공학석사 학위논문

신장 세포의 철분 과부하 독성을 완화하기 위한 아세틸 시스테인을 탑재한 리포솜에 관한 연구

Development of N-Acetyl Cysteine encapsulated liposomes to reduce iron overload-induced toxicity in Human Kidney Cells

2021 년 2 월

서울대학교 대학원

응용바이오공학과

이 두 희

신장 세포의 철분 과부하 독성을 완화하기 위한 아세틸 시스테인을 탑재한 리포솜에 관한 연구

Development of N-Acetyl Cysteine encapsulated liposomes to reduce iron overload-induced toxicity in Human Kidney Cells.

지도교수 이 강 원

이 논문을 공학석사 학위논문으로 제출함

2021 년 2 월

서울대학교 대학원

응용바이오공학과

이 두 희

이 두 희 의 공학석사 학위논문을 인준함

2021 년 2 월

위 원 장 송 용 규 (인)

부위원장 이 강 원 (인)

위 원 김 정 민 (인) 정 민

Abstract

Development of N-Acetyl Cysteine encapsulated liposomes to reduce iron overload-induced toxicity in Human Kidney Cells.

Doohee Lee
Department of Applied Bioengineering
Graduate School of Convergence Science and Technology
Seoul National University

When iron is overloaded in cell, it produces free radicals by Fenton reaction, induces lipid peroxidation, and eventually causes ferroptosis, a newly discovered cell death. So, several antioxidants were recommended as effective treatments for iron-related diseases. In this study, N-Acetyl Cysteine (NAC), as an effective antioxidant and a ferroptosis inhibitor, was investigated to treat iron overload-induced cell death. As a drug delivery system, a liposomal formulation was fabricated to encapsulate the NAC. It was studied whether the liposomal NAC reduces iron overload-induced toxicity in Human Kidney 2 (HK-2) cells. The liposomal NAC was made by the reverse phase evaporation method and size, zeta potential, morphology, and release profiles were studied for characterization. Cell viability assay (CCK-8) and Reactive oxygen species assay (DCFDA assay) were investigated to confirm the protective effect of liposomal NAC. They showed that liposomal NAC inhibited ferroptosis induced by a slow iron uptake (1mM Ferric ammonium citrate) and cell death caused by a rapid iron uptake (50μM Ferric ammonium citrate, 20μM 8-Hydroxyquinoline). Also, the liposomal formulation improved the protective effect of free NAC. These

results suggest that liposomal NAC more effectively protects human kidney cells from toxicity caused by various iron uptake models than free NAC.

Keywords: Liposome; Iron; N-Acetyl Cysteine; Ferroptosis; Kidney; Reactive oxygen species;

Student Number : 2019-22656

Table of Contents

List of Figures

Chapter 1. Introduction

Iron is one of the fundamental elements that need to be maintained homeostasis in our body. Iron overload is known to have some adverse effects on the heart, pancreas, kidney, etc (Patel and Ramavataram, 2012). Iron overload appears mainly in patients with acute anemia who should have frequent blood transfusions or iron supplementation treatment. Non-transferrin-bound iron (NTBI) is a critical factor in iron-overload-related diseases (Patel and Ramavataram, 2012; Brissot et al., 2012). Plasma iron is usually bound to transferrin. But, in iron overload conditions, saturated transferrin cannot afford elevated iron concentrations. The remaining iron, which is called an NTBI, bounds to albumin or small molecular weight molecules, such as citrate (van Swelm et al., 2020). NTBI increases the intracellular labile iron pool (LIP), and the enhanced LIP induces reactive oxygen species (ROS) by Fenton reaction. These ROS damages proteins, DNA, and cellular membrane and eventually leads to cell death (Patel and Ramavataram, 2012; Brissot et al., 2012; van Swelm et al., 2020). Some reports mentioned that ferroptosis seems to a leading cell death mechanism in iron overload-induced cell death (Wang et al., 2017; Fang et al., 2018). Ferroptosis is a recently discovered cell death mechanism that shows lipid peroxidation characteristics by the Fenton reaction of iron metabolism (Xie et al., 2016). Typically, patients use iron chelators to lower excessive plasma iron levels and prevent the toxicity of iron overload. However, iron chelators are reported to have side effects such as acute renal failure, liver failure, hearing loss, and visual loss (van Swelm et al., 2020; Musallam and Taher, 2012; Neufeld, 2010). So, various antioxidants such as curcumin and histidine have been studied as alternative treatments. (Kose et al., 2019; Vera-Aviles et al., 2018)

In this iron overload condition, N-Acetyl Cysteine (NAC) can be the candidate for protecting cells from iron overload-induced cell death. NAC is a well-known medication to treat paracetamol poisoning or mucolytic therapy and is selected as one of the World Health Organization's Lists of Essential Medicines (Tardiolo et al., 2018, Šalamon et al., 2019). Furthermore, it is low-priced, easily accessible, and has relatively few side effects and can act as a notable

antioxidant drug in its indirect and direct way (Tardiolo et al., 2018; Šalamon et al., 2019). Indirectly, NAC is converted to L-cysteine inside the cell, and L-cysteine is a rate-limiting factor to the synthesis of glutathione (GSH). So, a supply of NAC to cells can help replenish GSH, and synthesized GSH increases the antioxidant activity of the GPX4 enzyme (Lu, 2009; Wrotek et al., 2020; Atkuri et al., 2007). This mechanism of NAC can inhibit lipid peroxidation and eventually prevent ferroptosis (Xie et al., 2016). NAC also can scavenge free radicals directly because its thiol group can work as a source of reducing agents (Santus et al., 2014). However, NAC has a short half-life and low bioavailability, so NAC's use requires repeated and higher dosing (Olsson et al., 1988). Numerous studies have shown that NAC delivery by liposomal form makes the drug slowly released, prolongs blood circulation time, and improves bioavailability (Alipous et al., 2013; Mitsopoulos et al., 2008; Alipour et al., 2007). Furthermore, liposomes have a similar type of cell membrane, which in general promotes cellular uptake of hydrophilic molecules and have long been approved its safety by Food and Drug Administration (Eloy et al., 2014; Akbarzadeh et al., 2013).

