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Experimental study of realgar on tumor suppression in lymphoma model

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Abstract

Objective: To assess the tumor-suppressive effect of external application of realgar ointment on human lymphoma Raji cell transplantation mouse model.

Methods: Human lymphoma Raji cells were sub cultured and inoculated at dose of 1 x 10^7 cells to establish lymphoma xenografts mice model which was then assigned into group A (treated with 40% realgar ointment), B (20%), C (10%) and D (model group) followed by analysis of tumor volume and survival at 30,60 and 90 days.

Results: After treatment, tumor volume in group A, B and C was significantly decreased with increased tumor inhibition rates compared to group D. Meanwhile, the initial onset time of tumor suppressor in treatment group was significantly shortened (P< 0.05). The survival rate of mice in A group was significantly lower than that in the other three groups (P<0.0001), while the other three groups had no statistical difference (P>0.05). The concentrations of arsenic in group B (2658.86±1728.38 ug/L) were significantly higher than that in other groups (678.37±285.26 ug/L for group A, 522.55±285.29 ug/L for group C, 34.18±9.39 ug/L for group D) (P< 0.05). There were no significant differences regarding the level of alanine aminotransferase and creatinine value among four groups.

Conclusions: Realgar ointment has obvious anti-tumor effect on mouse model of human lymphoma transplanted tumor in a dose dependent manner, suggesting that it might be a novel approach for treating lymphoma.

Keywords: Realgar, As2S2, Chinese medicine, Lymphoma

Introduction

Lymphoma is not recorded in ancient Chinese medicine literature, but according to its clinical characteristics and related symptoms, it can be classified as "stone gangrene "," evil nucleus "," loss of glory "," phlegm nucleus "," defective carbuncle "," Yin gangrene "," saber with tassel" and so on. Traditional Chinese medicine believes that the etiology of this disease is mostly congenital endowment deficiency, or internal injury seven feelings, Qi invasion, guest in the viscera, eventually leading to Qi, blood and body fluid disharmony, viscera dysfunction and disease. The pathogenesis of malignant lymphoma has different medical knowledge, but the treatment is based on four basic factors: deficiency, phlegm, blood stasis and poison. In Traditional Chinese medicine books, it has been stated that "poison in the pulse" is an important contributing factor in the pathogenesis of lymphoma, as "Lingshu cold and heat" said: cold and heat in the neck axil, rat fistula cold and heat gas to make it. "Detoxification" is one of the important links in the treatment of malignant lymphoma. In the first part of this paper, we used

"detoxification" method to treat relapsed refractory lymphoma with detoxification traditional Chinese medicine compound. In traditional Chinese medicine, the use of realgar, arsenic and other products to attack poison is the most extreme embodiment of detoxification.

As one of the most representative drugs in detoxification Chinese medicine, realgar (As_2S_2) has been recorded in Shennong Herbal Classic as early as its internal and external use for skin diseases, ulcers, plague and malaria. "Compendium of Materia Medica" is known as "the medicine for killing and killing sores ". Compared with arsenic trioxide (arsenic trioxide), realgar has higher safety and lower toxicity. Realgar warm, flavor Xin, detoxification, dampness and phlegm, stasis and accumulation, the efficacy of the malignant lymphoma "phlegm "," blood stasis" and "poison" three basic factors. "Attack evil school" on behalf of the Jin Dynasty physician Zhang Zihe said:" Is there a disease accumulation of people, evil cannot come out, but can make up for it?" And "first on the attack of its evil, evil to go and vitality from the recovery is also." We believe that for malignant lymphoma, such as the disease of the disease, we can use realgar attack its evil to solve its poison, to dissipate phlegm and blood stasis, evil poison to give other methods of correction. Our present study aims to investigate whether realgar can be used to treat

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lymphoma using animal models to provide the evidences for its possible application in clinic.

