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In vitro and in vivo antitumor activity of oridonin nanosuspension

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ABSTRACT

The aim of the present study was to evaluate the antitumor activity of an oridonin (ORI) nanosuspension relative to ORI solution both in vitro and in vivo. ORI nanosuspension with a particle size of 897.2 ± 14.2 nm was prepared by the high pressure homogenization method (HPH). MTT assay showed that ORI nanosuspension could significantly enhance the in vitro cytotoxicity against K562 cells compared to the ORI solution, the IC_{50} value at 36 h was reduced from 12.85 μmol/L for ORI solution to 8.11 μmol/L for ORI nanosuspension. Flow cytometric analysis demonstrated that the ORI nanosuspension also induced a higher apoptotic rate in K562 cells compared to ORI solution. In vivo studies in a mouse model of sarcoma-180 solid tumors demonstrated significantly greater inhibition of tumor growth following treatment with ORI nanosuspension than ORI solution at the same dosage. The mice injected with ORI nanosuspension showed a higher reduction in tumor volume and tumor weight at the dose of 20 mg/kg compared to the ORI solution ($P < 0.01$), with the tumor inhibition rate increased from 42.49% for ORI solution to 60.23% for the ORI nanosuspension. Taken together, these results suggest that the delivery of ORI in nanosuspension is a promising approach for the treatment of the tumor.

1. Introduction

Oridonin (ORI, Fig. 1), is an active diterpenoid compound extracted from the medicinal herb *Rabdosia rubescens*, which is often used in Chinese traditional medicine (Zhang et al., 2003; Osawa et al., 1994). ORI is emerging as a promising antitumor agent, which has demonstrated significant activity against a wide variety of tumors and tumor cell lines including: liver, prostate, breast and cervical cancer cells, non-small cell lung cancer cells, acute promyelocytic leukemia, and glioblastoma multiforme, both in pharmacological experiments and in clinical trials (Chen et al., 2005; Fujita et al., 1988; Ikezoe et al., 2003; Gao et al., 2008b). However, the poor solubility of ORI in water has presented a serious obstacle for its practical use as a therapeutic agent. The usual approach for overcoming the problem of solubility is to prepare the formulation for injection in ethanol, propylene glycol and Tween 80 (Liu et al., 1998). However, adverse effects such as inflammation of the blood vessels and topical pain caused by delivery of miscible solvents by injection negate its clinical utility (Zhang et al., 2003). As a consequence, there is a growing need for developing a suitable formulation of ORI that can overcome this problem.

Recently the carrier-free drug nanosuspension has become an extensively utilized protocol to tackle formulation problems for insoluble compounds (Muller et al., 2001; Patravale et al., 2004). The most outstanding feature of drug nanosuspension is the significantly increased drug solubility and dissolution velocity that can be achieved due to the small particle size and enormous particle surface following nanosizing process, and consequently improved oral bioavailability (Liversidge and Cundy, 1995; Liversidge and Conzentino, 1995). Furthermore, because of their sufficiently small particle sizes and safe composition, nanosuspension can be administered through intravenous injection. Such strategies produce high drug content in a small volume and as a consequence markedly enhance the drug tolerance of the body (Muller and Peters, 1998). In addition, the aqueous nanosuspension, in general dispersed in water, can mitigate the side effects caused by miscible solvents. Several animal studies have already demonstrated that nanosuspension showed passive targeting similar to carrier nanoparticles after intravenous (i.v.) administration, resulting in uptake by the mononuclear phagocytic system (MPS) of the liver, spleen, lung, and bone marrow (Gao et al., 2008b; Ganta et al., 2009; Zirar et

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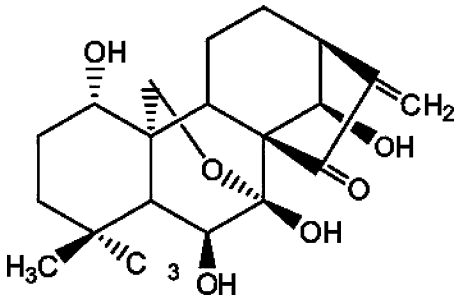

Fig. 1. The chemical structure of oridonin.

al., 2008). Thus nanotechnology promises to be beneficial for the treatment of diseases such as those caused by parasites residing the macrophages of the MPS and/or for the treatment of cancers in the organs mentioned above (Gao et al., 2008a; Date et al., 2007).

