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IN VITRO NEUROGICAL TOXICITY OF ARTEMISININ-BASED COMBINATION THERAPY AND MEDICINAL PLANTS

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ABSTRACT: *Introduction: The traditional medicine seems to be one of the factors associated with the occurrence of severe neurological adverse events described, after the use of artemisinin-based combination therapy. The aim of this study was to study the toxicity from the association of artemisinin-based combination therapy with the herbal medicine over the nerve cells. Material and Methods: Some nerve cells (N2a) were cultivated (0.5*10^5 cell/ml) and brought in contact with antimalarial preparations and / or antimalarial plants.The products used were artesunate100mg/amodiaquine 270mg (ASAQ) and artemether 80mg/lumefantrine 480mg (AL) as antimalarial drug and Sida acuta (PSA) and Enantia polycarpa (PEP) at 10µg/ml as antimalarial medicinal plant. After 5 days of incubation, a cell counting has been carried out with a hemocytometer. Results: A significant nerve cells destruction, compared to the control was observed for ASAQ between day 2 and day 4 ($p<0.001$). We also noted a significant difference between the control and AL, between day 1 ($p<0.05$) and day 3($p<0.001$), between the control and Sida acuta, at day 2 and day 5 ($p<0.001$). In the tubes treated with ASAQ and Sida acuta, cell mortality was greater than 30%. Finally, a cell destruction statistically significant in the tubes treated via the combination of antimalarials and traditional plants compared to the control tube was observed from day 3($p<0.001$). Discussion / Conclusion: Plants appeared to enhance the neurological toxicity of in vitro conventional antimalarials. The combination of conventional antimalarials with the traditional therapy, during malaria treatment should be avoided.*

KEY WORDS: cytotoxicity, antimalarials, nerve cells, artemisinin-based combination therapy, herbal medicine

INTRODUCTION

Since January 2007, a new protocol for the management of malaria has been adopted by the Ivorian National Program against malaria. The use of artemisinin-based combination therapy is recommended by this scheme. Those combinations seem efficient in the management of malaria. This treatment avoids the evolution to severe malaria. However, neurological adverse events such as vertigo, adynamia, convulsions and dyskinesias have been described. The mechanisms seem unknown in the occurrence of these lesions of the nervous system [1,2]. Kamagaté discussed this neurotoxicity [3]. Hydroartemisinin, a metabolite of artemisinin derivatives, appears to be neurotoxic and dose-dependent in experimental studies. However, severe cases seem rare in the human species. The reported cases are mostly non-severe neurological effects, more predominant in associations with amodiaquine, than in combinations with lumefantrine. Exposure times would be 2-5 days [3].

In addition, the risk factors for the occurrence of these neurological adverse effects were unknown. On the other hand, self-medication and misuse seem to be associated factors. In addition, the use of traditional treatment in combination with an antimalarial is common in our tropics. These drug combinations would probably be a factor favoring the occurrence of these adverse effects. What are the possible interactions between antimalarial and traditional treatment?

The aim of this study was to evaluate the toxicity of artemisinin-based combination therapy in association with antimalarial medicinal plant extracts on nerve cells.

MATERIALS AND METHODS

Cell lines

Mouse neuroblastoma cell lines (N2a cells or Neuro2a cells) from CDC Atlanta were used. They were thawed and cultured in T75 culture dishes (75 cm^2) in enriched MEM environment. This MEM culture environment contained 10% fetal calf serum (FCS) decomplemented with 1% antibiotic mixture (10 IU / ml penicillin, 10 mg / ml Sigma streptomycin) and 1% L-Glutamine (Sigma), to allow cell growth.

Products used and plant extract

Artesunate 100mg / amodiaquine 270mg (ASAQ) from the Winthrop laboratory and artemether 80mg / lumefantrine 480mg (AL) from the Novartis laboratory were used: as antimalarial drugs, and *Sida acuta* (PSA) and *Enantia polycarpa* (PEP) as antimalarial medicinal plant. These plants came from the medicinal plants markets of Abidjan (Adjamé Latin area and Abobo). They were antimalarial herbal remedies that had previously been shown to be effective against parasites [4-6]. The samples of Enantia Polycarpa bark (Annonaceae) and the leafy stem of Sida acuta (Malvaceae) were used for our study.

Cell culture

Trypsination of the cells was performed. The culturing involved a number of 0.5×10^5 cells / ml introduced into 2 ml of 10% MEM development environment, in 51 culture tubes T25 (25 cm^2) and incubated for 48 hours at 36°C - 37°C under CO_2 at 5%. At the end of the incubation, the tubes were removed.

