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Availability and toxicity of Fe(II) and Fe(III) in Caco-2 cells*

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Abstract: The objective of the present study was to compare the toxicity and availability of Fe(II) and Fe(III) to Caco-2 cells. Cellular damage was studied by measuring cell proliferation and lactate dehydrogenase (LDH) release. The activities of two major antioxidative enzymes [superoxide dismutase (SOD) and glutathione peroxidase (GPx)] and differentiation marker (alkaline phosphatase) were determined after the cells were exposed to different levels of iron salts. The cellular iron concentration was investigated to evaluate iron bioavailability. The results show that iron uptake of the cells treated with Fe(II) is significantly higher than that of the cells treated with Fe(III) (P<0.05). Fe(II) at a concentration >1.5 mmol/L was found to be more effective in reducing cellular viability than Fe(III). LDH release investigation suggests that Fe(II) can reduce stability of the cell membrane. The activities of SOD and GPx of the cells treated with Fe(II) were higher than those of the cells treated with Fe(III), although both of them increased with raising iron supply levels. The results indicate that both Fe(II) and Fe(III) could reduce the cellular antioxidase gene expression at high levels.

Key words: Iron availability, Caco-2 cells, Fe(II), Fe(III), Toxicity
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INTRODUCTION

Iron is an essential micronutrient involved in flesh oxygen transport and energy metabolism. Investigation of iron deficiency is carried out widely in the world (Glahn *et al*., 2002; Kloots *et al*., 2004). However, in the dietary sources, it may cause iron toxicity when adding iron excessively or choosing iron supplement unsuitably (Núñez *et al*., 2001). At present, the species of iron supplements are diverse, but mainly are Fe(II) and Fe(III). Except injection, most iron supplements must pass through the intestine. Therefore, the intestine is the major site of a barrier to large quantities of iron (Rossi *et al*., 1996), and its conditions are a direct basis to study how to choose the form of the iron supplement. Iron's cellular toxicity is dependent on, besides its valency, solubility and linkage, the intestine conditions and response time. Therefore, it is important to introduce an intestine model to study physiology and toxicology of iron in the intestine. In vitro digestion/Caco-2 cell model was established by Glahn *et al*.(1996; 1998), and was confirmed to be a useful tool of simulating the flesh intestinal tract iron absorption with nimble and highly effective characters. The Caco-2 cells are derived from a human colon carcinoma, and have similar characteristics of structure

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and biochemical functions to those of the intestinal epithelium (Walgren *et al*., 2000). The Caco-2 cells grown on porous polycarbonate membrane spontaneously differentiate into the intestinal epitheliums after confluence to express the characteristic of the continual monolayer (Glahn *et al*., 1998; Walgren *et al*., 2000). They are widely used for pharmaceutical, biochemical and toxicological studies as well as for investigation of crossing membrane transportation (Núñez *et al*., 2001; Zödl *et al*., 2004; Gan and Dhiren, 1997).

The accumulative toxicity of metals in cells may be produced via the cell transportation, which may cause reduction in cell viability and damage to DNA (Srigiridhar *et al*., 2001). In an investigation of the Caco-2 cell line, Núñez *et al*.(2001) discovered that increasing iron concentrations resulted in increased damage to DNA. Certain metals are observed on the specialized interference function of these cell monolayers. Shielding effect may be one of the main functions on the tight junctions of epithelial cells in human intestine (Watzl *et al*., 1999; Okada *et al*., 2000), and cadmium could reduce the tight junctions of Caco-2 cells in monolayer (Rossi *et al*., 1996). Furthermore, it was demonstrated that iron altered tight junction permeability of Caco-2 cells in monolayer cultures (Glahn *et al*., 1996), and it was believed that the iron toxicity displayed mainly the cell antioxidant function (Zödl *et al*., 2004; Zager *et al*., 1993). The iron could initiate lipid peroxidation of membranes, and produced malondial dehyde (MDA) to inactivate many kinds of enzymes and lead to cell injury. The Fe(II) is a main actor in the Fenton response, producing excessive free radical to attack cell membrane and depress stability or increase the penetrability of the membrane (Zhao, 1998; Xing *et al*., 2008). In addition, iron toxicity to cells is related to its valency and supply levels. The objective of the present study is to investigate toxicological effects of various concentrations of Fe(II) and Fe(III) on the Caco-2 cells.