The data in this paper extend our findings from previous studies on the use of ORI nanosuspension, in which we successfully prepared stable ORI nanosuspension and investigated their pharmacokinetics and tissue distribution. The results showed that the i.v. administration of ORI nanosuspension leaded to a delayed blood clearance of ORI compared to the ORI solution in rabbits, and showed good targeting to liver and spleen in mice (Gao et al., 2008b). In the present study, the in vitro and in vivo antitumor activity of ORI nanosuspension was investigated in comparison with free ORI solution.

2. Materials and methods

2.1. Materials

ORI (99% pure) was purchased from Shanxi Huike Plants Exploitation Co., Ltd. ORI solution was prepared using our previous method (Gao et al., 2008b); Pluronic F68 was purchased from Sigma. Lecithin was purchased from Beijing Shuangxuan Microbic Medium Co., Ltd. All culture media and supplements were purchased from Sigma. Water used in the experiments was deionized, and all organic solvents were of analytical reagent grade.

2.2. Animals and cell line

Female Kunming strain mice (20 ± 2 g) were supplied by the Shandong University Laboratory Animal Center (Jinan, Shandong, China). The animals were acclimatized to a temperature of $25 \pm 2\,^{\circ}C$ at a relative humidity of $70 \pm 5\%$ under natural light/dark conditions for 1 week with food and water ad libitum. All experimental procedures were performed in accordance with the guidelines for ethics and regulations for animal experiments as defined by the Department of Pharmaceutical Sciences, Shandong University, China.

Human leukemia cell line K562 cells was kindly supplied by the Department of Pharmacology, Shandong University, and the Sarcoma-180 (S-180) cell line (murine sarcoma) was kindly supplied by the Shandong Academy of Medical Sciences.

2.3. Preparation of ORI nanosuspension and particle size

ORI nanosuspension was prepared by the HPH method described previously (Gao et al., 2008b). Initially, the 100 mL ORI crude suspension (1%, w/v) was dispersed in the stabilizer solution and processed using a Heidolph homogenizer at 10,000 rpm for 1 min, the resultant suspension was then circulated for two cycles at 200 bar pressure and five cycles at 500 bar, followed by another 20 cycles at 1300 bar. The mean particle size of the obtained nanosuspension was 897.2 ± 14.2 nm, as measured by Zetasizer (3000SH, Malvern Instruments Ltd., UK). Finally, a freeze-dried ORI nanosuspension powder was obtained after lyophilization (LGJ 0.5, Beijing, China).

2.4. In vitro cytotoxicity of ORI nanosuspension against K562 cells

2.4.1. Cell culture

Human leukemia cell line K562 cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.4.2. MTT assay

K562 cells (50,000 per well) were seeded in 96-well tissue culture plates for 12 h and were then treated with the ORI nanosuspension or free ORI control solution (5–40 μM) for 12, 18, 24 and 36 h. The media were then removed and cells were washed with PBS. An MTT assay was performed by adding 10 μL MTT reagent (5 mg/mL, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO, USA) to each well and cells were then incubated for 4 h, after which culture media were replaced with 150 μL of DMSO. Light absorbance of the solution was measured at 570 nm on a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, USA). The cell inhibitory rate was calculated as follows: inhibitory rate = $(Abs_{570\text{control cells}} - Abs_{570\text{treated cells}})/Abs_{570\text{control cells}} \times 100\%$. All assays were done with six parallel samples. The IC_{50} value was defined as the drug concentration required to inhibit growth by 50% relative to controls.

2.5. Flow cytometry (FCM) detection

For DNA content analysis, K562 cells with different treatments were collected, pelleted, washed twice with PBS, fixed with 70% pre-cooled alcohol, and preserved at 4 °C. Before measurements, the cells were washed to eliminate alcohol, and resuspended in PBS containing 20 mg/L propidium iodide (PI) and 1 g/L ribonuclease A. Approximate 1×10^6 fixed cells were examined per experimental condition by flow cytometry (FACScan, Becton Dickison), and the percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-G_1) DNA divided by the total number of cells examined.