Aqueous extraction procedure of *Enantia Polycarpa* and *Sida acuta*

Enantia Polycarpa

Careful washing and drying of *Enantia polycarpa* bark samples was done. These samples were then crushed into small pieces and then crushed using a suitable grinder to obtain a very smooth powder. This bark powder of *Enantia polycarpa* (200g) was introduced into two liters (2L) of distilled water.A magnetic stirrer allowed to stir the mixture for 24 hours, then filtration of the obtained solution was carried out three times using hydrophilic cotton and Whatman paper. The solution was dried in an oven at a temperature of 40 ° C. A powder obtained was the aqueous extract of *Enantia polycarpa* bark powder (PEP).

Sida acuta

The aqueous extract of the leafy stems of *Sida acuta* has been prepared. A washing of two hundred grams (200g) of leafy stems of Sida acuta was performed, then they were introduced into two liters (2L) of distilled water. The mixture was boiled for 15 minutes. Cooling of the obtained solution was performed, followed by filtration, three times on hydrophilic cotton and Whatman paper. The filtered solution was then dried in an oven at a temperature of 40 ° C. The powder obtained, corresponded to the aqueous extract of leafy stems of Sida acuta (PSA).

Preparation of drugs

The artesunate 100mg / amodiaquine 270mg (ASAQ) and artemether 80mg / lumefantrine 480mg (AL) tablets were crushed. Solution of the obtained powder was made in 2% MEM.

Study of the cell toxicity

A mother concentration of 1 mg / ml was prepared for all our products. This concentration was reduced to 10 µg / ml by two dilutions to the tenth. In the T25 culture dishes, 5ml of the plants and drugs preparations were brought into contact with N2a cells. Artemether/ lumefantrine, artesunate / amodiaquine, Sida acuta and Enantia Polycarpa were first tested one by one. Then the products were tested 2 by 2 by combining antimalarial with traditional plants. Each experiment was performed in 5 boxes. The last 5 boxes were used as control; no product has been added to the culture environment. In total for the products individually tested, 25 boxes were used. To test some combinations and the control, 25 other boxes were also used.

After inoculation, the tubes were put in the oven between 36 ° C and 37 ° C under CO_2 at 5%. Observation of the cells was performed daily for 5 days under an inverted fluorescence microscope for the confluence of the cell layer. To know the effect of drugs and traditional plants on nerve cells, a daily count of the number of living and dead cells was performed using the hemocytometer.
In fact, according to Coulerie, the cytotoxicity of a product is established when it causes a cell death greater than 30% at a concentration of 10 µg / ml [7]. The experiments were performed in triplicate.

Statistical analysis

The statistical analysis was performed by using the Graph Pad Prism 5.0® software. The results were expressed as mean ± standard deviation (Mean ± SD). The difference between the groups was evaluated by an analysis of variance (ANOVA) followed, where appropriate, by the Turkey test or the Bonferroni post-test. The statistical significance was considered at $p< 0.05$.

RESULTS

Monotherapy effect on nerve cells

The effect of antimalarials and herbs on nerve cells compared to a cell control on which no treatment had been added, could be seen in Figure 1. We found that artesunate / amodiaquine caused significant cell destruction compared to the control, between day2 and day4 ($p <0.001$). There was a significant difference between the control and artemether / lumefantrine between day 1 ($p <0.05$) and day 3 ($p <0.001$), and between the control and the plant *Sida acuta*, at day 2 and day 5 ($p < 0.001$).This difference was also significant with the *Enantia Polycarpa* plant between day2 and day4 ($p <0.001$). We noted no difference from day 5 ($p> 0.05$).

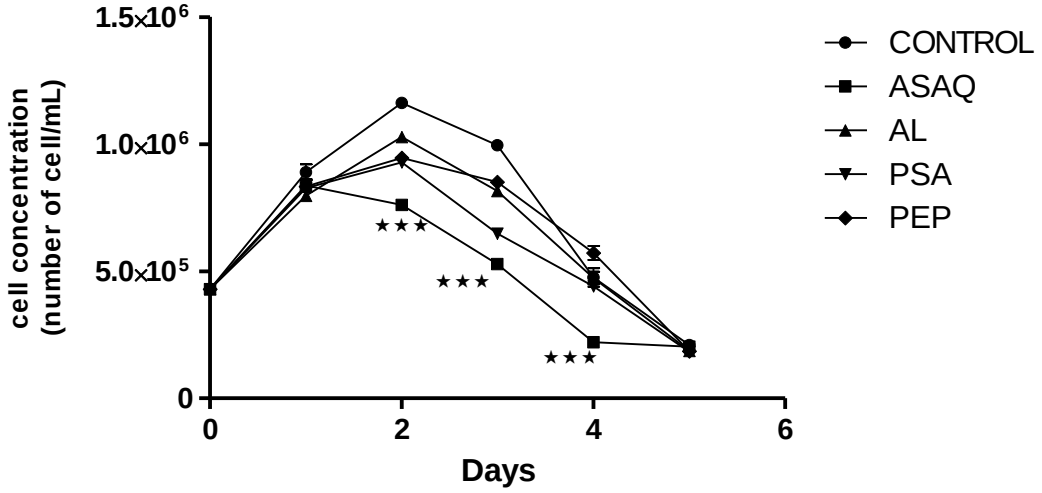

Figure 1: Effect of monotherapies on nerve cells (* p <0.05 ** p <0.01 *** p <0.001 compared to control, n = 5)

