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Letter

Impact of selenium content in fetal bovine serum on ferroptosis susceptibility and selenoprotein expression in cultured cells

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ABSTRACT — Ferroptosis, a mode of cell death involving iron-dependent lipid peroxidation, has attracted widespread attention in the development of anticancer drugs and toxicological studies as a potential mechanism of chemical-induced cytotoxicity. This process is regulated by several antioxidant enzymes, of which the selenium-containing glutathione peroxidase 4 (GPx4) is the prime regulator. However, accurately and reproducibly evaluating ferroptosis in cultured cells is challenging since numerous experimental factors in *in vitro* setting can influence the results. In the present study, we found that the expression levels of selenoproteins, such as GPx4 and GPx1, fluctuate across several cell lines depending on the selenium content of different origin of fetal bovine serum (FBS). Cells cultured in FBS containing higher selenium concentrations exhibited elevated GPx4 expression, and were resistant to ferroptosis induced by erastin and RSL3. These findings suggest that the variability of selenium content in different FBS batches can significantly influence the susceptibility of cells to ferroptosis, highlighting the importance of standardizing these factors to enhance the reproducibility of ferroptosis-related experiments.

Key words: Ferroptosis, Fetal bovine serum, Selenium, Glutathione peroxidase

INTRODUCTION

Regulated cell death, such as apoptosis, plays a crucial role in maintaining homeostasis in the body and is often induced by various cytotoxic chemicals (Berndt *et al*., 2024). Among the various modes of regulated cell death, ferroptosis, a non-apoptotic cell death characterized by iron-dependent lipid peroxidation, has attracted widespread attention in the development of anticancer drugs and toxicological studies as a potential mechanism of chemical-induced cytotoxicity (Stockwell, 2022). In the field of anticancer drug development, ferroptosis is considered to be a promising target to overcome anticancer drug resistance (Kuche *et al*., 2023). In addition, various toxic substances, including methylmercury, pesticides (e.g., imidacloprid on earthworms), and compounds generated by combustion (e.g., smoking), have been reported to modulate the sensitivity of cells to ferroptosis (Dong *et al*., 2022; Yang *et al*., 2024). Furthermore, ferroptosis is implicated in a variety of pathophysiological conditions, including neurodegenerative diseases such as Alzheimer's disease and organ damages such as in non-alcoholic steatohepatitis (Ma *et al*., 2022; Wang *et al*., 2022; Tsurusaki *et al*., 2019). Given its significance in pharmacology and toxicology, reproducible evaluation of ferroptosis is essential.

Ferroptosis is characterized by iron-mediated, unrestrained lipid peroxidation that leads to the destruc-

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tion of plasma membrane structures (Mishima and Conrad, 2022). To avert ferroptosis, cells employ several lipid peroxidation-surveillance system. Among them, glutathione peroxidase 4 (GPx4) is a prime regulator, reducing potentially toxic lipid hydroperoxides by consuming glutathione. Solute carrier family 7 member 11 (SLC7A11 also called xCT), which is responsible for the cellular uptake of cystine required for glutathione biosynthesis, also plays an important role in the inhibition of ferroptosis (Friedmann Angeli *et al*., 2014; Sato *et al*., 2000). In addition, independent of GPx4, ferroptosis suppressor protein-1 (FSP1) functions as crucial endogenous factors that suppress ferroptosis (Conrad *et al*., 2021; Bersuker *et al*., 2019; Doll *et al*., 2019; Mishima *et al*., 2022). Pharmacologically, ferroptosis can be inhibited by iron chelators and lipophilic radical trapping antioxidants such as α-tocopherol (Conrad *et al*., 2021).