2.6. In vivo antitumor activity of ORI nanosuspension in S-180 bearing mice

In vivo antitumor activity was evaluated using S-180 bearing KM mice. The S-180 tumor cells (3×10^6 cells per mouse) were inoculated subcutaneously to mice (female, 18–22 g, provided by Laboratory animal center of Shandong University) at the axillary region. At 24 h post-inoculation the S-180 bearing mice were randomly assigned to the following four groups (10 per group): negative control, ORI nanosuspension 10 mg/kg group, ORI nanosuspension 20 mg/kg group, and the free ORI solution 20 mg/kg group. The drugs were administered by injection through the tail vein, once daily for 14 days. The negative control group was treated with the same volume of 0.9% sodium chloride injections. The volume of the solid tumor was measured with a vernier caliper on day 4, 7, 11 and 14, and the tumor volume was estimated by measuring tumor size and using the following formula: tumor volume (mm^3) = $0.5 \times L \times W^2$, where L and W represent the largest diameter and the smallest diameter, respectively. Mice were sacrificed on the 15th day post-inoculation and the tumors were excised, weighed, and the tumor inhibition rate was calculated as follows: tumor inhibitory rate = (tumor weight of negative control

Table 1
The IC_{50} values (μmol/L) of ORI nanosuspension and ORI solution on K562 cells.

Formulation	12 h	18 h	24 h	36 h
ORI nanosuspension	13.84	12.65	11.72	8.11
ORI solution	18.54	16.07	15.38	12.85

group – tumor weight of tested group)/tumor weight of negative control group × 100%.

2.7. Statistical analysis of the data

Student's *t*-test and ANOVA were performed using Statgraphics plus 3.3 software with significance set at $P < 0.05$. Data are presented as the mean ± standard deviation for all treatments.

3. Results and discussion

3.1. In vitro cytotoxicity of ORI nanosuspension against K562 cells

The in vitro anticancer cytotoxic activity of ORI nanosuspension and the free ORI solution against K562 cells was expressed as % relative reduction of cell viability (Fig. 2). The IC_{50} value for ORI nanosuspension and the free ORI solution was calculated from the cytotoxicity data (Table 1). The ORI nanosuspension exhibited significantly higher inhibition rates against K562 cells compared to the ORI solution at the same concentration (Fig. 2). The activity of both the ORI nanosuspension and ORI solution increased in parallel with drug concentration and incubation times. The IC_{50} for both drug formulations were reduced with increased time of treatment. The IC_{50} of the ORI nanosuspension was much lower than that of the ORI solution after the same incubation time (Table 1).

Both incubation time and concentrations played a major role in the in vitro cytotoxicity of ORI. After longer incubation periods a larger number of cells entered the G_2 and M phases of the cell cycle during which ORI is known to be more effective (Zhang et al., 2003). It is generally considered that nanoparticles can be nonspecifically internalized into cells via endocytosis or phagocytosis (Storm et al., 1995). In addition, it is possible that the nanoparticles were not actively internalized by cells, but adsorbed non-specifically by pinocytosis after accumulating on the surface of the cells (Rieux et al., 2006). All of these can enhance the interaction between the drug and the cells. Apart from these, ORI nanoparticles, possessing a markedly increased solubility and dissolution rate, could induce sufficient molecular concentration around the cells. Therefore, for ORI nanosuspension, the increased cytotoxic effect may be a result of the presence of dissolved free ORI, or nanoparticles or the combination of both.

3.2. Cell apoptotic rate detected by FCM

As shown in Fig. 3, ORI nanosuspension treatment of K562 cells resulted in a markedly increased accumulation of sub-G_1 phase cells in a time- and dose-dependent manner. The mean apoptotic population of K562 cells was 2.43 ± 0.15% under control conditions, while for ORI nanosuspension treated cells apoptosis was increased to 4.70 ± 0.50%, 33.63 ± 0.66% and 42.86 ± 0.83% after 12 h of exposure to 10, 20 and 30 μmol/L, respectively. By 24 h of exposure to 10, 20 and 30 μmol/L, the apoptotic rate was 14.56 ± 1.23%, 36.7 ± 1.65% and 51.7 ± 1.71%, respectively. It was demonstrated that ORI nanosuspension induced a higher apoptotic rate compared with the ORI solution at the same dose. The apoptotic rate of K562 cells exposed to 20 μmol/L of ORI solution after 12 and 24 h was 16.26 ± 0.64% and 21.3 ± 2.00%, respectively; while for ORI nanosuspension the apoptotic rate was 33.63 ± 0.66% and 36.7 ± 1.65%, respectively.