Table I gave the percentage of cell mortality in the different tubes treated with the products. In the tube treated with artesunate / amodiaquine, mortality was greater than 30% from day2 to day4. This indicated, according to Coulerie that the product was toxic [7]. For the plant *Sida acuta*, the mortality was also greater than 30% at day3. This indicated a cellular toxicity of this plant on day3. We noticed a low cell mortality for artemether / lumefantrine and especially for the plant *Enantia polycarpa.*

Table I: Effect of monotherapies on nerve cell mortality (%)

Products	**Days of incubation (D)**						**Means ± SD**
	0	1	2	3	4	5	
MEM 2% (n=5)	0,58	0,5	2	1,37	0,65	0,1	0,92 ± 0,76
ASAQ (n=5)	0,58	5,22	34,95	48,07	54,75	2,91	29,18 ± 24,02
AL (n=5)	0,58	9,35	11,98	17,11	3,25	9,56	10,25 ± 5,01
PSA (n=5)	0,58	5,77	19,79	33,83	19,78	13,41	18,52 ± 10,32
PEP (n=5)	0,58	5,12	16,82	13,75	0	8,77	8,89 ± 6,70

SD=Standard Deviation

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Effect of therapies combination on nerve cells

The effect of the combination of antimalarials and traditional plants is observed in Figure 2. The curves all looked the same. However, we noted a statistically significant cell destruction in the tubes treated with the combination of antimalarials and traditional herbs compared to the control tube on day 1 and day 3 ($p < 0.001$). The destruction was more important in associations containing artesunate / amodiaquine.

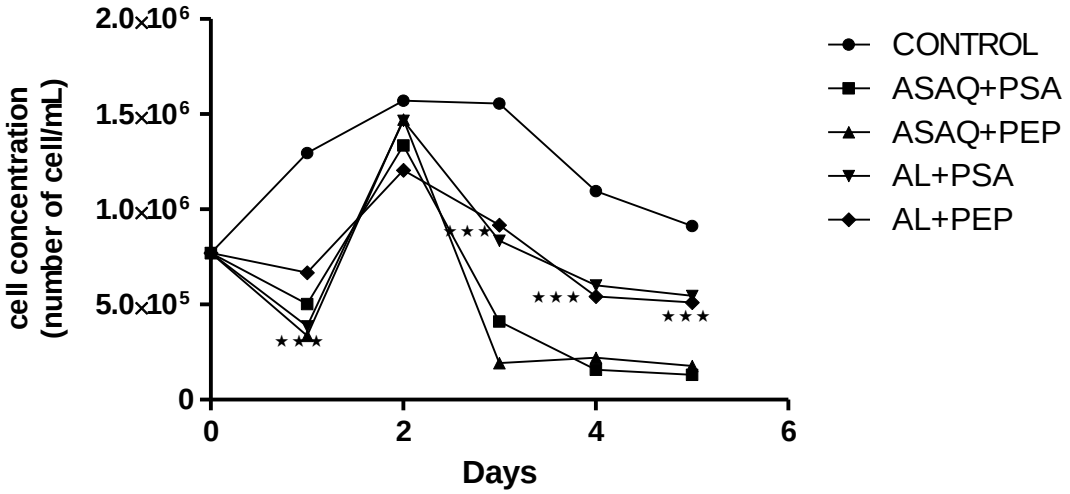

Figure 2: Effect of therapies combination on nerve cells (*** $p < 0.001$ compared to control, $n = 5$)

The percentage of cell mortality in the tubes treated with the products was shown in Table II. For all product combinations, cell mortality of nerve cell was>30% from day3. The combination of drugs and traditional plants could therefore be considered toxic from day3. The association of ASAQ with PSA had a toxicity comparable to the association of ASAQ with PEP as of day3.The association of AL with PSA had a toxicity comparable to the association of AL with PEP as of day3. We observed a higher mortality for combinations containing artesunate / amodiaquine by day1.