Materials

Medication

Realgar was purchased from Sanmenxia Yuhuangshan Pharmaceutical Co., Ltd., batch number: ly20120903046. Sesame oil was from Shanghai Santian Food Co., Ltd. Yellow Vaselin was from Jiangxi Yipusheng Pharmaceutical Co., Ltd.

Cell

Human Raji Burkitt`s lymphoma cell line was purchased from Beijing Hongxin Stem Cell Biotechnology Co., Ltd

Animals

BALB/c nude mice, female (SPF grade) was purchased from Shanghai Zesheng Technology Development Co., Ltd., production license No. (Shanghai)2020-0004.

Cellular resuscitation

Raji cells were removed from the -196℃ liquid nitrogen tank before the experiment, and quickly put into a constant temperature tank preheated to 37℃, then melted and sterilized and transferred into the sterile operating table.

Cell passage

The resuscitated Raji cells were inoculated in RPMI-1640 medium (Jiangsu Lihan Biotechnology Co. Ltd) including 10% fetal bovine serum and cultured in a 37℃-incubator containing 5% CO^2.

Establishment of Mouse model for human lymphoma transplantation

43 female BALB/C nude mouse aged 6-8 weeks (20-30 g) were used to establish the model. The logarithmic phase Raji lymphoma cells were washed with PBS and subcutaneously inoculated into the right anterior armpit at a density of 1×10^7 cells. After inoculation, the volume of tumor was measured and recorded once a day after a palpable mass appeared. A successful model was established when the length of the subcutaneous tumor was more than 10 mm.

Animal grouping

38 mice were randomly divided into 4 groups: group A (40% realgar ointment treatment) (n=10); group B (20% realgar ointment treatment) (n=10); group C (10% realgar ointment treatment) (n=8); group D (model control group) (n=10).

Preparation of Realgar Ointment

First, the 80℃ of Yellow Vaselin was melted and weighted (1000 g) followed by addition of 250 g sesame oil and being stirred well. 50 g realgar powder was added into 450 g molten and mixed and stirred until it cools to room temperature to get 10% concentration realgar ointment. 100 g realgar powder was added into 400 g molten to obtain 20% realgar ointment; 200 g realgar powder was added into 300 g molten mixture to obtain 40% realgar ointment.

Realgar ointment treatment

Group D was treated with yellow Vaseline-sesame oil mixed ointment, group A was treated with 40% realgar ointment (high concentration group), group B was treated with 20% realgar ointment (medium concentration group) and group C was administrated with 10% realgar ointment (low concentration group) once a day. Undergone continuous administration until the tumor disappeared or died or performed euthanasia 90 days after administration.

Animal observation

The survival state, living habits and skin condition of the mice were observed daily. The mice weight was measured every 1-3 days and recorded on the experimental observation table. The observation period was recorded until the tumor disappearance or mouse death or performed euthanasia 90 days after administration.

Tumor volume: The maximum vertical transverse diameter (W) and the maximum tumor diameter (L) of mice were recorded every 1-3 days with a vernier micrometer. Tumor volume in mice computational formula：$V=LW^2/2$.

Survival rate: the survival rate of mice in each group was recorded 30 days ,60 days and 90 days after medication.

Tumor inhibition rate: the tumor inhibition rate was calculated for 30 days ,60 days and 90 days in three groups of A, B, C. tumor inhibition rate computational formula = (1-the average tumor volume in the administration group / the average tumor volume in the control group) ×100% respectively.

Detection of Arsenic Concentration and Biochemical Indicators in Whole Blood

When the first mouse died (21 days after continuous administration), 3 mice were randomly selected from each group to measure the whole blood arsenic concentration, serum alanine aminotransferase (ALT) and creatinine values (CREA).

Detection of whole blood arsenic concentration: 100μl whole blood of mice was collected and 600 μl 67% nitric acid was added and boiled for 3 hours. After cooling, 2.5 ml pure water was added and then centrifuged at 4000 rpm for 25 min followed by measuring arsenic concentration using plasma mass spectrometer (Mass Spectrometry provided by Shanghai Institute of Life Sciences, Chinese Academy of Sciences).

