

**Tackling Regulated Cell Death Improves the
Quality of DCD Grafts: Mechanism and
Application**

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Doctor of Philosophy

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Statement of Originality

I, Qian Chen, affirm that the work presented in this document is my original work and contains no unauthorised or plagiarised content. All sources used in this PhD thesis have been fully and properly cited, and any ideas or information obtained from external sources have been thoroughly summarised and integrated into my own words and understanding. Some experiments in Chapters 4, 5, 6 and 7 were performed together with students Shujing Zhang, Jade Phillips, Jiashi Sun, and Yiyue Li under my day-to-day supervision. Prof. Jianteng Gu and Dr. Xiangfeng Liu provided animal kidney, liver, and human lung samples at the Department of Anaesthesiology, Southwest Hospital, Military Medical University, Chongqing, China.

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Abstract

Graft availability of donation after circulatory death (DCD) is significantly limited by ischaemia reperfusion (IR) injury. α_2 -adrenergic receptor agonist dexmedetomidine (Dex) and noble gases xenon (Xe) and argon (Ar) have been demonstrated to reduce IR. This project investigated whether combining different concentrations of Xe, Ar, and Dex supplemented in the University of Wisconsin (UW) preservation solution provided better protection for cold storage of DCD grafts.

Human lung tissues preserved at 4°C for 24 h in the UW preservation solution supplemented with 0.1 nM Dex exhibited improved lung morphology and decreased apoptotic and necroptotic cell death caused by cold storage. Dex supplementation also reduced the activation of NLRP3 inflammasome and enhanced membrane self-resealing in pulmonary epithelial cells (A549 cell line) following hypothermic hypoxia-reoxygenation injury. In the case of kidney and liver grafts from DCD pig donors, when preserved at 4°C for 24 or 72 h in UW solutions supplemented with Dex, Xe and Ar, their quality significantly improved by reducing apoptosis and necroptosis and enhancing the antioxidant protein Glutathione Peroxidase 4 (GPX4). Among the options evaluated, the solution supplemented with 0.1 nM Dex combined with 50% Ar demonstrated the best results in maintaining the quality of kidney and liver grafts during cold preservation. *In vitro*, 0.1 nM Dex and 50% Ar effectively inhibited the translocation of thioredoxin-interacting protein (TXNIP), thereby increasing the expression of anti-oxidative stress proteins and inhibiting ferroptosis in hepatocytes (HepG2 cell line) subjected to hypothermic hypoxia-reoxygenation injury.

Our findings highlight the potential of targeting regulated cell death pathways as therapeutic strategies for DCD grafts. Inhibition of regulated cell death by supplementing 0.1nM Dex and 50% Ar in the graft preservation solution, we observed improvements in the quality of DCD kidney and liver grafts. These findings may have promising implications for expanding the donor pool and enhancing recipient outcomes following transplantation.

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Chapter ONE

Introduction

Organ transplantation is an appropriate therapy for patients with end-stage organ dysfunction and failure. However, a significant mismatch exists between the demand for transplantation and the limited supply of donor grafts, therefore imposing constraints on the broader implementation of this critical therapeutic intervention. One possible approach to tackle this change is the utilisation of grafts obtained from donation after circulatory death (DCD), which can potentially enhance the supply of organs. However, DCD grafts have been reported to be linked to an increased risk of primary graft dysfunction (PGD) or failure due to ischaemia-reperfusion (IR) injury. As a result, the use of DCD grafts is significantly restricted.

Using organs from donation after brain death (DBD) donors and organs from DCD donors presents distinct challenges. Brain death usually triggers a systemic inflammatory response that affects the quality of the donated organs, which contributes to organ dysfunction. In addition, the number of suitable DBD organs is limited, leading to challenges in meeting the high demand for transplantable organs. Compared to DBD, The primary challenge with DCD is the prolonged warm ischaemia time and more serious IR injury, leading to increased rates of PGD and transplant failure. DCD grafts also exhibit a distinct transcriptome signature characterised by cell death pathways rather than the conventional concerns of inflammatory events. Regulated cell death, including Rip kinase-mediated necroptosis, Gasdermin D-mediated pyroptosis, and iron-dependent ferroptosis, have been implicated in developing post-transplant complications caused by IR injury. Activation of these cell death pathways leads to increased transcription of immunoregulatory molecules and proinflammatory cytokines, exacerbating immune and inflammatory responses. Developing therapeutic strategies that effectively attenuate regulated cell death is crucial for the safe and wide use of DCD grafts. Nevertheless, while DBD organs may generally exhibit better outcomes, DCD and

extended criteria donor (ECD) organs contribute significantly to addressing organ shortages, expanding transplant opportunities, and providing life-saving options for patients in need.

Noble gases argon (Ar) and xenon (Xe) have been demonstrated to have potent organoprotection properties. Additionally, dexmedetomidine (Dex), a selective α_2 -adrenergic receptor agonist, has been shown to mitigate IR injury by reducing oxidative stress and inflammatory responses. These therapeutic interventions may reduce IR injury and improve the outcomes of organ transplantation. Therefore, the present study aims to investigate the impact of regulated cell death on the quality of lung, kidney, and liver grafts and explore the potential therapeutic effects and mechanisms of Dex and noble gases (Xe and Ar) in reducing IR injury.

In the *ex vivo* study, human lung tissues were preserved in the University of Wisconsin (UW) solution supplemented with 0.1 nM Dex at 4 °C for 24 h to evaluate the protective effects. Kidney and liver grafts from DCD pigs were preserved in the UW solution at 4 °C for 24 or 72 h. Different concentrations of Dex, Xe or Ar (as shown in **Table 1.1**) were supplemented into the UW solution to select the optimal concentration that inhibits regulated cell death in DCD grafts. In the *in vitro* study, human lung epithelial cells (A549 cell line), human kidney tubular cells (HK2 cell line), and human hepatocellular carcinomas (HepG2 cell line) were preserved in the UW solution at 4 °C for 24 h and then re-cultured in cell culture medium at 37 °C to simulate IR injury. The protective effects of Dex, Ar, and Xe were investigated. An overview of this study is illustrated in **Figure 1.1**. The summary of this PhD project are outlined in **Table 1.2**.

Table 1.1 Graft preservation solutions prepared for porcine DCD grafts

Group	Dex concentration	Gases applied to UW solution*	Inflation time	Flow rate
1	—	50% N ₂	20 min	1 L/min
2	0.1 nM	50% N ₂	20 min	1 L/min
3	0.1 nM	30% Xe	20 min	1 L/min
4	0.1 nM	30% Ar	20 min	1 L/min
5	0.1 nM	15% Xe + 15% Ar	20 min	1 L/min
6	0.1 nM	50% Xe	20 min	1 L/min
7	0.1 nM	50% Ar	20 min	1 L/min
8	0.1 nM	25% Xe + 25% Ar	20 min	1 L/min

*Balanced with 45% oxygen, 5% carbon dioxide and 20% nitrogen (if applicable).

In our investigation of lung transplantation, we evaluated the protective effect of Dex during the cold preservation of human lung tissues. The results indicated that the supplementation of Dex in the preservation solution significantly improved lung morphology and decreased lung apoptosis and necroptosis. The *in vitro* study further demonstrated that Dex significantly inhibited the activation of NLRP3 inflammasome and attenuated necroptosis. In addition, Dex played an important role in preserving lysosome architecture and improving membrane self-resealing function in cells subjected to hypothermic hypoxia-reoxygenation.

In the context of kidney transplantation, our research demonstrated that hypothermic hypoxia-reoxygenation triggered apoptosis, necroptosis, and pyroptosis in renal tubular cells. In addition, hypothermic hypoxia-reoxygenation increased the release of inflammatory mediators and damage-associated molecular patterns (DAMPs), including HMGB1. In the *ex vivo* study using DCD pig models, we observed significant damage to the kidney tubular architecture following warm ischaemia and prolonged cold storage for either 24 or 72 h. This damage was accompanied by increased oxidative stress and the activation of apoptosis and

necroptosis pathways. The combined supplementation of Dex with either Xe or Ar significantly enhanced graft quality by reducing the activation of apoptotic and necroptotic execution proteins. Among the various solutions tested, the preservation solution supplemented with Dex and 50% Ar demonstrated the most effective outcomes for maintaining graft quality during cold storage in our experimental setting.

In the context of liver transplantation, our investigation revealed severe injury to liver grafts obtained from DCD pig models following prolonged cold storage. However, when Dex and Xe/Ar were supplemented into the UW preservation solution, a significant reduction in the activation of multiple regulated cell death was observed. Moreover, the supplementation of Dex and Xe/ Ar significantly mitigated glycogen depletion, reticulin framework collapse, iron deposition, and oxidative stress. Notably, the solution supplemented with Dex and 50% Ar exhibited the most potent organoprotective effects in preserving the quality of liver grafts post-cold storage. In addition, *in vitro* experiments demonstrated that the combination of Dex and 50% Ar significantly inhibited the translocation of thioredoxin-interacting protein (TXNIP) from the cytosol to nuclei, This, in turn, increase the expression of anti-oxidative stress proteins, effectively inhibiting ferroptosis in hepatocytes subjected to hypothermic hypoxia-reoxygenation injury.

In summary, in this PhD project, we evaluated the impacts of prolonged cold ischaemia on human lung tissues, as well as kidneys and livers from DCD pig models. We focused on investigating the mechanism underlying oxidative stress, regulated cell death, and inflammatory responses during cold preservation. In addition, we investigated the impacts and mechanisms of regulated cell death on IR injury through *in vitro* hypothermic hypoxia-

reoxygenation cell cultures. Our findings indicated that the supplementation of Dex and noble gases (Xe or Ar) into organ preservation solution represents a potent protective strategy. This combination significantly reduced regulated cell death and inflammatory responses. Notably, this study revealed that the combination of 0.1 nM Dex and 50% Ar yielded the most improvement in the quality of DCD kidneys and livers. However, further research, including pre-clinical transplant studies and clinical trials, is needed to validate the efficacy of this preservation strategy.

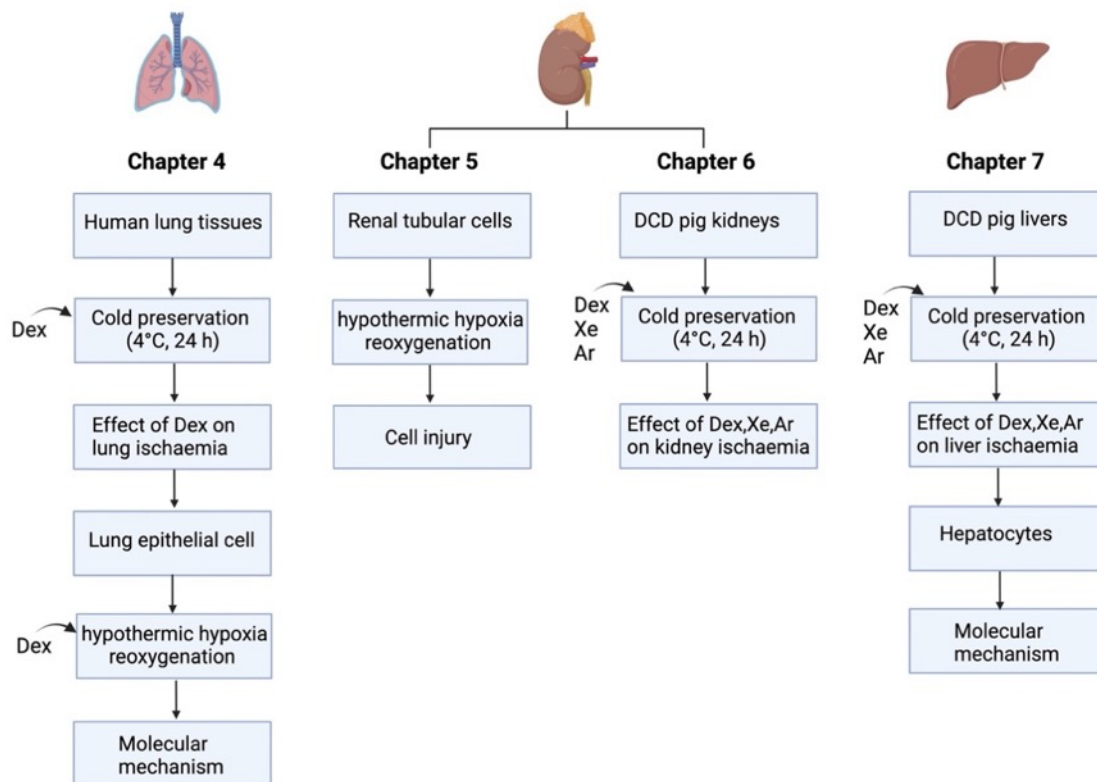


Figure 1.1 Study Overview.