Therefore, in this research, we investigated the correlation between iron loading and ferroptosis mechanism in Human Kidney 2 (HK-2) cells. HK-2 cells were used because iron overload is known to induce acute renal failure as it produces oxidative stress to kidney cells (Patel and Ramavataram, 2012; van Swelm et al., 2020; Yang and Stockwell, 2016). And we developed liposomes that encapsulate NAC using the reverse phase evaporation (REV) method. The REV method can increase the encapsulation efficiency of hydrophilic drugs by making a water-in-oil (W/O) emulsion (Eloy et al., 2014; Akbarzadeh et al., 2013; Szoka and Papahadjopoulos, 1978). To confirm the feasibility in pharmaceuticals, we checked out the size, zeta potential, morphology, and release profiles of liposomes. Finally, we investigated whether liposomal NAC reduces iron overload-induced toxicity more effectively than free NAC using cell viability assay and Reactive oxygen species assay. This liposomal NAC can be a good candidate for treating iron-overload-related diseases, considering its price, safety and mechanisms. Figure 1A shows the overall schematic illustration of how liposomal NAC protects cells from toxicity caused by cellular uptake of iron.

Chapter 2. Experimental Methods

2.1 Materials

The HK-2 (human kidney) cells were purchased from Korea Cell Line Bank (Gyeonggi-do, Korea). N-Acetyl-L-cysteine (NAC), sodium bicarbonate, cholesterol, Ammonium iron(III) citrate (FAC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 8-Hydroxyquinoline (8-Hq), Liproxstatin-1, Ferrostatin-1, Necrostatin-1, Z-VAD-FMK were all obtained from Sigma-Aldrich Company (St Louis, USA). Chloroform (99.8%), dimethyl sulfoxide (DMSO, 99.8%) were obtained from Samchun (Gyeonggi-do, Korea).

2.2 Preparation of Liposomal NAC

Liposomes were fabricated as referring to the previously reported reverse phase evaporation method (Szoka and Papahadjopoulos, 1978) but with modifications. The organic solvent was prepared by dissolving 7mM of DPPC and 3mM of cholesterol in chloroform. The aqueous solution was then prepared by dissolving 0.067g/mL of NAC in 1X DPBS with 0.7M sodium bicarbonate. And two solvents were separately added in glass vials to make an aqueous-to-organic phase ratio of 1:3. The solution was sonicated using a 6mm probe tip sonicator (VC-750, Sonics and Materials, Newtown, USA) for 5 minutes with pulse-on for 5 seconds and pulse-off for 10 seconds at an amplitude of 40% to make a water-in-oil emulsion. After the sonication, the cloudy homogeneous solution was formed, and the homogeneous state was confirmed to be maintained for more than 30 minutes. Then, the nitrogen gas was slowly purged into the solution to evaporate chloroform for overnight. The remaining viscous gel was formed. The aqueous solution was added to rehydrate it and then vigorously mixed by homogenizer (HS-30E, DAIHAN, Daejeon, Korea) until the transparent liposome solution was seen. The liposome solution was sequentially extruded through a polycarbonate filter (Avanti Polar Lipids, Alabama, USA) with 400nm and 200nm of diameter using a Mini-Extruder (Avanti Polar Lipids, Alabama, USA) at above the phase transition temperature of DPPC (41°C). The centrifugal ultrafiltration method was performed by an Amicon Ultra-15 Centrifugal filter unit (MWCO 50kDa, Merk Millipore, Massachusetts, USA) to purify the

liposomes from the unentrapped drug. The liposomes were loaded into the centrifugal filter device and centrifuged at 5000g for 30min. Control liposomes were fabricated using the above method without drugs. Finally, the made liposomes were stored at 4°C in darkness.

2.3 Measurement of size intensity and zeta potential of liposomes

A Zetasizer Nano (Malvern, UK) was used to determine the size distribution and zeta potential of liposomes. Samples were maintained with a fixed scattering angle of 173° at 25°C, and DI water was used to disperse the samples.

2.4 Morphology of Liposomal NAC.

The morphology of liposomes was analyzed using a High Resolution - Transmission Electron Microscope (JEM-3010, JEOL Ltd, Japan). The 5μL of liposomes was dropped on a copper grid, and negative staining was done by 5μL of 2% w/v of sodium phosphotungstate solution.

2.5 Release profile of drugs.

After purification of liposomes using the ultrafiltration method, the purified liposomes were put into a dialysis bag (1000MWCO). The dialysis bag is immersed in DPBS 5mL at 37°C in darkness and gently shakes with 80 rpm. At each time point, the 10μL of the sample was taken, and the amount of released drug was detected. The amount of drug was measured in triplicate at the 200nm UV-Vis Absorbance using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.6 Cell culture

The RPMI 1640 media (Welgene, Gyeongsan), which has 1% penicillin/streptomycin and 10% Fetal bovine serum (FBS), was made to culture HK-2 cell lines. The media was renewed every 2 to 3 days, and an incubator was maintained an atmosphere of 5% CO2 at 37°C.

2.7 Measurement of cell viability

A cell counting kit 8 (CCK-8, Dojindo Inc, Tokyo, Japan) was used to investigate cell viability. The 1.5*10^4 of cells were seeded per well in a 96 well plate. And it was maintained in an RPMI media without FBS. After overnight, compounds were added into cells, and cells were incubated for a specific time. After the treatment, the media was replaced with a 110μL of RPMI media, which has 10μL of CCK-8 solution. The experiment was done in the darkness. Cells were incubated in an incubator for 3 hours, and cell viability was measured at the 460nm absorbance by a microplate reader (Synergy H1, BioTek, USA).

2.8 Quantification of reactive oxygen species

The amount of ROS released in cells was measured by the DCFDA/H2DCFDA Cellular ROS assay kit (ab113851, Abcam, Cambridge, UK). At first, 1.5*10^4 cells were seeded per well in a black 96 well microplate. After the attachment of cells, the drug of interest was treated. After a specific period, the media was removed and washed with DPBS. The cells were stained with 25μM of DCFDA solution for 45min in an incubator. After staining, the DCFDA solution was removed and washed once, and 200μL of DPBS was added. The fluorescence intensity can be measured using a fluorescence plate reader at Ex/Em = 485/535nm.

2.9 Statistical Analysis

GraphPad Prism 8 (San diego, USA) was used, and data are presented as mean ±SEM. A one-way ANOVA test was used to determine significant differences.