MATERIALS AND METHODS

Cell culture

Caco-2 cells were obtained from the Institute of Biochemistry and Cell Biology (SIBS, CAS, Shanghai, China) and were used at Passages 20~43. The cells were maintained in 25-cm^2 flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) non-essential amino acids, 20 mmol/L 2-[4-(2-hydroxyethyl)-1-piperazine]-ethanesulfonic acid (HEPES), 2 mmol/L L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The culture medium was replaced with a fresh medium every 2~3 d (Peng *et al*., 2008). After being nearly confluent, the cells were washed with phosphate-buffered saline (PBS) to remove any unattached cells. The attached cells were harvested using ethylenediaminetetraacetic acid (EDTA) solution. The cells were seeded in 24-well plates at a density of approximately 250000 cells/ml. The cells were used for further studies and were exposed to known concentrations of iron in the culture medium and incubated for 22~24 h. Cytological and biochemical assessments were carried out on the cell lysate and culture medium for determination of markers of cell damage.

Preparation of iron solutions

Ferric chloride was dissolved in deionized water and sterilized by using a 0.22-μm filter. The solutions were diluted with the medium (1:20, v/v) to give the concentrations of 0.25, 0.5, 1.0, 1.5, 3.0 and 5.0 mmol/L in the culture medium.

Proliferation and viability index

After collection of cells with trypsin/EDTA, cell proliferation was measured using a hemocytometer according to the trypan blue method. Viability index was calculated as the ratio of the number of living cells to the total number of cells. Dead cells were detected by a blue staining counted in a microscope.

MTT (3-[4,5-dimethyl-2-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromid) uptake

After the cells were exposed to iron, the medium was aspirated and 1 ml phenolred-free medium with 0.4 mg/ml MTT (Sigma, USA) was added to each well and incubated for a period of 4 h. The medium was aspirated and the blue formation was dissolved in 1 ml dimethyl sulphoxide (DMSO) (Sigma, USA). Finally, the absorption was measured at 552 nm using a spectrophotometer (Bio-Red-680, Bio-Red, USA) (Okada *et al*., 2000).

LDH (lactate dehydrogenase) release

LDH activity was measured in an aliquot of culture medium according to the method of Guzzie and Gad (Zhang, 2004). The LDH Testing Kit was bought from Jiancheng Biochemical Co., Ltd. (Nanjing, China). The change in absorbency was measured by the spectrophotometer at 540 nm.

Enzyme assays

The alkalinity phosphatase activity was measured by the alkalinity phosphatase Testing Kit, and the superoxide dismutase (SOD) activity by the SOD Testing Kit. Glutathione peroxidase (GPx) activity was determined spectrophotometrically. Oxidation of NADPH was continuously monitored using GSH-GSSG glutathione reductase model at 340 nm. All Testing Kits were bought from Nanjing Jiancheng Biochemical Co., Ltd. (Nanjing, China).

Intracellular iron concentration

The cells were treated with different levels of iron and washed twice with 2 ml D-Hank after being carried out from nutrient liquid. Then the cells in various wells were harvested using trypsin-EDTA solution, and the cell lysate was removed into the 5 ml epoxy epoxide tube (EP). The cells were then dried at 100 °C for 24 h. Prior to analysis, wet digestions were carried out with 5 ml concentrated HNO_3 and approximately 5 drops of 30% (v/v) H_2O_2 on a heating plate at 80 °C (Zödl *et al*., 2003). The digestion residual was dissolved with 1.4 mol/L nitric acid solution and added with the deionized water to 5-ml flasks. Intracellular iron uptake was measured by inductively coupled argon plasma-mass spectrometer (ICP-MS) (Aglient 7500a, America). The average relative standard deviation (*RSD*) for sample analysis was below 5%.