The protective effect of Dex on human lung tissue cold ischaemia injury, the molecular mechanism was evaluated through an *in vitro* hypothermic hypoxia-reoxygenation model (Chapter 4). *In vitro* renal tubular cell injury under hypothermic hypoxia-reoxygenation (Chapter 5), and the protective effect of Dex, Xe, and Ar on DCD kidney grafts from DCD pigs (chapter 6). The protective effect of Dex, Xe, and Ar on DCD liver grafts from DCD pigs, and the molecular mechanism was examined by an *in vitro* hypothermic hypoxia-reoxygenation model (Chapter 7).

Table 1.2 The summary of the main achievements of this PhD project

Lung	<i>ex vivo</i>	Cold preservation induced necroptosis in human lung tissues.
		0.1 nM Dex improved lung morphology and decreased lung necroptosis.
	<i>in vitro</i>	Dex inhibited NLRP3 inflammasome activation, attenuated necroptosis, and repaired plasma membrane of lung epithelial cells induced by hypothermic hypoxia reoxygenation.
Kidney	<i>ex vivo</i>	Cold preservation induced necroptosis in DCD pig kidneys.
		0.1 nM Dex combined with 50% Ar improved renal morphology and reduced necroptosis.
	<i>In vitro</i>	Hypothermic hypoxia-reoxygenation induced regulated cell death of renal tubular cells.
Liver	<i>ex vivo</i>	Cold preservation induced regulated cell death in DCD pig livers.
		0.1 nM Dex combined with 50% Ar mitigated glycogen depletion, reticulin framework collapse, iron deposition, oxidative stress, and reduced regulated cell death in DCD pig livers.
	<i>in vitro</i>	Dex combined with 50% Ar significantly reduced ferroptosis through inhibiting TXNIP translocation.

Chapter TWO

Background

Organ transplantation is one of the advancements of modern medicine. It is impossible without such therapy to prolong the life span in some patients who have end-stage function or failure of organs.

2.1 Organ transplantation in the modern era

2.1.1 Short history of organ transplantation

Organ transplantation is one of the most successful advances in medical history, providing numerous patients with a last chance for survival. The history of organ transplantation dates back to the 18th century when Dr Alexis Carrel performed several kidney transplant surgeries on dogs. Although the transplanted kidney only survived for a short period before rejection, this study established revascularisation techniques for kidney transplant and described the complexity of immune rejection [1]. In 1933, Dr. Yurii Voronoy of Ukraine performed the first successful human kidney transplantation surgery. However, only after 2 days the transplanted kidney was immunologically rejected [2]. Donor-recipient matching is an important advance in organ transplantation history. This was discovered by British surgeon Peter Medawar, who was awarded his Nobel Prize for Physiology or Medicine in the year 1960 [3].

In 1954, Dr. Joseph Murray and his colleagues successfully conducted the first kidney transplantation surgery. The donated kidney was obtained from a twin brother of the recipient. The patient lived without immunologic rejection for eight years after the surgery [4]. In 1963, Dr. James Hardy and his colleagues performed the first lung transplantation surgery, and the transplanted lungs were obtained from a DCD donor. The recipient survived for 18 days without immunologic rejection [5]. Dr. Thomas Starzl and his colleagues performed the first liver transplantation in the same year [6]. In 1967, Dr. Christiaan Barnard and his team performed the first heart transplantation, and the transplanted heart was from a DBD donor [7]. Since the 1970s, kidney and liver transplantations have been widely accepted and conducted, and lung and pancreas transplantations have become popular since the 1980s [8].

The developments of surgical techniques and immunosuppressant drugs significantly improved the survival rates of organ transplantations. Graft preservation strategies, such as static cold storage and perfusion systems, significantly prolonged organ preservation periods, and increased the survival rate of transplanted organs [9]. In recent years, efforts have been made to reduce the risk of organ rejection and research alternative organs, such as artificial organs [10]. Meanwhile, efforts are being made to tackle organ shortage through regenerative medicine to increase organ availability for transplantation [11]. These advancements in surgical and preservation techniques and ongoing research have significantly contributed to organ transplantation.

2.1.2 Challenges in Organ Transplantation

One of the biggest challenges in organ transplantation is graft shortage, which is predominant due to increasing demand for organ transplants and a limited supply of acceptable donated organs [12]. For example, in 2020 alone, approximately 95,000 patients were awaiting a kidney, while only around 21,000 kidney transplants were performed annually in the United States [12]. Similarly, in 2018, there were over 12,000 patients on the liver transplant waiting list, but only approximately 8,000 liver transplants were performed [13]. The demand for lung transplants remains high, with approximately 2,000 transplants performed annually; however, the number of available lungs is significantly lower than this demand [14].

The efficacy of a transplant surgery is directly influenced by ischaemia injury. Ischaemic time is when a graft loses blood flow and oxygen after being retrieved from a donor until revascularised in the recipient [15]. Prolonged warm ischaemia leads to organ injury and reduces the viability of organs. To mitigate the effects of ischaemia during transport, retrieved

organs are often stored at low temperatures (4°C) using a preservation solution [9]. While cold storage delays metabolic processes, it results in cellular injury over extended periods, potentially affecting transplanted organ function [16]. Ongoing research has focused on developing alternative preservation methods to address these challenges. One technique is normothermic perfusion [17], which keeps the organs at body temperature while perfusing them with warm oxygenated blood and nutrients. This technique aims to increase the safe transport of organs and reduce ischaemia injury. Additionally, modified preservation solutions are explored as potential alternatives to improve organ preservation quality [18, 19].

In conclusion, future research efforts should be dedicated to increasing organ donations, exploring alternative organ sources, improving graft preservation techniques, and advancing immunosuppressive therapies and post-transplant care. All these may overcome the challenges associated with long-term graft survival and further improve the success of organ transplantation.

2.2 Organ donation

2.2.1 Donation after brain death (DBD)

Organ donors who are certified as brain-dead based on neurological criteria (referred to as donation after brain death, DBD) have their circulatory and respiratory functions with the help of ventilator support [20]. During organ retrieval, the circulation is stopped by clamping the aortic arch and draining the vena cava. Subsequently, a cold preservation solution is flushed through the distal aorta to minimise anaerobic metabolism and reduce the risk of warm ischaemia [16]. DBD donors significantly contribute to the organ supply and play an important

role in meeting the high demand for organs [21]. However, it is important to consider both the benefits and disadvantages of utilising organs from DBD donors in organ transplantation.

It has been recognised that the organs procured from DBD donors have higher quality due to the donor circulation still functioning when brain death is declared. This ensures a continuous supply of oxygen and nutrients to the organs until they are retrieved. Therefore, the organs experience a shorter duration of warm ischaemia, leading to improved organ quality and better transplant outcomes [22]. Traditionally, warm ischaemic time is defined as the duration from irreversible cardiac arrest to the end of cold perfusion. However, current knowledge suggests that organ hypoperfusion starts earlier because the circulatory and respiratory functions gradually decline after treatment withdrawal. This means that warm ischaemia may already occur before cardiac arrest is confirmed [23]. Therefore, while the recorded warm ischaemic time may be in minutes, the actual "functional" warm ischaemic time may be beyond 1 h, considering the gradual decline in organ function before complete cessation.

Brain death often accompanies significant hemodynamic changes, leading to organ injury [24]. During brain stem death, catecholamines are simultaneously released in the body, leading to a hyperdynamic cardiovascular response. This phenomenon is referred to as a "catecholamine storm." As a result, there is an increase in cardiac output due to hypertension and bradycardia, leading to blood pressure increase [25, 26]. After that, there is a decrease in serum catecholamine and peripheral vascular resistance, resulting in cardiovascular failure and hypovolemia. These hemodynamic changes impair organ perfusion and contribute to organ injury. Meanwhile, the immune system can be severely disrupted during brain death, which leads to a rapid and uncontrolled release of pro-inflammatory cytokines, such as interleukin

(IL)-1, IL-6, tumour necrosis factor (TNF)- α , and interferon (IFN)- γ [27]. These cytokines may trigger an aggressive immune response after transplantation, increasing the risk of acute rejection [27, 28]. In addition, the systemic inflammation caused by cytokine release exacerbates IR injury, leading to a higher risk of PGD and delayed graft function (DGF) [28].

2.2.2 Donation after circulatory death (DCD)

Donation after circulatory death (DCD) is defined as retrieving donor organs for transplantation after death using circulatory criteria [29]. The utilisation of DCD significantly mitigates the ongoing organ shortage in many countries [23]. Existing evidence indicated that kidneys obtained from DCD donors had comparable survival rates and lifespans to those obtained from DBD donors [30]. In addition, organs such as the liver and lungs, which are more susceptible to warm ischaemia, can also be successfully transplanted from DCD donors with reasonable survival rates [31, 32]. However, one of the current challenges in the practice of DCD is identifying patients as appropriate potential DCD donors and minimising ischaemia injury in a professional, ethical, and legally acceptable way [32].

Organs from DCD donors typically experience ischaemia longer than organs from DBD donors [33]. Warm ischaemia injury, although most severe between the time of cardiorespiratory arrest and the end of cold perfusion, actually begins during cardiorespiratory function decline. Therefore, in the case of a DCD organ, the beginning of warm ischaemic time is now defined as when the systolic arterial pressure drops below 50 mmHg, arterial oxygen saturation decreases below 70%, or both [34]. Compared to organs from DBD, prolonged warm ischaemia in organs from DCD donors increases the risk of graft failure, DGF, and other ischaemic complications in recipients. Thus, if the warm ischaemic time is prolonged, the

excised organ may not be appropriate for transplantation. Therefore, it is reasonable to explore strategies for mitigating warm ischaemia injury and identify effective means of achieving this goal.

2.2.3 Outcomes of organs from DBD and DCD donors

Compared to organs from DBD donors, DCD donor organs are generally believed to experience significant damage due to prolonged warm ischaemia (**Figure 2.1**). This impairment in organ function contributes to a reduction in the survival rate of transplanted organs [35]. In the context of kidney transplantation, the use of organs from DCD donors is associated with an increased incidence of acute tubular necrosis, leading to the necessity of post-transplant dialysis for over 50% of the recipients [36]. DCD livers may carry a higher risk of primary nonfunction, resulting in poorer outcomes for the recipients. Additionally, DCD livers exhibit an elevated risk of anastomotic and intrahepatic biliary strictures [37]. Conversely, DCD lung transplantation demonstrates acceptable results, with outcomes at least comparable to those of DBD lung transplantation [38, 39]. DCD pancreas transplantation has been less extensively studied compared to other organs, mainly due to concerns related to postoperative dysfunction and pancreatitis. Nonetheless, previous data indicated similar rates of graft thrombosis and pancreas loss between DCD and DBD pancreas transplants [40, 41].

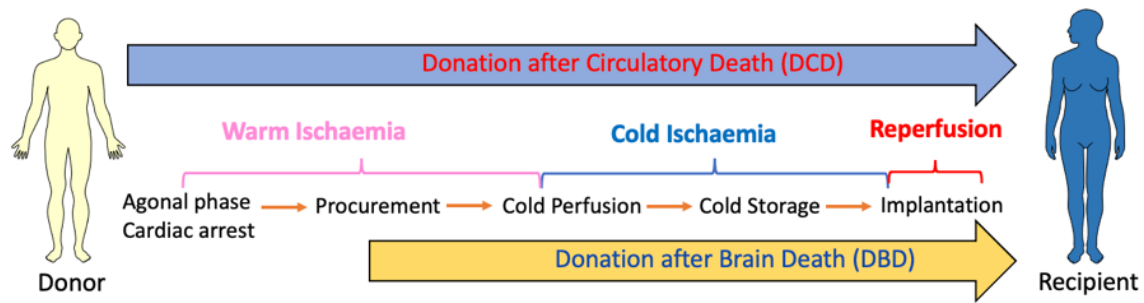


Figure 2.1 Ischemia Reperfusion During Organ Donation and Implantation.

Grafts from donation after circulatory death (DCD) donors experienced longer warm ischaemic time than those grafts from donation after brain death (DBD) donors due to cardiorespiratory failure before cardiac arrest.

2.3 Ischaemia reperfusion (IR) injury in organ transplantation

2.3.1 mechanisms of IR injury

IR injury is characterised by organ injury due to the blood supply to an organ is restored after a period of interruption [42]. This interruption is unavoidable regardless of the grafts from DBD or DCD donors (**Figure 2.2**). IR injury is a complex inflammatory condition influenced by various factors, including hypoxia, oxidative stress, leukocyte extravasation, activation of cell death pathways, and immune responses [42]. Warm ischaemia occurs when a donor organ experiences a loss of blood flow until the perfusion with a cold preservation solution. Cold ischaemia results from preserving the donor organ in a cold preservation solution at 4°C until blood flow is restored to the graft during implantation in the recipient. Reperfusion occurs when blood flow is re-established to the organ in the recipient [43].

