A novel realgar-indigo naturalis formula more effectively induces apoptosis in NB4 cells

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Abstract: Realgar as a kind of arsenic agent is currently used to treat APL in China. The effectiveness and low toxicity of realgar have been verified, lower than arsenic trioxide. Although the therapeutic efficacy of realgar is blocked severely by its poor insolubility in water. In our lab, we addressed this problem by obtaining realgar bioleaching solution (RBS) from microbiological leaching technique. To develop a tradition Chinese medicinal formula (TCMF) for clinical application realgar is usually used with other herbs. However, treated realgar with RBS has not been evaluated in TCMF contain realgar. In the present study we used NB_4 to investigate the effects of novel Realgar-Indigo naturalis formula (FRBS) on cell proliferation and apoptosis. We used MTT assay to measure anti proliferative activity of FRBS. We further study the effects of FRBS on cell growth and apoptosis according flow cytometry, DNA fragmentation assay and Fluorescence microscopy and Western blot. The results revealed that FRBS significantly inhibited growth in a dose-dependent manner, and induced apoptosis in NB4 cells. NB4 cell inhibitory response to FRBS at 2μg ml^{-1} of arsenic concentration was twofold higher, dissimilar to RIF, and induced apoptosis more effectively. Further, a higher expression of caspase-3, caspase-9 and cytochrome C from increased from FRBS. RBS can substitute the traditional realgar powder in RIF in order to provide a novel and promising Realgar-Indigo naturalis formula to treat acute promyelocytic leukemia.

Keywords: Realgar bioleaching solution (RBS), apoptosis, acute promyelocytic leukemia (APL).

INTRODUCTION

Since thousands of years, realgar (AS_4S_4) is a mineral arsenical has been used in traditional and clinical treatments in China and India (Gao *et al.,* 2000). Externally, it is used to cure skin problems of carbuncle, lump and furuncle, even to treat bites by insects or snakes etc., and internally it is a remedy for convulsive epilepsy, malaria and abdominal pain resulting from parasitic infection. Nowadays realgar is also clinically used to treat acute promyelocytic leukemia (APL) (Wang *et al*., 2007; Lu *et al*., 1999; Xiang *et al*., 2007; Ting *et al*., 1984) and other forms of cancer in China (Baláž *et al*., 2010; Soignet *et al*., 1998; Xu *et al*., 2000; Gupta *et al*., 2013). Therefore, realgar currently turns to the focus of toxicant researches. Unfortunately, realgar is poorly soluble, thus resulting in very low bioavailability and it has been used on a large dose with severe safety risk from excessive arsenic intake. In our lab, a kind of microbiological leaching technique is used to dissolve realgar to obtain realgar bioleaching solution (RBS) (Zhang *et al*., 2007). RBS has been demonstrated to exhibit high therapeutic efficacy of anti-tumor and low toxicity *in vitro* and *in vivo* in the past decade (Zhang *et al*., 2010; Wang *et al*., 2013; Xie *et al*., 2014; Zhi *et al*., 2015). In fact, realgar is usually in combination with other herbs to treat various diseases in traditional Chinese medicine (TCM). Nearly 100,000 formulae in TCM have been recorded, 10% of which contain realgar (Wang *et al*., 2013). However, whether the therapeutic effect of TCM formula containing realgar can be improved after replacement realgar with RBS is still unknown.

Realgar-Indigo naturalis formula (RIF) has been proved to treat APL effectively since 5-year overall survival rate of 86.88% (Xiang *et al*., 2007). This formula is composed of realgar, *Indigo naturalis*, *Salvia miltiorrhiza* and *Radix psudostellariae*. Realgar in combination with other three herbs exerts synergistic effect to treat APL (Wang *et al*., 2007). In this paper, this formula has been chosen to act as a preventative to explore the therapeutic efficacy of TCM formula containing realgar after replacement realgar with RBS.

Here, we report the effects of a novel Realgar-Indigo naturalis formula (FRBS) including RBS, *Indigo naturalis*, *Salvia miltiorrhiza* Bge and *Radix pseudostellariae* on apoptosis induction in human APL cell line of NB_4, and

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RIF including realgar, *Indigo naturalis*, *Salvia miltiorrhiza* and *Radix pseudostellariae*, which is used in clinical treatment in China currently, is regarded as positive control. We tested FRBS effects on the proliferation of NB_4 cells and our findings revealed that there are evidences on promotion of therapeutic efficacy for anti-APL which contributed to replacing realgar with RBS in RIF. Further, it provided cues on TCM containing realgar to be acceptable for clinical and medical use.