RESULTS

Effects on cell viability

The MTT assay value, cell number and viability index decreased with the increase of iron treatment levels, except that the MTT value increased slightly when the iron supply level increased from 0.25 to 0.5 mmol/L (Table 1). And the MTT assay values of all Fe(II) treatments were lower than those of all Fe(III) treatments, especially at iron supply level >1.5 mmol/L (Table 1). These results indicate that Fe(II) is much more powerful to decrease cell viability and proliferation than Fe(III) (P<0.05).

Effects on cell membrane stability

LDH release assay is an important method to assess cell membrane stability. The values of LDH release increased significantly with rising iron supply level, and Fe(II) had much stronger effects on membrane stability than Fe(III) at iron level >1.0 mmol/L (Fig.1a), implying that high level of Fe(II) is more harmful to cell membrane than that of Fe(III). The intracellular iron concentration of Caco-2 cells was found to increase with elevation of iron supply levels, and at the same iron level, more iron was accumulated in the cells with Fe(II) treatment than in those with Fe(III) treatment (Fig.1b). Significant differences were observed at iron supply levels of 0.25, 1.5 and 3.0 mmol/L.

Table 1 Toxicological parameters in Caco-2 cells after iron exposure[#]

Iron concentrations (mmol/L)	MTT assay value		Proliferation ($\times10^5$)		Viability index	
	$FeSO_4$	$FeCl_3$	$FeSO_4$	$FeCl_3$	$FeSO_4$	$FeCl_3$
0.25	1.13±0.11	1.10±0.17	2.98±0.07	3.06±0.13	96.2±0.5	97.8±1.0
0.5	1.27±0.07	1.34±0.09	3.06±0.06	3.04±0.16	95.2±0.3	95.2±0.5
1.0	1.23±0.05	1.27±0.18	2.87±0.06	3.09±0.09	94.3±0.2	94.1±1.0
1.5	0.94±0.04	1.23±0.14*	2.46±0.12	2.83±0.21	90.3±2.3	93.8±3.8
3.0	0.88±0.08	1.19±0.07*	2.13±0.10	2.67±0.10*	82.1±0.5	90.1±2.2*
5.0	0.72±0.02	1.14±0.08*	2.00±0.06	2.68±0.14*	77.1±2.5	88.2±2.5*

[#]Values represent mean±*SEM*, n=6; *P<0.05, Fe(III) vs Fe(II) at the same concentration according to the Duncan's test

Effects on cell enzyme activities

At all treated levels, alkaline phosphate and GPx activities of cells treated with Fe(II) were considerably higher than those of cells treated with Fe(III), although there were no significant differences observed among different Fe(II) levels (Table 2). At low iron levels (<1.5 mmol/L), SOD activities of cells treated with Fe(II) were over 5 times higher than those of cells treated with Fe(III), whereas at iron supply levels >1.5 mmol/L, slight higher SOD activities were noted in the Fe(III) treated cells than in the Fe(II) treated ones. Interestingly, SOD activities of cells treated with Fe(III) increased gradually when iron level increased from 1.5 to 3 mmol/L.