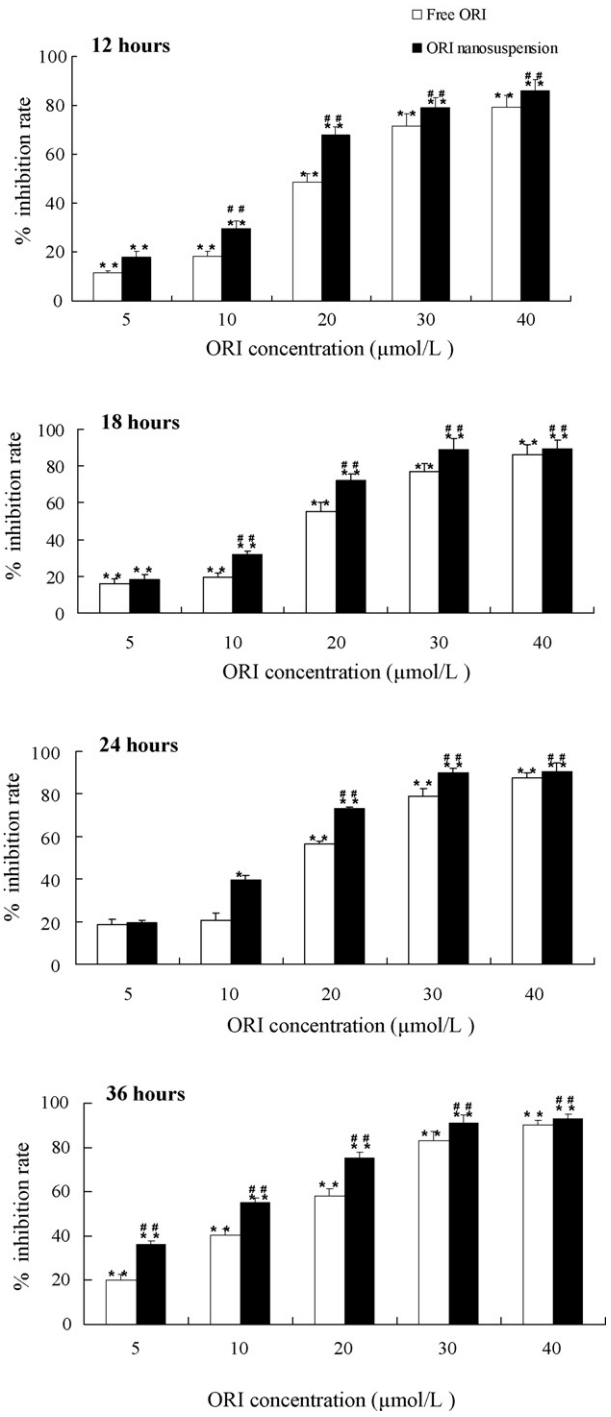

Fig. 2. MTT assay showing that ORI nanosuspension and free ORI solution treatment inhibit growth of K562 cells in a time- and dose-dependent manner. Results are expressed as mean ± standard deviation ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* control group, $^{\#\#}P < 0.01$ *vs.* the same dose of free ORI solution group.

The results of FCM were consistent with the previous studies by Liu et al. (2005), which also reported that K562 cells treated with ORI presented the special sub-G_1 peak of apoptotic cells, and the apoptotic rates increased in a dose-dependent manner. In the present studies, we found that the apoptotic rate of cells treated with the ORI nanosuspension was significantly higher than that of ORI solution at the same dose and incubation time, which may be explained by the uptake effect of tumor cells in response to drug nanoparticles. This result appears to be consistent with that of the in vitro cytotoxicity experiments.