During ischaemia, the lack of oxygen prevents the production of sufficient ATP through oxidative phosphorylation in mitochondria. As a result, organs cannot rely on the metabolism of sugar, lipids, and amino acids to maintain ATP levels [16]. However, organs continue to

consume ATP, rapidly declining the intracellular ATP: ADP ratio. This decline impairs cellular functions and even results in cell death. During cold storage, the demand for ATP by organs is significantly reduced, causing a gradual decrease in the ATP: ADP ratio. However, anaerobic glycolysis is still necessary for cell survival and maintaining essential functions even in low temperatures [16]. Therefore, the intracellular ATP level at the end of warm ischaemia plays a crucial role in determining the subsequent tolerance to cold ischaemia. During reperfusion, re-establishing blood flow gives a sudden influx of oxygen, leading to a rapid oxidation of succinate in mitochondria. This change generates an amount of reactive oxygen species (ROS), causing significant damage to lipids, proteins, DNA, and overall cellular function [16].

ROS have been identified as the primary elements responsible for IR injury [44-46]. Superoxide and hydroxyl radicals are the main ROS in cells, and their cytotoxicity comes from lipid peroxidation-induced membrane damage and the formation of other free radicals that cause DNA damage [47]. Under normal physiological conditions, the body has several antioxidant mechanisms, including superoxide dismutase (SOD), glutathione, and catalase, which help counteract the effects of ROS [48]. However, ROS overproduction during IR injury disrupts the balance between oxidative and antioxidative mechanisms, leading to oxidative stress and even cell death [48]. Previous studies showed that increased ROS levels mediated neutrophil adhesion at the blood-endothelial cell interface, contributing to necrosis and initiating a feedback loop that further increased ROS production and exacerbated cellular injury [49]. Meanwhile, neutrophils generate significant amounts of extracellular superoxide due to membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The superoxide is then converted to H_2O_2 through disproportionation. Excessive H_2O_2 production generates hypochlorous acid and hydroxyl radicals, further contributing to the deterioration

of organ function [50]. ROS overproduction also impairs cellular calcium homeostasis by releasing intracellular calcium from the endoplasmic reticulum and increasing extracellular calcium entry through voltage-gated calcium channels or store-operated calcium entry channels. Increased calcium activates calcium-dependent enzymes, such as proteases and phospholipases, leading to cellular damage and dysfunction [51].

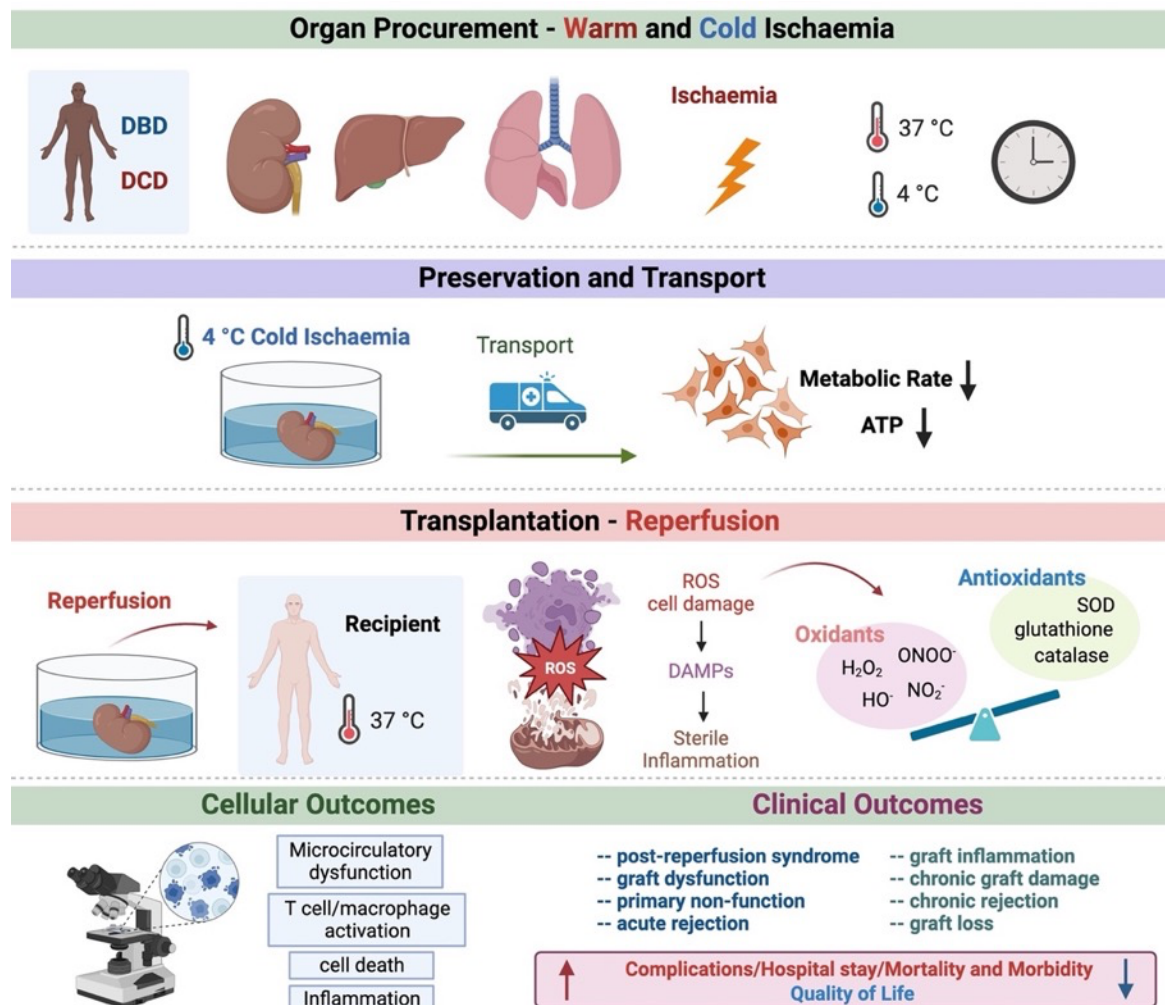


Figure 2.2 IR injury in organ transplantation.

Grafts such as kidney, liver, and lungs from DBD or DCD donors experience warm ischaemia when they lose blood flow until the end of perfusion with a cold preservation solution. These grafts are subsequently preserved in a cold preservation solution at 4°C and transported to the recipients. The low temperature during preservation reduces the metabolic rate and the demand for ATP by cells. Upon reperfusion, when blood flow is restored to the organ in the recipient, mitochondria produce

an abundance of ROS. The imbalance between oxidative and antioxidative levels releases damage-associated molecular patterns (DAMPs) from oxidative stressed cells, triggering sterile tissue inflammation. IR injury causes microcirculatory dysfunction, activation of T cells and macrophages, cell death, and inflammation. These processes increase the risk of post-reperfusion syndrome, graft dysfunction, primary non-function, acute rejection, and other complications, ultimately impacting the quality of life for the recipients.

2.3.2 Cold preservation

In recent years, the focus of organ transplantation has shifted from primarily improving immunosuppression to prioritising the mitigation of IR injury for better post-transplant outcomes in recipients. Preservation techniques, such as temperature control, modified preservation solutions, and perfusion settings, were developed to reduce cellular metabolism and provide organoprotection against IR injury [9, 52].

Static cold storage is the current gold standard technique for organ preservation [9, 52]. Without blood flow, cells rapidly switch from aerobic to anaerobic metabolism. Anaerobic metabolism requires 19 times more glucose substrate to produce ATP than aerobic metabolism. This metabolic shift leads to the rapid depletion of intracellular glucose substrate and increases the accumulation of metabolites such as lactic acid [53]. Organ cold preservation aims to minimise these changes by reducing the metabolic rate to 10% of normal body temperature [54]. In clinical settings, various organ preservation solutions (**refer to Table 2.1**) are currently used to mitigate IR injury [55]. These solutions contain physiological buffers, such as phosphate or citrate, to maintain intracellular pH. They also include large molecules, such as mannitol or raffinose, to preserve intravascular osmotic pressure, thereby minimising swelling.

The UW solution was developed in 1988 by the Belzer and Southard team at the University of Wisconsin. This solution was designed for the perfusion and preservation of intra-abdominal organs. The UW solution has a high concentration of K^+ (125 mmol/l) and a low concentration of Na^+ (29 mmol/l), closely resembling the composition of intracellular fluid in organs. A high concentration of K^+ inhibits the outward movement of K^+ from cells according to the concentration gradient [56]. Furthermore, the UW solution is formulated with hydroxyethyl starch, effectively maintaining fluid inside the vascular compartment and mitigating extracellular oedema. Lactobionic acid and raffinose, two impermeable compounds, can decrease tissue oedema. Glutathione eliminates oxygen-free radicals and maintains the integrity of cellular membranes structural. Adenosine functions as a substrate for the biosynthesis of ATP and plays a crucial role in supporting the energy metabolism of organs. Allopurinol, a xanthine oxidase inhibitor, protects against ischaemia injury [57, 58]. The UW solution is now recognised as a preservation solution that has undergone clinical validation for organ preservation [59]. Recent clinical research has been on integrating additives to facilitate compositional advancements, whereas there have been a few investigations involving alterations to the original constituents of the UW solution.

The Collins solution and Euro-Collins (EC) solution were developed by Collins and his research team in 1969 for organ preservation. Initially, these solutions were used for organ preservation with a designated time constraint of 6 to 8 h [60]. One notable benefit of the EC solution, in contrast to the Collins solution, is its ability to eliminate the influence of Mg^{2+} on phosphatase deposits [61]. The application of EC solution modifies the typical intracellular/extracellular ratio of Na^+ and K^+ , impacting the energy reduction mechanism facilitated by the Na^+/K^+

ATPase *via* changes in ion concentrations. Nevertheless, elevated levels of K^+ may lead to membrane depolarisation, thereby affecting the proper functioning of smooth muscle and endothelial cells in the blood arteries [62]. Moreover, both the Collins solution and EC solution are composed of phosphate buffer systems, which have the potential to induce lactate generation in the presence of high osmotic circumstances. This lactate production may disrupt the osmotic equilibrium inside and outside the cells, resulting in cellular swelling. Using Collins and EC solutions may cause adverse effects, contributing to a progressive decline in their clinical application [63].

The Histidine-Tryptophan-Ketoglutarate (HTK) organ preservation solution is extensively used in clinical settings. The primary purpose of this solution is to preserve solid organs, such as the heart, liver, and kidney [64]. The HTK solution is an isotonic solution including a mixture of components to provide a suitable environment for organ preservation. The HTK solution has essential components such as histidine, tryptophan, and ketoglutarate, which are vital in maintaining cellular metabolism and protecting organs during cold storage [65]. Unlike the UW solution, the HTK solution has a reduced viscosity and increased fluidity, which is more convenient for cold perfusion of the transplanted organs [66]. A retrospective study reviewed 400 patients who received liver grafts preserved with HTK and UW between 2007 and 2015. The results showed no significant difference in graft and recipient survival between the two solutions; however, using the HTK solution resulted in a lower incidence of post-transplant biliary stricture compared with the UW solution [64].

The Celsior solution, developed by Dr. Menasche and his team, has been used in clinical settings as an innovative approach to organ preservation. The solution was first used to

preserve hearts and showed acceptable outcomes. In addition, it has been found that the Celsior solution provided good clinical outcomes in preserving abdominal organs, particularly in liver preservation. The formulation of the Celsior solution came from the UW solution. In contrast to the UW solution, the Celsior solution is characterised by its high Na^+ and low K^+ concentration and low viscosity [67]. The Celsior solution effectively decreases cell swelling, oxidative damage, and Ca^{2+} overload. Mannitol in the solution has been demonstrated to reduce cellular oedema effectively, and antioxidants such as glutathione protect against oxidative stress caused by ROS overproduction [67]. Comparative analysis indicated that the Celsior solution exhibited performance comparable to other organ preservation solutions in the transplant utilisation rate [68].

The EC and UW solutions are the two most commonly used organ preservation solutions to preserve kidneys, livers, and pancreas. These solutions belong to the intracellular fluid (ICF)-type solution, which characterises low Na^+ and high K^+ concentrations. This environment can prevent cellular oedema by maintaining intracellular ion concentrations during cold preservation [9]. However, during reperfusion, there is a high risk of hyperkalemia due to the flushing of the preservation solution from the graft into the circulation of the recipient [9]. Therefore, recent developments focus on developing low- K^+ and high- Na^+ solutions, such as the HTK solution [69] and the Celsior solution [70]. Although these solutions are not extensively used in clinics, they aim to reduce the risk of hyperkalemia. For cardiac preservation, solutions with a high K^+ concentration are frequently used to induce cardiac arrest and rapidly reduce cardiometabolism, thereby increasing the effectiveness of cold preservation [71]. For lung preservation, the low K^+ /dextran solution, also known as Perfadex

solution, includes sugar, electrolyte and organoprotective contents that can protect pulmonary endothelium, resulting in excellent postoperative lung graft outcomes [72].