Chapter 3. Result and Discussion

3.1. Characterization of liposomal NAC

The liposomes were fabricated using a REV method making a W/O emulsion to entrap more hydrophilic drugs. At first, an organic phase (phospholipids and cholesterol) and an aqueous phase (drugs) were separated (Fig 1B). And then, the W/O emulsion was made by sonication (Fig 1C). The homogeneous state was maintained for more than 30mins, and it showed that the formation of the W/O emulsion is stable enough (Szoka and Papahadjopoulos, 1978). After evaporation of the organic solvent, a stable gel was formed because the liposomes are in a solid gel phase at a temperature lower than the phase transition temperature of DPPC (41°C) (Fig 1D). Finally, the liposomes were fabricated after rehydration and serial extrusions (Fig 1E).

After the formation of liposomes, the morphology was confirmed by Transmission electron microscopy (TEM) (Fig 2A). The morphology was spherical enough, and the size results were smaller than 200nm because of the serial extrusions. The ultrafiltration process purified liposomes, and the release profile of purified liposomes in DPBS (pH 7.4) at 37°C was analyzed (Fig 2B). After releasing drugs rapidly for 2 hours, the sustained release was shown, and about 40% of the drug was released for 24 hours. And then, the size and zeta potential of liposomal NAC were measured by a dynamic light scattering (DLS). The Z-Average of size was confirmed to be 157.7nm, and PDI is 0.217, which is small and homogeneous enough (Fig 2C). The presented data showed a size smaller than 200nm, which was similar to the size determined by TEM (Fig 2C). This range of liposome size is appropriate to enhance circulation time in the blood (Liu et al., 1992). The zeta potential was a slightly negative charge (-14.5mV), and 30% of cholesterol content seems to lower the surface charge of liposomes. (Fig 2D) (Magarkar et al., 2014).

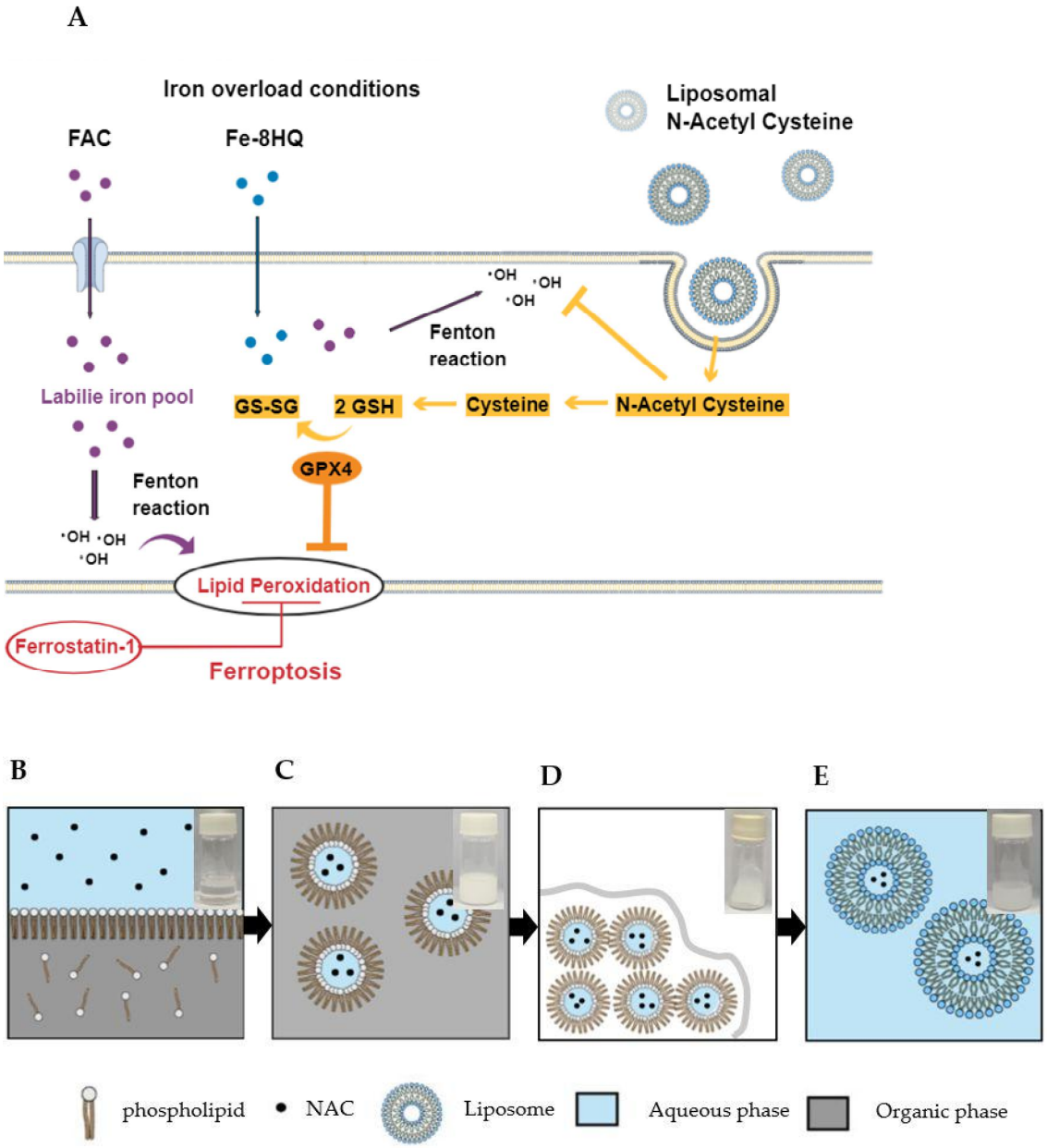

Figure 1. **(A)** Schematic illustration that shows the protective effect of Liposomal NAC on cell death induced by a slow or rapid increase of labile iron pool. **(B)** Schematic illustration and images that show the overall reverse phase evaporation method. The organic phase (phospholipids and choelesterol) and the aqueous phase (drugs) were separated. **(C)** After sonication, the homogeneous milky solution was seen that shows the W/O emulsion. **(D)** The organo-gel was formed after evaporation of the organic solvent. **(E)** The transparent solution of liposomes was seen after serial extrusions.