GPx4 is a member of selenoproteins and has a selenocysteine residue at its active center. Selenoproteins, including GPx4, require trace element selenium for their translation, which is mainly supplied by selenoprotein P (SeP), a major selenium transport protein produced in the liver (Saito *et al*., 2004). We recently found that SeP expression in peripheral tissues contributes to the preservation of cellular GPx4 levels in an autocrine manner, through the formation of a cycling selenium storage system in the extracellular space (Zheng *et al*., 2024). In human hepatoma HepG2 cells, SeP expression was found to increase in a selenium supply-dependent manner, and could also be inhibited by several compounds, including sulforaphane and epigallocatechin (Ye *et al*., 2023; Mita *et al*., 2021). Although the importance of selenium in the expression of selenoproteins and its involvement in ferroptosis have been established, the significance of selenium level in the culture medium, particularly in fetal bovine serum (FBS), has not been thoroughly investigated.

FBS contains selenium and bovine SeP is considered its major source. SeP passes through the blood-placental barrier and is thought to supply selenium from the mother to the fetus. Bovine SeP contained in FBS is an essential nutritional factor for the survival of cultured cells (Yan and Barrett, 1998). Indeed, serum-free medium results in reduced cellular GPx levels (Saito and Takahashi, 2002). Therefore, when using culture media without FBS, it requires a supplementation of inorganic selenium source. In contrast, supplementation with sufficient amounts of inorganic selenium, such as sodium selenite, increases GPx4 expression and confers ferroptosis resistance to cells (Kitabayashi *et al*., 2022).

Since blood selenium concentrations correlate with selenium level in food intake (Rayman, 2008), it is possible that selenium concentrations in FBS may also vary depending on the rearing environment. This difference in the selenium level in FBS is also expected to potentially affect the result of ferroptosis in cultured cells via modulating the expression level of selenoproteins, including GPx4. In this study, we compare the selenium concentration in FBS obtained from multiple origins and its effect on the expression of selenoproteins in cultured cells and the resulting susceptibility to ferroptosis. Our findings demonstrated the importance of using FBS with a constant selenium concentration to maintain experimental reproducibility.

MATERIALS AND METHODS

Reagents

FBS were obtained from the sources as follows. No.1 from Sigma (SA, USA. Lot.BCCC5944), No.2 from Cosmobio (Tokyo, Japan. 29061723FBS), No.3 from Biosera (Cholet, France. Lot.S00OV), No.4 from Biosera (Lot.S00KH), No.5 from Nichirei (Tokyo, Japan. 22F00B), No.6 from Nichirei (22D222), and No.7 from Funakoshi (Tokyo, Japan. S00Q0). The production areas are shown in Table 1. All other reagents used were of the highest grade commercially available. Human SeP was purified as we reported previously (Saito *et al*., 1999).

Cell culture and treatment of chemicals

SH-SY5Y (EC94030304-F0) cells were obtained from KAC (Kyoto, Japan) and HepG2 (JCRB1054) cells were obtained from JCRB cell bank (Tokyo, Japan). HT-1080 cells were obtained from the Conrad Laboratory. All cells were cultured in high-glucose DMEM (08458-16, Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, L-glutamine (584 mg/L), sodium pyruvate (110 mg/L) and 1% penicillin-streptomycin solution. The cells were cultured in a humidified CO_2 incubator at 37°C.

Cell viability

After seeding cells in with DMEM supplemented with 10% of each FBS in a 96-well plate, they were incubated with or without of selenite for 24 hr. Following the incubation, the cells were treated with erastin or 1S, 3R (RSL3) for an additional 24 hr. The media were then replaced with media containing 10% alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific), and incubated for 2 hr. Fluorescence intensity was measured using a microplate reader (SpectraMax iD5, Molecular Device, MA, USA) at Ex 544/Em 585 nm. The data are shown as a percentage (%) of the control,

Table 1. Metallomics analysis of FBS by ICP/MS.

