Combinational Cytotoxicity of Non-toxic Strontium Compounds with Uric Acid toward to Kidney Cells

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Abstract

In this study, we used non-toxic strontium (Sr) compounds: SrCO₃, Sr(NO₃)₂ and Sr(OH)₂ to investigate their combinational cytotoxicity with uric acid. Our results indicate that the anion effect on the combinational cytotoxicity of non-toxic Sr compounds with the polar organic compounds in regard to kidney cells of live species is very important, for example, uric acid. Our results showed that the overall the combinational cytotoxicity order of these three Sr compounds are Sr(OH)₂ << SrCO₃ < Sr(NO₃)₂. Preliminary quantum chemistry calculation also confirmed that the high toxicity is caused the most stable complex of Sr(NO₃)₂ with Uric anion (U⁻) in aqueous solution, Sr²⁺(U⁻)₂.

Keywords

Combinational Cytotoxicity; Strontium; Uric Acid; Complexes

Introduction

Strontium forms 0.02-0.03% of the earth’s crust and is present in igneous rocks in amounts averaging 375 ppm [1]. Of the naturally occurring strontium compounds, only the minerals strontianite (strontium carbonate) and celestite (strontium sulfate) are of economic importance [2]. Human Toxicity Excerpts: inorganic salts of strontium, such as chloride, sulfate and carbonate are remarkably benign. In large oral doses they presumably exert local osmotic effects & so tend to induce vomiting & diarrhea [3]. Strontium commonly occurs in nature, comprising about 0.034% of all igneous rock, as well as in the form of the sulfate mineral celestite (SrSO₄) and carbonate strontianite (SrCO₃). Foods containing strontium range from very low e.g. corn (0.4 ppm and oranges (0.5 ppm) to high, e.g. cabbage (45 ppm), onions (50 ppm) and lettuce (74 ppm) [3].

Strontium Ranelate (SrCl₂) is a medicine for osteoporosis; it was first approved by the European Medicines Agency (EMA) in 2004 for female patients, and in 2012 for male patients; until now it has not been approved by the FDA in the USA. The Periodic Safety Update Report (PSUR) of the EMA, after the analysis of 7,500 female patients, found that their risk of heart disease was increased by 60% [4]. Strontium levels in food and drinking water are not high enough to be able to cause these effects. Certain deep-sea creatures incorporate strontium into their shells as strontium sulfate [5]. The uptake of high strontium concentrations is generally not known to constitute a great danger to human health. In one case, someone experienced an allergic reaction to strontium, but there have been no similar cases since. For children, exceeding strontium uptake may be a health risk because it can cause problems with bone growth. Strontium salts are not known to cause skin rashes or other skin problems of any kind. When
strontium uptake is extremely high, it can cause disruption of bone development. But this effect can only occur when strontium uptake is in the thousands of ppm range.

The US Environmental Protection Agency (EPA) has developed a lifetime health advisory of 4 mg/L (Lifetime HAL) for strontium levels in drinking water. One-Day HAL and Ten-Day HAL are 25 mg/L [6]. Researchers in China have found that Sr, Ca, Mg, Si and Li can reduce the risk of death by blood channel sickness-related causes [7]. Several companies have packaged the drinking water containing Sr for sale in the market; they claim that this drinking water can prevent blood tube sickness, and this is one of the sources for the living system [8]. According to the government regulation in the People's Republic of China, water containing Sr above 10 mg/L and 0.2 mg/L is called medical strontium water and drinking strontium water, respectively [9]. In Luoyang City, people have discovered a spring source that contains strontium concentration up to 21.78 mg/L; while in Xinchou City, people have discovered a spring source that contains strontium concentration up to 2.135 mg/L [10]. According to the records [11] of Sr in the human body, Sr in the urine, derived from the kidney, is near ND (less than 0.03 ug/L) and its average value is less than 50 ug/L in the blood; the highest is found in blood platelets of 1880 ug/L. In nature, the source of Sr is the soil, and plants can uptake Sr with water and save them in their cells. For example, Sr in grapes and orange is near 30 mg/kg; in cabbage, 60 mg/kg; in lettuce, 20mg/kg; in cucumber, 25 mg/kg; in apples, 15 mg/kg; and in tomatoes and beans, less than 10 mg/kg. The dairy uptake for an adult people is about 2 mg; therefore if we uptake Sr from normal food and high Sr drinking water, our kidney may be harmed.