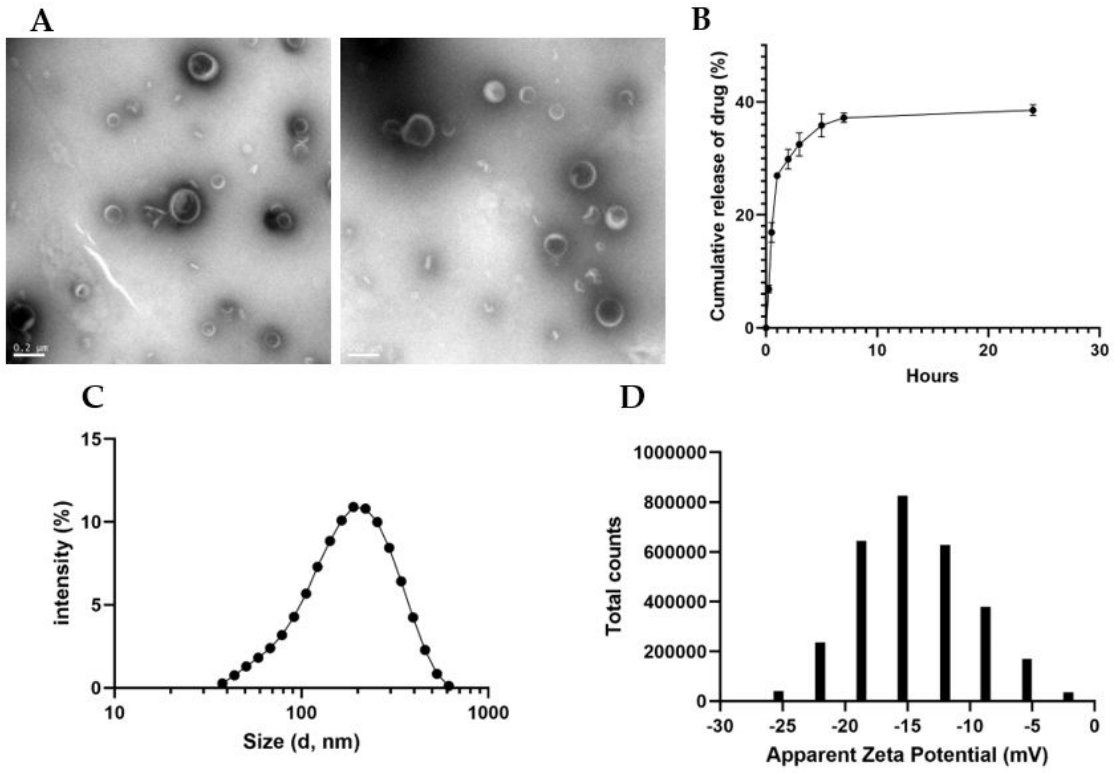

Figure 2. Characterization of Liposomal NAC. **(A)** The morphology of liposomes was confirmed by TEM image. **(B)** The release profile of liposomes in DPBS at 37 °C. **(C)** DLS data showing the size distribution and **(D)** Zeta potential distribution. Data are presented as the mean ± SEM (n=3).

3.2. The iron-mediated cytotoxicity and the involvement of ferroptosis according to the iron uptake rate in HK-2 cells.

It has not been studied what cellular death mechanisms are associated with iron toxicity in kidney cells. Therefore, we studied what mechanisms of iron toxicity in cells are involved to find the proper way to prevent it. So, we tested the toxicity of iron overload on HK-2 (Human Kidney) cells. Ferric ammonium citrate (FAC) is usually used to establish an iron overload model in vitro because it is a more stable form of ferric citrate, which is one of the NTBI (Messner et al., 2013; WHEBY and UMIPIERRE, 1964; Grootveld et al., 1989). The cell viability assay results showed that FAC has toxicity in HK-2 cells concentration and time-dependent (Fig 3A). And the involvement of ferroptosis in iron overload-induced renal cell death has not been studied (van Swelm et al., 2020). A ferroptosis inhibitor was added before treating FAC (1mM) for 48 hours in cells to verify whether the ferroptosis is the main cause of FAC-induced cell death. Ferrostatin-1 is known to be a specific ferroptosis inhibitor (Skouta et al., 2014). It showed that Ferrostatin-1 prevented cell death induced by the slow accumulation of iron (Fig 4A). However, necrostatin-1 (necrosis inhibitor) and Z-VAD-FMK (apoptosis inhibitor) had little or no protective effect on the iron toxicity (Fig 4B-C). It was a similar result from other researches that tested on hepatocytes and cancer cells (Wang et al., 2017; Fang et al., 2018). Although some studies insisted that necrosis is also involved in iron overload-induced cell death (Yang et al., 2017), these results showed that ferroptosis is the main cause of FAC-induced cell death.

Another widely used iron overload model is to use the FAC with 8-Hydroxyquinoline (8-Hq) because 8-Hq facilitates the cellular uptake of iron when bonding to Fe ions (Messner et al., 2013, 2009). The rapid uptake of iron, which is shorter than 2 hours, can minimize the cellular defense responses to iron overload conditions (Messner et al., 2013). So, we used this model to evaluate the direct ROS scavenging effect of liposomal NAC. Figure 3B shows that 8-Hq increased the cytotoxicity of iron within 2 hours compared to adding iron alone or 8-Hq alone. The Ferrostatin-1 was also treated before adding FAC (50μM) and 8-Hq (20μM) to confirm the relevance of ferroptosis in this iron overload condition (Fig 4D). Interestingly, unlike the results from a significant

protective effect of Ferrostatin-1 on FAC-induced cell death, Ferrostatin-1 did not have a protective effect on cell death induced by FAC and 8-Hq. This result shows that rapid accumulation of iron does not induce ferroptosis not only in hepatocytes but also in kidney cells (Fang et al., 2018). Necrostatin-1 (necrosis inhibitor) and Z-VAD-FMK (apoptosis inhibitor) also did not recover cell death (Fig 4E-F). However, when NAC was treated overnight, it showed that NAC recovered cell death in a dose-dependent manner (Fig 3C). Considering that NAC is an antioxidant, cell death might be because of a sudden increase of free radicals by rapid cellular uptake of iron ions. However, research will be needed on why ferroptosis inhibitor, acting by inhibiting lipid peroxidation, cannot recover this cell death.