	Se (μg/L)	Mg (mg/L)	P (mg/L)	S (mg/L)	Ca (mg/L)	Fe (μg/L)	Cu (μg/L)
1 (France)	8.3	28.8	108.1	449.8	134.2	2649	138.3
2 (Netherlands)	14.3	24.6	95	403.9	119.8	2091.5	131.1
3 (Colombia)	42.3	30.5	107.3	527.5	146.5	2328.7	143.2
4 (Guatemala)	11	28.1	102.6	481.4	131.4	2805.5	149.5
5 (Netherlands)	15.4	27.2	107	454.3	134.3	2611.8	160.9
6 (Panama)	20.3	26.7	85.7	469.7	138.2	1752.3	123.7
7 (France)	9.2	30.3	114.5	490.5	147.9	2902.7	144.6

Each FBS was subjected to ICP/MS analysis and quantitative values are shown.

with S.D.

ICP-MS analysis

Aliquots of each FBS (10 μL) were mixed with 70% HNO_3 (500 μL) and incubated for 18 hr at 50°C to degrade organic molecules. After dilution with water, ICP/MS/MS analysis was performed to determine the total amount of trace elements. The instrument used was Agilent 8900 (Agilent, CA, USA). The measurement was conducted double-blind, and the regions of origin are listed in no particular order.

SDS-PAGE and Western blotting

Cells were harvested with SDS buffer, and protein concentration was determined using DC protein assay reagents according to the manufacturer's instructions (Bio-Rad, CA, USA). Loading buffer (containing glycerol, 2-mercaptoethanol and SDS) was added and an aliquot of protein was denatured at 95°C for 10 min. The protein-samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Blocking was performed by incubation in TBS-T containing 5% skim milk for 1 hr. The membrane was rinsed with TBST and incubated with the indicated primary antibodies (anti-GPX1 [ab22604], and anti-GPX4 [ab125066] were from Abcam, Cambridge, UK; anti-GAPDH [015-25473] was from Wako Pure Chemical, Osaka, Japan). Anti-SeP antibody was established previously (Mita *et al*., 2017). The membrane was incubated with a secondary antibody combined with HRP for 1 hr. Chemiluminescence was detected using an ImmunoStar LD kit (FUJIFILM Wako, Osaka, Japan) and Luminograph (ATTO, Tokyo, Japan). GAPDH or CBB-stain of the membrane were used as the loading control.

Statistical analysis

Statistical significance was assessed by GraphPad Prism (10.2.2) using multiple comparisons ANOVA with Dunnett's test, and or Tukey's test. The type of statistical test is noted in the figure legend.

RESULTS

Selenium and trace elements contained in the different batches of FBS

While the global average soil selenium concentration is estimated to be around 0.32 mg/kg, it varies by region (Jones *et al*., 2017). For example, based on previous reports, France, with soil selenium levels from 0.1 to 0.7 mg/kg, is classified as a low selenium intake region by the WHO standard (Mombo *et al*., 2016). In the present study, we measured the amount of trace elements contained in FBS derived from seven different origins by inductively coupled plasma mass spectrometry (ICP/MS) (Table 1). These results showed that the two FBS lots of French origin (No.1 and No.7) had the lowest selenium content among the sample tested, FBS from Guatemala (No.4) also had relatively low selenium levels. Selenium levels varied up to 5-fold between the lowest and highest samples, indicating great variation in selenium content. These findings show that the trace element levels, especially selenium, in FBS varies by region, potentially influencing selenoprotein expression and susceptibility to ferroptosis in cultured cells.

Culturing cells in low selenium FBS reduces basal GPx expression, and reduces the production of SeP in HepG2 cells

Next, we cultured cells in FBS with a variety of selenium levels. We used SH-SY5Y cells, a human neuroblastoma cell line, as neuronal cells are particularly dependent on SeP for a selenium source (Solovyev, 2015; Scharpf *et al*., 2007; Hill *et al*., 2003). Differences in GPx expression in the cells cultured in each FBS were evaluated.

Although cells were viable in all FBS (data not shown), cellular selenoprotein expression was varied. GPx4 showed reduced basal expression in low-selenium FBS condition, specifically in lots No.1 and 7 (Fig. 1A).