Table 2.1 Composition of Cold Preservation Solutions

Solutions	UW	EC	HTK	Celsior	Perfadex
K⁺ (mEq/L)	125	115	10	15	6
Na⁺ (mEq/L)	29	10	15	100	138
Cl⁻ (mEq/L)	20	15	32	71	142
Ca²⁺ (mEq/L)	--	--	0.015	0.25	--
Mg²⁺ (mEq/L)	5	--	4	13	0.8
Buffer	Phosphate	Phosphate	Histidine	Histidine	Phosphate
Antioxidant	Glutathione Allopurinol	--	Mannitol Tryptophan α -ketoglutarate	--	--
Glucose	--	180	--	5	10
Amino acids	--		Histidine Tryptophan	--	--

2.3.3 Machine perfusion

Modern organ reconditioning methods have revolutionised organ transplantation, offering innovative approaches to enhance organ viability and transplant outcomes. Machine perfusion techniques have become popular and have a significant advantage in mitigating IR injury.

Using cold machine perfusion systems (hypothermic machine perfusion, HMP) has earned significant attention. Although static cold storage offers simplicity and convenience during transportation from the donor hospital to the recipient hospital, using a machine that circulates the cold preservation solution may lead to better organ preservation. Continuous cold perfusion has the potential to significantly improve the quality of transplanted organs by effectively flushing out accumulated metabolites from small capillaries. HMP is particularly beneficial for grafts retrieved from DCD donors for further assessing, reconditioning, and optimising their physiological state before engraftment. Previous clinical trials suggested that, compared to static cold storage, HMP enhanced the quality and success of kidney transplants by reducing the risk of delayed graft function and improving graft survival in the first year after transplantation [73-75]. In addition, HMP is a promising technique for liver transplantation. HMP holds significant promise as an effective strategy for preserving DCD or ECD liver grafts during transplantation, resulting in lower serum markers of liver damage, shorter hospital stays, lower biliary complications, and increased graft survival [76-78].

Another technique is normothermic machine perfusion (NMP). This is particularly useful for preserving fatty livers, which are vulnerable to damage due to intrahepatic fat globules that can affect hepatocytes and liver microcirculation [79]. In addition, recent preclinical data showed significant advantages of normothermic perfusion over cold storage, especially for livers obtained from DCD donors with prolonged warm ischaemia periods [80]. *Ex vivo* lung perfusion represents excellent outcomes for organ preservation at normothermic temperatures. Research indicated that normothermic perfusion had the potential to "repair" lung cells that would otherwise be considered unsuitable for transplantation, thereby expanding the pool of available lungs for transplantation [81].

Evidence suggests that normothermic regional perfusion of abdominal organs from DCD donors may improve organ quality and reduce IR injury [82-84]. In the DCD process, a double-balloon catheter is inserted through the femoral artery into the aorta to isolate the abdominal aorta. At the same time, blood is drained through the ipsilateral femoral vein. Subsequently, the catheter is connected to an extracorporeal membrane oxygenator (ECMO) circuit, which enables the perfusion of warm and oxygenated blood to the abdominal organs. The organs can receive sufficient ATP by supplying oxygenated blood to enhance their ability to withstand subsequent cold ischaemia [85]. Preliminary studies have shown that this strategy improved the quality of kidneys and livers obtained from DCD donors [86]. As the number of DCD donors increases, normothermic regional perfusion may become a common practice.

These advanced machine perfusion techniques provide several advantages over traditional static cold storage. They enable continuous monitoring, assessment, and intervention, allowing medical professionals to optimise organ conditions before transplantation. The ability to recondition organs through HMP and NMP has shown promising results in improving graft function, reducing the risk of complications, and expanding the pool of viable organs for transplantation. As research and technology in organ perfusion continue to advance, these reconditioning methods will play a crucial role in improving transplant outcomes and addressing the persistent challenges of organ shortages in transplantation.

2.4 Cell death pathways in organ transplantation

Several regulated cell death pathways have been reported related to organ IR injury in the context of transplantation, such as necroptosis [87], iron-dependent ferroptosis [88], and

GSDMD-mediated pyroptosis [89]. Necroptosis has been examined in transplanting all main solid organs, such as the kidney, liver, heart, and lungs [90]. It has been reported that inhibiting necroptosis in kidney [91] and liver grafts [92] increased their survival without rejection. Ferroptosis is notable in liver transplantation and current research demonstrated that the ferroptosis inhibitor ferrostatin-1 inhibited hepatocyte ferroptosis and may become a promising treatment [93]. Overall, preventing the adverse effects of cell death during transplantation is a promising approach to improving organ quality.

2.4.1 Apoptosis

There are two pathways that activate apoptosis: intrinsic and extrinsic pathways (**Figure 2.3**). The interaction between death receptors and their ligands activates the extrinsic apoptotic pathway. This interaction leads to the formation of the death-inducing complex (DISC), which consists of the cytoplasmic tail of the death receptor (FAS) and FAS-associated proteins. The DISC regulates the cleavage and activation of caspase-8, resulting in mitochondria injury [94]. On the other hand, intracellular stress usually activates the intrinsic apoptotic pathway, which is mediated by mitochondrial translocation of Bcl-2-associated X protein (BAX) and Bcl-2 antagonist killer 1 (BAK), followed by the activation of the mitochondrial permeability transition pore (MPTP) [95]. The intrinsic and extrinsic apoptosis pathways ultimately rely on activating the executioner protein caspase-3 and -9. Caspase-3 can be cleaved to generate p12 and p17 subunits, which initiate DNA fragmentation and degradation of the cytoskeleton and nucleoproteins [95, 96].

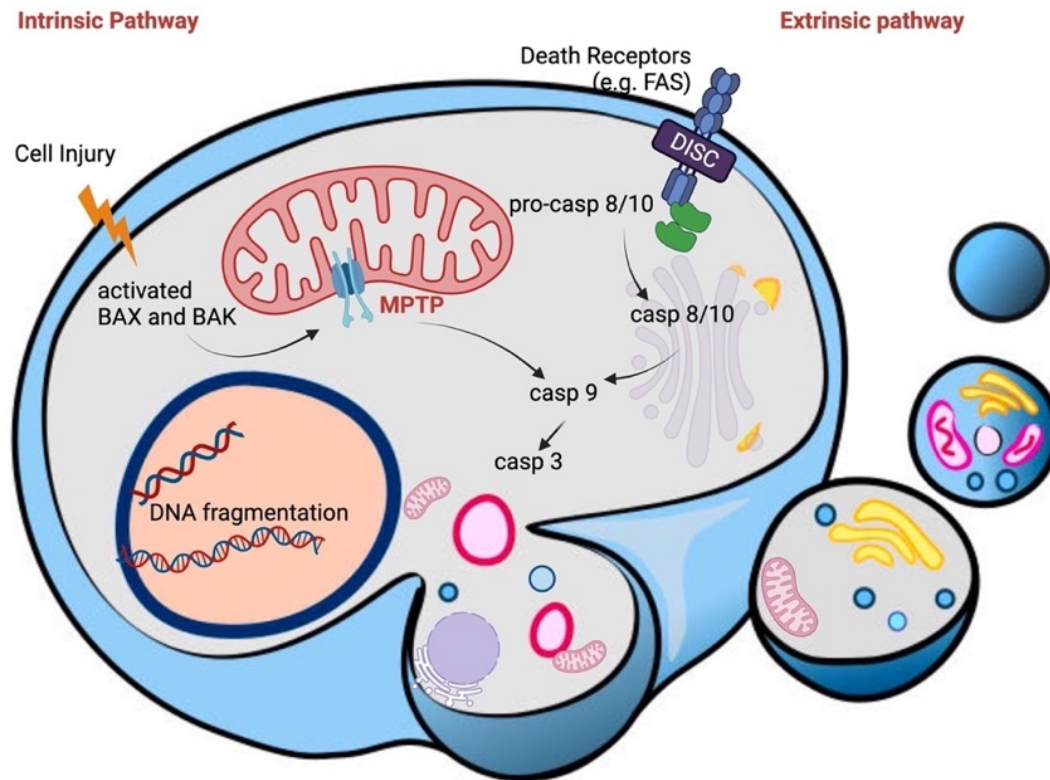


Figure 2.3 Intrinsic and Extrinsic Pathways of Apoptosis.

Cell injury (such as IR injury) activates the intrinsic apoptosis pathway by activating BAX and BAK to form mitochondrial permeability transition pores (MPTP). The extrinsic pathway is triggered by the activation of death receptors (e.g., FAS/FASL) to form a death-inducing complex (DISC) and then active caspase-8/10. The activation of caspase-9 and 3 ultimately initiates both intrinsic and extrinsic pathways.

Caspase activation has been found to be increased in the models of kidney, liver, and lung IR injury. In kidney transplantation, administering caspase-3 siRNA intravenously or through cold perfusion has been shown to reduce renal cell death and inflammation [97]. Similarly, inhibiting caspase-3 before IR in lung transplantation has been found to enhance lung function and decrease cell death in rats [98]. In rat liver transplantations, adding DEVD-FMK in the preservation solution to inhibit caspase-3 and -7 has been shown to reduce microvascular injury and improve the survival of transplanted organs [99]. Studies also reported that

administering a pan-caspase inhibitor during cold perfusion improved liver function in rats [100]. Moreover, supplementation of pan-caspase inhibitors in the preservation solution yielded similar results for preserving human livers [101]. In heart transplantation, studies have reported that administering a caspase-3 inhibitor into either the donor or recipient before transplantation decreased coronary artery vasculopathy (CAV) in mice [102]. These findings suggested that caspase inhibition may effectively reduce apoptosis in transplanted organs.

2.4.2 Necroptosis

Necroptosis is a form of regulated cell death characterised by plasma membrane rupture and the release of cellular contents. Necroptosis plays a role in graft dysfunction post-transplant, such as DGF and primary non-function (PNF) in transplant recipients [103]. Necroptosis is initiated by the activation of various cell surface death receptors, including FAS, tumour necrosis factor receptor (TNFR), and toll-like receptors (TLRs), independently of caspase activation [104, 105]. The TNF-mediated necroptosis pathway has been most thoroughly investigated (**Figure 2.4**). Briefly, upon stimulation by TNF- α , TNFR is activated, leading to the binding of TNF- α to TNFR-associated death protein (TRADD) and Fas-associated death domain (FADD), ultimately forming complex I in the cytoplasm. Complex I then activates receptor-interacting protein kinase 1 (Rip1). When the activation of caspase-8 is inhibited, Rip1 and Rip3 interact, leading to the phosphorylation of Rip3. This phosphorylated form of Rip3, known as p-Rip3, then phosphorylates the mixed lineage kinase domain-like (MLKL) protein. The fully p-MLKL or its latent N-terminal 4-helix bundle (4HB) domain binds to liposomes containing phosphatidylinositol phosphates (PIPs). This interaction enables p-MLKL to recruit and insert into the plasma membrane, damaging membrane integrity and releasing inflammatory factors and danger-associated molecular patterns (DAMPs) [106].

A study demonstrated that the knockout Rip3 gene (Rip3^{-/-}) resisted renal IR injury in mice [91]. Rip3^{-/-} kidneys showed lower histological injury scores, reduced neutrophil infiltration, decreased fibrosis, and less vascular damage than control transplanted kidneys. Moreover, mice receiving Rip3^{-/-} kidneys had prolonged survival without experiencing acute or chronic rejection [91]. In liver transplantation, early allograft dysfunction (EAD) significantly affects the outcomes of recipients. Studies have reported that patients who developed EAD after liver transplantation exhibited significantly higher phosphorylated MLKL (pMLKL) expression in their livers compared to those who did not develop EAD. Furthermore, liver grafts with high pMLKL expression showed significantly elevated levels of transaminases and lactate dehydrogenase 24 h after transplantation [107]. In the lung transplantation studies, it was found that supplementing a necroptosis inhibitor (Nec-1) to the cold preservation solution resulted in a substantial reduction in pulmonary oedema and necrosis following transplantation in rats [108].