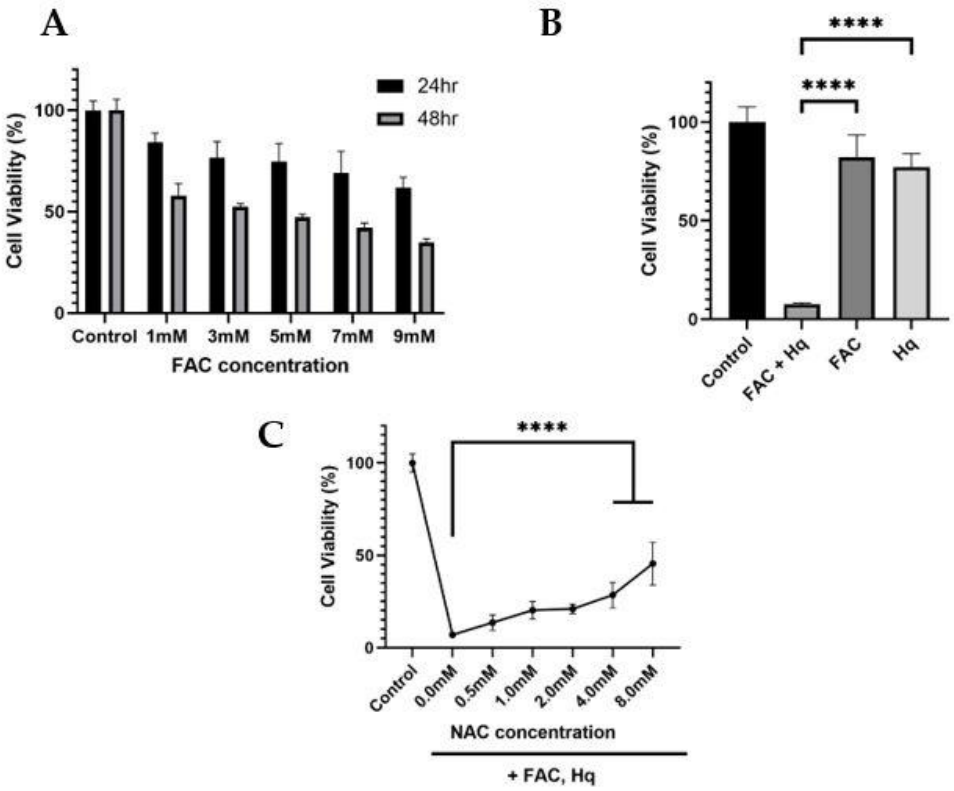

Figure 3. The iron-mediated cytotoxicity in HK-2 cells and NAC has a significant protective effect on cytotoxicity induced by rapid iron uptake. **(A)** FAC reduced cell viability in a concentration and time-dependent manner. **(B)** Hq increased the cytotoxicity of FAC within 2hr. 50μM FAC and 20μM Hq were treated for 2hr, and it was compared with the FAC treated group and then, Hq treated group. **(C)** NAC increased cell viability in a concentration manner. After NAC was added, 50μM FAC and 20μM Hq were treated for 2 hr. Data are presented as the mean ±SEM (n=5, **** : $P<0.0001$. Significant statistical differences were verified using a one-way ANOVA test.)

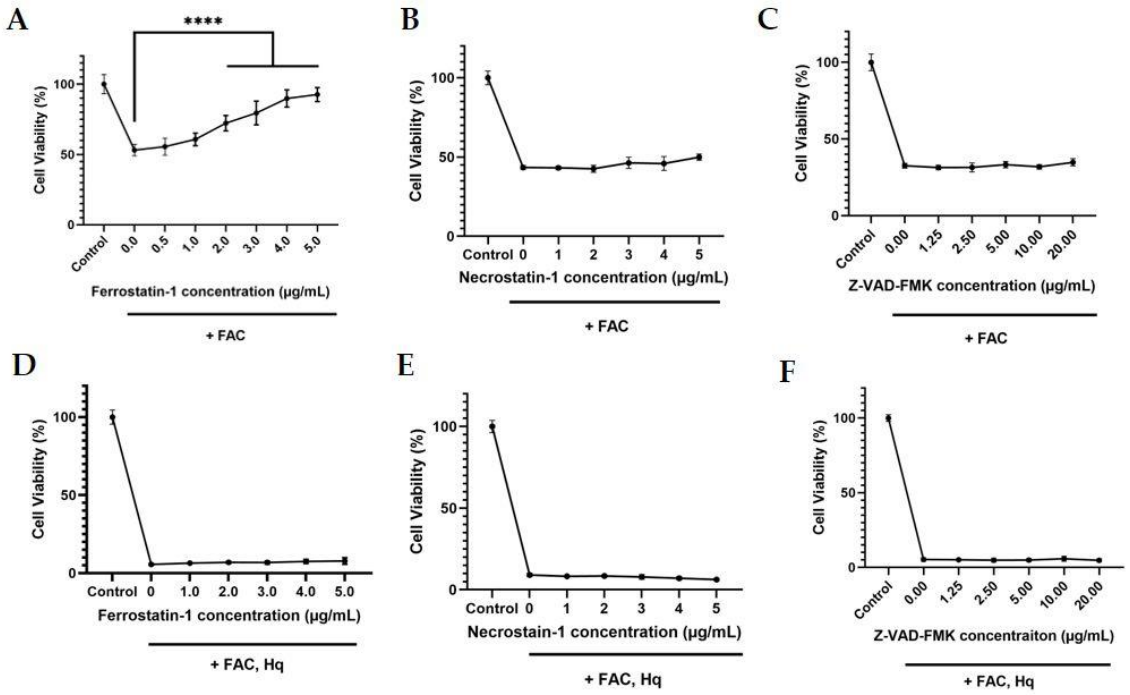

Figure 4. The involvement of ferroptosis in iron overload-induced cell death. **(A)** Ferrostatin-1 prevented FAC-induced cell death. **(B)** Necrostatin-1 and **(C)** Z-VAD-FMK have no effect on FAC-induced cell death. After inhibitors were added, 1mM FAC was treated for 48hr. **(D)** Ferrostatin-1 and **(E)** Necrostatin-1 and **(F)** Z-VAD-FMK have no effect on Fe-Hq induced cell death. After inhibitors were added, 50μM FAC and 20μM Hq were treated for 2hr. Data are presented as the mean ±SEM (n=5, **** : $P<0.0001$. Significant statistical differences were verified using one-way ANOVA test.)

