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THE POTENTIAL THERAPEUTIC EFFECT OF SHIKONIN IN HEMATOLOGICAL NEOPLASIAS

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The Potential Therapeutic Effect Of Shikonin In Hematological Neoplasias

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Abstract

Hematologic malignancies (HM) are increasing and present different etiology, clinical findings and prognosis according to their lineage. Although many investigations, the treatment of these diseases is still not effective and it is important to continue studying other drugs and strategies to improve treatment response and to minimize side effects. Shikonin (SHK), the most important component of *Lithospermum erythrorhizon*, has been described as having multiple biological functions, including antitumor effects. Several studies have showed the potential therapeutic in innumerous solid cancers, including lung adenocarcinoma, colorectal cancer and glioma, decreasing cell proliferation and inducing apoptosis. It seems that SHK cause oxidative damage and, consequently, apoptosis. However, the exact molecular mechanisms of action have not been completely defined, mainly in HM. Thus, the aim of this study was to evaluate the potential therapeutic effect of Shikonin in hematological malignancies and to evaluate some molecular mechanisms of SHK, mainly the induction of oxidative stress.

For this purpose, three HM cell lines were used: HEL cells, an Erythroleukemia cell line; NB-4 cells, an Acute Promyelocytic Leukemia cell line; and H929 cells, a Multiple Myeloma cell line. Cells were cultured in absence and presence of different concentrations of SHK in single and daily dose administration schemes and metabolic activity was analyzed by Rezasurin assay. Cell death was determined by optical microscopy and flow cytometry (FC). The caspases activation, mitochondrial membrane potential and cell cycle was evaluated by FC, using the ApoStat probe, the JC-1 dye and PI/RNase solution, respectively. Peroxides and reduced glutathione (GSH) levels were measured to evaluate the oxidative stress, using fluorescent probes (DHR-123 and mercury orange, respectively).

Our results showed that SHK reduce metabolic activity in a time, dose, administration scheme and cell type dependent manner, being the NB-4 cells the more sensitive. The administration of SHK in a daily dose seems to reduce more accentuated the metabolic activity,

especially in NB-4 cells. SHK induces cell death mainly by apoptosis, which may be related with the increase in activated caspases expression levels and the decrease of mitochondrial membrane potential. The cell cycle analysis confirmed the SHK's cytostatic and cytotoxic effect. These results could be mediated by an oxidant state induced by SHK since treated cells exhibited a higher peroxides/GSH ratio.

Concluding, SHK might be used as a new therapeutic approach in different hematological malignancies. However, the therapeutic efficacy may depend on the cell type and on schedule of drug administration used.

Resumo

As neoplasias hematológicas estão a aumentar e apresentam diferentes etiologias, características clínicas e prognóstico, de acordo com a sua linhagem. Apesar dos inúmeros estudos, o seu tratamento não é completamente eficaz e, por isso, é importante continuar a investigar outros fármacos e outras estratégias de tratamento para melhorar a resposta e minimizar os efeitos adversos do tratamento. A *Shikonin* (SHK), o componente mais importante da planta *Lithospermum erythrorhizon*, tem sido apontado como tendo várias funções biológicas, nomeadamente efeitos anti-tumorais. Estudos têm mostrado o potencial terapêutico em várias neoplasias sólidas, nomeadamente adenocarcinoma pulmonar, cancro coloretal e glioma, por diminuição da proliferação celular e indução de apoptose. O efeito citotóxico da SHK parece estar associado à lesão oxidativa e, consequentemente, apoptose. Todavia, o mecanismo molecular exato não está completamente definido, principalmente nas neoplasias hematológicas. Assim, o objetivo deste estudo foi avaliar o potencial terapêutico da *Shikonin* nas neoplasias hematológicas e avaliar alguns mecanismos moleculares, nomeadamente o stresse oxidativo.

Para o efeito, utilizaram-se as células HEL (Leucemia Eritroblástica Aguda), as NB-4 (Leucemia Promielocítica Aguda), e as H929 (Mieloma Múltiplo). Estas células foram incubadas na ausência e presença de concentrações crescentes de SHK, em administração única e fracionada, e a atividade metabólica foi avaliada pelo ensaio da resazurina. Posteriormente, a morte celular foi analisada por microscopia ótica e por citometria de fluxo (CF). Foi ainda avaliada a ativação das caspases, o potencial de membrana mitocondrial e o ciclo celular por CF através da marcação com a sonda ApoStat, o corante JC-1 e a solução IP/RNase, respetivamente. Os níveis de peróxidos e a concentração de glutationa reduzida foram medidos para avaliar o stresse oxidativo, utilizando sondas fluorescentes.

Os nossos resultados mostraram que a SHK induz diminuição da atividade metabólica, dependente do tempo, da dose, do esquema de administração e da linha celular, sendo as células NB-4 as mais sensíveis. A administração fracionada mostrou-se mais eficaz na redução da atividade metabólica, especialmente nas células NB-4. Além disso, a SHK induziu morte por apoptose, o que pode estar relacionado com o aumento dos níveis de expressão de caspases ativadas e com a diminuição do potencial de membrana mitocondrial. A análise do ciclo celular confirmou os efeitos citostático e citotóxico induzidos pela SHK. Estes efeitos podem ser mediados por stresse oxidativo, uma vez que se verificou aumento da razão peróxidos/glutationa reduzida nas células tratadas com SHK.

Concluindo, estes resultados sugerem que a Shikonin poderá ser útil como nova abordagem terapêutica em neoplasias hematológicas. Contudo, a sua eficácia terapêutica poderá depender do tipo de neoplasia e do esquema de administração utilizado.