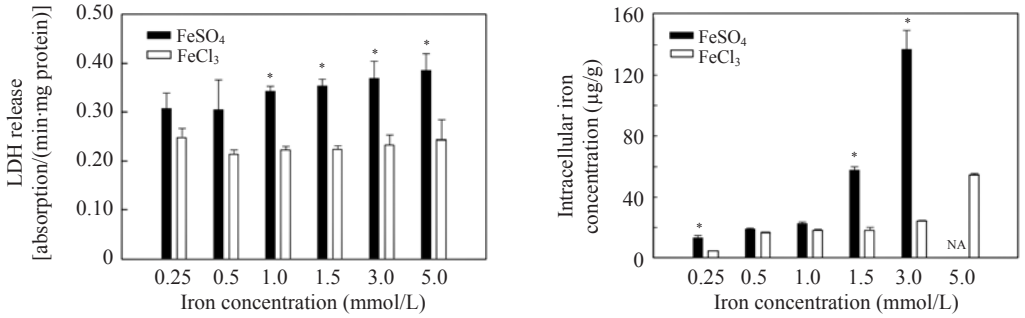

Fig.1 (a) Membrane stability (LDH release) and (b) intracellular iron concentration (cells were dissolved in HNO_3 after broken up by ultrasonic, and then intracellular iron uptake was measured by ICP-MS) of Caco-2 cells after incubation with 0.25, 0.5, 1.0, 1.5, 3.0 and 5.0 mmol/L of $FeSO_4$ and $FeCl_3$. Values represent mean±*SEM*, *n*=6; *Significant difference between the same concentration of Fe(II) and Fe(III) at *P*<0.05; NA: Not analyze

Table 2 Effects of increasing iron levels on enzyme activities (U/grot)#

Iron concentrations (mmol/L)	Alkaline phosphatase activity		SOD activity		GPx activity	
	$FeSO_4$	$FeCl_3$	$FeSO_4$	$FeCl_3$	$FeSO_4$	$FeCl_3$
0.25	89.0±37.8*	22.2±1.5	14.9±7.3*	2.4±1.5	193±110	149±172
0.5	66.9±6.4*	15.2±7.2	62.8±13.6*	5.5±0.7	382±116	192±105
1.0	87.2±15.2	49.9±21.2	48.7±15.2*	4.0±5.1	991±255*	108±25
1.5	96.9±24.3*	40.3±15.4	57.4±12.2*	8.9±3.2	492±264	469±183
3.0	77.4±10.3	55.5±12.8	61.3±19.3	83.6±13.8	855±146*	520±15
5.0	85.5±12.2	111.2±39.2	83.2±10.0	101.6±33.7	1118±32*	606±25

#Values represent mean±*SEM*, *n*=6; **P*<0.05, Fe(II) vs Fe(III) at the same concentration according to the Duncan's test

DISCUSSION AND CONCLUSION

MTT assay is an important measuring of cellular toxicity in immunology, and was firstly used in cellular immunology by Mosmann (Zödl *et al*., 2005). MTT can reflect the sensitivity of live cells to extrinsic stimulation; therefore, it also has an important value to assess cell viability. Here, the result of MTT assay indicates that at high iron levels, Fe(II) has stronger effects on decreasing Caco-2 cell viability than Fe(III), which is similar to previous reports (Walgren *et al*., 2000). When pre-confluent and post-confluent Caco-2 cells were treated with 100~3000 μmol/L Fe(II), the cell viability index and proliferation were decreased with increasing iron concentration (Zager *et al*., 1993). The study of Vero monkey kidney cells treated with different forms of iron [the half maximal effective concentration (EC_{50}): 5.5 and 22 mmol/L, respectively] also found that the proliferation of cells treated with Fe(II) decreased about fourfold as that of cells treated with Fe(III) (García-Alfonso *et al*., 1996).