It is common sense that the toxicity of an Sr compound depends on its counter anion; for example the Sr compound with the highest toxicity is SrCrO4. In the study of Van Esch, they found that being fed food with 300 mg SrCl2·H2O and 50mg Sr(NO3)2 /kg led to no observable significant effect [11]. According to the study of water quality in the Chia-Nan Plain area in Taiwan (Chiayi, Tainan and Yenlin counties), the Sr in the underground water ranged from 0.062 to 6.22 mg/L, while the As (V) in the same area ranged from 0.002 to 0.247 mg/L, and As (III) ranged from 0.033 to 0.783 mg/L. In many sample points the As is ND but the Sr is detected in most sampling points [12].

Another research on the redox condition of groundwater in Taiwan also showed that the higher As area is located in Chia-Nan Plain, at 60% in which the As is higher than 0.05 mg/L, the regulated concentration for drinking water by Taiwan EPA [13]. Prof. Fung-Jou Lu [14] found that Blackfoot disease is not caused solely by As; the role of humic acids is also important. Later studies also show that the interactions between As with humic acids can be affected by Al, Fe and Mn, and also that humic acids enhance the cytotoxicity effects of arsenic trioxide on human cervical cancer cells.

In Taiwan, the uric acid in blood is about 3.5~7.0mg/dL for men and 2.6~6.0mg/dL for women; it is almost 10 times higher than in animals. In most cases, the producers use fructose instead of sucrose in popular cold drinks; this would explain these results since fructose can be transformed into uric acid in the human body. The uric acid in human blood which is higher than 70 mg/L can lead to “Uremia”, “Hyper-uricemia” and “Gout” in Taiwan. Strontium ranelate is an effective drug for Gout afflicting people. All these conditions require urine dialysis; the percentage of urine dialysis was 435 per million Taiwanese in 2005. In North Taiwan, for example in Taipei, the percentage is 258 per million people; while it is 480 per million people in Tainan. Reasons for urine dialysis are sugar-urine disease 40%, kidney disease, high-blood pressure and unknown factors are 60%. This causes NT$ 330 billion in cost for the public health system.

The above illustrations indicate that the interaction between Sr and uric acid is like As and humic acid. The resulting affect of As and humic acid in the human body is Blackfoot, and we have proposed that the resulting affect of Sr and uric acid in the human body is kidney disease. Mammals, amphibians and some fishes can release urea in urine, while birds and reptiles release uric acid. In this paper, we try to evaluate the cytotoxicity for our human kidney through the Vero cell (kidney cell line from Africa green monkey) in regard to Sr salts and uric acid solution. Considering the solubility of Sr salts, the sulfate mineral celestite (SrSO4) is not soluble in water, while that of carbonate strontianite (SrCO3) in water is rather higher. Sr(NO3)2 is usually made for smoke-fire products and is found in natural water. In this study, we used non-toxic Sr compounds of SrCO3 Sr(NO3)2 and Sr(OH)2 to investigate their combinational cytotoxicity with uric acid. Our results provide a possibility of the harmful pathway of Sr in the urinary system for human.
Methodology

Vero cell lines were purchased from Bioresource Collection and Research Center (BCRC, Taiwan), the code number is BCRC60013. Trypsin versene solution (TVS) was purchased from HyClone Co. (USA). Minimum Essential Medium Eagle (MEM M0643), Fetal bovine serum (FBS), uric acid, Cell Growth Determination Kit (MTT), pyruvate and sodium bicarbonate were purchased from Sigma Chemical Co. Enzyme linked immnosorbent assay (ELISA) was performed on a DTNEX MRX-ELISA reader. The linear OD is 0.100 ~ 4.000; wavelength is 340 ~ 850 nm and the sensitivity is ± 0.0005 OD. Strontium ion concentration was determined by Thermo Fisher S4 Scientific atomic absorption spectrophotometer. UV spectra was performed on THERMO G10s UV-Vis spectrometer.