Keywords

- Leukemia
- Multiple Myeloma
- Oxidative Stress
- Apoptosis
- Anticancer agents
- Shikonin

Abbreviations

A – Aggregates

AML – Acute Myeloid Leukemia

APC – Allophycocyanin

APL – Acute Promyelocytic Leukemia

AV – Anexin V

DHR-123 – Dihydrorhodamine-123

EL – Erythroleukemia

FC – Flow Cytometry

GSH – Reduced Glutathione

IC_{50} – Half-maximal inhibitory concentration

JC-1 – 5,5’,6,6’-tethrachloro-1,1’3,3’-tethraethylbenzimidazolcarbocyanine iodide

HM – Hematologic malignancies

M – Monomer

MDS – Myelodysplastic Syndrome

MIF – Mean intensity fluorescence

MO – Mercury Orange

PBS – Phosphate-buffered saline

PI – Propidium Iodide

ROS – Reactive Oxygen Species

SHK – Shikonin

Ψmit – Mitochondrial membrane potential

I. Introduction

Hematologic malignancies (HM) emerge from a disturbance in normal hematopoietic process (1). According to the lineage, lymphoid and myeloid, and cell maturity of hematopoietic progenitor cell, the World Health Organization (WHO) has developed a consensus-based classification for HM (2). However, in population studies, hematological neoplasias are categorized in four groups: Leukemia, Myeloma, Hodgkin Lymphoma and Non-Hodgkin Lymphoma (3), with various etiology, incidence, clinical findings, prognosis and survival (4). These neoplasias are increasing (5) and their treatment is still not effective, although many investigations. There are patients who do not respond, have weak response or become resistant. Thus, it is important to investigate other drugs and strategies to improve treatment response and minimize side effects.

Shikonin (SHK) is the most important component of *Lithospermum erythrorhizon*, it has been used in traditional Chinese medicine for the past centuries. It is still listed in the Pharmacopoeia of the People's Republic of China 2010 (6) as a traditional Chinese medicine for clinical application (7). It has been described as having multiple biological functions, antibacterial, anti-inflammatory and antitumor effects (8). Several studies have showed the potential therapeutic in innumerous types of solid cancers, including lung adenocarcinoma, colorectal cancer, breast cancer, hepatocellular carcinoma and glioma (9). Various molecular mechanisms have been discussed as targets of SHK (7), including inhibition tumor cell growth and induction cell death, both modulated by numerous signaling pathways. Some studies have been demonstrated that SHK can induce oxidative stress, which leads to apoptosis in osteosarcoma (8), gastric cancer (9), glioma (10) and hepatocellular carcinoma (11).

Reactive oxygen species (ROS) are a collective term to radical and non-radical molecules, which are originated from free oxygen. However, these molecules, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), are more chemically reactive (12). Anti-oxidants, the ROS scavengers, are divided in enzymatic and non-enzymatic defenses.

Enzymatic defenses include superoxide dismutase, glutathione peroxidase, and catalase, while non-enzymatic defenses include reduced glutathione (GSH), vitamin C, and E (13). The oxidative stress results from ROS accumulation, due to increase ROS production and/or decrease antioxidant defenses. Low concentration of ROS can stimulate cell proliferation, differentiation and survival (13), but high oxidative stress levels lead to oxidative damage of macromolecules, which contributes to genomic instability and/or cell death (13). Consequently, it leads to cellular senescence or transformation and triggering a series of pathological processes, such as carcinogenesis (14). On the other hand, drugs that induce ROS accumulation can induce cell death. As cancer cells are more sensitive to oxidative stress than normal cells, normal cells are less affected, providing a good therapeutic window (15). There are many antineoplastic agents that lead to ROS accumulation, even in hematological neoplasias, such as anthracyclines (16).

Apoptosis is one of the most relevant programmed cell death and typically there are two apoptosis signal transduction pathways: intrinsic and extrinsic pathway (17). The extrinsic pathway is initiated at plasma membrane by death receptor ligation (receptor pathway) and ends with activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 (18). The intrinsic or mitochondrial pathway is initiated by stress signals through release of apoptogenic factors such as cytochrome c from the mitochondrial intermembrane space to cytosol, which leads to effector caspase-3 activation (19).

Despite many targets of SHK action already described, the molecular mechanisms have not been completely understood, especially in hematological neoplasias. Thus, the aim of this study is to evaluate the potential therapeutic effect of Shikonin in *in vitro* models of three different hematological malignancies and to evaluate some molecular mechanisms of SHK, mainly oxidative stress.

II. Material and Methods

2.1 Cell culture

We used three hematological neoplasias cell lines: HEL cells, an Erythroleukemia cell line positive for $JAK2^{V617F}$; NB-4 cells, an Acute Promyelocytic Leukemia cell line with the translocation t(15;17)(q22,q12) and H929 cells, a Multiple Myeloma cell line, all obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ; Germany).

Cells were cultured in RPMI-1640 medium (Gibco, Portugal) supplemented with 10% (NB-4 and HEL) or 20% (H929) of fetal bovine serum, 2 mM glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in humidified air containing 5% CO2.

The Shikonin (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma).

2.2 Metabolic Activity

Cells were incubated at a density 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929 cells). The cells were cultured in absence (control) and presence of increasing concentrations of SHK, ranging from 10 nM to 10000 nM. We also evaluated the daily administration (24 h, 48 h and 72 h) scheme, using the dose of 25 nM and 250 nM.

Metabolic activity was analyzed at 24 h, 48 h and 72 h by rezasurin assay. Briefly, rezasurin, a blue and weakly fluorescent compound, is reduced by dehydrogenase enzymes in metabolically active cells in resorufin, a red and highly fluorescent compound. Then, the optical density was measured at 570-600 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments). The metabolic activity was calculated in percentage, compared to control wells. Metabolic activity of daily dose scheme (25 nM# and 250 nM#) was compared with a single dose scheme, 75 nM and 750 nM. Each experiment was repeated 5 times.

2.3 Cell death evaluation

Cell death was determined by optical microscopy, using the May-Grünwald Giemsa staining and by flow cytometry (FC), using the Annexin-V (AV) and propidium iodide (PI) double staining. For the analysis, the cells were treated with 250 nM (H929 cells) and 500 nM (NB-4 and HEL) of SHK, and incubated during 48 h.

For optical microscopy assays, cells were transferred to slides fixed, stained and evaluated under light microscopy, using a Nikon Eclipse 80i equipped with a Nikon Digital Camera DXm 1200F for morphological analysis (amplification 500x).

