20%, artesunate/sulfamethoxypyrazine 16% and artesunate/amodiaquine 1%. This renal toxicity occurred within 2 to 4 days. Furthermore, cases of multi-visceral attack were described after taking artesunate [17] or artesunate/amodiaquine [18]. But, this renal toxicity seems to be dose-dependent or accumulative. Artemether/lumefantrine seems to be less nephrotoxic in the study reported. The cases of renal failure described after taking artemether / lumefantrine could be related to the haemolysis responsible for the glomerular obstruction.

4.2. Toxicity of medicinal plants to Vero Cells.

Sida Acuta (*Malvaceae*) showed Cell toxicity in Day 4 and Day 5, while Konate *et al* [19] reported an acute toxicity (Lethal dose 50 (LD_{50})= 3.2 g/kg) in rats, which is qualified as low according to Hodge and Stener scale. For sub-chronical toxicity, after one week treatment, a significant body weight difference was noticed between the treated group and control ($p<0.01$). Additionally, in this study, the respective value of creatinine and urea in rats showed a significant decrease in these biomarkers in the group treated with extracts of *Sida Acuta* compared to control group ($p<0.05$). As for *Enantia Polycarpa* (*Annonaceae*) extracts, no cytotoxicity was noticed. In contrast, Anosa *et al* [7] revealed in their study an acute toxicity in rats, an intra-peritoneal LD_{50} value of 186 mg/kg. Moreover, the plant did not cause death, even when administered orally at higher doses of 2000 to 4000 mg/kg.

4.3 Toxicity to Vero Cells as a result of combination therapy of antimalarial drugs and traditional plants

The mixture of antimalarial drugs and traditional plants extracts showed cell toxicity. In several investigations on severe side effects of antimalarial drugs, the combination of these compounds was mentioned. A study performed by Die Kacou [5] showed that 20% of cases of hepatonephritis were due to ACT combined with traditional herbal recipe. Particular occurrence of hepatonephritis could be due to self-medication and/or misuse of antimalarial drugs either associated or not with fluoroquinolones. Those molecules having a similar structure to that of amino-alcohols (lumefantrine, quinine), whose hepatic and renal toxicity is well-known as well as that of artemisinin. Ultimately, the risk of hepatonephritis occurrence is nine fold by self-medication. The occurrence of renal impairment could be explained due to accumulative or additive toxicity of combined molecules.

5. Conclusion

The present study showed cytotoxicity of drug combination artesunate/amodiaquine, to Vero Cells. In addition, a pronounced toxicity was noticed in combining ACT with traditional medicines. Further studies will elucidate the mechanisms involved.

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