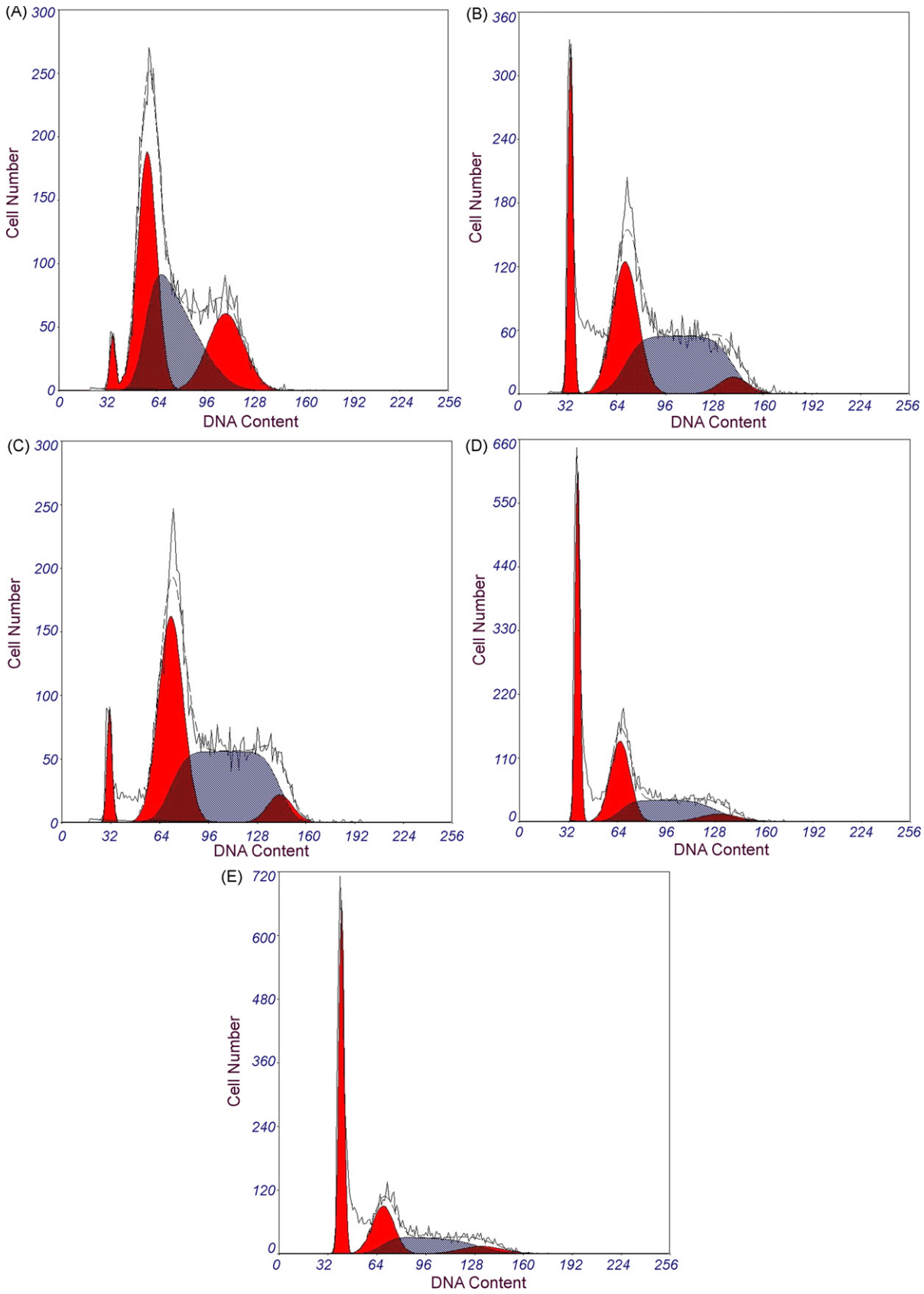

Fig. 3. Flow cytometric analysis of DNA fragmentation for K562 cells after treatment with free ORI solution and ORI nanosuspension. (A) Negative control; (B) free ORI solution (20 μmol/L, 12 h); (C) ORI nanosuspension (10 μmol/L, 12 h); (D) ORI nanosuspension (20 μmol/L, 12 h); (E) ORI nanosuspension (30 μmol/L, 12 h); (F) free ORI solution (20 μmol/L, 24 h); (G) ORI nanosuspension (10 μmol/L, 24 h); (H) ORI nanosuspension (20 μmol/L, 24 h); (I) ORI nanosuspension (30 μmol/L, 24 h). The apoptotic rates calculated by flow cytometry are shown in (J). Data represent mean ± standard deviation from three independent experiments. $^{**}P < 0.01$ *vs.* negative control group, $^{\#\#}P < 0.01$ *vs.* free ORI solution 20 μmol/L group.