Statistical processing

SPSS 21.0 software was utilized to analyze data which were displayed as mean ± SD for measurement data and assessed by ANOVA or student's t test. Classification variable data were tested by $\chi 2$ or Fisher accurate. Kaplan-meier method is used to draw survival curve and analyze survival. $P < 0.05$ indicates a significance.

Results

Comparison of tumor volume before administration

Before administration, there was no statistical difference (P>0.05) regarding the tumor volume of mice in each group (Table 1).

Comparison of maximum volume of transplanted tumor after treatment

After the intervention, the tumor volume of the mice continued to increase in all groups, which reached a maximum on 23, 27, 38 and 64 days on average, then the tumor body shrinks (Figure 3 for the tumor growth curve). Compared to the control mice, the maximum tumor volume was significantly decreased in groups A and B (P<0.05), indicating that high and medium doses of realgar could significantly inhibit tumor growth. Although the tumor body of the C group was smaller than that of the control group, no statistical difference was found (P>0.05) (Table 2).

Comparison of initial onset time of tumor inhibition

There was no significant difference among A, B and C group (p<0.05). It showed that realgar needs to reach a certain concentration in vivo to play a tumor suppressor role. The onset rate of higher concentration realgar showed a faster trend, but there was no significant difference in the onset time among A, B and C group (p>0.05) (Table 3).

Tumor suppressor analysis

The tumor inhibition rate in group A, B, and C at 30,60 and 90 days after administration was shown in Table 3-6. Thirty days after administration, high, medium and low concentrations of realgar ointment showed inhibitory effects on transplanted tumors with an inhibition rate of 95.1%,39.4% and 18.6%, respectively. However, compared with control mice, there was no significant difference in tumor volume (p>0.05). 60 days after administration, group B and C continued to show tumor suppression, with tumor suppression rate of 97.3% and 97.5%, respectively. Compared to the control mice, tumor volume was significantly reduced (p<0.05); Because of the high mortality in the group A, there is no statistical comparison after 90 days administration. All the tumor bodies in group B subsided as tumor suppression rate reached 100%, group C had a tumor suppression rate of 94.2% compared with control mice, so group B had a higher 90-day survival rate (p<0.05). Despite tumor suppressor efficiency in group A, but all died when the tumor was not completely subsided; group C of mice showed promising tumor inhibition at 60 days, however, there was still tumor in 90 days observation period.

Comparison of survival rates

Thirty days after administration, mice in group B, C and D all survived (100% survival); 3 of the group A mice survived (42.9% survival); 60 days after administration, only one mouse survived in group A, 6 in group B, 4 in C group and 7 in group D. The 60-day survival rate of group A (14.3%) was significantly different from that of group B (85.7%) and group D (100%) (p<0.05); 90 days after administration, no mice survived in group A group, 6 in group B group, 3 in group C and 5 in group D. Compared with the other three groups, group A (0%) had a significantly lower 90-day survival rate (p<0.05) (Table 7).

Daily observation showed that group A mice had skin sclerosis, necrosis, limited activity, mental retardation, weight loss and finally went death, which was not observed in group B and C. The results showed that high concentration of realgar had obvious toxicity by skin administration, but the toxicity of medium and low concentration was not obvious. (Figure.2)

Survival time and survival curve

The survival time of group A mice was significantly lower than that of the other three groups (p<0.0001), It is proved that high concentration of realgar has obvious toxicity to mice. During the observation period, there was no significant difference in survival time among other three groups (p>0.05). Maybe after expanding the sample size, the survival time of B, C and D groups would reach different. We will continue to observe this in subsequent experiments (Figure 3, Table 8).

Comparison of total arsenic concentration

When the experimental mice died for the first time (21 days after administration), 3 mice were randomly selected to measure the whole blood arsenic concentration by mass spectrometry. The whole blood arsenic concentration in group B (medium concentration group) was significantly higher than that in other three groups (p<0.05). For group A mice, due to high drug concentration, the application of local skin hardening, necrosis, may explain the blood concentration in group A mice was lower than group B (Table 9).