3.3. Protective effect of Liposomal NAC on iron overloaded HK-2 cells.

The protective effect of liposomal NAC was confirmed using a cell viability assay and cellular ROS assay. Before treating 1mM FAC for 48hr on cells to establish the slow iron uptake model, the various liposomal NAC concentration was added overnight. The result showed that 157.5μg/mL (about 0.25mM lipid concentration) of liposome increased cell viability from 52.3% to 67.38% (Fig 5A). Interestingly, a higher concentration of liposomes did not have a significant protective effect on cells. Therefore, further experiments were done with 157.5μg/mL concentration. The control liposomes without NAC, and free NAC containing the same amount of drug in liposomes were compared with liposomal NAC under the same conditions. Contrary to the control liposomes and free NAC, which did not show a significant protective effect, liposomal NAC recovered the FAC-induced cell death from 53.9% to 68.4% (Fig 5B). When treating FAC 50μM and Hq 20μM, liposomal NAC also increased cell viability from 7.2% to 19.4% and with a slightly more improved effect of free NAC (Fig 5C). And then, the amount of intracellular ROS was quantified using a DCFDA assay kit to verify the anti-oxidative effect of liposomes (Fig 5D). The Fe-Hq complex induced ROS generation within 30min, but control liposomes and free NAC had no significant scavenging effect. In contrast, liposomal NAC inhibited ROS generation that showed significant differences. These results suggested that liposomal NAC increases the protective effect of free NAC by facilitating the cellular uptake of hydrophilic drugs. According to the results, liposomal NAC can be newly proposed as the iron poisoning treatments. However, further research will need to be conducted on what mechanisms NAC acts and whether liposomal NAC has an in vivo protective effect.

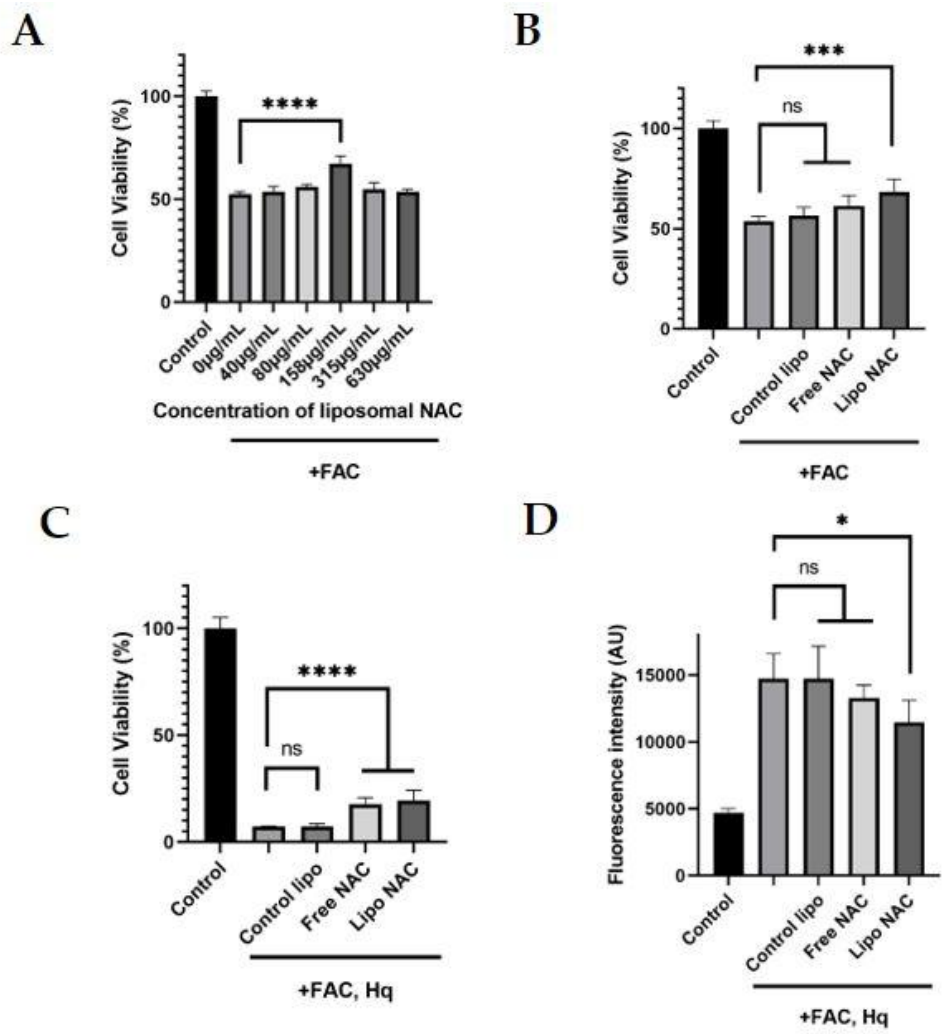

Figure 5. The protective effect of liposomal NAC was shown using Cell Viability assay and ROS assay in HK-2 cells. **(A)** Various concentration of liposomal NAC was added before FAC was treated. **(B)** Control liposome (157.5μg/ml), free NAC (1.5mM), liposomal NAC were added before FAC was treated. 1mM FAC was treated for 48hr. **(C)** Control liposome, free NAC, liposomal NAC were added before 50μM FAC, 20μM Hq was treated for 2hr. Cell viability was measured using the CCK-8 assay. **(D)** Control liposome, free NAC, liposomal NAC were added before 50μM FAC, 20μM Hq was treated for 30min. Intracellular ROS was quantified using a DCFDA assay. Data are presented as the mean ±SEM (n=5, * : $P<0.05$, *** : $P<0.001$, **** : $P<0.0001$, ns : no significance. Significant statistical differences were verified using one-way ANOVA test.)

Chapter 4. Conclusion

As a result, we fabricated liposomes that encapsulate antioxidant NAC using a reverse phase evaporation method. And the iron overload model was established in HK-2 cells according to the cellular uptake rate of iron. Although ferroptosis is involved in the slow accumulation of intracellular iron, the ferroptosis seems to be not involved in the rapid iron uptake condition. However, the liposomal NAC increased the cell viability and decreased ROS generation in both iron overload models. These results imply that liposomal NAC can protect kidney cells from iron overload-induced toxicity, whether or not ferroptosis is involved. Also, liposomal formulation improved the effect of free NAC by increasing the cellular uptake of hydrophilic drugs. This research would help determine the mechanism of cell death caused by iron overload and how to treat related diseases.