Preparation of medium for Vero Cell Lines

1 L of MEM was mixed 10% FBS, 1.5 g sodium bicarbonate, 5 mL pyruvate, then the pH was adjusted to be 7.2.

Population of Cell Lines

The purchased cell lines were filled in a frozen tubule at -70°C. We unfroze the tubule in a 37°C water bath, and then poured the contents into a 25T flask filled with MEM with Fetal bovine serum (FBS). The cell line solution was transferred to numerous dishpans for incubation at 37°C under 5% CO2 for several days. We refreshed the MEM in the dishpan every 2-3 days. The full cultured cell dishpan was cleaned by phosphate buffered saline (PBS) 2-3 times, and then 0.25% of the TVS was added to suspend cells in the solution. For sampling purposes, 1 mL of cell suspension solution was added into a new incubation flask filled with medium to initiate the incubation of the next generation.

MTT (3-(4,5-dimethyl)-[3H]-2,5-diphenyltetrazoliumbromide) is a yellow compound which can be reduced by mitochondria dehydrogense of live cells to derive colored formazan. MTT used in this study is Sigma M2128. Lysing buffer was prepared by mixing 100 mL DI water and 30 of SDS. Then 50 mL of DMF was added, followed by a pH adjustment to 4.2; 0.25% of Tripsin-EDTA was added to the full cultured cell dish (10 cm) to suspend cells and adjust the cell number to be 2×10^5/1 mL. Then we placed the cell suspension solution into a 96 well ELISA sample pan; 50 μL of cell solution was added to each well. Then the pan was placed in an incubator under a environment of 5% CO2 at 37°C overnight and 50 μL was added for another 24 h.

In our previous study on the MTT cytotoxicity test for MSWI baghouse extracts, we found that the best mixing ratio of liquid sample to MEM is 1:9. Therefore we kept the strontium solutions to MEM as 1:9. The Sr solutions of Sr(NO3)2, Sr(OH)2 and SrCO3 were mixed with uric acid under ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. The mixed Sr/uric acid solutions were performed by UV and MTT tests. To test the cell survival ability for our Sr sample, 0.5 mg/mL of MTT in PBS was prepared. Then 50 μL MTT was added to each well and incubated for 2 h under 5% CO2 at 37°C. Then 100 μL of Lysing buffer was added into each well to sit overnight at RT. The surviving cell percentage was determined by ELISA reader at 570 nm wavelength.

Quantum Chemistry Calculation

The geometries of Sr-Uric acid complexes were calculated using Paramters Number Model 3 [15].

Results and Discussion

The used Sr compounds and uric acid are all non-toxic for Vero cells (Figure S1-S4). However, from results in Figure 1-3, toxic effects are observed after mixing Sr(NO3)2 and SrCO3 with uric acid. The Sr(OH)2 still remained non-toxic. The most toxic ratios of Sr to uric acid ranged from 0.375 to 1.5. Due to the mixing of various mL of 100 PPM Sr(OH)2 with 100 PPM Uric Acid in MEM, the total mixed volume was 10 mL. Therefore the Sr uptake from drinking water would be below 26.25 ~ 105 mg/L. The mixed ratio of the sample to MEM was 1:9; therefore the active factor was 0.1, so the effective Sr in the drinking water would be below 2.625 ~ 10.5 mg/L. After dilution with MEM by 10 times, the toxic effects in all of the experimental sets were removed, as shown in Figure S5-S7. Therefore the dilution by 10 times is necessary for the Sr and uric acid mixture, so that the safer concentration of Sr in drinking water would be below 0.26 ~ 1.05 mg/L. These observations are consistent with the PRC regulation.
level of 0.2 mg/L for drinking water. The lifetime health advisory of 4 mg/L recommended by the EPA in the USA might be significantly too high. Although the averaged Sr in underground water is less than 1 mg/L in the USA [15] and in Taiwan, Sr in the pipe water sampled in Pingtung City (Taiwan) is determined to be 0.38 mg/L. However, it is ND to 0.54 mg/L in Pingtung County and 7.22 mg/L for Kantian Sea water. Since agriculture is very important for Pingtung County, the Sr for animal livestock shall be noted in the future.