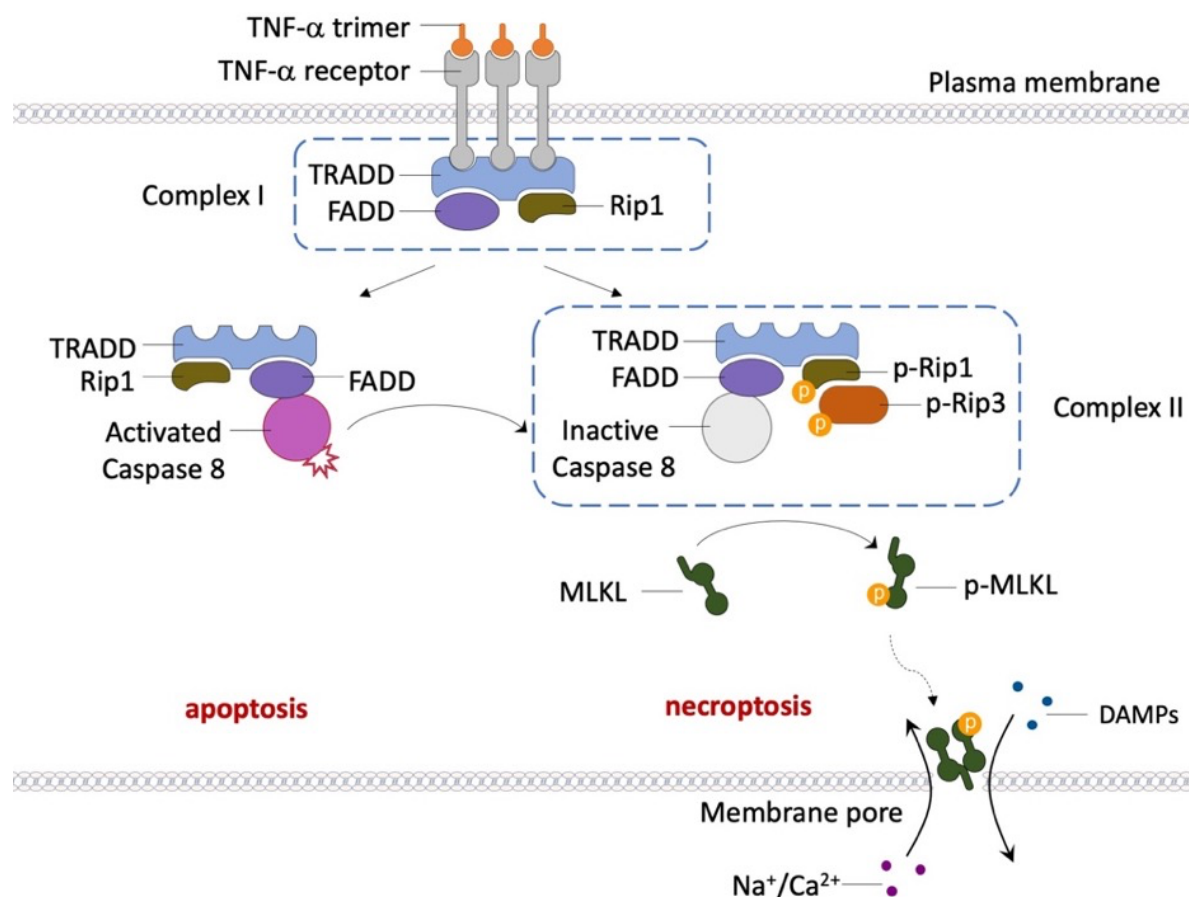


Figure 2.4 The Molecular Mechanism of Necroptosis.

Upon the stimulation of the TNF-α primer, the TNF-α receptor combines with Rip1, FADD, and TRADD, forming complex I. Then, FADD binds to the activated caspase 8, resulting in apoptosis. When the caspase-8 is inhibited, Rip1 and Rip3 combine and are phosphorylated, forming complex II. This complex promotes the oligomerisation of MLKL by phosphorylating it. The oligomeric form of p-MLKL then transfers towards the plasma membrane from the cytosol, forming membrane pores, ultimately leading to Na⁺/Ca²⁺ influx and DAMPs release.

2.4.3 Ferroptosis

Ferroptosis is characterised by the accumulation of lipid peroxides and the release of iron-dependent ROS in cells [109]. The initiation of ferroptosis is accompanied by the accumulation of toxic lipid peroxides, specifically phospholipid hydroperoxides, within the cell membrane. This process is induced by the iron-dependent uncontrolled oxidation of polyunsaturated fatty

acids (PUFAs) in the lipid bilayer [88, 109]. The iron-dependent promotion of lipid peroxidation is a significant characteristic of ferroptosis. Proteins, including transferrin, transferrin receptor 1 (TFR1), and ferritin, are crucial in regulating intracellular iron concentration. The disruption of iron homeostasis increases the labile iron pool (LIP), leading to ROS overproduction through the Fenton reaction [110]. In addition, many factors modulate intracellular glutathione (GSH) levels during ferroptosis [111]. System Xc⁻ is a cystine/glutamate antiporter system crucial in transporting cystine and glutamate. The inhibition of system Xc⁻ decreases cysteine availability, thereby reducing the levels of GSH. The decline in GSH concentrations significantly decreases antioxidant capacity, facilitating lipid peroxidation. Furthermore, glutathione peroxidase 4 (GPX4), a significant indicator of ferroptosis, is an essential enzyme for eliminating lipid hydroperoxides and protecting cells from oxidative stress. The GPX4 relies on reduced GSH as a cofactor to catalyse the conversion of lipid hydroperoxides into alcohols. The depletion of GSH results in the inactivation of GPX4 during the process of ferroptosis, which subsequently leads to the accumulation of lipid peroxides and cell death [112].

According to recent studies, specific components related to ferroptosis, such as GPX4 and the system Xc⁻ transporter, were involved in transplanted kidneys. Animal models have shown that inhibiting ferroptosis through modulating GPX4 or system Xc⁻ transporter enhanced graft survival [113, 114]. Similar to kidney transplantation, studies have demonstrated that the preoperative serum ferritin level is an independent risk factor for recipients with liver transplantation, and ferroptosis significantly contributed to the development of hepatic IR injury [115, 116]. While there is limited evidence regarding ferroptosis in lung transplantation, it has been suggested that IR-induced ferroptosis in the early stage triggers excessive inflammation, leading to lung injury in animal models [117]. Consequently, these findings

highlight the potential involvement of ferroptosis in organ transplantation, emphasising the need for further research to elucidate underlying mechanisms.

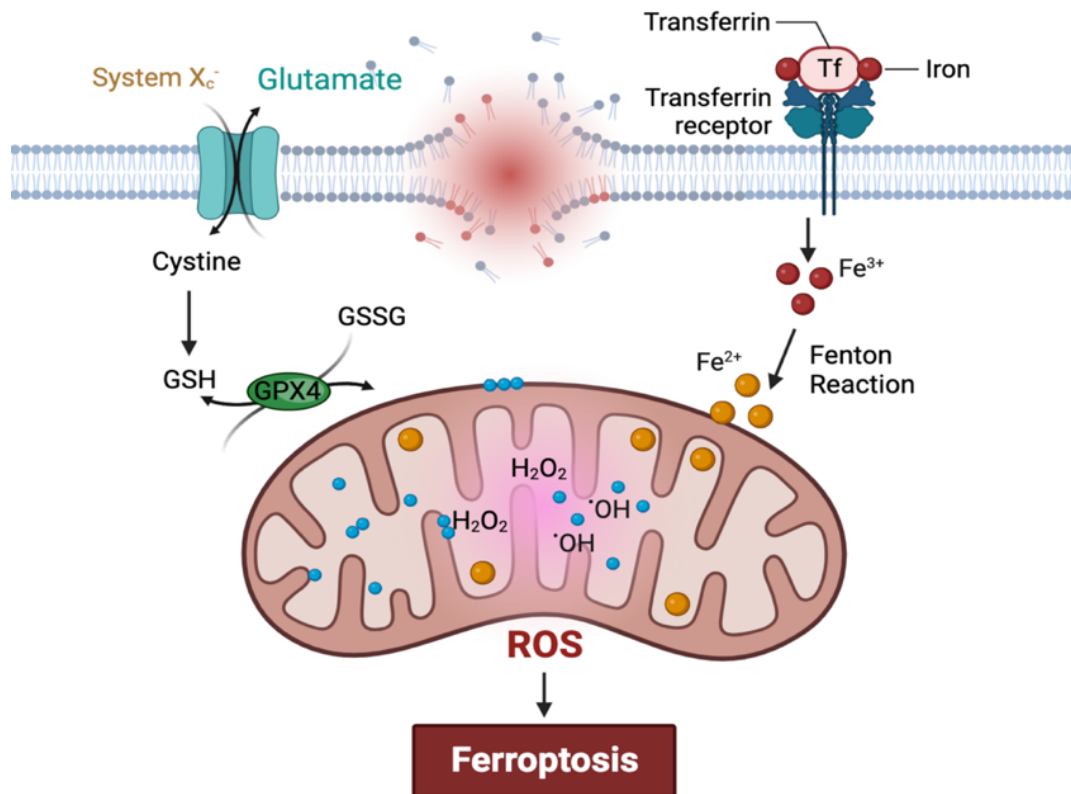


Figure 2.5 The Molecular Mechanism of Ferroptosis.

Transferrin imports Fe³⁺ (red ball) from the extracellular environment to the cytoplasm through TfR1 recognition, and then Fe³⁺ is converted to Fe²⁺ (yellow ball) through Fenton Reaction. The excess iron catalyses the production of ROS that contributes to lipid peroxidation within the cell membrane. When system X_c⁻ is inhibited, the source of cysteine is reduced, and then the amount of GSH decreases. GSH depletion causes the inactivation of GPX4, which results in the accumulation of lipid peroxides and subsequent ferroptosis.

2.4.4 Pyroptosis and NLRP3 inflammasome

Pyroptosis is a highly inflammatory form of regulated cell death that releases pro-inflammatory cytokines and forms pores in the plasma membrane [118]. The critical molecular machinery involved in pyroptosis is the NLRP3 inflammasome, a multi-protein complex that detects cellular stress and initiates the inflammatory cascade [119]. Two main pathways, the canonical and non-canonical pathways, have been identified for pyroptosis (**Figure 2.6**). In the canonical pathway, immune cells recognise pathogen-associated molecular patterns (PAMPs) or DAMPs through pattern recognition receptors (PRRs). This recognition triggers NLRP3 inflammasome assembly, which consists of NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and pro-caspase-1 [120]. Upon activation, NLRP3 undergoes conformational changes and recruits ASC, activating caspase-1. Activated caspase-1 cleaves gasdermin D (GSDMD), a critical effector protein in pyroptosis. Cleaved GSDMD forms pores in the plasma membrane, resulting in cell swelling, membrane rupture, and release of intracellular contents. The inflammatory cytokines, such as IL-1 β and IL-18, promote inflammation and contribute to the inflammatory response associated with pyroptosis. The non-canonical pyroptosis pathway is typically initiated by detecting cytosolic lipopolysaccharide (LPS). Upon sensing cytosolic LPS, caspase-4, -5, or -11 are activated, leading to a series of downstream events of pyroptotic cell death.

Additionally, mitochondrial dysfunction can lead to the release of mitochondrial DNA, which acts as a danger signal and triggers inflammasome activation. Although there is limited clinical research on the role of pyroptosis in organ transplantation, a previous study conducted in rodents suggested that the expression of pyroptosis biomarkers, NLRP3 and caspase-1, was higher in donated hearts from DCD mice than DBD mice after warm ischaemia. These elevations were decreased after treating donors with NLRP3 [121]. Furthermore, the increased expression of the NLRP3 inflammasome, induced by the HMGB1-TLR4 pathway, was also involved in kidney injury and sterile inflammation in high-risk donated kidneys [122]. Interestingly, in a rat lung graft normothermic perfusion model, it was observed that circulating leukocytes derived from donated lungs played a crucial role in impairing the quality of lung grafts through the activation of pyroptosis. When a caspase-1 inhibitor was administered to inhibit leukocyte pyroptosis, the release of immunological molecules and pro-inflammatory cytokines was significantly reduced. This, in turn, reduced IR injury and the incidence of PGD following lung transplantation [123]. These findings highlight the potential of targeting pyroptosis as a therapeutic strategy to reduce organ injury.

Notably, activation of the NLRP3 inflammasome accompanying pyroptosis can also be activated during necroptosis and ferroptosis. Recent findings indicated that specific necroptosis-related proteins may trigger the activation of NLRP3 inflammasome. For example, the necroptotic executor MLKL may act as an intrinsic stimulator of the NLRP3 inflammasome, activating NLRP3 in a GSDMD-independent manner and subsequently secreting inflammatory cytokines [124]. Additionally, inhibition of caspase-1 resulted in direct activation of NLRP3 by MLKL *via* K⁺ efflux in response to intracellular dsDNA. Previous studies have shown that MLKL regulated the NLRP3 inflammasome by directly activating the adapter protein ASC or NF- κ B

transcription factor. Therefore, the NLRP3 inflammasome may be involved in the inflammatory response triggered by necroptosis [125]. Furthermore, a potential association between the NLRP3 inflammasome and ferroptosis has been reported. The inhibitory effect of GPX4 on NLRP3 inflammasome activation has been demonstrated through GSDMD and caspase-11-dependent mechanisms [126]. This observation suggested that GPX4 may have an inhibitory influence on pyroptosis. Moreover, initiating the NLRP3 inflammasome may also induce several types of regulated cell death, such as ferroptosis and necroptosis, leading to inflammatory and immunological reactions. The process of stimulation and activation within this cycle is believed to substantially impact the progression of acute organ damage and dysfunction after transplantation.