For FC analysis, cells were stained simultaneously with AV (BD Pharmingen), labelled with allophycocyanin (APC) and with PI (Biolegend). Annexin-V presents a natural affinity for phosphatidylserine in presence of calcium. In apoptotic cells, phosphatidylserine migrates from the inner to the outer layer of the cytoplasmatic cell membrane and AV can detect the redistribution of this phospholipid by FC. In necrotic cells, the membrane becomes permeable and small charged molecules such as PI enter in the cell. PI has a high affinity to DNA and the binding results in an increase of their fluorescence (20). Therefore, this assay discriminates among live cells (AV-/PI-), early apoptotic cells (AV+/PI-), late apoptotic/necrotic cells (AV+/PI+) and necrotic cells (AV-/PI+). Cells were co-stained with AV-APC and PI using the manufacturer's recommended protocol. Briefly, cells were washed with ice-cold Phosphate-buffered saline (PBS) (centrifuged at 500xg for 5 min), ressuspended in 100 µL of binding buffer and incubated with 5 µL of AV-APC solution and 5 µL of PI solution for 15 min in the dark. After incubation time, cells were diluted in 300 µL of binding buffer, and analyzed by FC. Flow cytometry analysis was performed using a six-parameter, four-colour FACSCalibur™ flow cytometer (Becton Dickinson, USA). For each assay, $1x10^6$ cells were used and at least 10,000 events were collected by acquisition using CellQuest software (Becton Dickinson, USA) and analyzed using Paint-a-gate software (Becton Dickinson, USA). Results

are expressed in % ± standard error of the means (SEM) of at least five independent experiments.

2.4 Activation of caspases

Activation of intracellular caspases was evaluated by flow cytometry, using ApoStat probe (R&D Systems, USA). Cells were incubated for 48 h in the absence or in the presence of 250 nM (H929 cells), 500 nM (NB-4 and HEL) of SHK. The $1x10^6$ cells were ressuspended in 1000 µL of PBS and incubated with 1µg of ApopStat probe according manufacture instructions and were analyzed by FC, using the equipment and the programs described in section 2.3. Results are represented in % ± SEM of at least five independent experiments.

2.5 Cell cycle evaluation

Cell cycle was evaluated by flow cytometry using PI and RNase detection kit (Immunostep, Spain). As mentioned previously, PI is a fluorescent dye that stains DNA in permeable cells. The fluorescence intensity, read by FC, is proportional to the DNA quantity of each cell, allowing us to determine the relative proportion of cells in the G_0/G_1 phase (fewer amount of DNA), S phase (coincident with DNA replication) and G_2/M phase (double DNA of the G_0/G_1 phase) (21). Given that apoptotic cells undergo the process of DNA fragmentation, these cells are represented as an apoptotic peak, that has the fewest DNA quantity (22). Cells were incubated as indicated above. At 48 h, cells were washed with PBS, ressuspended in 200 µL of ice ethanol, and incubated for 30 min in the dark at 4°C. Then, cells were washed with PBS, ressuspended in 300 µL of PI/RNase solution and analyzed by FC, with the equipment already described. The results were analyzed using Modfit software (Becton Dickinson, USA) and expressed by % ± SEM of cells at Sub-G0/G1, G0/G1, S and G2/M of at least 5 independent experiments.

2.6 Mitochondrial membrane potential evaluation

Mitochondrial membrane potential (Ψmit) was evaluated using the dye 5,5',6,6'-tethrachloro-1,1'3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). This lipophilic cationic probe exists in a monomeric form (M) emitting at 527 nm (green fluorescence) and is able to reversibly form aggregates (A), which emits 590 nm, greenish-orange fluorescence, as the mitochondrial membrane becomes more polarized. In apoptotic cells, mitochondrial membrane potential collapses, and JC-1 cannot accumulate within the mitochondria, remaining in the monomeric form in cytosol. These cells, exhibit a higher monomer/aggregate ratio of JC-1 (M/A) than viable cells (23). Succinctly, the cells were ressuspended in 1000 μL of PBS and were incubated with JC-1 at final concentration 5 μg/mL for 15 min at 37ºC, in the dark. At the end of the incubation period, the cells were washed twice in cold PBS, ressuspended in a total volume of 300 μL, and analyzed by FC. Results are expressed in mean ± SEM of monomer/aggregate ratio of JC-1 and this ratio was calculated as the fraction of mean intensity fluorescence (MIF) observed for each molecule.

2.7 Oxidative stress evaluation

The oxidative stress was evaluated by FC using two different dyes. The dye dihydrorhodamine-123 (DHR-123) was used to measure peroxides (H_2O_2) levels and the dye mercury orange (MO) (1-(4-Chloromercuriophenylazo)-2-naphthol) to measure GSH concentration.

DHR-123 is a nonfluorescent, noncharged dye that easily penetrates cell membranes. Once inside the cell, DHR-123 reacts with intracellular oxidants to yield rhodamine 123, a fluorescent cationic compound, which localizes to the mitochondria (23). Cells cultured in the absence and in the presence of SHK were incubated with 5 μM DHR-123 for 15 min at 37ºC in the dark. Cells were then washed twice with PBS, ressuspended and fluorescence was detected

by flow cytometry. MO and GSH reaction occurred faster than with thiols protein, and can be excited using the 488 nm line of an argon laser. After a short incubation time, more than 75-80% of the GSH had reacted, while at least an 8 h period was required for the mercury orange to react with protein SH groups (24). Briefly, after incubation, cells were washed with PBS (centrifugation at 300x during 5 min) and incubated for 15 min at 37ºC with MO at final concentration of 40 µM. Cells were then washed and ressuspended in PBS, and analyzed by FC. Results were expressed in MIF ± SEM of at least 5 independent experiments.

2.8 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (USA). Student's t test and two-way ANOVA were used to compare the different groups. A p value < 0.05 was considered statistically significant. Results are expressed as the mean ± SEM of at least 5 independent experiments. The half maximal inhibitory concentration (IC_{50}) was determined from log dose-response curves in Graph Pad Prism using non-linear regression analysis.