References

1. Patel, M., & Ramavataram, D. V. (2012). Non transferrin bound iron: nature, manifestations and analytical approaches for estimation. Indian journal of clinical biochemistry : IJCB, 27(4), 322 - 332. https://doi.org/10.1007/s12291-012-0250-7
2. Brissot, P., Ropert, M., Le Lan, C., & Loréal, O. (2012). Non-transferrin bound iron: a key role in iron overload and iron toxicity. Biochimica et biophysica acta, 1820(3), 403 - 410. https://doi.org/10.1016/j.bbagen.2011.07.014
3. van Swelm, R., Wetzels, J., & Swinkels, D. W. (2020). The multifaceted role of iron in renal health and disease. *Nature reviews. Nephrology*, *16*(2), 77 - 98. https://doi.org/10.1038/s41581-019-0197-5
4. Wang, H., An, P., Xie, E., Wu, Q., Fang, X., Gao, H., Zhang, Z., Li, Y., Wang, X., Zhang, J., Li, G., Yang, L., Liu, W., Min, J., & Wang, F. (2017). Characterization of ferroptosis in murine models of hemochromatosis. Hepatology (Baltimore, Md.), 66(2), 449 - 465. https://doi.org/10.1002/hep.29117
5. Fang, S., Yu, X., Ding, H., Han, J., & Feng, J. (2018). Effects of intracellular iron overload on cell death and identification of potent cell death inhibitors. Biochemical and biophysical research communications, 503(1), 297 - 303. https://doi.org/10.1016/j.bbrc.2018.06.019
6. Xie, Y., Hou, W., Song, X., Yu, Y., Huang, J., Sun, X., Kang, R., & Tang, D. (2016). Ferroptosis: process and function. Cell death and differentiation, 23(3), 369 - 379. https://doi.org/10.1038/cdd.2015.158
7. Musallam, K. M., & Taher, A. T. (2012). Mechanisms of renal disease in β-thalassemia. Journal of the American Society of Nephrology : JASN, 23(8), 1299 - 1302. https://doi.org/10.1681/ASN.2011111070
8. Neufeld E. J. (2010). Update on iron chelators in thalassemia. Hematology. American Society of Hematology. Education Program, 2010, 451 - 455. https://doi.org/10.1182/asheducation-2010.1.451
9. Kose, T., Vera-Aviles, M., Sharp, P. A., & Latunde-Dada, G. O. (2019). Curcumin and (-)- Epigallocatechin-3-Gallate Protect Murine MIN6 Pancreatic Beta-Cells Against Iron Toxicity and Erastin-Induced Ferroptosis. Pharmaceuticals (Basel, Switzerland), 12(1), 26. https://doi.org/10.3390/ph12010026

10. Vera-Aviles, M., Vantana, E., Kardinasari, E., Koh, N. L., & Latunde-Dada, G. O. (2018). Protective Role of Histidine Supplementation Against Oxidative Stress Damage in the Management of Anemia of Chronic Kidney Disease. Pharmaceuticals (Basel, Switzerland), 11(4), 111. https://doi.org/10.3390/ph11040111
11. Tardiolo, G., Bramanti, P., & Mazzon, E. (2018). Overview on the Effects of N-Acetylcysteine in Neurodegenerative Diseases. Molecules (Basel, Switzerland), 23(12), 3305. https://doi.org/10.3390/molecules23123305
12. Šalamon, Š., Kramar, B., Marolt, T. P., Poljšak, B., & Milisav, I. (2019). Medical and Dietary Uses of N-Acetylcysteine. *Antioxidants (Basel, Switzerland)*, *8*(5), 111. https://doi.org/10.3390/antiox8050111
13. Lu S. C. (2009). Regulation of glutathione synthesis. Molecular aspects of medicine, 30(1-2), 42 - 59. https://doi.org/10.1016/j.mam.2008.05.005
14. Wrotek, S., Sobocińska, J., Kozłowski, H. M., Pawlikowska, M., Jędrzejewski, T., & Dzialuk, A. (2020). New Insights into the Role of Glutathione in the Mechanism of Fever. International journal of molecular sciences, 21(4), 1393. https://doi.org/10.3390/ijms21041393
15. Atkuri, K. R., Mantovani, J. J., Herzenberg, L. A., & Herzenberg, L. A. (2007). N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency. *Current opinion in pharmacology*, *7*(4), 355 - 359. https://doi.org/10.1016/j.coph.2007.04.005
16. Santus, P., Corsico, A., Solidoro, P., Braido, F., Di Marco, F., & Scichilone, N. (2014). Oxidative stress and respiratory system: pharmacological and clinical reappraisal of N-acetylcysteine. COPD, 11(6), 705 - 717. https://doi.org/10.3109/15412555.2014.898040
17. Olsson, B., Johansson, M., Gabrielsson, J., & Bolme, P. (1988). Pharmacokinetics and bioavailability of reduced and oxidized N-acetylcysteine. European journal of clinical pharmacology, 34(1), 77 - 82. https://doi.org/10.1007/BF01061422
18. Alipour, M., Buonocore, C., Omri, A., Szabo, M., Pucaj, K., & Suntres, Z. E. (2013). Therapeutic effect of liposomal-N-acetylcysteine against acetaminophen-induced hepatotoxicity. Journal of drug targeting, 21(5), 466 - 473. https://doi.org/10.3109/1061186X.2013.765443
19. Mitsopoulos, P., Omri, A., Alipour, M., Vermeulen, N., Smith, M. G., & Suntres, Z. E. (2008). Effectiveness of liposomal-N-acetylcysteine against

LPS-induced lung injuries in rodents. International journal of pharmaceutics, 363(1-2), 106 - 111. https://doi.org/10.1016/j.ijpharm.2008.07.015

20. Alipour, M., Omri, A., Smith, M. G., & Suntres, Z. E. (2007). Prophylactic effect of liposomal N-acetylcysteine against LPS-induced liver injuries. Journal of endotoxin research, 13(5), 297 - 304. https://doi.org/10.1177/0968051907085062
21. Eloy, J. O., Claro de Souza, M., Petrilli, R., Barcellos, J. P., Lee, R. J., & Marchetti, J. M. (2014). Liposomes as carriers of hydrophilic small molecule drugs: strategies to enhance encapsulation and delivery. Colloids and surfaces. B, Biointerfaces, 123, 345 - 363. https://doi.org/10.1016/j.colsurfb.2014.09.029
22. Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S. W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., & Nejati-Koshki, K. (2013). Liposome: classification, preparation, and applications. Nanoscale research letters, 8(1), 102. https://doi.org/10.1186/1556-276X-8-102
23. Yang, W. S., & Stockwell, B. R. (2016). Ferroptosis: Death by Lipid Peroxidation. Trends in cell biology, 26(3), 165 - 176. https://doi.org/10.1016/j.tcb.2015.10.014
24. Szoka, F., Jr, & Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proceedings of the National Academy of Sciences of the United States of America, 75(9), 4194 - 4198. https://doi.org/10.1073/pnas.75.9.4194
25. Liu, D., Mori, A., & Huang, L. (1992). Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochimica et biophysica acta, 1104(1), 95 - 101. https://doi.org/10.1016/0005-2736(92)90136-a
26. Magarkar, A., Dhawan, V., Kallinteri, P., Viitala, T., Elmowafy, M., Róg, T., & Bunker, A. (2014). Cholesterol level affects surface charge of lipid membranes in saline solution. Scientific reports, 4, 5005. https://doi.org/10.1038/srep05005
27. Messner, D. J., Rhieu, B. H., & Kowdley, K. V. (2013). Iron overload causes oxidative stress and impaired insulin signaling in AML-12 hepatocytes. Digestive diseases and sciences, 58(7), 1899 - 1908. https://doi.org/10.1007/s10620-013-2648-3