2.2 Comparison of apoptosis, necroptosis, ferroptosis, and pyroptosis

	Apoptosis	Necroptosis	Ferroptosis	Pyroptosis
Morphological Feature	Plasma membrane blisters, cell shrinkage, nuclear condensation and chromatin margination, apoptotic bodies	Cell swelling, plasma membrane disruption, cytoplasmic vacuolisation, mitochondrial damage	Shrunk and electron-dense mitochondria, increased mitochondrial membrane density, decreased mitochondrial volume, plasma membrane disruption	Cell swelling, plasma membrane disruption, cell lysis, cytoplasmic vacuolisation, formation of pyroptotic bodies (inflammatory bodies)
Biochemical Feature	Caspase activation	Rip-regulated, MLKL phosphorylation	Iron metabolism	Inflammasome activation, caspase-1 and GSDMD activation
Activation Condition	DNA damage, ROS overload,	Severe oxidative	Depletion of GSH, Dysfunction of GPX4,	LPS, NLRP3 and other

	death receptor activation, infection and immune response, developmental process	stress, Ca ²⁺ overload, death receptor activation	Iron accumulation, lipid peroxidation	inflammasome bodies
Key genes	Caspase-3, Bcl-2, Bax, Fas	Rip1, Rip3, MLKL, CYPD, LEF1	GPX4, SCL7A11, P53, FSP1, ACSL4, VDAC2/3, TFR1, FTH1, FTL	Caspase-1, IL-1, IL-18, GSDMD
Relevant Disease	Cancer, Autoimmune disease	Infection, neurodegeneration disease, inflammatory disease, trauma, toxins	Cerebral stroke, cancer, acute kidney/liver injury, neurodegeneration disease	Infection, inflammation, neurodegeneration disease, cardiovascular disease, cancer

2.4.6 Cell death induced-plasma membrane injury and membrane resealing

Regulated cell death, such as necroptosis and ferroptosis, can induce plasma membrane injury, which refers to the disruption or permeabilisation of the outer membrane of cells [127, 128]. The intracellular contents, such as enzymes and cytokines, can be released, exacerbating organ injury and inflammation. Plasma membrane resealing is an essential mechanism that allows cells to maintain cellular integrity and survival [129, 130]. Minor membrane pores less than a nanometer in diameter may spontaneously reseal. Membrane pores with a diameter of more than a few nanometers cannot be resealed but require the help of membrane repair pathway activation [131]. Three membrane resealing pathways have been identified: lipid-patching, endocytosis & exocytosis, and macro-vesicle shedding [131]. Regarding membrane pores induced by necroptosis and ferroptosis, current evidence showed that macro-vesicle

shedding and exocytosis participated in membrane resealing, which could maintain cellular integrity and reduce cell death [132, 133]. There are several critical factors needed for membrane resealing [131, 134] during regulated cell death: (1) Internal membrane lipids from Golgi and endoplasmic reticulum (ER) as raw materials to fill in the plasma membrane pores; (2) membrane fusion proteins, including Annexin, endosomal sorting complexes required for transport (ESCRT), and dysferlin as catalysis to promote membrane repair; (3) cytoskeletal proteins like F-actin and tubulin as “bridges” and “roads” to transfer membrane perfusion proteins and internal membrane lipids to the wounded plasma membrane.

Recently, it has been reported that ESCRT-III machinery played a central role in repairing damaged plasma membranes by ensuring extracellular shedding of membrane pores in necroptotic cells [133, 135]. When cell death pathways are active, cytoskeletal proteins like F-actin and alpha-tubulin are rapidly anchored on the inner side of the wounded plasma membrane for transporting ESCRT-III to the damaged areas. Subsequently, the ESCRT-III outwardly deforms the damaged plasma membrane, followed by forming membrane micro-vesicles that are shed off to reseal plasma membrane pores. Finally, when the membrane pores are resealed, the ESCRT-III detaches from the plasma membrane and back to the cytoplasm (**Figure 2.7**). Therefore, promoting the plasma membrane resealing function of cells during organ transplantation can be a potential therapeutic strategy to improve transplant outcomes by preventing the release of intracellular contents *per se*.

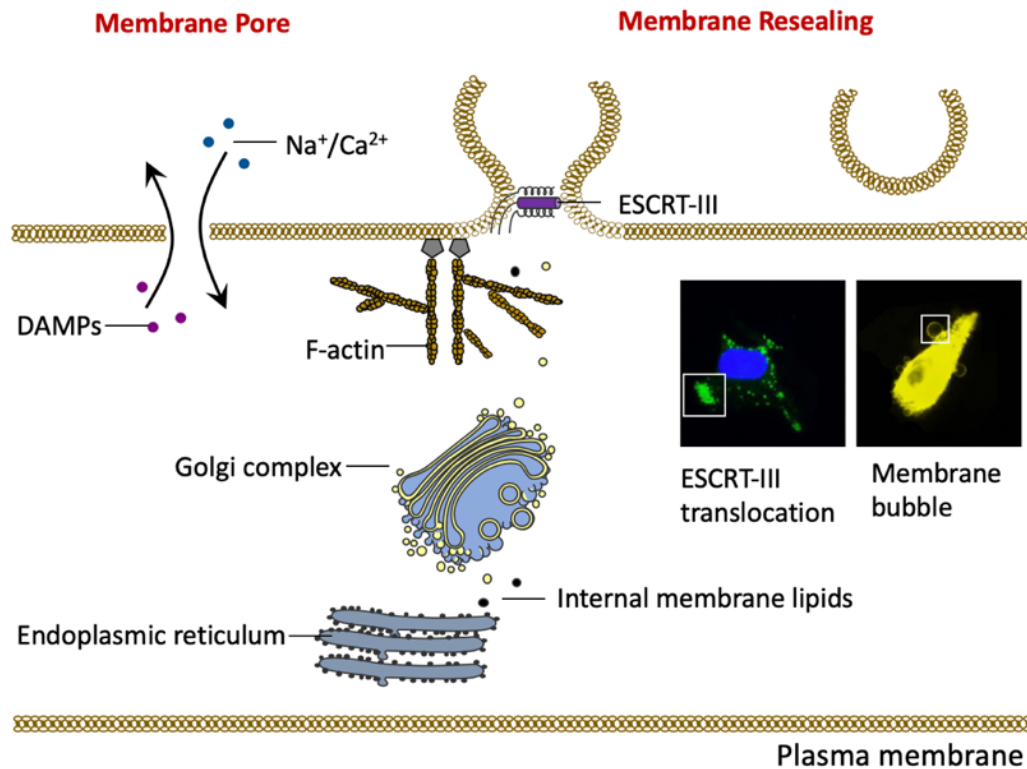


Figure 2.7 Membrane Resealing Pathway during Necroptosis.

Membrane pores form due to necroptosis. Cytoskeletal proteins move to the cell periphery, promoting ESCRT-III accumulation at the wounded area. The ESCRT-III deforms the wounded plasma membrane into outwardly forming pinched vesicles to close and remodel the injured area with the help of internal membrane lipids provided by the Golgi complex and endoplasmic reticulum. When the membrane pore closes, the ESCRT-III dissociates from the plasma membrane, and the necroptotic cell can be resuscitated.

2.5 Dexmedetomidine and graft protection

Dexmedetomidine (Dex) is a highly selective α_2 -adrenergic receptor ($\alpha_2\text{AR}$) agonist and exerts sedative and analgesic effects by stimulating $\alpha_2\text{AR}$ in the brain [136]. $\alpha_2\text{AR}$ has three subtypes (A, B and C), with the $\alpha_{2A}\text{AR}$ and $\alpha_{2C}\text{AR}$ subtypes widely distributed throughout the central nervous system (CNS), while the $\alpha_{2B}\text{AR}$ subtype is distributed at a low concentration in certain regions of the CNS and most peripheral tissues [137]. In 1999, the United States Food and Drug Administration (FDA) authorised Dex for sedation and analgesia use in intensive care

(ICU). In 2008, the FDA approved Dex for pre- and intraoperative sedation in patients without tracheal intubation. Then, in 2009, it was approved for sedating patients undergoing general anaesthesia, tracheal intubation, and mechanical ventilation [138]. Dex has a specific effect as a sedative in that it induces sedation without producing substantial respiratory depression. In addition, Dex has also been used to reduce the need for anaesthetics and analgesics, such as opioid drugs, in the treatment of pain [139].

The underlying mechanism of the protective effect of Dex may be mediated by parasympathetic excitation, resulting in a dampened inflammatory response and oxidative stress [140, 141]. Specifically, Dex inhibits the excitability of the central nervous system by promoting acetylcholine (ACh) release and increasing parasympathetic activation, which regulates the immune system by reducing T cells and natural killer cell activity [140, 142]. Dex also regulates several physiological functions, such as neurotransmitter release, insulin secretion, and renal sodium reabsorption, by facilitating chemical channels and modulating genetic transcription [139]. Clinical and basic research recently found that Dex reduced oxidative stress and inflammatory responses, activating anti-apoptotic pathways, and protecting cells from IR injury [143, 144].

During kidney ischaemia, pretreatment with Dex decreased TLR4 expression in renal tubular cells, alleviated tubular cell oedema and death, and enhanced renal tubular function [145]. Through inhibiting vasopressin secretion, Dex improved diuresis at low doses in dogs [146]. Dex also inhibited the release of inflammatory cytokines, such as TNF- α , IL-1, IL-6, IL-8, and HMGB1, by activating the PI3K/Akt [147], ERK1/2 [148], and PKC signalling pathways [149]. In clinical studies, patients who received Dex as part of an analgesic regimen experienced a

significant increase in urine output within 24 h after surgery [150]. In addition, serum creatinine was decreased in patients treated with Dex, which persisted for one week following surgery [151]. Dex also increased the glomerular filtration rate, attenuating the ischaemic-related histological injury [152]. These results support the hypothesis that Dex may prevent IR-induced kidney injury.

Studies have also shown that the anti-inflammatory and anti-apoptotic effects of Dex are the underlying mechanisms in reducing liver IR injury [153]. In patients undergoing hepatectomy, studies validated the potential protective effects of preoperative injection of Dex on livers [154]. Hepatic IR can cause systemic stress during hepatectomy by blood flow occlusion. In the initial phase (the first 2 h of hepatic IR injury), hepatic Koopfer cells were activated and released oxidative substances, resulting in liver necrosis and apoptosis. During the second phase (the subsequent 6 h or more), neutrophils infiltrate other organs, such as the intestines and lungs, to cause damage. Dex may have anti-systemic stress by decreasing catecholamine, aldosterone, cortisol, and ACTH plasma levels during anaesthesia and surgery [155]. Other mechanisms by which Dex reduces transplanted liver damage include reducing nitric oxide synthesis levels, inhibiting epinephrine secretion, thereby alleviating liver ischaemia injury, reducing the number of neutrophils, and subsequently reducing endothelin-1 and proteases by reducing Intercellular Adhesion Molecule 1 (ICAM-1) [156].

The lung is highly susceptible to inflammation and IR injury. IR injury is always associated with increased oxidative stress, enhanced lipid peroxidation, and diminished antioxidant capacities, damaging pulmonary endothelial cells and epithelial cells and resulting in lung oedema [157]. In recent years, it has been observed that Dex has a lung-protective effect in numerous cases

of pulmonary IR injury by modulating the mechanism of pulmonary vasoconstriction and reducing the release of inflammatory cytokines. For instance, Dex can directly activate $\alpha_{2B}AR$ in vascular smooth muscle to enhance pulmonary vasoconstriction, thereby increasing the ventilation-to-perfusion (V/Q) ratio and oxygenation function [158]. In addition, Dex can increase the blood concentration of nitric oxide, decrease pulmonary shunting, and raise the partial pressure of oxygen (PaO₂) [159]. Dex can also substantially reduce the concentration of TNF- α and IL-6, inhibiting lung inflammation and minimising lung injury [160].

2.6 Noble gas and graft protection

2.6.1 Xenon (Xe)

Xe, with an atomic number of 54 and atomic weight of 131.3g/mol, exists in the atmosphere at > 0.0875 ppm and has a boiling point of -108.1°C. Its density and viscosity are 3.2 and 1.7 times greater than air, respectively [161]. The desirable features of Xe have led to many labelling it as an almost 'ideal' anaesthetic. It has a minimum alveolar concentration (MAC) of 63% in humans, and due to its low blood gas and brain-blood coefficients, rapid induction and emergence are possible with little to no toxicity and adverse effects on haemodynamic parameters in adults and children [162, 163]. Xe has been documented to have the added potential benefit of reducing anaesthesia-induced postoperative cognitive dysfunction in elderly patients compared to other inhalational anaesthetic agents that are commonly used, making it desirable for the ageing surgical patient population [164]. Furthermore, compared to widely used inhalational anaesthetic gases or propofol, Xe has been noted to lead to a higher mean arterial pressure, lower heart rate and cardiac output [165, 166].