Both Fe(III) and Fe(II) can initiate lipid peroxidation and increase of MDA, but they had different effects (Glahn *et al*., 1996). Here, we found that the damage of membrane stability was affected by Fe(II)

more than by Fe(III). The reason might be that Fe(II) can perform the Fenton reaction directly, and produce a great number of free radicals, subsequently inducing lipid peroxidation (Glahn *et al*., 1996); however, the reaction speed caused by Fe(III) was much slower (Glahn *et al*., 1998; Chamnongpol *et al*., 2002). The study of the Fe(III) toxicity shows that Fe(III) bound to nitrilotriacetate (at molar ratios of 1:1) could decrease membrane stability of Caco-2 cells, whereas Fe(III)/citric had no obvious effects (Walgren *et al*., 2000). And Fe(III)/NTA caused more oxidative damage than Fe(III) salts (Cai *et al*., 1998). Fe(III)/NTA was even more effective in inducing lipid peroxidation in rabbit small intestinal microvillus membrane vesicles than Fe(II) ascorbate (Walgren *et al*., 2000), which might be due to action of NTA. However, NTA alone did not decrease viability (via MTT assay) in E9 and MV5 cells (Zödl *et al*., 2005). Those results indicate that toxicological effects of Fe(III) to cells may be related to other specific participants. Lipid peroxidation caused by both Fe(III) and Fe(II) was complex (Zhao, 1998).

A higher intracellular iron concentration in Caco-2 cells was found when incubated with Fe(II) than that with Fe(III), which was one of major causes for membrane damage. In this work, we demonstrated that the intracellular iron concentration in Caco-2 cells incubated with Fe(II) was higher than that with Fe(III) at all iron treated levels (Fig.1b). Intracellular free iron can initiate oxidative stress leading to cellular injury (Mccord, 1996). The results of enzyme activity assay also indicate that with increasing iron level, both iron forms could elevate activities of antioxidative enzymes, e.g., SOD and GPx. Nevertheless, other studies suggested that the SOD activities of Caco-2 cells were not affected by iron supplementation (Zödl *et al*., 2005). It was reported that oral iron supplementation decreased the activity of MnSOD in rat colon mucosa (Kuratko, 1998). In the intestinal epithelial cell line IEC-6 which had been exposed to iron ($FeSO_4$) up to 2 mmol/L for 48 h, an increase in MnSOD activity was observed (Kuratko, 1999). The higher activities of SOD and GPx in Fe(II) treated cells might be resulted from the expression of Fe(II) induced oxidative stress genes (Zödl *et al*., 2003). Compared to Fe(II), the ability of Fe(III) induced oxidative stress was relatively low, especially at low concentrations. Greater activities of SOD and GPx at higher iron levels suggest that the oxidative stress in cells was increased due to the increasing iron levels (Baker and Baker, 1992). The cells could consume antioxidative enzymes to resist oxidative stress at low iron levels. Different from Fe(II), Fe(III) can damage cells by producing free superoxide negative ion (Chamnongpol *et al*., 2002). In this study, we found that the Fe(III) treated cells used intrinsic SOD to rid free superoxide negative ion as iron concentration increased from low levels to 1.5 mmol/L, which might cause the increasing catalase level with iron concentration in the medium decreased (Okada *et al*., 2000), thereby depleting GPx. In high iron levels, both Fe(II) and Fe(III) may induce the expression of antioxidative genes. Although it seems that the high concentration of iron (>1.5 mmol/L) rarely occurs in vivo, in some situations, it is still impossible to avoid, for instance, in high iron contamination mining area or adding too much iron to the fortification food. Furthermore, in the animal feed, it is true that high concentration as much as 200×10^{-6} iron is always supplemented, especially in China.

In conclusion, we found that iron form and concentration have different effects on iron uptake and toxicity to Caco-2 cells, and that the toxicological effects of Fe(II) to Caco-2 cells are higher than those of Fe(III). Fe(II) can alter membrane stability and penetrability, resulting in higher LDH release values at the same treat level as Fe(III). At low iron levels, cells can use intrinsic antioxidative enzymes to resist iron oxidative damage. At high levels, iron can induce expression of cell antioxidative genes. However, in this work, the Caco-2 monolayer was exposed to "pure" solution of iron salts, which may not be comparable to the real intestinal environment. Therefore, further studies in conditions closer to a physiological environment are needed.

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