28. WHEBY, M. S., & UMPIERRE, G. (1964). EFFECT OF TRANSFERRIN SATURATION ON IRON ABSORPTION IN MAN. The New England journal of medicine, 271, 1391 - 1395. https://doi.org/10.1056/NEJM196412312712704

29. Grootveld, M., Bell, J. D., Halliwell, B., Aruoma, O. I., Bomford, A., & Sadler, P. J. (1989). Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. The Journal of biological chemistry, 264(8), 4417 - 4422.

30. Skouta, R., Dixon, S. J., Wang, J., Dunn, D. E., Orman, M., Shimada, K., Rosenberg, P. A., Lo, D. C., Weinberg, J. M., Linkermann, A., & Stockwell, B. R. (2014). Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. Journal of the American Chemical Society, 136(12), 4551 - 4556. https://doi.org/10.1021/ja411006a

31. Yang, F., Li, Y., Yan, G., Liu, T., Feng, C., Gong, R., Yuan, Y., Ding, F., Zhang, L., Idiiatullina, E., Pavlov, V., Han, Z., Ma, W., Huang, Q., Yu, Y., Bao, Z., Wang, X., Hua, B., Du, Z., Cai, B., … Yang, L. (2017). Inhibition of iron overload-induced apoptosis and necrosis of bone marrow mesenchymal stem cells by melatonin. Oncotarget, 8(19), 31626 - 31637.

32. Messner, D. J., Sivam, G., & Kowdley, K. V. (2009). Curcumin reduces the toxic effects of iron loading in rat liver epithelial cells. Liver international : official journal of the International Association for the Study of the Liver, 29(1), 63 - 72.

33. Hoesel, L. M., Flierl, M. A., Niederbichler, A. D., Rittirsch, D., McClintock, S. D., Reuben, J. S., Pianko, M. J., Stone, W., Yang, H., Smith, M., Sarma, J. V., &Ward, P. A. (2008). Ability of antioxidant liposomes to prevent acute and progressive pulmonary injury. Antioxidants &redox signaling , 10 (5), 973 - 981. https://doi.org/10.1089/ars.2007.1878

국문초록

신장 세포의 철분 과부하 독성을 완화하기 위한 아세틸 시스테인을 탑재한 리포솜에 관한 연구

이두희

응용바이오공학과

서울대학교 융합과학기술대학원

철분이 체내에 과부하되면 Fenton 반응에 의해 활성산소를 생성하여 각종 장기에 부작용을 일으킨다. 그래서 여러 가지 항산화제가 철 관련 질병의 효과적인 치료법으로 연구되었다. 본 연구에서는, 효과적인 산화 방지제로서, 철 과부하로 인한 부작용을 완화하기 위해 아세틸 시스테인을 연구하였다. 약물 전달 시스템으로서, 리포솜이 약물을 캡슐화하도록 제작되었다. 아세틸 시스테인을 탑재한 리포솜이 인간 신장 (HK-2) 세포에서 철분 과부하 유발 독성을 감소시키는지를 연구했다. 리포솜은 Reverse Phase evaporation 방법에 의해 만들어졌다. 그리고 리포솜의 크기, 제타 전위와 모양을 확인하고 약물의 방출 실험을 진행하였다. 세포 생존률(CCK-8)과 활성 산소의 감소 양(DCFDA 검사)을 실험해 리포솜의 보호 효과를 확인했다. 실험 결과는 리포솜이 느린 철 흡수에 의해 유발된 페롭토시스를 억제한다는 것을 보여주었다. 또한 급속한 철 흡수에 의한 독성으로부터 세포를 보호하는 것을 확인하였다. 또한, 리포솜은 약물의 보호 효과를 증진 시켜준다는 것 또한 확인하였다. 이러한 결과는 아세틸 시스테인을 탑재한 리포솜이 다양한 철분 흡수 모델에 의해 유발되는 독성으로부터 인간의 신장 세포를 효과적으로 보호한다는 것을 시사한다.

주요어 : 리포솜, 철분, 아세틸 시스테인, 페롭토시스, 신장, 활성 산소

학번 : 2019-22656

Acknowledgement

석사 과정을 무사히 마치고 학위 논문을 제출하게 되었습니다. 지난 시간 동안 저에게 큰 도움을 주신 분들께 미흡하지만, 감사의 말씀 전하고자 합니다.

먼저, 저에게 석사 연구 과정을 지도해주신 이강원 교수님께 감사 인사드립니다. 많이 부족한 실력이지만 교수님의 지도가 있어 연구란 무엇인지 많이 배울 수 있었고 무사히 학위 논문을 마칠 수 있었습니다. 제가 연구에서 많은 실패를 겪으면서 힘들어할 때마다 도움을 주시고 격려해주신 배채원, 김수진, 그리고 김창헌 선배님께도 감사 인사드립니다. 선배님의 말씀을 들으면서 연구의 방향에 대해서 많은 고민을 할 수 있었고 용기를 얻을 수 있었습니다.

그리고 무엇보다도 제가 힘들어할 때마다 저를 위해서 기도해주시고 용기를 북돋워 주신 어머님, 아버님께 감사 인사드립니다. 힘든 상황이 올 때마다 어머님, 아버님의 사랑과 지지를 생각하며 버텨낼 수 있었습니다. 감사합니다.

석사를 졸업한 뒤에도 많은 고난과 역경이 기다리고 있겠지만 그럴 때마다 저에게 도움을 주신 분들의 은혜를 항상 기억하며 용기를 내겠습니다. 감사합니다.