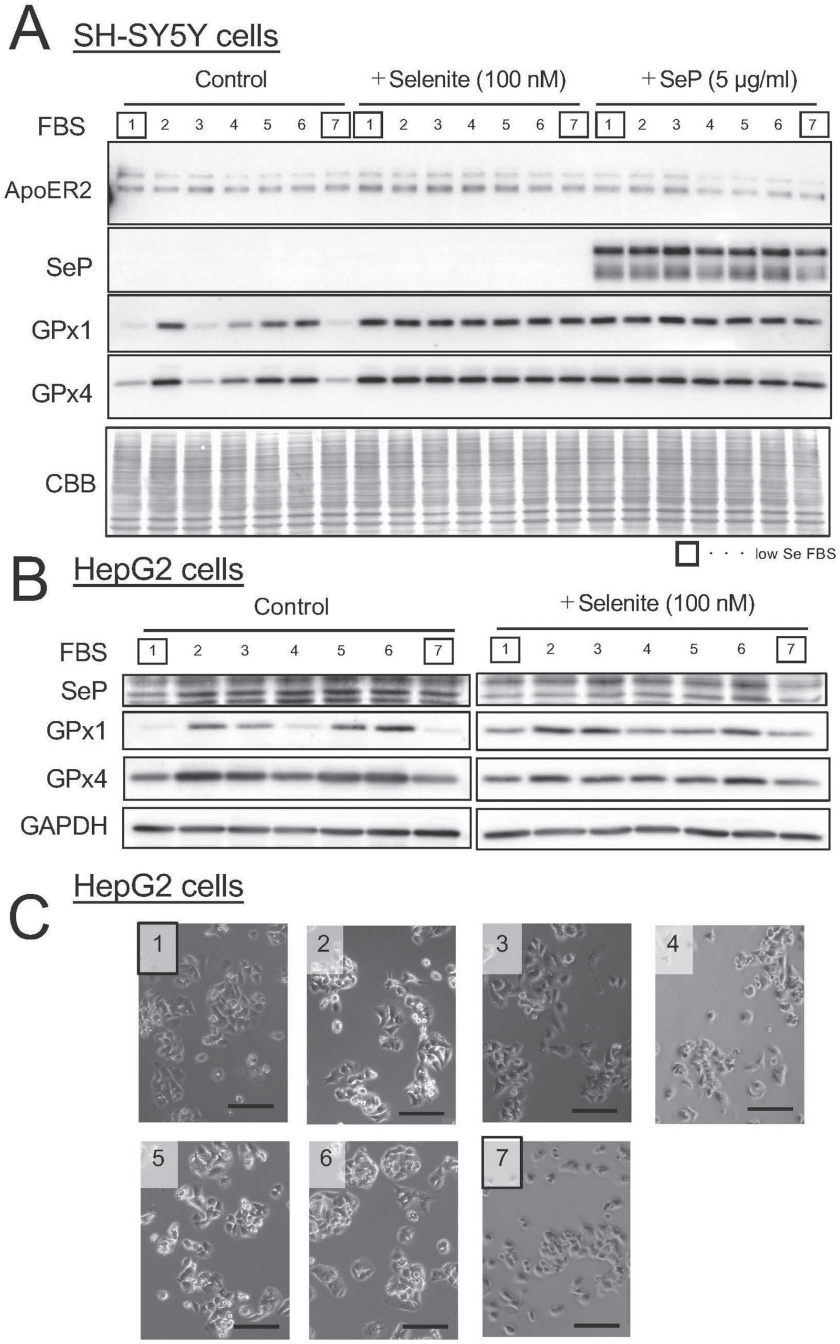

Fig. 1. Effect of different FBS batches on glutathione peroxidase (GPx) expression in cultured cells. (A) SH-SY5Y cells were cultured for 24 hr in DMEM containing 10% of different baches of FBS (#1-7), the cells were treated with selenite (100 nM) or selenoprotein P (SeP, 5 μg/mL) for 24 hr in the presence of 10% of each FBS. The cells were harvested and subjected to Western blotting and detected with each antibody. CBB stain was used as loading control. (B) HepG2 cells were cultured for 24 hr in DMEM containing 10% of FBS (#1-7), the cells were treated with selenite (100 nM) for 24 hr, and the cells were collected and Western blotting was performed. (C) The morphological appearance of HepG2 cells cultured in DMEM containing 10% of each FBS. The scale bar indicates 200 μm.