Biochemical testing

After 21 days of administration, 3 mice were randomly selected for biochemical test and the results showed no obvious difference among group A, B and C in serum alanine aminotransferase (ALT) and creatinine (CREA) (P>0.05), and three different concentrations of realgar ointment had no

significant effect on liver and kidney function (Table 10).

Discussion

In modern medicine and pharmacology, the antitumor mechanism of realgar mainly includes targeting tumor cells and indirectly inhibiting tumor cell growth. At present, the mechanism of realgar anti-lymphoma is mainly focused on inhibiting cell proliferation and promoting apoptosis. Jiang Shuang [1] and other studies have observed that realgar acts on Raji cells to destroy the adhesion substance and subcellular structure of cell membrane and lead to tumor cell apoptosis. A previous study by Shilili [2] has shown that realgar blocks the G1 cycle of lymphoma cells, and up-regulates the expression of apoptosis-related molecules, at the same time inhibits the expression of Bcl-2 proteins and promotes apoptosis of lymphoma cells through the mitochondrial dependent pathway. In the study of solid tumors, realgar reduces neovascularization in tumor tissue, leading to necrosis [3]. The mechanism may be that realgar can downregulate the expression of vascular endothelial growth factor (VEGF) through inhibition of PI3K/AKT pathway to inhibit the angiogenesis in the tumor environment, leading to the inhibition of the distal metastatic of the tumor [4].

Several experimental studies on the anti-lymphoma effect of realgar are mostly focused on in vitro cell experiments, but animal experiments are rare, so the purpose of this experiment is to observe the anti-lymphoma effect of realgar external application in animals. Realgar is used as toxic drug, and external ointment is made as intervention route mainly for convenient administration. After 90 days of observation, it was found that the external application of realgar ointment could effectively inhibit the growth of subcutaneous lymphoma in nude mice, in which the 20% realgar ointment treatment presented the highest tumor inhibition rate and the highest survival rate with all 6 mice survived within 90 days. The effect of realgar's inhibition on tumor growth was higher than that of 10%, that is, realgar inhibited the growth of lymphoma transplanted tumor in a certain dose dependent manner, which was consistent with the results of inhibiting the proliferation and promoting apoptosis of lymphoma cells in vitro [1,2][5][7]. This study proves that realgar has tumor inhibition effect on human lymphoma.

In the course of the experiment, 20% and 10% of the mice treated with realgar ointment had no obvious drug-related skin damage, while in 40% of realgar ointment group, the mice died within 90 days of administration. All mice in each group were randomly selected to measure the level of ALT and CREA in peripheral blood and showed that there was no obvious abnormality, which indicated that realgar ointment administered through the skin has no obvious effect on liver and kidney function. The 40% concentration of realgar ointment may die by causing local skin necrosis secondary infection in mice.

As one of the most representative drugs in detoxification Chinese medicine, realgar has been proved to be effective in other blood tumors such as promyelocytic leukemia and myelodysplastic syndrome, and there is also effective evidence in lymphoma in vitro. However, it has not been reported in animal models of lymphoma. Our present study proves that realgar can inhibit tumor of lymphoma transplanted in animals by skin administration, which provides a reliable basis for the clinical treatment of lymphoma and makes it possible for realgar to be used in the treatment of some skin lymphoma in clinic. However, the exact molecular mechanism of anti-lymphoma by realgar is not investigated in our present study which is the main study limitation and we plan to investigate this in the future. In addition, the dosage and curative effect of oral administration are also worth further exploration; the bioavailability of Shuifei realgar is how, whether it can be used further, and whether its curative effect is better; whether realgar and other anti-tumor drugs can be used to detoxify and synergize compound preparation all requires further investigation.