In regard to the anion effect in ionic liquids on cytotoxicity for 10 of 27 tested anions, the remaining 17 anions from their test kit no significant effect was found, and the toxicity order of anions is \( \text{OH}^- > \text{NO}_3^- > \text{CO}_3^{2-} \) [16, 17]. Recently the toxicological and ecotoxicological risk potentials of ionic liquids were determined by IPC-81 rat leukemia cell line and WST-1 assay was employed [18].
We also used UV-Vis to identify the stable mixed products of Sr compounds with uric acid in MEM solution shown in Figure S8-S10. Absorptions of peak 200 nm are commonly seen in any water-base solution; they result from the photo-degradation of water molecules, and 230 nm could be caused by the absorption of mono-anion form of uric acid [19] in neutral pH. The 280 nm could be the monohydrate uric acid clusters [20]. Under a low uric acid loading volume, the enhancement order concerning the formation of uric acid clusters in different Sr compounds is: Sr(OH)2 << SrCO3 < Sr(NO3)2. We could not even detect the peak of 280 nm in Sr(OH)2/uric acid solution in Figure S10 due to the formation of mono-anion ions needing to lose a proton from uric acid molecule. Since the OH- can extract the proton from NH- group of uric acid molecules, the formation of uric acid mono-anion ions in Sr solution is faster than the formation of uric acid clusters.

The results have shown that the relative formation ratio between mono-anion form and clusters of uric acid changed after uric acid was mixed with Sr compounds in MEM. So we have defined a “uric acid toxic parameter (Y)” for all system studied in this paper:

\[ Y = \frac{I_{200}}{I_{230} - I_{280}} \]

We found that as Y < 0, the cell survival percentage is reduced to a negative value; the monohydrate uric acid clusters have a higher concentration than that of uric acid mono-anion ions. Moreover, the toxicity of uric acid clusters is higher than that of uric acid mono-anion ions. The plot of Y value with mixing ratio of Sr compounds to uric acid in MEM solution are shown in Figure S11-S13. Comparing with Figure 1-3, the changing trend of the toxicity and Y value are similar in all three Sr/Uric acid systems.

A preliminary quantum calculation of the geometries of Sr-Uric acid complexes was carried to understand the contribution of the complexes’ geometries on the cytotoxicity. Figure 4 shows the calculated results of the most stable complex of Sr(NO3)2 with Uric anion (U-) in aqueous solution is Sr2+(U-)2. Figure 5 shows the calculated results of the most stable Sr(OH)2/Uric acid (U) mixtures in aqueous solution is SrU2(OH)2. Figure 6 shows the calculated results of the most stable SrCO3/Uric acid mixtures in aqueous solution is SrU2CO3. The formation a monoanion of uric acid to provide a binding site with Sr cations has been identified experimentally [21]. Our results confirm that the cytotoxicity of the Sr/Uric acid solution is contributed from the formation of complexes rather than from the formation of Sr clusters.

**Conclusions**

Our results indicate that the anion effect on the combinational toxicity of non-toxic Sr compounds with the polar organic compounds in live species is very important, for example the uric acid. The toxicity source of such a mixing system results from the formation of a higher concentration of uric acid clusters than that of uric acid mono-anion ions. Overall, the combinational cytotoxicity order of these three Sr compounds is: Sr(OH)2 << SrCO3 < Sr(NO3)2. Our results confirm that the cytotoxicity of the Sr/Uric acid solution is contributed from the formation of complexes rather than from the formation of Sr clusters.

**REFERENCES**


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