There has been a great interest in the renoprotective properties of Xe. Pre-conditioning with 70% Xe gives rise to significant functional recovery with earlier creatinine normalisation and prolonged kidney survival time after unilateral severe warm IR injury in mice [167]. This benefit persists regardless of whether it is applied to donors or recipients. This is likely a result of Xe upregulating Hypoxia-inducible factor (HIF)-1 α in the mammalian target of the rapamycin (mTOR) pathway, leading to an increase in hypoxia-tolerating factors erythropoietin (EPO) and vascular endothelial growth factor (VEGF), an effect seen in inhalation of subanaesthetic doses. In *in vitro* models of hypoxia-glucose deprivation, Xe preconditioning exhibited remarkable cell-protective properties compared to other noble gases, of which Ar exhibited no effects and helium was found to be cytotoxic to renal tubular cells [168].

Xe has increasingly been shown to have anti-inflammatory effects. In a recent study using a mouse model of lupus nephritis, 5 weeks of 2 h of daily exposure to 70% Xe/oxygen was compared to 70% nitrogen/oxygen in female mice after induction of the development of accelerated and severe lupus nephritis [169]. The Xe group showed better overall renal function and histological appearances with reduced neutrophil infiltration. These effects were proposed to be mediated by inhibiting apoptosis, ROS formation, reduced glomerular deposition of IgG and component C3, and enhanced renal HIF-1 α expression. Proteomic analysis studies also showed a downregulated renal NLRP3 inflammasome-mediated cellular signalling in the Xe group.

2.6.2 Argon (Ar)

Ar, with an atomic number of 18 and an atomic weight of 39.95g/mol, makes up nearly 1% of the atmosphere and, therefore, is more abundant than the other noble gases. It has a boiling point of -185.9°C and is mainly extracted from liquefied air. Ar exhibits anaesthetic properties only under hyperbaric conditions of greater than 10 atmospheres, with its narcotic effects first noted in self-experiments by deep sea divers in the 1930s [161]. It is generally well-tolerated with no record of toxicity. The mechanism by which Ar induces anaesthesia is suggested to be via stimulation of γ -Aminobutyric acid sub-type A (GABA_A) receptors. This finding derived from experiments in rats investigating the narcotic potency of Ar after pretreatment with GABA-receptor antagonists, which showed that the narcotic potency of Ar increased after pretreatment with GABA_A-receptor antagonists [170].

Evidence of an Ar-saturated storage solution in protection against IR injury in the setting of renal transplantation is less established compared to Xe. Nonetheless, it has been suggested that treatment of the graft preservation solution could also lead to better early functional recovery, graft quality and survival. Creatinine clearance post-transplantation was significantly higher on Days 7 and 14 in the group with Ar-saturated cold-storage solution in comparison to groups with storage solution saturated with air or nitrogen and when compared to Xe. Urinary albumin was also significantly lower than other groups [171]. In a pig model of heterotropic renal autotransplantation, the supplementation of pure Ar in preservation solution showed acute and long-term (21 days) positive impacts on IR injury with significantly better-preserved tissue characteristics with fewer hallmarks of IR injury in the histology [172]. Many therapeutic benefits were seen in the Ar group, such as improved functional recovery shown by creatinine clearance and sodium excretion, earlier diuresis and improved

postoperative survival at 21 days. In the Ar group, authors noted increased heat shock protein (Hsp)27 expression and, therefore, a simulated antioxidant cell response, which aligns with its known properties in preventing apoptosis [173].

In a recent study using a model of multiorgan failure through aortic cross-clamping in rabbits, attenuated renal injuries were seen in the group administered a mix of Ar/oxygen (70/30%) at the time of reperfusion [174]. This finding was thought to be due to HMGB1 systemic release inhibition, the levels of which were reduced in the blood. A similar effect was seen when the HMGB1 inhibitor glycyrrhizin was administered pre-reperfusion to a group that inhaled nitrogen/oxygen (70/30%). This mechanism would be plausible, given that HMGB1 activates innate immunity following ischaemic cell death. Nonetheless, a previous study also found that postconditioning either with 70% Xe or Ar did not give rise to any significant differences in renal graft function, graft injury or survival compared to the control group in a pig model of autotransplantation [175]. In this study, inhalation of the respective gases began 5 min before reperfusion and continued for 2 h post-reperfusion. As this is a method of postconditioning, it would mean that the deleterious IR injury molecular cascade has already started, and postconditioning could be easily implemented in clinical practice. These findings suggest the possibility of longer periods of organoprotective gas inhalation having more profound effects and, therefore, should be investigated in further studies to characterise the concentration-response relationship and cumulative effects.

2.7 Hypothesis and Aims

2.7.1 Hypothesis

Based on the current literature, we hypothesise that *ex vivo* grafts (kidney, liver, and lung) obtained from DCD donors undergo cold preservation, resulting in warm and cold ischaemia injury to grafts. These injuries, in turn, lead to graft damage and sterile inflammation of the tissues. Dex and noble gases supplemented in the graft preservation solution may protect DCD grafts from IR injury by reducing the activation of regulated cell death, such as apoptosis, necroptosis, and ferroptosis. In addition, to simulate the clinical scenario of IR injury during transplantation, we implemented an *in vitro* model using lung epithelial cells, hepatocytes, and tubular cells subjected to hypothermic hypoxia-reoxygenation. We hypothesise that the saturation of Dex and noble gases (Xe and Ar) in the graft preservation solution can mitigate IR-induced regulated cell death through molecular mechanisms of anti-oxidative stress, anti-inflammation, and enhancement of plasma membrane self-resealing function.

2.7.2 Aims

1. The first objective is to examine the activation of the necroptosis pathway and plasma membrane disruption in human lung epithelial cells (A549 cell line) subjected to hypothermic hypoxia-reoxygenation conditions. We aim to explore the effects of Dex on the recruitment of membrane resealing-related proteins, which may improve plasma membrane resealing function and subsequently reduce necroptosis-induced membrane injury.
2. The second objective is using the human kidney tubular cells (HK2 cell line) to investigate the impact of hypothermic hypoxia-reoxygenation on the activation of various regulated cell

death pathways, including apoptosis, necroptosis, ferroptosis, and pyroptosis. We aim to examine the inflammatory responses during the reoxygenation stage.

3. Our 3rd objective is to use a pig DCD model to examine the potential benefits of applying different concentrations of Xe and Ar with 0.1nM Dex into the UW solution. We aim to determine whether this combination provides better protection to mitigate IR injury and reduce regulated cell death, therefore improving cold preservation of graft quality.

4. Our 4th objective is using a pig DCD model to investigate whether the Dex, Xe, and Ar supplementation to the UW preservation solution provides increased protection against ischaemia injury during liver cold preservation. This includes reducing liver structural damage, inhibiting the activation of regulated cell death, and reducing oxidative stress. In addition, using human hepatocellular carcinomas (HepG2 cell line), we also aim to assess whether combining these agents in a cold preservation solution reduces hypothermic hypoxia-reoxygenation injury by inhibiting regulated cell death and improves antioxidant functions in the cells.

Chapter THREE

Methodology

In vitro and *ex vivo* experiments with various experimental techniques were applied in my PhD study project.

3.1 Cell culture

The human alveolar epithelial cells (A549 cell line), human proximal tubular cells (HK2 cell line), and human hepatoblastoma Cells (HepG2 cell line) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in a 25 cm² cell culture flask (Sigma-Aldrich, Poole, UK) with fresh Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium; Gibco, Billings, USA) or Dulbecco's Modified Eagle Medium (DMEM; Gibco, Billings, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Paisley, UK) and 1% penicillin-streptomycin (PS; Invitrogen, Paisley, UK) in a humidified incubator at 37°C with 5% CO₂ balanced with air. The medium was changed every two days following washing with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, USA). When the flask reached 80% confluency, the cells were washed with PBS and dissociated with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, USA) for 5 min at 37°C. Neutralised the trypsin-EDTA with cell culture medium, and then the cells were translocated into a centrifuge tube (Invitrogen, Paisley, UK) and centrifuged at 1500 rpm for 5 min for subculturing.

3.2 TLQ treatment

The following substances were used in the various experiments.

E. coli-derived human TNF-alpha protein (TNF- α): TNF- α (R&D system, Minnesota, USA) was diluted in sterile PBS containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, Poole, UK) to 0.1 mg/mL. The cells were treated with the final concentration of TNF- α at 150 ng/mL accordingly [176].

LCL161: LCL161 is a small molecule second mitochondrial activator of caspase (SMAC)

mimetic, potentially inhibits IAPs and shows both pro-apoptotic and anti-proliferation effects. In the present study, LCL161 (MedChemExpress, Cambridge, UK) was diluted in DMSO (Sigma-Aldrich, Poole, UK) to 1 mM. In the experiments, the cells were treated with diluted LCL161 at 10 nM [176].

Q-VD-Oph: Q-VD-Oph is a potent pan-caspase inhibitor and potentially inhibits apoptosis. In the present study, Q-VD-Oph (MedChemExpress, Cambridge, UK) was diluted in DMSO (Sigma-Aldrich, Poole, UK) to 1 mM. In the experiments, the cells were treated with diluted LCL161 at 10 nM, as reported previously [176].

After verifying the health and confluency, the A549 cells were washed with pre-warm sterile PBS and were then treated with TLQ composed of 150 ng/mL TNF- α , 10 nM LCL161, and 10 nM Q-VD-Oph for 5, 15, 30, 60, and 120 min respectively at 37°C to induce necroptosis.

3.3 Hypothermic hypoxia-reoxygenation treatment

The hypothermic hypoxia-reoxygenation model was used in the present study to simulate graft IR injury. After the health and confluency of the cells were verified, A549, HK2, and HepG2 cells were washed with PBS. The cells were then applied with the pre-cold University of Wisconsin (UW) cold storage solution (Bridge to Life Ltd, Columbia, USA) and kept in a gas chamber flushed with 95% N₂ and 5% CO₂ and quickly transferred the chamber into the fridge at 4 °C for 24 h to simulate the cold storage stage. The cells were washed with PBS and incubated with fresh pre-warmed RPMI1640 or DMEM medium supplemented with 10% FBS and 100U/mL PS according to cell line types. The cells were then transferred to an incubator at 37°C with air balanced with 5% CO₂ to simulate the reperfusion stage (**Figure 3.1**).

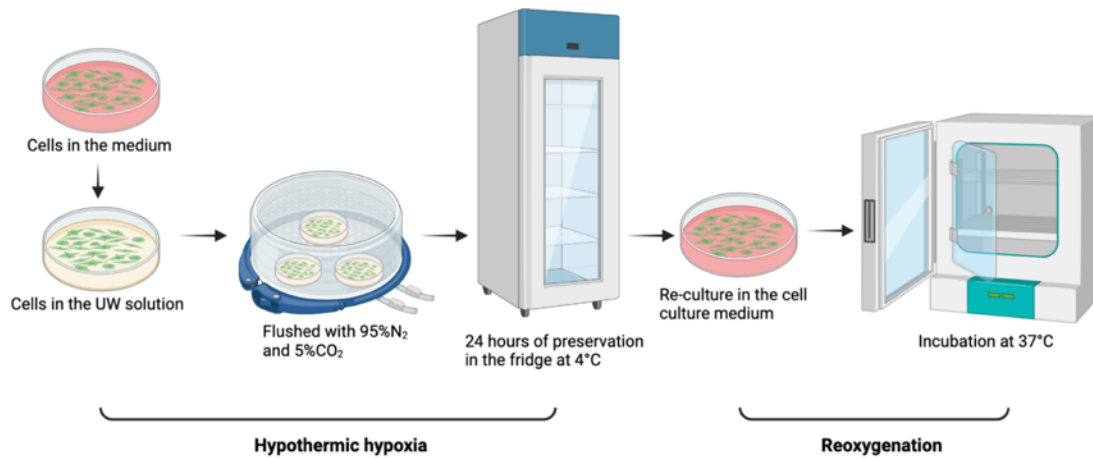


Figure 3.1 Procedures of hypothermic hypoxia-reoxygenation.

The cell culture medium was changed to cold UW solution and moved the cells to a chamber flushed with 95% N₂ and 5% CO₂ at 4°C in a fridge for 24 h, then re-cultured with the cell culture medium in an incubator at 37°C for different periods.

3.4 Animals and graft cold preservation

3.4.1 Cold preservation solutions

The following substances were used in the present study.

Dexmedetomidine: Dexmedetomidine (Dex; Sigma-Aldrich, Poole, UK) was diluted in sterile saline to 0.1 µM for storage. The 0.1 µM Dex was applied to the UW solution with a ratio of 1:1000 to the final concentration at 0.1 nM reported previously [177].

Gas mixtures: The gas mixtures were prepared in cylinders with 45% O₂ and 5% CO₂ and balanced with N₂ (Heruijia BioMed, Chongqing, China). Eight gas mixtures as follows were used in this study:

- 1) 50% N₂, 45% O₂, and 5% CO₂.
- 2) 0.1 nM Dex, 50% N₂, 45% O₂ and 5% CO₂.
- 3) 0.1 nM Dex, 30% Xe, 45% O₂, 20% N₂, and 5% CO₂.