III. Results

Metabolic Activity Evaluation

Our results showed that SHK reduce metabolic activity in a time, dose and cell type dependent manner. As we can see in figure 1A-C, SHK decreases metabolic activity in all cell lines with a single dose administration, being the NB-4 cells the most sensitive cells and the H929 cells the lowest. The decreased metabolic activity was more evident in the acute promyelocytic leukemia cell line, NB-4 cells (Figure 1A), were SHK show an IC_{50} between 500 nM and 750 nM at 24 h. However, revealing the importance of incubation time, the IC_{50} at 48 h was slightly less than 100 nM and at 72 h the IC_{50} was between 10 nM and 25 nM. In HEL cells (Figure 1B), we also observed a reduction on metabolic activity, but these cells required higher doses to reach the IC_{50} in comparison with NB-4 cells. Analyzing the dose-response curve, the IC_{50} was approximately 7500 nM at 24 h, approximately 500 nM at 48 h and between 100 nM and 250 nM at 72 h. In H929 cells, a multiple myeloma cell line, (Figure 1C), SHK also decreased metabolic activity but it was needed much higher doses to achieve IC_{50}. The IC_{50} at 24 h was between 5000 nM and 10000 nM. However, for some concentrations, it was observed a moderately reversion of SHK effect at 48 h. After 72 h of exposure, the IC_{50} was between 500 nM and 750 nM. The IC_{50} calculated for the different cell lines at 24 h, 48 h and 72 h of exposure using non-linear regression analysis is represented at table 1.

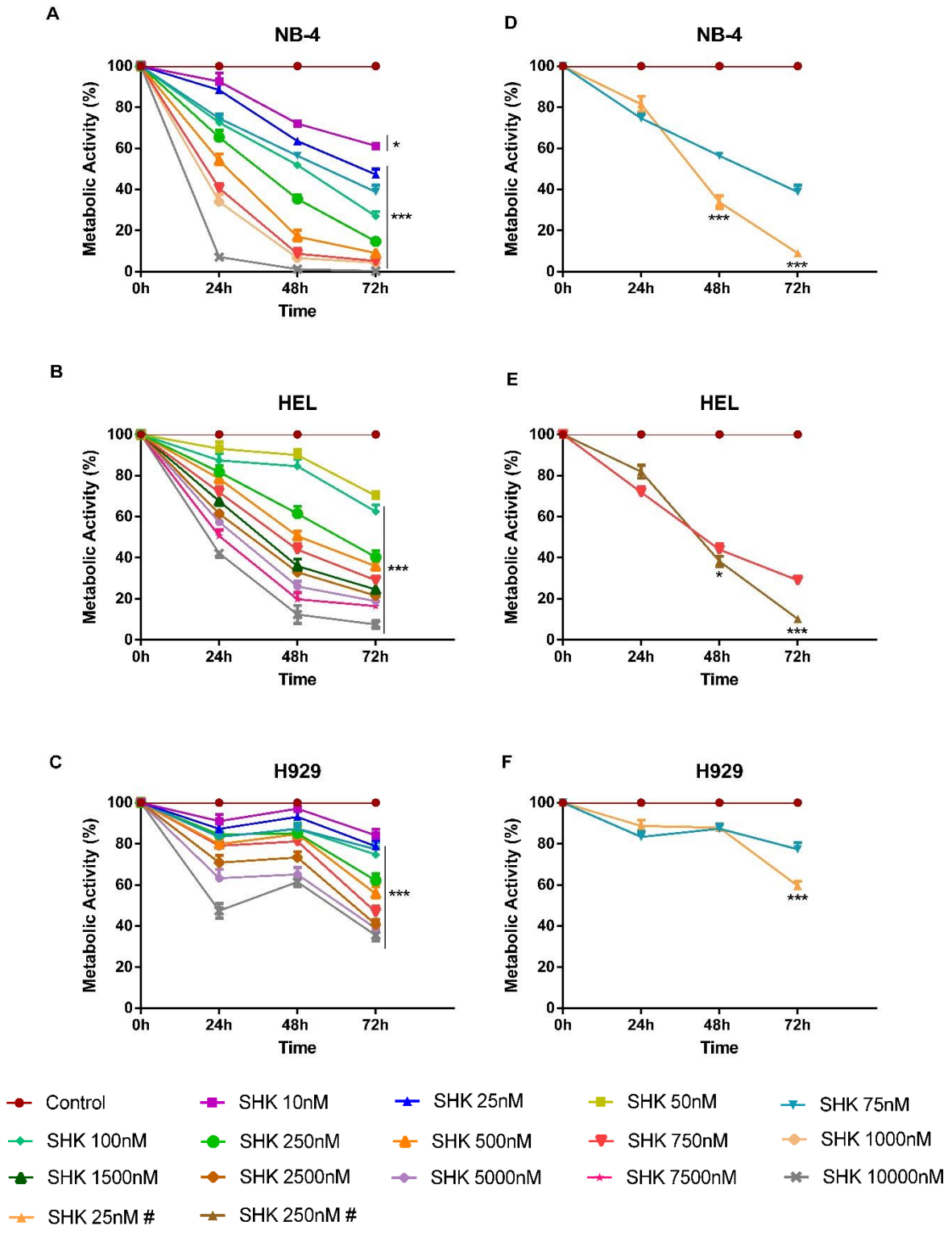

Figure 1 – Dose-response curves of Shikonin in single and daily dose administration in hematological cell lines. NB-4, HEL and H929 cells were cultured during 72 h in absence (control) and in presence of increasing concentrations of Shikonin (SHK) in a single dose (A, B, C). The cells were also cultured in presence of 25 nM# (NB-4 and H929 cells) or 250 nM# (HEL cells) in daily dose administration scheme (D, E, F). Dose response

curves were established by rezasurin assay each 24 hours, as described in Methods and Materials. The results were expressed in cell percentage (%) normalized to control and represents the mean ± SEM of at least 5 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Table 1 – Half-maximal inhibitory concentration (IC_{50}) calculated

Cell line	IC_{50} (nM)		
	24 h	48 h	72 h
NB-4	469.2	83.7	29.0
HEL	3932.0	694.9	293.4
H929	5953.0	7008.0	872.6