In conclusion, the traditional Chinese medicine realgar has anti-lymphoma effect in mice model, suggesting that it might be used as a novel approach for the treatment of lymphoma in clinic. However, large cohort clinical study is required to confirm the finding in order to provide more solid evidence for its potential application in clinic.

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Tables and Figure legends

Table 1. **Tumor volume of mice before administration（x±s）.**

Groups	n	Tumor volume /cm³
A	7	0.371±0.112
B	7	0.364±0.105
C	5	0.355±0.094
D	7	0.415±0.092

Note: Single factor ANOVA, F=0.452, P>0.05, LSD test for multiple comparison, the results showed that there was no significant difference in the mean volume of tumor in the three groups of mice

Table 2. **Maximum volume of transplanted tumor after administration（x±s）.**

Groups	n	Tumor volume /cm³
A	7	1.656±1.541[a]
B	7	2.916±2.926[b]
C	5	5.784±5.689
D	7	9.011±8.102

Note:a, A group compared with control group, P=0.014; b, B group compared with control group p=0.038.

Table 3. **Initial onset time of tumor suppressor（x ±s）.**

Groups	n	Time/days
A	7	23.0± 21.5a
B	7	27.4± 12.2b
C	5	38.4± 19.3c
D	7	63.9± 23.6

Note: The test method adopts One Way-single facto-ANOVA, F=2.569, P=0.08. Multiple comparisons were performed by LSD test. a) A group vs control group, P=0.039; b) B group vs control group, P=0.018.

Table 4. **Tumor suppression rate after 30 days administration（x±s）.**

Groups	n	Tumor volume /cm³	tumor suppression rates /%	P
A	3	0.454 ±0.161	95.1	0.054
B	7	1.841±2.096	39.4	0.233
C	5	2.475±2.228	18.6	0.602
D	7	3.039±1.474		

Table 5. **Tumor suppression rate after 60 days administration（x±s）.**

Groups	n	Tumor volume /cm³	tumor suppression rates /%	P
B	6	0.187±0.205	97.3	0.025
C	4	0.179±0.260	97.5	0.042
D	7	7.036±7.468		

Table 6. **Tumor suppression rate after 90 days administration（x±s）.**

Groups	n	Tumor volume /cm³	tumor suppression rates /%	P
B	6	0.000±0.000	100.0	0.029
C	3	0.196±0.339	94.2	0.076
D	5	3.355±3.652		

Table 7. **Survival rate of mice after 30,60,90 days (χ2 test).**

Groups	Total	30 天			60 天			90 天		
		Survival	Death	Survival rate /%	Survival	Death	Survival rate /%	Survival	Death	Survival rate /%
A	7	3	4	42.9	1	6	14.3[ab]	0	7	0.0[cde]
B	7	7	0	100.0	6	1	85.7	6	1	85.7
C	5	5	0	100.0	4	1	80.0	3	2	60.0
D	7	7	0	100.0	7	0	100.0	5	2	71.4

Note: a)60 days of administration, A group vs B group, p=0.029; b)60 days, A group vs D group p=0.005; c)90 days, A group vs B group p=0.005; d)90 days, A group vs C group p=0.045; e)90 days, A group vs D group, p=0.021.

Table 8. **Survival time（x±s）.**

Groups	n	Survival time /days
A	7	36.3±19.3[a]
B	7	84.4±14.7
C	5	78.0±15.1
D	7	84.4±10.5

Note: The test method adopts One Way-single facto-ANOVA, F=16.08, P<0.0001. Multiple comparisons were performed by LSD test. a) A group vs other 3 groups, p<0.0001.

Table 9. **Detection of arsenic concentration in mice （x±s）.**

Groups	n	Plasma arsenic concentration ug/L
A	3	678.37±285.26
B	3	2658.86±1728.38[abc]
C	3	522.55±285.29
D	3	34.18±9.39

Note: a) B group vs A group, p=0.026; b) B group vs C group.