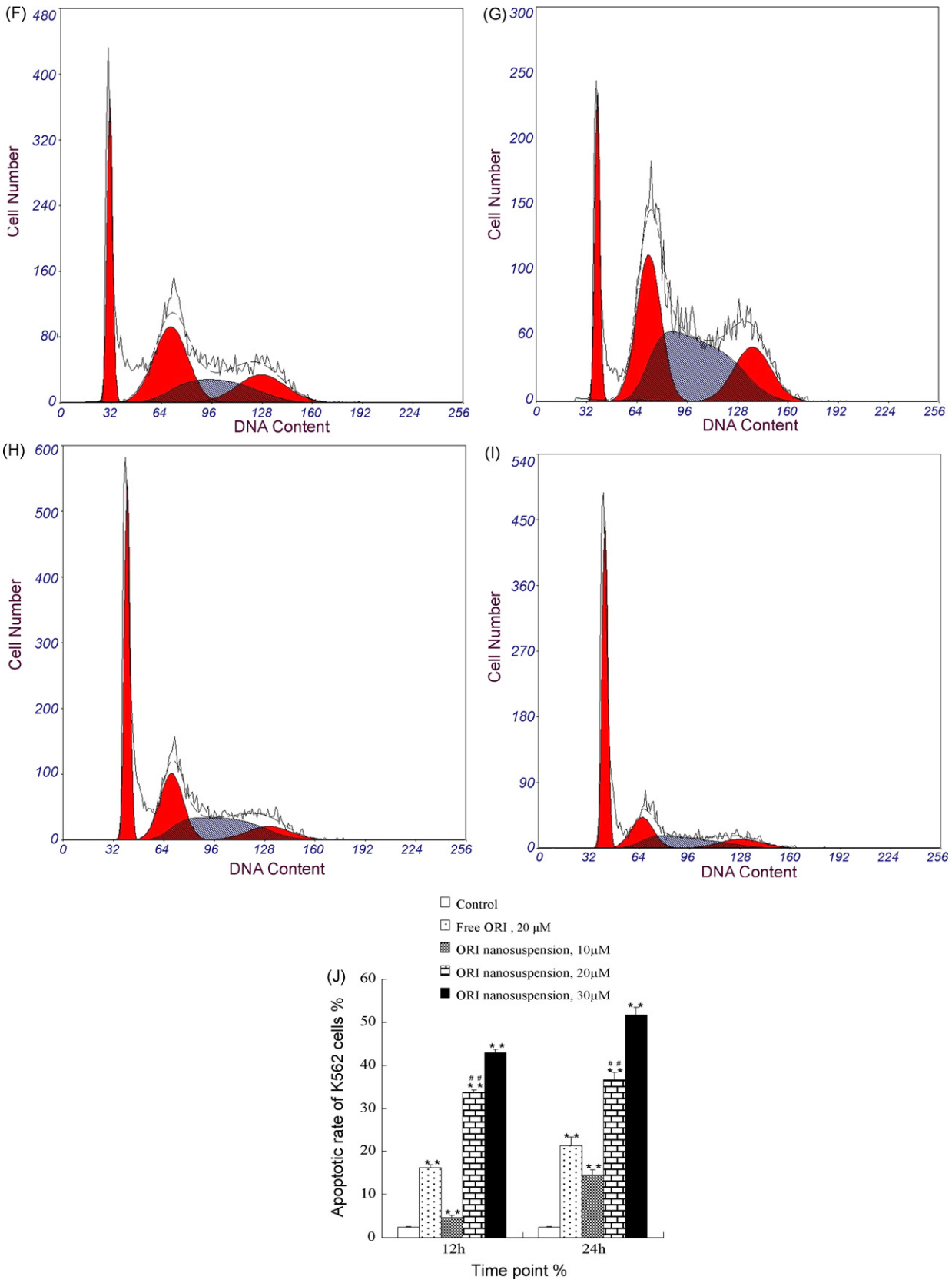

Fig. 3. (*Continued*).