Daily administration scheme is a common strategy used in clinical practice to minimize the therapeutic toxicity. In this context, we tested the SHK effect using daily dose administration scheme, represented in figure 1 D-F. SHK reduced even more the metabolic activity in all cell lines, when compared with cells untreated (control) and treated with the same cumulated concentration in single dose. This effect was statistically significant in all cell lines, but once again more prominent in NB-4 cells (Figure 1 D). SHK administrated in daily dose, decreased the metabolic activity from approximately 40% in single dose to approximately 10% after 72 h of exposure in NB-4 cells. In HEL and H929 cells, incubation time was particularly determinant to decrease metabolic activity in comparison with cell treated in single dose. In HEL cells (Figure 1E), the metabolic activity decreased near 5% (from approximately 45% achieved in single dose to 40% in daily dose) at 48 h, but at 72 h it was observed a significant decreased in metabolic activity from approximately 20% (30% in single dose to 10% in daily dose). The incubation time effect was very pronounced in H929 cells (Figure 1F), at 48 h it was not observed difference between cells treated with single or daily dose. However, our results showed a decreased in metabolic activity from approximately 80% to 60% in daily dose scheme at 72 h.

Cell death evaluation

We evaluate cell death by optical microscopy, using the May-Grünwald Giemsa staining and it was observed nuclear fragmentation, blebbing and apoptotic bodies, both typical morphological features of apoptosis (Figure 2) (25).

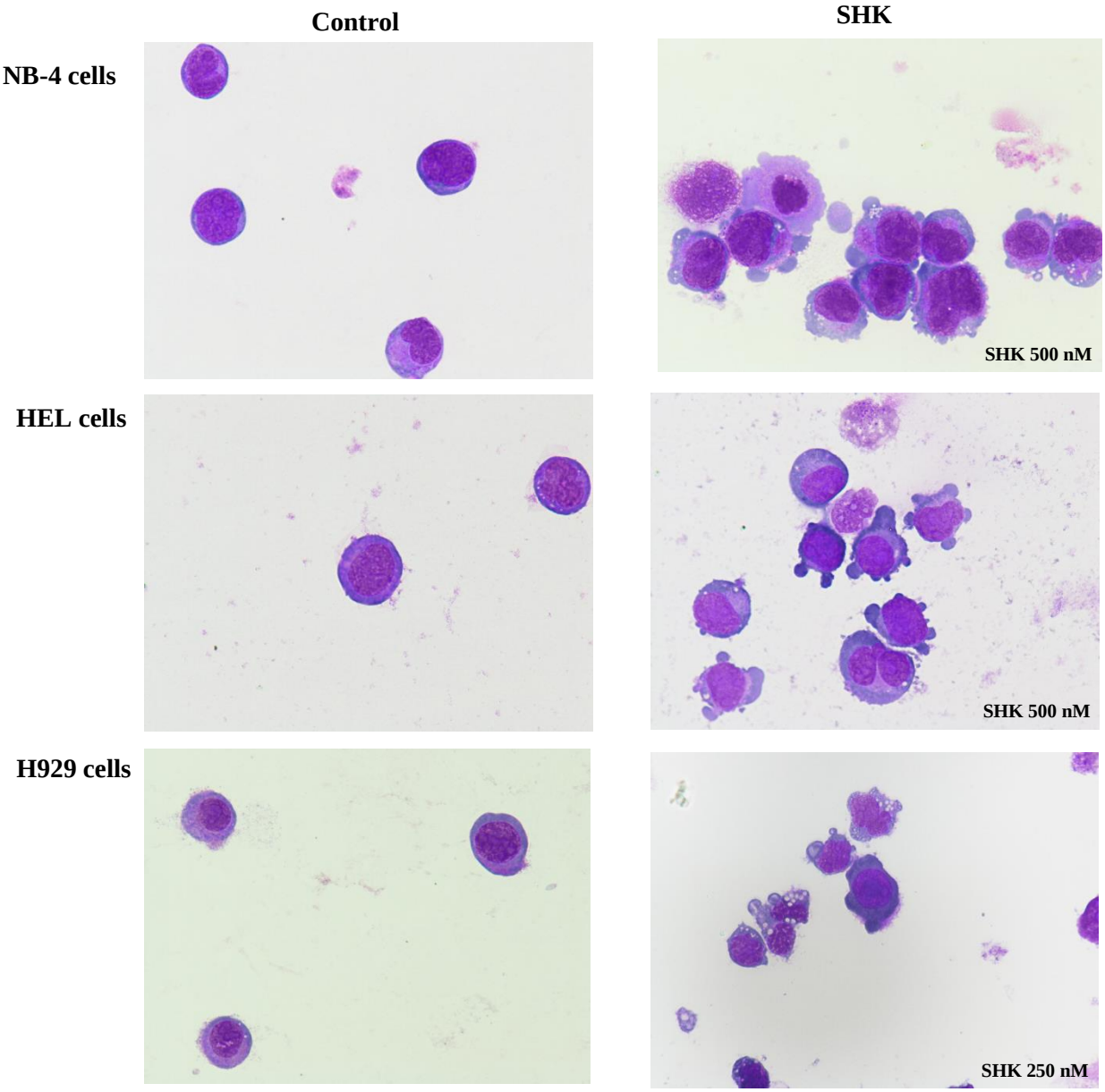

Figure 2 – Cell analysis by optical microscopy, in NB-4, HEL and H929 cells treated with Shikonin. Cells were incubated at a density 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929). NB-4, HEL and H929 cells were cultured in absence (control) and in presence (500 nM at NB-4and HEL cells, and 250 nM at H929 cells) of Shikonin (SHK) during 48 h. Then, cells were stained using a May-Grünwald-Giemsa stain being after transferred to slides and fixed. Finally, the smears were analyzed using a light microscope (amplification 500x).

To confirm the SHK's cytotoxic effect we also analyzed cell death by FC, using AV/IP double staining. SHK induce a significant decrease in live cells with an increase in apoptotic and necrotic cells, when compared with control cells (Figure 3A). After that to confirm the apoptosis induction, caspases activation was determined by FC, using ApoStat probe. We observed an increase of caspases positive cells (Figure 3B), being the differences statistically significant in all cell lines.