GPx1 also showed decreased expression in these lots. However, little change was observed in the expression of low-density lipoprotein receptor-related protein 8 (ApoER2), the main receptor/importer for SeP (Mizuno *et al*., 2023) suggesting no relationship between receptor-dependent uptake of SeP and selenium content in FBS. When sodium selenite or purified human SeP protein was supplemented to the media, the expression levels of GPx1 and GPx4 consistently increased, suggesting that the reduced GPx expression in the low selenium FBS conditions was due to the limited selenium availability (Fig. 1A).

Next, we also tested the effects of different FBS on human hepatoma HepG2 cells, which produce SeP. The expression of intracellular SeP, GPx1, and GPx4 was lower in the conditions cultured in lots No.1, 4, and 7 of FBS (Fig. 1B). Such as cell proliferation and density, were largely unaffected by the type of FBS (Fig. 1C). Similarly, the addition of sodium selenite to the medium increased selenoprotein expression, highlighting the importance of selenium content in FBS for maintaining basal expression of selenoproteins in cultured cells.

Effect of FBS with different selenium level on susceptibility to ferroptosis

To examine the effect of different FBS on ferroptosis, SH-SY5Y cells were treated with an xCT inhibitor erastin. The results showed that the cells cultured with low selenium FBS (No.1 and 7) exhibited higher sensitivity to ferroptosis. In particular, cells cultured with FBS No.1, which had the lowest selenium content, showed the highest sensitivity to erastin (Fig. 2A). In contrast, almost no