MATERIALS AND METHODS

Materials

Realgar was obtained from Shimen County, Hunan Province, China, and purified through traditional methods. MTT (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoli-um bromide) and PI (Propidium Iodide) were purchased from Solarbio (Cat. No. M8180). RPMI 1640 was obtained from Invitrogen Corporation (Ct.No.31800-022). Newborn calf serum was purchased from Limited Liability Corporation of Sijiqing Bio-engineering Material in Hangzhou.

Cells and cell culture

NB4 cell line was purchased from the Institute of Cancer Research of Gansu province in China. NB4 cells were cultured in RPMI medium containing 10% Newborn calf serum and 100 IU/ml penicillin and 100 IU/ml streptomycin in an incubator at 37℃ and 5% CO_2 (Xie *et al*., 2014).

Preparation for formula

RBS was prepared according to the protocol reported by Zhang *et al*. (Zhang *et al*., 2010). Total arsenic concentration was routinely analyzed by inductively coupled plasma-actomic emission spectrometry (ICP-AES; Jobin-Yvon Ultimate 2R).

FRBS include RBS, *Indigo naturalis*, *Salvia miltiorrhiza* and *Radix psudostellariae* at a proportion of 14.5%, 33.5%, 40% and 12% were prepared according to the method of Huang and Wang, and the percentage of realger were 7.8% and 14.5% in RBS formula and in RIF formula, respectively (Huang *et al*., 2005).

Determination of inhibitory effect on cell proliferation

The MTT assay used to measure the cell proliferation inhibitory effects of FRBS on NB4 cells was done according to our previously reported (Xie *et al*., 2014).

Flow cytometry analysis of apoptosis and cell cycle distribution

Apoptosis was identified and quantified by flow cytometry. NB4 Cells were treated with various concentrations of FRBS for 24 h. At the end of the incubation, all cells was done according to our previously reported (Xie *et al*., 2014)

DNA fragmentation assay

Following incubation with different FRBS at 2μg/ml for 24 h, approximately 1×10^6 cells were harvested after centrifugation, washed twice with ice-cold PBS buffer, and then was done according to previously reported (Wu *et al*., 2006)

Western blot analysis

Cells (1×10^6 cells) exposed to FRBS were collected into tubes and then washed with PBS. Cell lysates containing 15μg proteins were separated on SDS-PAGE and transferred to nitrocellulose filters. The blots were probed with the rabbit anti-human caspase-3, caspase-9, cytochrome C antibody diluted by 1: 500 and incubated at 4°Covernight, then incubated with a secondary antibody diluted by 1: 5000 at room temperature for 1 hour. The immunoblots were subsequently reprobed with a 1:5000 dilution of an anti-β-actin antibody and developed as previously described (Wu *et al*., 2014).

Fluorescence microscope characterization

Cells (1×10^6 cells) exposed to FRBS were collected into tubes and washed with PBS. The samples were then incubated at room temperature for 5 minutes and allowed to be stained by Hoechst 33258 according to Fan (Fan *et al*., 2004).

RESULTS

Inhibitory effects of FRBS on NB4 cell proliferation

NB_4 cells proliferation *in vitro* was inhibited significantly by FRBS at arsenic concentration ranges from $0.125\mu g.ml^{-1}$ to $4\mu g.ml^{-1}$ (table 1). It reduced NB_4 cell viability significantly with FRBS during treatment time; however the inhibitory rate reached to the maximal value after 24h treatment with FRBS.