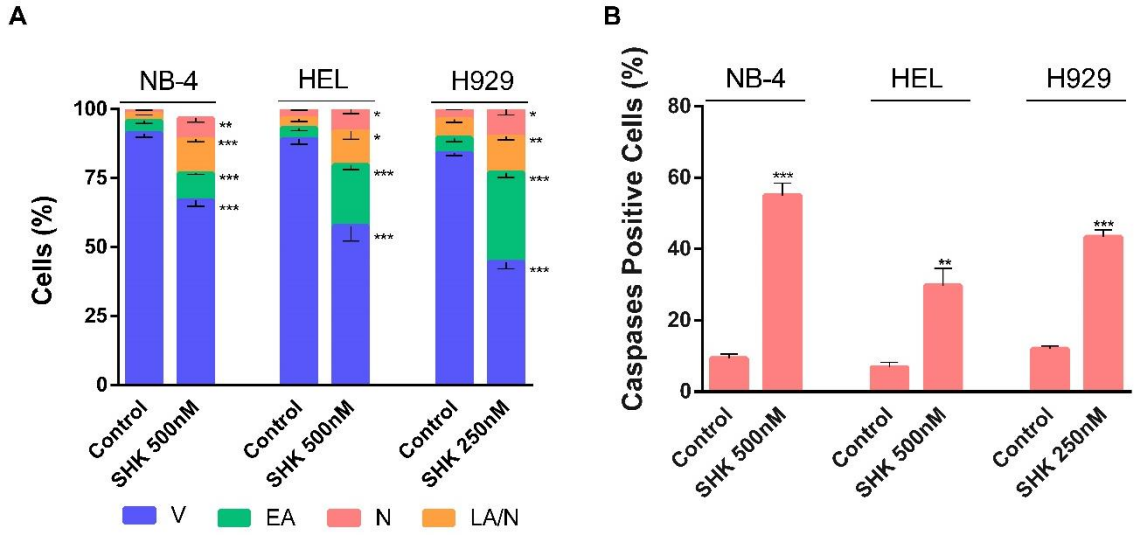

Figure 3 – Analysis of the cytotoxic effect of Shikonin in hematological neoplasias cell lines. Cells were incubated at a density 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929) and cultured in absence (control) and in presence of Shikonin (SHK) during 48 h. NB-4 and HEL cells were treated with 500 nM and H929 were treated with 250 nM. Cell death was determinated by flow cytometry (FC) using Annexin-V and Propidium Iodide double staining and results are expressed as percentage (%) of viable cells (V), early apoptotic cells (EA), late apoptotic/necrotic cells (LA/N) and necrotic cells (N). The activation of caspases was evaluated by FC using the ApoStat probe, and is represented as % cells with activated caspases (B). *p<0.05; **p<0.01; ***p<0.001.

Cell cycle analysis

Evaluation of cell cycle distribution allows the determination of SHK antiproliferative effect. In addition, by the same technique, we could confirm the presence of cells undergoing apoptosis. The cytostatic effect of SHK was only observed in NB-4 cell line, with an increase of cells in S phase comparing with control cells ($p<0.01$) (Figure 4).The Sub-G_0/G_1 peak was observed in all cell lines, which corresponding to DNA fragmentation typical of apoptotic cells, confirming the cytotoxic effect induced by SHK.

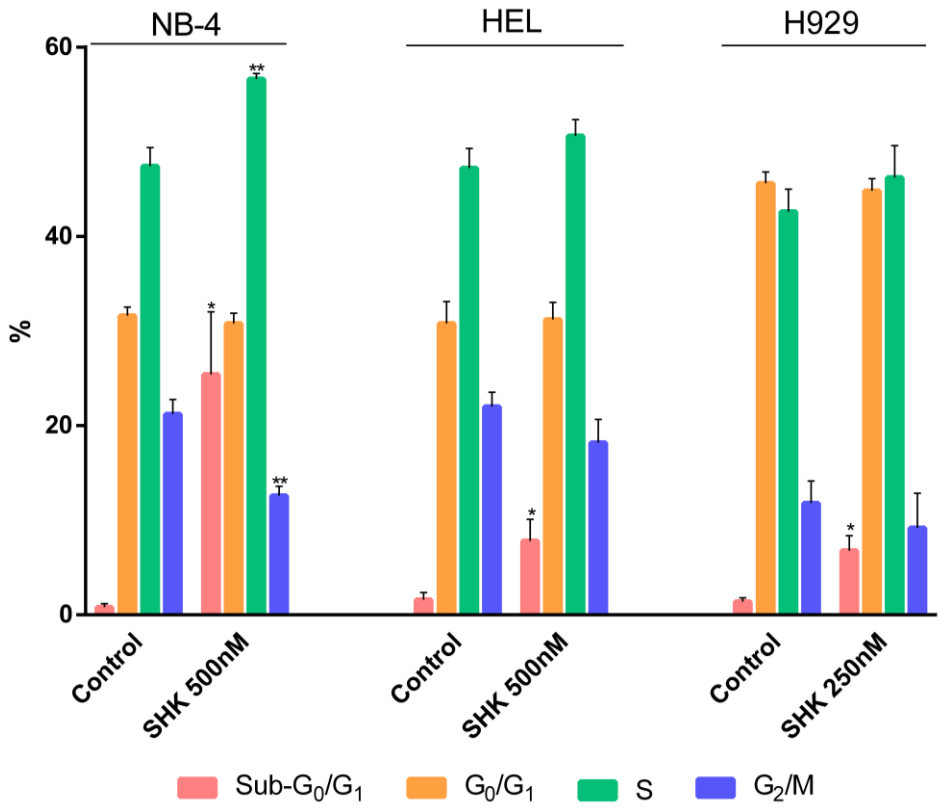

Figure 4 – Analysis of Shikonin's effects in cell cycle by flow cytometry in hematological neoplasias cell lines. Cells were incubated at a density 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929). NB-4, HEL and H929 cells were cultured in absence (control) and in presence (500 nM at NB-4 and HEL cells, and 250 nM at H929 cells) of Shikonin (SHK) during 48 h. The SHK's effects were analyzed by FC, using propidium iodide and RNase. Data are expressed by the percentage (%) of cells in sub-G_0/G1 peak, G_0/G_1 phase, S phase and G_2/M phase and represent the mean ± SEM of at least 5 independent experiments. *$p<0.05$; **$p<0.01$.