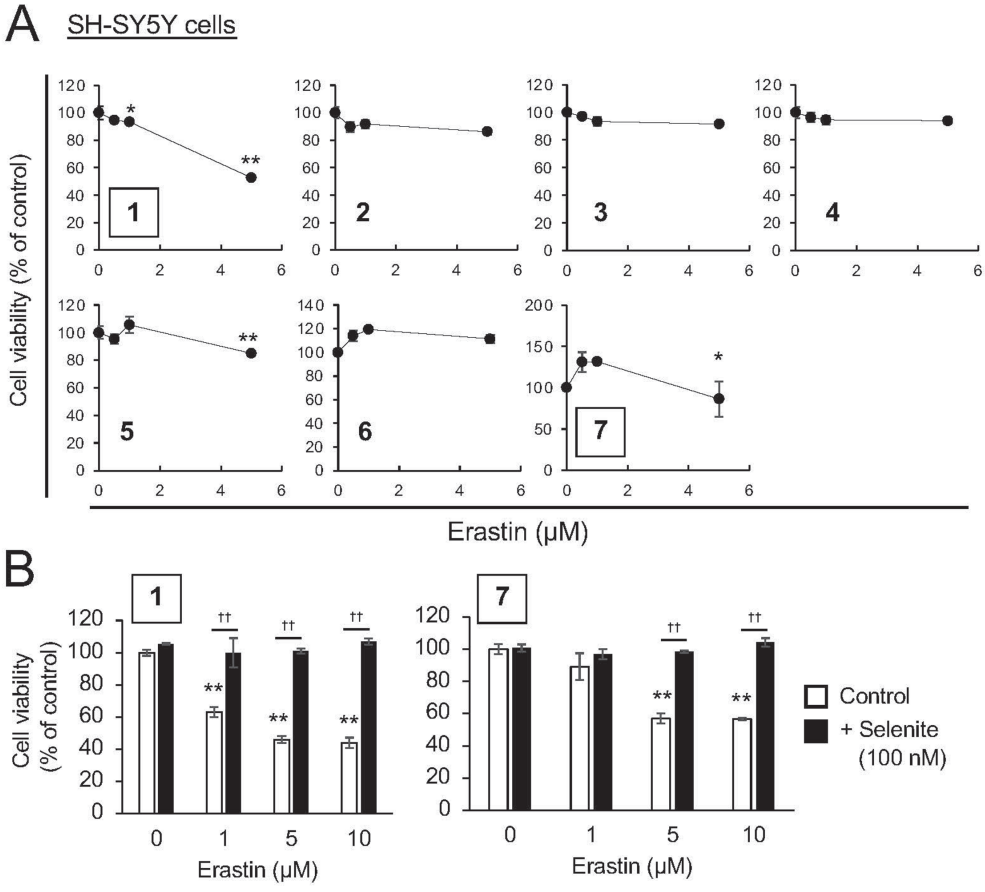

Fig. 2. Effect of different FBS batches on the susceptibility of SH-SY5Y cells to ferroptosis. (A) SH-SY5Y cells precultured in DMEM containing 10% of FBS (#1-7, 24 hr) were treated with the indicated concentration of erastin for 24 hr. The cell viability was determined by alamarBlue assay (Mean ± S.D., n=3, *p<0.05 vs erastin 0 μM, **P<0.01 vs erastin 0 μM; ANOVA, Dunnet's test was used). (B) SH-SY5Y cells precultured in DMEM containing 10% of FBS (#1 and 7) with or without of selenite (100 nM) for 24 hr were treated with indicated concentration of erastin for 24 hr. The cell viability was evaluated. (Mean ± S.D., n=3, **P<0.01 vs erastin 0 μM, ††P<0.05 vs control; Tukey's test was used).