Table II: Effect of Therapies combinations on Nerve Cells Mortality (%)

Products	**Days of incubation (D)**						**Means ± SD**
	0	1	2	3	4	5	
MEM 2% (n=5)	0,33	0,39	0,48	0,16	0,23	0,56	0,36 ± 0,17
ASAQ+PSA (n=5)	0,33	59,02	12,58	71,99	83,72	83,47	62,16 ± 29,51
ASAQ+PEP (n=5)	0,33	75,29	6,29	88,76	81,39	82,35	66,82 ± 34,17
AL+PSA (n=5)	0,33	68,23	4,19	44,29	39,76	36,69	38,63 ± 22,91
AL+PEP (n=5)	0,33	49,21	23,54	41,53	51,40	45,10	42,16 ± 11,08

SD = Standard Deviation

DISCUSSION

At concentration of 10 µg/ml, artesunate / amodiaquine and artemether / lumefantrine showed toxicity on nerve cells. However, artesunate / amodiaquine seemed more neurotoxic than artemether / lumefantrine. In the drug information leaflets, neurological disorders have been described for artesunate / amodiaquine [8]. The most cited effects were peripheral neuropathy, vertigo, neuromyopathy, and rarely the extra-pyramidal syndrome. For arthemether/lumefantrine, the leaflets described effects such as headache, dizziness, paresthesia, hypoesthesia [8] In the literature, severe neurological adverse effects have been described with amodiaquine. Indeed, oro-facial dyskinesias have been described with amodiaquine [1, 2]. Intoxication with amodiaquine would result in syncope, spasticity, convulsions and involuntary movements such as dyskinesia [9]. Neurological events have also been described after taking artesunate [10]. In addition, artemether / lumefantrine would be able to cause neurological effects such as ataxia [11]. Amodiaquine, artemether and lumefantrine seem to have a neurological tropism. 4-aminoquinolines such as chloroquine and amodiaquine accumulate in the optic nerve and retina, and may be responsible for retinopathy [12]. The neurotoxicity of artemisinin derivatives is thought to be related to a common active metabolite, dihydroartemisinin. This effect would be dose-dependent [12]. This severe and fatal neurotoxicity has been found in dogs, rats and monkeys for high doses of artemisinin derivatives [13, 14]. Injectable forms seem more neurotoxic than oral forms. Cumulative doses greater than 960 mg of artemether would increase the risk of neurotoxicity, as well as at least 2 to 5 days of exposure to artemisinin

derivatives [12]. Our study confirmed this delay. This finding suggests that artemisinin derivatives have cumulative toxicity in relation to treatment duration extension or concomitant treatment associated with misuse [15]. In addition, the neurological tropism of the various molecules (dihydroartemisinin, lumefantrin and amodiaquine) could partly explain this neurotoxicity. Lumefantrine, a structural analogue of mefloquine, would affect the cholinergic system through neuronal inhibition of calcium homeostasis [3; 12]. Shorter delays of occurrence, and/or with low doses would rather suggest immunoallergic mechanisms. Further studies should be done to confirm this.

The plant *Enantia polycarpa* seemed less neurotoxic than the plant *Sida acuta.* However, the therapies combination with the plants were all neurotoxic. Those containing artesunate / amodiaquine appeared to be more neurotoxic with comparable toxicity for artesunate/ amodiaquine and Sida acuta and artesunate / amodiaquine and *Enantia polycarpa* combinations. Plants seemed to enhance the neurological toxicity of conventional antimalarials. We could mention a toxic additive effect. This additive effect was evident from the first day of exposure. This would then lead us to ask patients to avoid the association of ACTs with traditional therapy. Indeed, this association would increase the risk of neurological toxicity.

The most common side effects, according to users of medicinal plants on social networks, appeared to be fatigue, insomnia with transit slow-down [16]. A patient using *Sida acuta* to treat bartonellosis, at a dose of ⅓ cp × 2 / day for 10 days, presented with neurological signs such as vertigo, confusion, disconnection from reality, fatigue, headache, somnolence [17].

Neurological effects have been described in interactions between medicinal plants and drugs. The plants involved were *Zingiber officinal*, *Panax ginseng*, *Allium sativum* and *Ginkgo biloba*. The drugs concerned immunosuppressants (cyclosporine, tacrolimus), psychotropic drugs (midazolam, amitriptylline) cardiotonics (digoxin), antiretrovirals (indinavir) and anticoagulants. The mechanisms of these interactions would be pharmacokinetic or pharmacodynamic [18, 19]. Neurological toxic effects would involve secondary active metabolites on muscarinic receptors (cholinomimetic or cholinolytic) [20-22].

CONCLUSION

Alltogether, this study showed that therapies combination with both plants were neurotoxic, with greater toxicity for combinations containing artesunate-amodiaquine. According to previous studies antimalarials containing artemisinin may be toxic to nerve cells. This toxicity would be dose-dependent with a mechanism of additive toxicity. But further studies would confirm this assertion.

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