Mitochondrial membrane potential evaluation

We also studied the mitochondrial involvement in cell death induced by SHK, by evaluating the mitochondrial membrane potential of treated and untreated cells (control), by FC using the JC-1 dye. As figure 4C showed, SHK significant increases M/A JC-1 ratio in treated cells, suggesting that this drug decreases mitochondrial membrane potential.

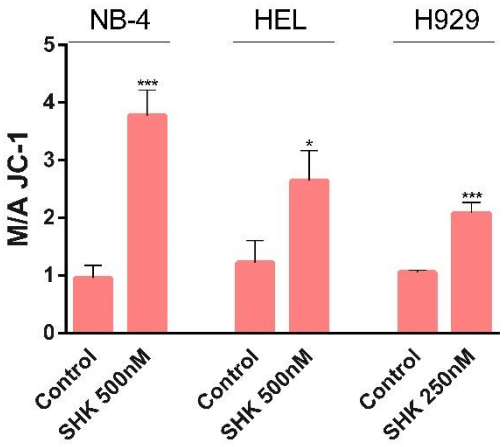

Figure 5 – Mitochondrial membrane potential evaluation in NB-4, HEL and H929 cells treated with SHK. Cells were incubated at a density of 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929) and cultured in absence (control) and in presence of Shikonin (SHK) during 48 h. NB-4 and HEL cells were treated with 500 nM and H929 were treated with 250 nM. The mitochondrial membrane potential (ψmit) were analyzed by FC using JC-1 fluorescent probes, as describe in material and methods. JC-1 probe coexist in monomeric (M) or aggregate (A) forms depending on the mitochondrial membrane potential. An increase in the monomer/aggregate ratio (M/A ratio) indicates a decrease in the mitochondrial membrane potential. Results are expressed in mean ± SEM of monomer/aggregate ratio of JC-1, of at least 5 independent experiments, and this ratio was calculated as the fraction of MIF observed for each molecule. *p<0.05; ***p<0.001.

Oxidative stress evaluation

The levels of peroxides and GSH (Figure 6) were analyzed to investigate if cytotoxic effects were mediated by oxidative stress. We observed that SHK significantly increases peroxides levels in all cell lines, and this oxidant state was accompanied by a decrease in antioxidant defenses, measured by GSH levels. This oxidative stress induced by SHK, is confirmed by a higher peroxides/GSH ratio, when compared with control. This ratio was higher in NB-4 cells.

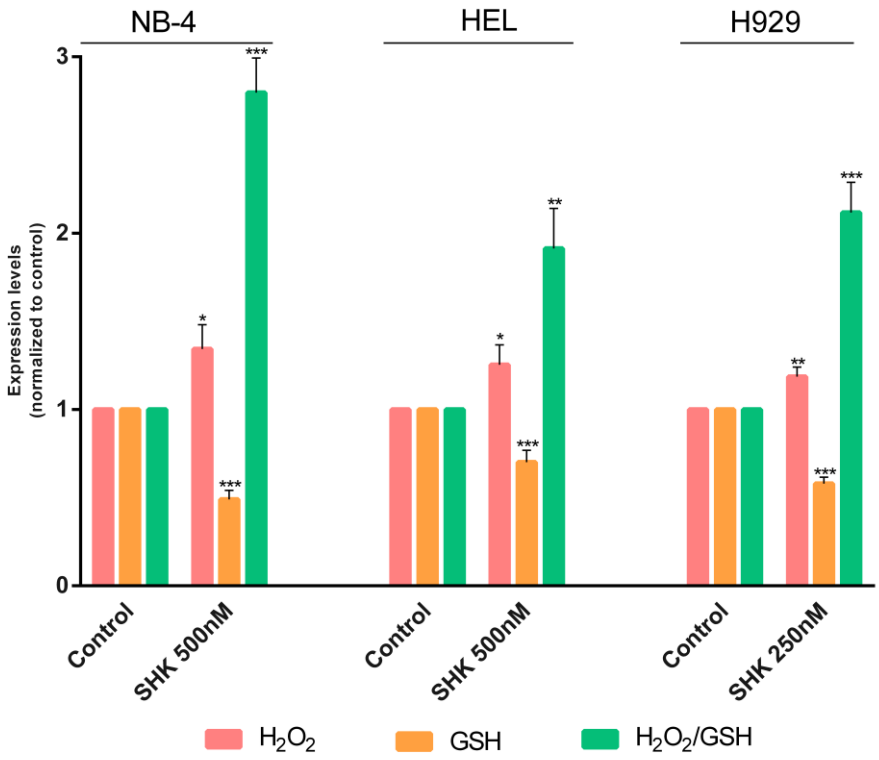

Figure 6 – Oxidative stress induced by Shikonin were analyzed in hematological neoplasias cell lines. Cells were incubated at a density 0.3 x 10^6 cells/ml (NB-4 cell line), 0.4 x 10^6 (HEL cell line) and 0.5 x 10^6 cells/ml (H929) and cultured in absence (control) and in presence of Shikonin (SHK) during 48 h. NB-4 and HEL cells were treated with 500 nM and H929 were treated with 250 nM. Peroxides (H_2O_2) levels and reduced glutathione (GSH) concentration were measured by Flow Cytometry (FC) using dihydrorhodamine-123 (DHR-123) and mercury orange, respectively. Results are expressed as mean intensity fluorescence (MIF) normalized to control and represents mean ± SEM at least 5 independent experiments. *p<0.05; **p<0.01;*** p<0.001.

IV. Discussion

Despite great advances in chemotherapy, the standard treatment of hematological malignancies is still not effective and drug resistance and relapse remain a major problem (28,29). Recently, natural compounds extracted from dietary food, vegetables or traditional Chinese herbs have received much attention for cancer chemoprevention and even for cancer therapy (30,31). These herbs are considered safe agents with long clinical applications in Chinese traditional medicine (11). Shikonin, a plant-derived naphthoquinone, has been highly used with numerous biological functions (8). Recently, SHK has been recognized as a promising chemotherapeutic agent for the treatment of osteosarcoma (8), hepatocarcinoma (11), gastric cancer (9) and lung cancer (32). In the present study, we evaluated the potential therapeutic in three different hematological neoplasias cell lines in culture: erythroleukemia, acute promyelocytic leukemia and multiple myeloma.