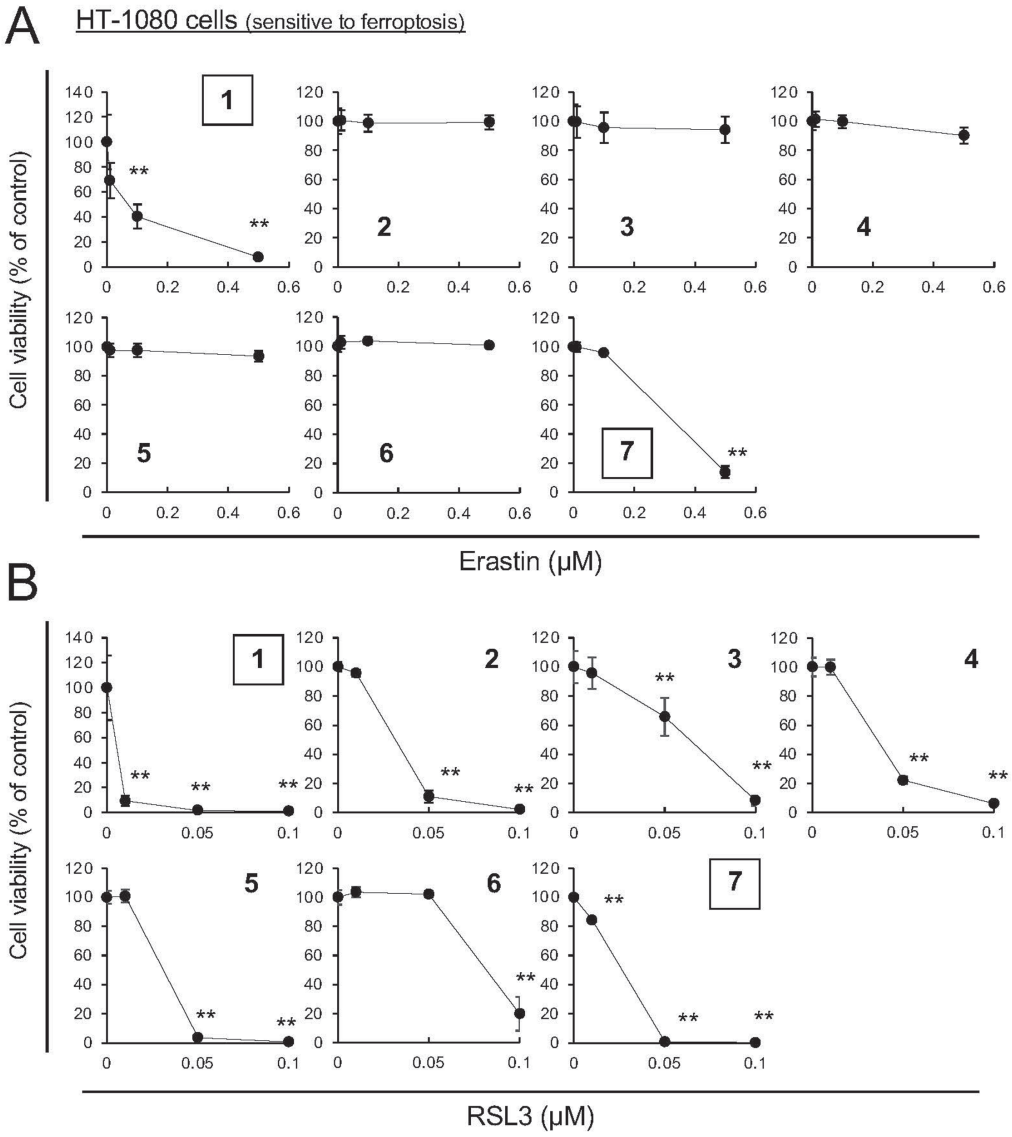

Fig. 3. Effect of different FBS batches on the susceptibility of HT-1080 cells to ferroptosis. HT-1080 cells precultured in DMEM containing 10% of each FBS (#1-7, 24 hr) were treated with the indicated concentration of (A) erastin or (B) RSL3 for 24 hr. The cell viability was determined by alamarBlue assay (Mean ± S.D., n=3, **P<0.01 vs erastin 0 μM; ANOVA, Dunnet's test was used).

cell death was observed in the cells cultured with the other FBS lots, even when treated with erastin up to 5 μM (Fig. 2A). Additionally, the erastin-induced ferroptosis in the cells cultured with No. 1 and 7 FBS was inhibited by the addition of selenite supplementation (Fig. 2B). These results suggest that selenium level contained in FBS greatly influences the sensitivity of the cells to ferroptosis.

Furthermore, we examined the effect of different FBS on the susceptibility of HT-1080 cells, commonly used in ferroptosis studies, to ferroptosis induced by erastin and a GPx4 inhibitor RSL3. Consistent with the results using SH-SY5Y cells, sensitivity to ferroptosis increased when cells were cultured in the low selenium FBS (No.1 and 7) (Fig. 3). In summary, ferroptosis sensitivity can vary drastically depending on selenium content in FBS.

DISCUSSION

This study highlights the impact of selenium content

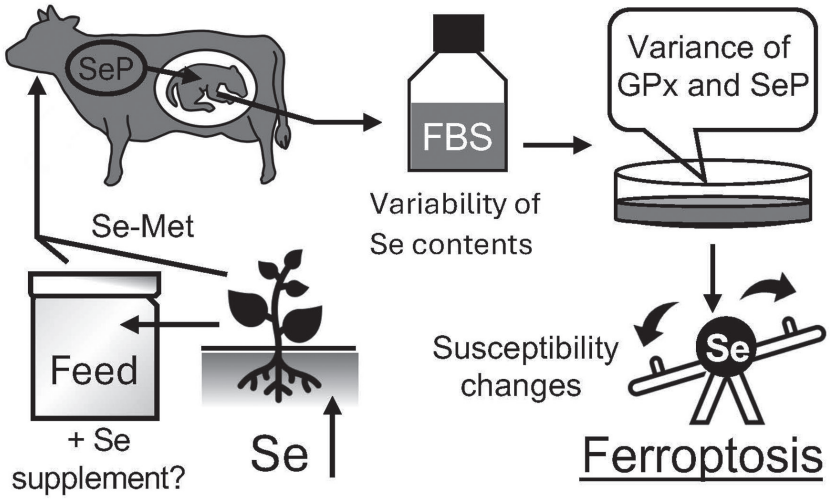

Fig. 4. Pathways by which selenium is supplied to cultured cells. Selenium in the soil accumulates in plants and enters the diet as selenomethionine (Se-Met) as the major form. Sodium selenite/selenate and Se-Met (in yeast) can also be added as a food supplement. These selenium sources are taken by bovine and converted to SeP. This is included up to FBS, which greatly affects cellular selenium supply and base-level GPx expression. Finally, these culture conditions affect susceptibility to ferroptosis, and thus it is important to set appropriate conditions for reproducibility.