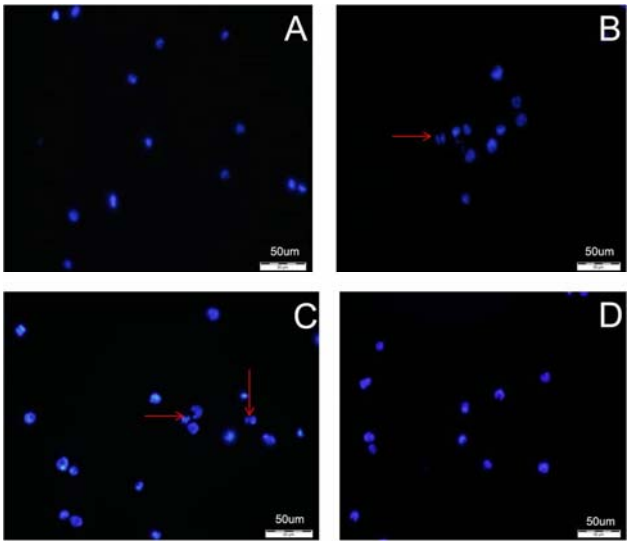

Fig. 1: The effects of FRBS on apoptosis in NB_4 cells after treatment for 24 hours. Hoechst33258 stained cells untreated (A), treated with 2μg/ml (B), 4μg/ml (C) and

realgar-Indigo naturalis formula containing arsenic concentration at 2μg/ml (D) for 24 h were observed by fluorescence microscope. Fragmented nuclei (red arrow) were indicated as arrows, arrow heads.

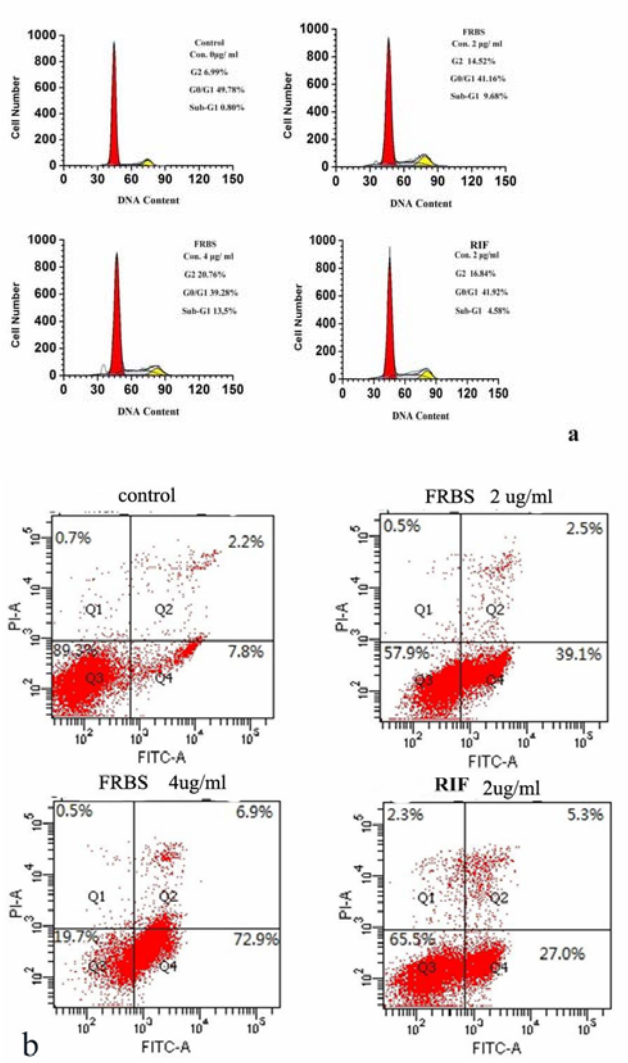

Fig. 2: Effects of 4μg/ml or 2μg/ml (Arsenic) of FRBS and 2μg/ml (Arsenic) of realgar-Indigo naturalis formula on cell cycle (2a) and apoptosis (2b) of NB4 assessed by flow cytometry after 24 h treatment, untreated cells acted as control.

Assessment of induction apoptosis effects of the FRBS

After Hoechst 33258 staining, we used florescence microscope to observe specific morphological changes of the cell induced by the FRBS. After FRBS treatment, all NB_4 cells shrank (red arrow). Typical apoptotic features were observed in the NB_4 cell lines, including broken of cell nucleus. Similar morphological changes were also noted in NB_4 cells treated with RIF containing arsenic concentration at $2\mu g.ml^{-1}$ (fig. 1).