We demonstrated that SHK has cytotoxic and cytostatic effect in a time, dose and cell type dependent manner. The cell line more sensitive to SHK was NB-4, an APL cell line, followed by HEL, an EL cell line, and H929, the MM cell line, was the lowest sensitive. Multiple carcinogenesis mechanisms implied in cell lines could be different, predisposing to distinct sensitivities to SHK. One possible reason could be related with different hematopoietic progenitor cell lineage, myeloid (NB-4 and HEL cells) versus lymphoid (H929 cells). In human histiocytic lymphoma, Wiench B *et al.* showed a cytotoxic effect of SHK also with rezasurin assay, being the IC_{50} of 0.3 μM at 24 h of exposure (29). Another study using HL-60 cells, an APL cell line without translocation t(15;17)(q22,q12), determined a IC_{50} between 1.0 to 1.5 μM at 48 h (33), which is 10 times higher than we observed in NB-4 cells (in our study, the IC_{50} at 48h in NB-4 cells was approximately 100 nM). This IC_{50} variance between the two APL cell lines, NB-4 and HL-60, could be probably associated to genetic differences between these cell lines. However, further studies are needed to disclose the exact reason.

In addition, we used a daily administration scheme to explore SHK cumulative dose effect. This administration scheme seems to be more effective, because it reduces even more the metabolic activity in all cell lines compared with the single dose. The fractionated scheme could improve pharmacokinetics/pharmacodynamics and constitute a valid option to reduce the potential side effects of SHK, since allow decreasing the concentrations used. Futurity, it also could be tested if SHK could be used as adjuvant of conventional therapy, since pharmacological combination may minimize drug toxicity and improve quality of life of patients.

Most current therapeutics decrease cell proliferation and induce programmed cell death, specially apoptosis (17), as their main therapeutic targets. With this in mind, we investigate the mechanism of cell death induced by SHK. Our results showed an increase of cell population in apoptosis, in all three cell lines, but also in necrosis. SHK induces cell death predominantly by apoptosis, which was also supported with appearance of a peak sub-G_0/G_1, correspondent to DNA fragmentation, and morphological features of apoptosis visible at optical microscopy. Similar findings have already been described in *in vitro* hematological malignancies, such as other model of human acute promyelocytic leukemia (HL-60 cell line) (33), in two models of chronic myelogenous leukemia (K562 and LAMA84) (34) and in human lymphoma U937 cells (35). Similarly to what was seen in leukemia, bladder and cervical cancer cells (33,36,37), we observed an increase on caspases positive cells, which confirm cell death by apoptosis. Both apoptosis signal transduction pathways lead to activation of initiators and effectors caspases (27). However, in our study it was not possible to discriminate if extrinsic apoptotic pathway is activated. As it is known, the intrinsic apoptotic pathway is initiated by various stimuli, which change the mitochondrial membrane permeability (19). Consequently, there are a release of sequestered pro-apoptotic proteins to cytosol and a decrease of mitochondrial membrane potential, resulting ultimately in apoptosis. Considering this, we evaluated Ψmit by FC, using

JC-1 dye and our results indicated a higher monomer/aggregate ratio of JC-1 in treated cells with SHK, which occurs in apoptotic cells (23). In agreement, previous results in other leukemic cells have reported that SHK induced apoptosis mitochondria-mediated, and activated caspases (34). In addition to apoptosis, as it was mentioned earlier, cell death analysis demonstrated an increase of cell population in necrosis. Recently, low necroptosis, a form of regulated necrosis, have been associated to cancer growth and drug resistance (38). This form of cell death has been reported in solid and hematological cancers treated with SHK (39-41). In this context, necroptosis is an important mechanism to explore in future works.

Furthermore, we observed that SHK arrest NB-4 cells in S phase. Because SHK in these cells present both, cytostatic and cytotoxic effects, this could be another reason that leads to the highest sensitivity observed in NB-4 cells to SHK, compared to HEL and H929 cells, where SHK only present a cytotoxic effect. The SHK influence in cell cycle has also observed in malignant melanoma (42) and lung adenocarcinoma (32).

Although SHK could induce cell death, mainly by apoptosis, which is caspases and mitochondria mediated, it is not clear the exact mechanism how SHK acts. The permanent oxidative stress leads to damage of macromolecules, triggering a series of pathological processes (13). According with this, a number of chemotherapeutic agents that directly induce oxidative stress have been approved for treatment of several types of malignancies, including hematological (14,43). Our results showed that SHK increases peroxides levels and decrease GSH content, one of the major non-enzymatic antioxidant defenses (13). A higher peroxides/GSH ratio in treated cells compared with control cells confirmed the oxidant state induced by SHK. Our results are coincident with another work where chronic myelogenous leukemia cells were pretreated with L-N-acetylcysteine, a free radical scavenger, showed a markedly block in ROS production and a significant decrease in apoptosis after SHK treatment (34). Gong *et al.* (11) demonstrated that SHK increase oxidative stress in hepatocarcinoma cells

and this could be the trigger to mediated apoptosis by the lack of Ψmit. Similarly, oxidative stress induced by SHK was also found in colorectal carcinoma (44) and in osteosarcoma (8) cells.

Since studies with SHK have been so promising, a clinical trial has already been done. Nineteen patients with late-state lung cancer, who were not candidates for surgery, radiotherapy or chemotherapy were treated with a shikonin-containing mixture. The authors demonstrated a reduced lung cancer growth and no harmful effects on peripheral system were observed (45).

Concluding, Shikonin might be used as a new therapeutic approach in different hematological malignancies, since this drug reduce metabolic activity and induce cell death by apoptosis, through oxidative stress. However, the therapeutic efficacy may depend on the cell type and schedule of drug administration used. Undoubtedly, additional studies are needed to clarify some molecular mechanisms of Shikonin and to evaluate its potential therapeutic effect in hematological malignancies.

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