in FBS on cellular selenoprotein expression, which subsequently influences susceptibility of cultured cells to ferroptosis. Given the active global research on ferroptosis, it is crucial to consider the variation in selenium content in FBS to enhance study reproducibility (Fig. 4). Also, maintaining a constant selenium concentration in FBS during cell culture is important for achieving reproducible results in ferroptosis studies.

Plants fix selenium in the soil to produce selenocysteine and selenomethionine (White, 2018), and some of the selenium undergoes methylation. Selenomethionine is utilized through the same pathway as methionine (Fig. 4) (Stadtman, 1987). Selenomethionine absorbed from the intake of plants is used by animals as a selenium source after desulfurization (White, 2018), and is then converted to SeP in the liver and circulates in the body (Schrauzer, 2000). Therefore, it is likely that majority of selenium in FBS is derived from SeP (Yan and Barrett, 1998); however, this has not been accurately confirmed and needs to be evaluated by establishing antibodies against bovine SeP. The SeP antibody we previously established and used in this study is human-selective and did not react with bovine SeP (data not shown) (Mita *et al*., 2017). Therefore, to accurately measure SeP in bovine serum, it is more reliable to verify the results using techniques such as LC-ICP/MS, like in the present study.

Interestingly, selenium levels in FBS did not always correspond with GPx and SeP expression levels in cells. For example, FBS No. 3 had the highest selenium concentration in this study, yet GPx and SeP expression was relatively low in SH-SY5Y and HepG2 cells. Therefore, it is possible that differences in components between FBS other than selenium content may also contribute to the expression level of selenoproteins, although identifying which FBS components independent of selenium level affect selenoprotein expression remains challenging. For example, the inhibitory effect of FSP1 on ferroptosis via lipid radical scavenging is independent of selenium. Therefore, selenium in FBS and GPx levels are not always an indicator of susceptibility to ferroptosis, such as vitamin K, substrates for FSP1 in FBS would be considered as well (Mishima *et al*., 2022).

Additionally, it is important to note that selenium levels in cultured cell experiments are much lower than those in human blood. For example, FBS No. 1 used in this study (8.3 µg/L of selenium) would yield a selenium concentration of 0.83 µg/L in media containing 10% FBS, which is approximately one-tenth lower than the concentration in human plasma (73.3 µg/L) (Hać *et al*., 2002). This artificial effect, used in *in vitro* experiments, results in a selenium-deficient state in cultured cells. Indeed, when supplementation of selenite or SeP at physiological human plasma concentration (5 µg/mL) was used (Kikuchi *et al*., 2019), GPx

expression of the cells in this study increased to the upper limit, which increased resistance to ferroptosis (Fig. 1A and 2B). Although low selenium conditions are useful for studying the effects of increased susceptibility to ferroptosis, it should not be noted that *in vitro* results may not always be applicable *in vivo*.

Taken together, our study highlights the importance of FBS selection in ferroptosis-associated studies. Instead of direct measurement of selenium level, evaluation of basal GPx protein expression level in the cultured cells, which is less equipment-intensive than ICP/MS, could be a good indicator partly enhancing the selenium level in FBS. Since protein expression levels of selenoproteins are mainly regulated at the translation level, including the selenocysteine incorporation step, evaluation of their mRNA levels is not useful for evaluating the effect of selenium level in different FBS (Bulteau and Chavatte, 2015). We hope these findings will contribute to developing reproducible and accurate ferroptosis studies in the scientific field.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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