Table 2
The in vivo antitumor effects in S-180 bearing mice ($n = 10$).

Group	Dose (mg/kg)	Tumor weight (g)	Inhibition rate (%)
Negative control		2.091 ± 0.398	
ORI nanosuspension	10	$1.283 \pm 0.209^{**}$	36.45
ORI nanosuspension	20	$0.803 \pm 0.273^{**,\#\#}$	60.23
ORI solution	20	$1.161 \pm 0.216^{**}$	42.49

** $P < 0.01$ *vs.* negative control group.
$^{\#\#}$ $P < 0.01$ *vs.* ORI solution 20 mg/kg group.

3.3. *In vivo antitumor effects in S-180 bearing mice*

The in vivo antitumor effect of ORI nanosuspension and ORI solution was assessed in S-180 bearing mice. Table 2 and Fig. 4 listed the tumor weight, tumor inhibition rate and tumor volume of all the tested groups. Both ORI nanosuspension and ORI solution yielded significantly enhanced tumor inhibition compared with the negative control group with a marked reduction of the tumor volume and tumor weight. The tumor inhibition rate of ORI nanosuspension was significantly higher than that of the free ORI solution at the same dose of 20 mg/kg. Piloerection and diminished vigor were observed in the ORI solution treated mice. In stark contrast, mice treated with the ORI nanosuspension remained vigorous, had a healthy appearance and were normal groomed throughout the entire experiment.

Numerous investigations have shown that nanoparticulate drug delivery systems can increase antitumor efficacy while reducing systemic side effects (Brigger et al., 2002; Mattheolabakis et al., 2009). It has been acknowledged that the nanoparticles could escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and then accumulate in certain solid tumors by the so-called enhanced permeation and retention (EPR) effect (Brannon-Peppas and Blanchette, 2004). In addition, our previous studies demonstrated that ORI nanosuspension showed a longer retention time than ORI solution, which might produce a prolonged exposure of the tumor cells to the antitumor drugs. Therefore, for the ORI nanosuspension, drug nanoparticulates could arrive at the tumor site through EPR effect and might also sustain the therapeutic concentration over time. The aqueous nanosuspension formulations also avoided the adverse effects caused by the use of ethanol, propylene glycol and Tween 80 (Gao et al., 2008b).

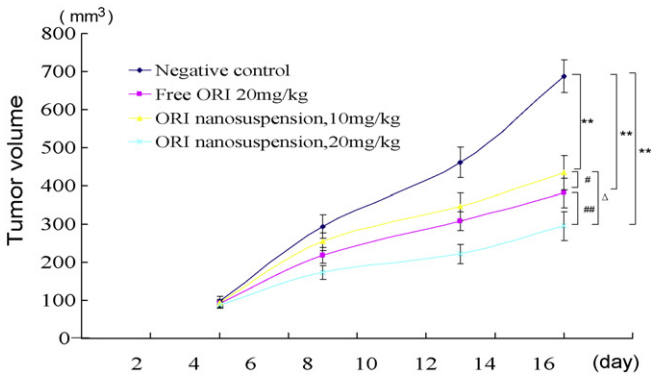

Fig. 4. Effects of ORI nanosuspension and ORI solution on tumor volume of S-180 bearing mice. The results showed that ORI nanosuspension and ORI solution treatment could inhibit the tumor growth significantly. At the same dosage (20 mg/kg), the tumor volume in ORI nanosuspension group was significantly smaller than those in ORI solution group. $n = 10$ mice. $^{**}P < 0.01$, *vs.* control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the free ORI solution group, $\Delta P < 0.01$ *vs.* ORI nanosuspension 20 mg/kg group.

4. Conclusion

The present study demonstrates that when formulated as a nanosuspension, ORI showed stronger antitumor activity compared to the free drug. Cytotoxicity tests using K562 cells showed that the ORI nanosuspension was more cytotoxic than ORI solution. FCM analysis further indicated that the ORI nanosuspension induced a higher apoptotic rate in K562 cells. Similarly, in mice, the ORI nanosuspension was better tolerated, and induced noticeable antitumor effects compared to the ORI solution. Based on these results, it can be concluded that nanosuspension of ORI that was developed in this work may be an effective drug delivery strategy, especially for relatively insoluble anticancer drugs.

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