The cell distribution in various phases of cell cycle was analyzed after FRBS treatment for 24h. The proportions of NB_4 cell lines tested in the G1 phase decreased and increased in G2 phase (fig. 2a).This result suggested that FRBS induces apoptosis specifically through delay in G1 phase cell in the cell cycle. fig. 2b shows the typical apoptosis cell treated with FRBS at arsenic concentration from $2\mu g.ml^{-1}$ to $4\mu g.ml^{-1}$ for 24h. The number of apoptotic cells increased with arsenic concentration.

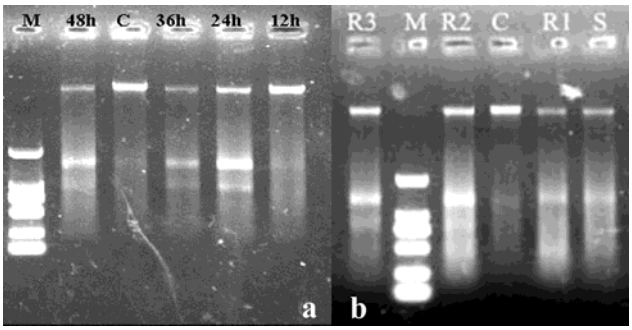

Fig. 3: DNA fragmentation in NB_4 cell treated with FRBS on the different treatment time (3a) and concentration (3b). Lane R3-R1: FRBS (4μg/ml, marker, 2μg/ml, 1μg/ml), M, C, S stands for marker, control, realgar-Indigo naturalis formula containing arsenic concentration at 2μg/ml, respectively.

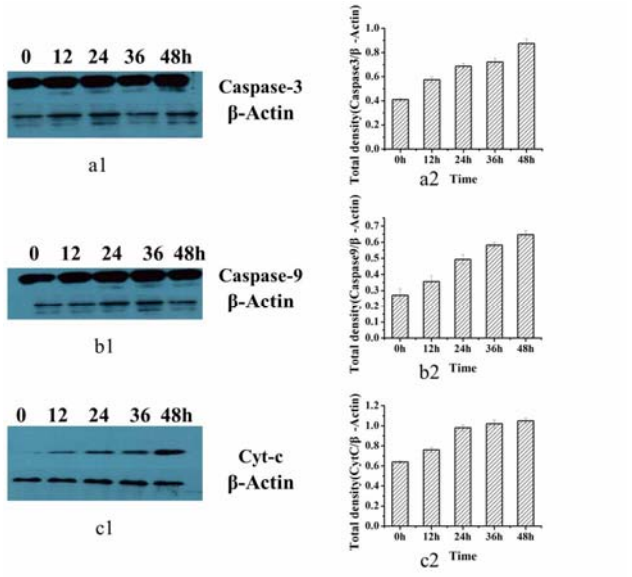

Fig. 4: Western blotting analysis on caspase-3, caspase-9 and cytochrome C in NB_4 cells. Total cell lysates of NB_4 were collected after FRBS treatment for 0 h, 12h, 24h, 36 h, 48h (a, b and c) Columns means bars.

DNA content distribution clearly indicated that exposure of all NB_4 cells to FRBS brought on the appearance of cells with a fractional DNA (sub-G1). In contrast, the positive control group, RIF containing arsenic concentration at $2\mu g.ml^{-1}$ only induced apoptosis to a lesser degree (fig. 2b).

Nucleosomal DNA laddering in NB_4 cells is a common event in apoptotic cascade (Alison *et al*., 1995). As shown

Table 1: Inhibitory rate of FRBS at different arsenic concentration on NB4 cell growth (x±s)

	Arsenic Con.	n	OD570nm			Inhibitory rate%		
	μg.ml-1		12h	24h	36h	12h	24h	36h
FRBS	0.125	3	1.205±0.022	1.453±0.017	1.328±0.014	14.5±0.004	10.52±0.021	5.7±0.021
	0.25	3	1.196±0.012	1.42±0.026	1.31±0.012	15.2±0.013	12.56±0.013	7.1±0.008
	0.5	3	1.182±0.022	1.31±0.01	1.26±0.029	16.2±0.027	19.33±0.03[a]	10.6±0.023
	1	3	1.162±0.004	1.251±0.007	1.241±0.022	17.6±0.003	22.97±0.002[a]	11.9±0.019
	2	3	1.147±0.005	1.229±0.008	1.211±0.009	18.7±0.011	24.30.004[a]	14.05±0.01
	4	3	0.972±0.004*	1.057±0.017*	1.077±0.002*	31.1±0.005	34.91±0.011[a]	23.56±0.02
control		3	1.41±0.028	1.624±0.099	1.409±0.081			