Table 10. **ALT and CREA detection in （x±s）.**

Groups	n	ALT （IU/L）	CREA （mmol/L）
A	3	46.67±6.35	6.67±5.69
B	3	47.00±25.12	75.67±93.61
C	3	55.67±22.74	12.67±9.45
D	3	44.00±5.20	29.00±29.82

Note: ALT comparison F=0.254, P=0.857. CREA comparison F=1.198, P=0.371.

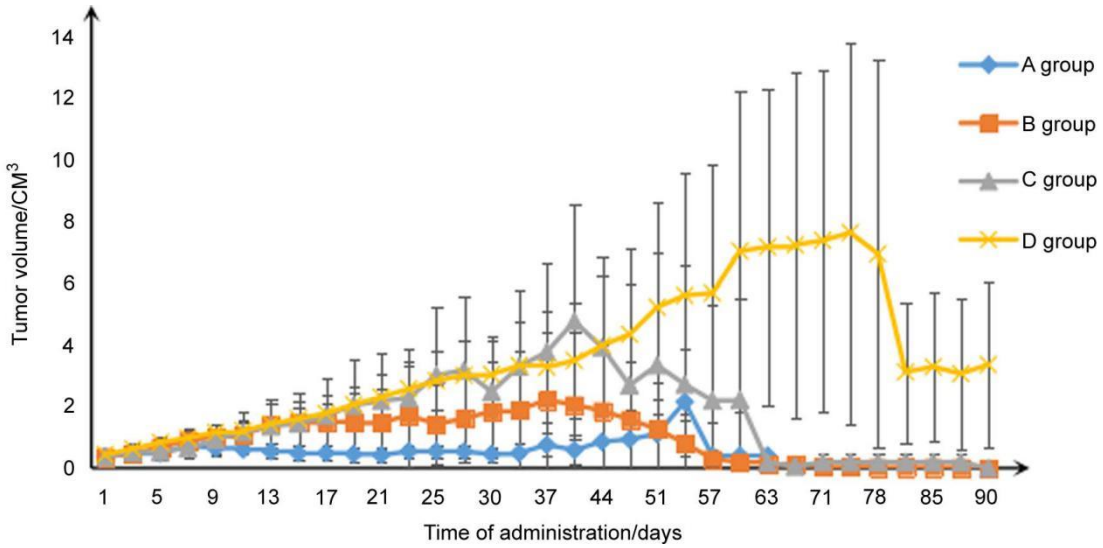

Figure 1. **Growth curve of tumor**

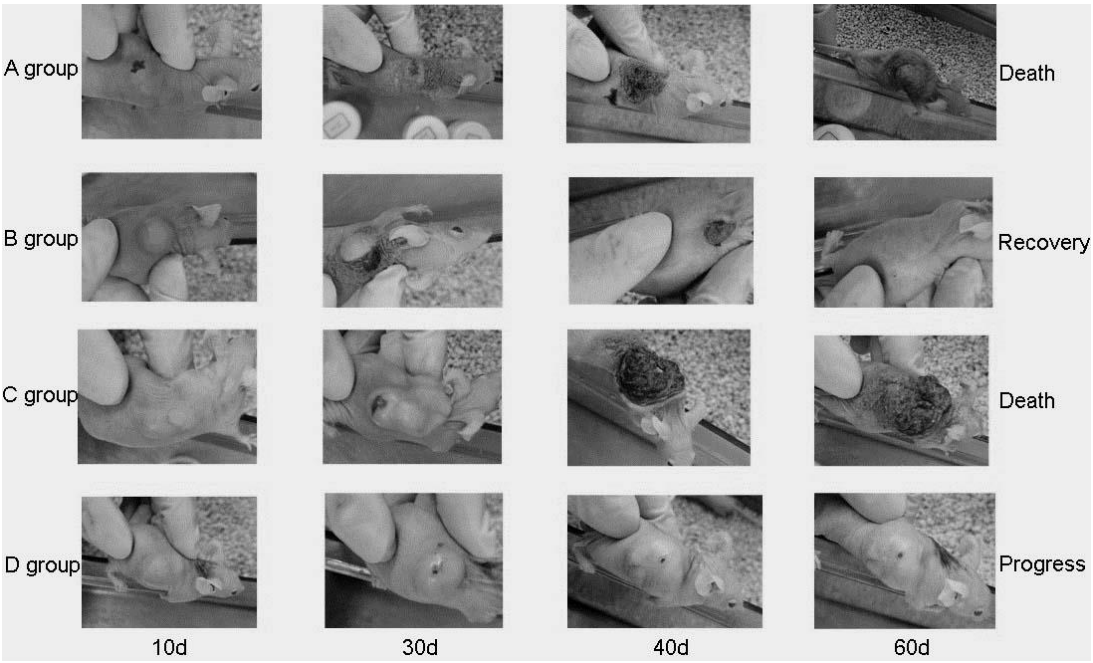

Figure 2. **Representative images for survival status of mice.**

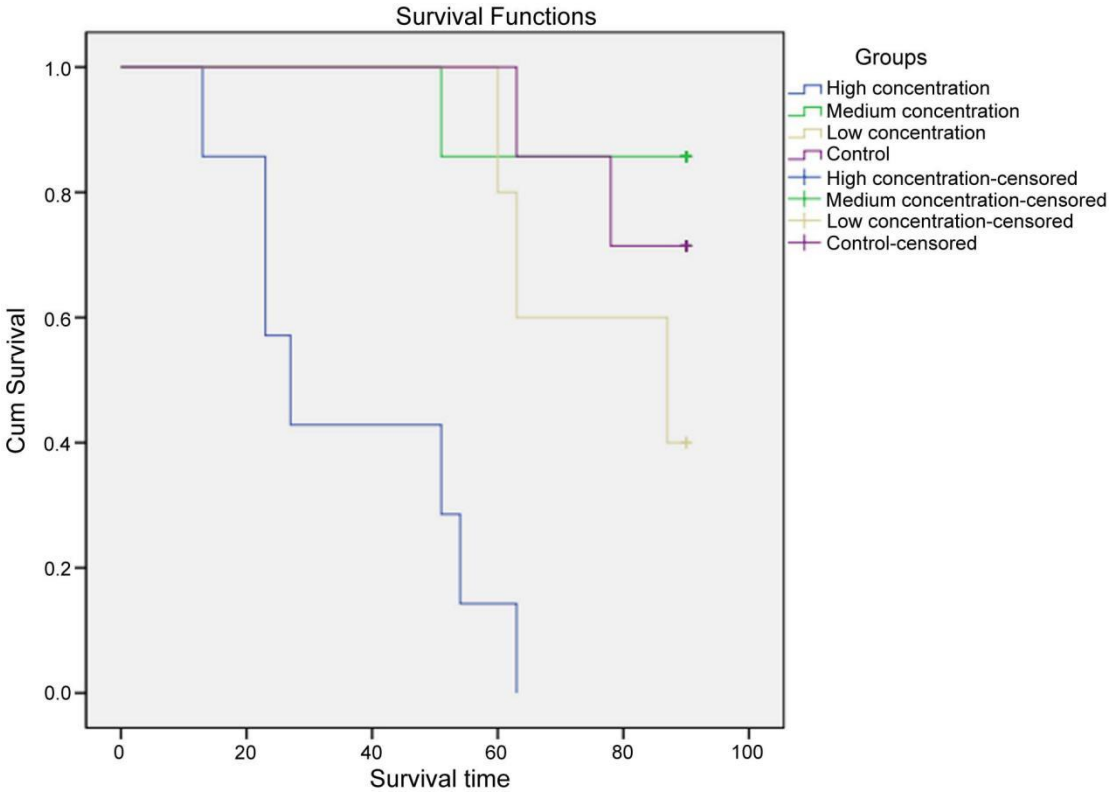

Figure 3. **Kaplan-meier survival curve.**