*Differences obtained at levels of a $p<0.01$ were considered significant.

in fig. 3. The results showed that after treatment with FRBS at various arsenic concentrations for 24h (fig. 3b), the DNA extracted from NB4 cells displayed the characteristic nucleosomal ladder of DNA fragments, suggesting the assumed apoptosis. In contrast, similar DNA ladders appeared for NB_4 cell lines after all FRBS treatments for 12h, 24h, 36h (fig. 3a).

Caspases-3 and caspases-9 are cysteinyl aspartate proteinases that cleave substrate proteins at aspartate residues (Knudson *et al*., 1997). As expected, FRBS increased the expression of caspase-3 and caspase-9 in NB4 cells (fig. 4a-b), indicating that a caspase-mediated pathway involved in FRBS-induced apoptosis. In addition, the expression of cytochrome c significantly increased in a time dependent manner, indicating that mitochondria participated in FRBS-induced apoptosis in NB_4 cells (fig. 4c).

DISCUSSION

Apoptosis is a form of cellular suicide is essential for the development and homeostasis of all multi cellular organisms. For cancer cells, they can reduce cell death by the inhibition of apoptosis (Wu *et al*., 2006; Bode *et al*., 2002). Previous works reveal that realgar can act effectively to induce apoptosis in cancer cells (Xiao *et al*., 2005; Zhang *et al*., 2015; Adisak *et al*., 2014), realgar bioleaching solution can significantly improve anti-tumor activity of realgar (Wang *et al*., 2013). In the present work, realgar in RIF was replaced by RBS to form a novel FRBS, and our results supported that FRBS functioned more effectively than RIF, suggesting that FRBS is a promising anti-APL drug candidate.

Our data convincingly demonstrated that FRBS at 2μg.ml^{-1} of arsenic concentration displayed the higher inhibition rate in NB_4 growth than raw realgar at the same arsenic concentration by about two-fold (fig. 2a). Further, in the present study, FRBS after replacement realgar with RBS also at the same arsenic concentration of 2μg ml^{-1} induced higher level of apoptosis in NB_4 cells than RIF by 27 % (fig. 2b). Those results supported FRBS functioned more effectively than that RIF did.

Our research illustrated that FRBS induced NB_4 of APL cell lines apoptosis dose-dependentment. The Fluorescence microscope assay showed that typical apoptosis body appeared at a high dose of FRBS at arsenic concentration of 4μg ml^{-1}(fig. 1). After FRBS treatment, NB_4 cells with sub-G1 increased (fig. 2a), then apoptosis cells were dramatically induced (fig. 2b), nucelosomal DNA laddering displayed (fig. 3). These results are consistent with anti-APL effects of RIF. In addition, the expression of caspase-3, caspase-9 and cytochrome c in NB_4 cells increased after FRBS treatment (fig. 4). It revealed that FRBS at least initiated a mitochondrial-mediated, caspase-dependent intrinsic apoptosis pathway in NB_4 cells. It is deserved to notice that FRBS can markedly inhibit NB_4 cell growth (table 1), and it is at least partially contributed to FRBS therapeutic efficacy for anti-APL. Previous work in our lab has demonstrated that RBS up-regulate AQP9 to resulting in more arsenic intake and hence more effective that raw realgar in K_{562} cells (Wang *et al*., 2013). RBS can suppress ras/MAPK over-activated by inducing ROS (Zhi *et al*., 2015; Liu *et al*., 2013). Therefore, the mechanism of FRBS after replacement realgar with RBS in RIF on anti-APL still needs much more investigation in our future works.

In summary, the successful substitute of RBS for realgar in RIF in the present study through hydrometallurgy technology provided a potent anti-APL medication for clinical use, but also a good model for study of other TCM formula containing realgar for improving their therapeutic efficacy. On the other hand, this study also offers some references to the further useful preclinical trial in exploring this novel realgar-Indigo naturalis formula.

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