- 4) 0.1 nM Dex, 30% Ar, 45% O₂, 20% N₂ and 5% CO₂.
- 5) 0.1 nM Dex, 15% Xe, 15% Ar, 45% O₂, 20% N₂ and 5% CO₂.
- 6) 0.1 nM Dex, 50% Xe, 45% O₂ and 5% CO₂.
- 7) 0.1 nM Dex, 50% Ar, 45% O₂ and 5% CO₂.
- 8) 0.1 nM Dex, 25% Xe, 25% Ar, 45% O₂ and 5% CO₂.

Gases were bubbled to 250 mL UW cold storage solutions supplemented with 0.1 nM Dex at 1 L/min for 20 min using a gas-washing bottle (ZAUBEE, Chengdu, China). Gas flow was monitored by a gas flowmeter (ZAUBEE, Chengdu, China) throughout. All solutions were kept at 4 °C in sealed glasses wrapped with aluminium foil to avoid light (**Figure 3.2A**).

3.4.2 Animals and graft extraction

The experimental protocol was approved by the Third Military Medical University Biomedical Ethics Committee (Approval No. AMUWEC20210209), and adhered to the International Guidelines for Animal Experimentation[178] and followed the ARRIVE guidelines. Male 15-17 kg experimental mini pigs were obtained from the Laboratory Animal Centre of Army Medical University, Chongqing, China. Doctors from the Department of Anesthesiology of Southwest Hospital, Third Military Medical University, Chongqing, China, performed all surgical procedures on animals.

The animals fasted for 8 h. Local lidocaine anaesthetic cream was applied to the skin behind the ear in the dorso-cranial aspect of the neck and subcutaneously injected with 500 µg/kg midazolam (Pfizer, New York, USA) through the auricular vein for sedation [179]. Animals were then admitted to the animal operating room, where they received non-invasive blood

pressure (BP), blood oxygen saturation (SPO₂), and electrocardiogram (ECG). 1-2 L/min oxygen was given *via* an animal face mask. After a peripheral intravenous line was established through the left auricular vein, anaesthesia was induced with intravenous injection of 20 µg/kg atropine (Mingsheng Medicine, Zhejiang, China), 2 mg/kg propofol (AstraZeneca, Cambridge, UK) and 100 µg fentanyl (Renfu Medicine, Hubei, China)[180]. Heparin (Dongcheng Pharma, Shandong, China) at 300 IU/kg was administered intravenously 5 min before cardiac death induction [179].

Cardiac death was induced by intravenous injection of 600 mg propofol (AstraZeneca, Cambridge, UK) and then 20 mg atracurium (Nhwa Pharmaceutical Co., Ltd, Jiangsu, China). The cardiac death was confirmed when the circulatory arrest for 5 min [181]. The clamshell thoracotomy was performed to expose the lungs and heart. The left atrial appendage was incised for venting blood. A 12-F catheter (Byram Healthcare, New York, USA) connecting the infusion apparatus was inserted into the main pulmonary artery and flushed the pulmonary circulation with 500 mL warm saline with 5000 U heparin. Meanwhile, the precava and postcava were incised for venting blood, and another 12-F catheter was inserted into the aorta; 1000 mL warm saline with 10000 U heparin flushed the renal and hepatic vascular [181]. After flushing, the pigs were left at room temperature for *in situ* warm ischaemia. The kidney and liver were extracted 30 min after cardiac death (**Figure 3.2B**).

3.4.3 Graft cold preservation

The extracted kidneys and livers were washed with cold saline, then sectioned into 0.5-1.0 cm slices, and placed in a tube containing prepared cold UW preservation solution. Sealed the tube and transferred the tube to a box with ice water and stored in a fridge at 0-4 °C for 24 or

72 h. Non-cold storage control grafts were fixed using 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) or stored in liquid nitrogen immediately after extraction (**Figure 3.2C**).



Figure 3.2 Procedure of pig graft cold storage.

(A). The UW solution was supplemented with 0.1 nM Dex and then bubbled with gas mixtures for 20 min at 1L/min. The prepared UW solutions were in glass bottles wrapped with aluminum foil to avoid light and kept in a fridge at 0-4 °C. (B). Experimental mini pigs were sedated and anaesthetised; cardiac death was induced by injection of 600mg propofol and 20 mg atracurium. The circulatory was flushed with heparin water, and then the kidneys and livers were extracted and kept in cold saline. (C). Kidneys and livers were sectioned into 0.5-1 cm slices and preserved in a tube containing prepared cold UW storage solution. Sealed the tube and transferred the tube to a box with ice water and stored in a fridge at 0-4 °C for 24 or 72 h.

3.5 Histology

3.5.1 Paraffin wax embedding and sectioning

Kidney and liver samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) for 24 h at room temperature. Dehydrated the tissues in 70% ethanol (Sigma-Aldrich, Poole, UK) for 4 h, 90% ethanol for 3 h, and 100% ethanol for 3 h, and then merged the tissues in xylene (Sigma-Aldrich, Poole, UK) for 3 h. Embedded the tissues in paraffin wax for 1 h at 60°C and sectioned them into 5µm slices using a microtome. Slices were floated in distilled water at room temperature and then mounted onto gelatine-coated glass slides. Slices were stored in a slide box and ready for use (**Figure 3.3**).

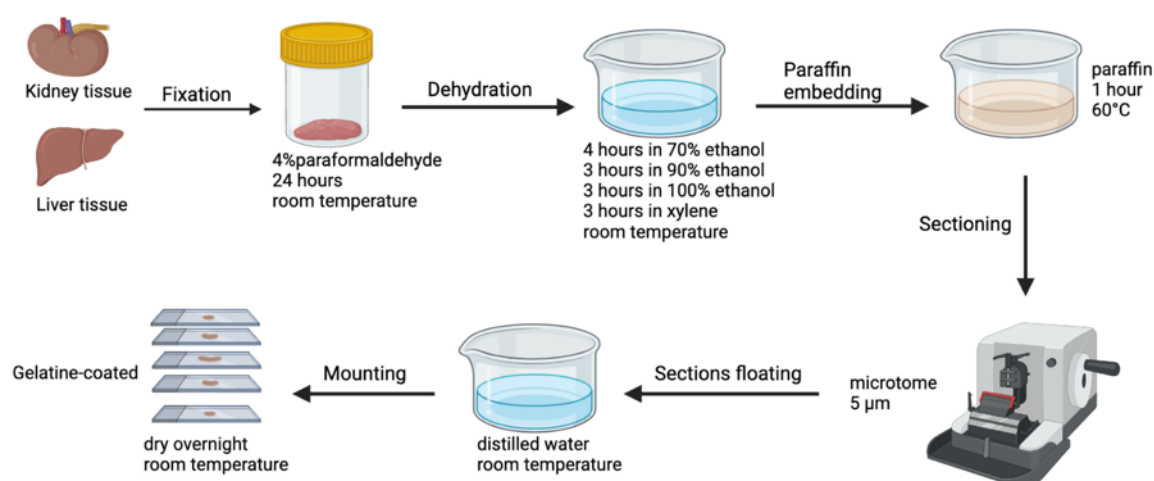


Figure 3.3 Procedure of tissue paraffin embedding and sectioning.

Cold-preserved kidney and liver tissues were fixed in 4% paraformaldehyde for 24 h at room temperature. Then they were dehydrated in graded ethanol and xylene by the following steps: 1. 4 h in 70% ethanol; 2. 3 h in 90% ethanol; 3. 3 h in 100% ethanol; 4. 3 h in xylene at room temperature. Tissues were then embedded in paraffin for 1 h at 60°C and sectioned into 5 µm slices using a microtome. Slices were floated in distilled water at room temperature and then mounted onto gelatine-coated glass slides overnight for drying.

3.5.2 Deparaffinised and antigen-retrieval

Paraffined tissue sections were deparaffinised by merging into xylene for 30 min and then rehydrated by graded 100%, 95%, 70%, 50% ethanol and deionised water. Slides were then moved to a cooker pot with citrate antigen-retrieval buffer (Santa Cruz, Dallas, USA) and boiled for 30 min. Then, the slides were cooled down with cold tap water (**Figure 3.4**). Drew a circle on the slide around the tissue with a hydrophobic barrier pen before use.

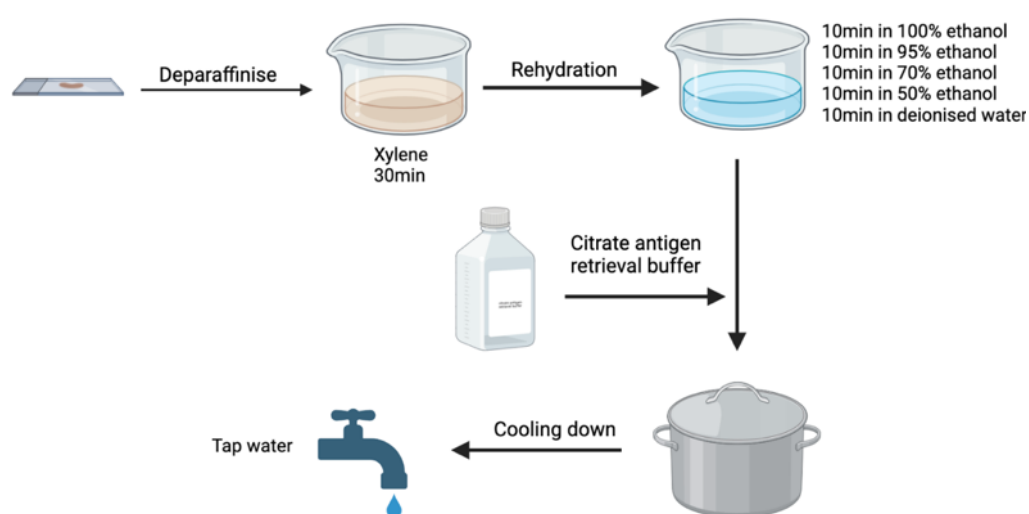


Figure 3.4 Procedure of tissue deparaffinised and antigen-retrieval

Paraffined tissue sections were deparaffinised by merging into xylene for 30 min at room temperature and then rehydrated in graded ethanol and deionised water by following steps: 1. 10 min in 100% ethanol; 2. 10 min in 95% ethanol; 3. 10 min in 70% ethanol; 4. 10 min in 50% ethanol; 10 min in deionised water. After rehydration, slides were moved to a cooker pot with citrate antigen-retrieval buffer and boiled for 30 min. Then, the slides were taken out and cooled down with cold tap water.

3.5.3 Hematoxylin and eosin (H&E) staining

The sections were deparaffinised, nuclei were stained with haematoxylin (Sigma-Aldrich, Poole, UK) for 3 min, and the extracellular matrix and cytoplasm were stained with eosin (Sigma-Aldrich, Poole, UK) for 30 sec. The sections were then rinsed and dehydrated through

graded alcohols. At last, the sections were mounted in synthetic resin (Sigma-Aldrich, Poole, UK). All sections were visualised under a widefield microscope at x 20 magnification (Zeiss Axio Observer, Jena, Germany), and 10 pictures were captured randomly for each section. Damage to the renal cortex or livers was scored from 0 to 4 degrees [182]. The main damage criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Detailed scoring is shown in **Table 3.1**. Tubular and glomerular cell death rating was calculated in individual tubules/ glomeruli (10 tubules/picture, 2 glomeruli/picture), and an average was calculated for the samples.

Table 3.1 Kidney and liver damage scoring criteria.

Score	0	1	2	3	4
Damage area	Normal	<25%	25-50%	50-75%	>75%

3.5.4 Periodic Acid Schiff (PAS) staining

The PAS staining kit was purchased from Abcam (ab150680, Cambridge, UK). After washing with distilled water, the slides were incubated in Schiff's solution for 30 min. Then, the sections were rinsed and stained in hematoxylin for 3 min. After applying the bluing reagent for 30 sec and the light green solution for 2 min, the sections were dehydrated through graded alcohols and mounted in synthetic resin. All sections were visualised under a widefield microscope at x 20 magnification (Zeiss Axio Observer, Jena, Germany).

3.5.5 Reticulum staining

The reticulum staining kit (ab236473, Cambridge, UK) was obtained to demonstrate reticular fibres histologically. Reticular fibres are present throughout the body, with a particular

abundance in the liver, and aid in assessing the architectural structure of hepatic plates. The staining procedure followed the instructions provided by the manufacturer. Briefly, the slides were first washed with distilled water before being sequentially exposed to various solutions: acidified potassium permanganate for 1 min, potassium metabisulfite for 1 min, ferric ammonium sulfate for 30 sec, ammonium silver for 1 min, 20% formalin for 3 min, gold chloride for 5 min, sodium thiosulfate for 1 min, and nuclear fast red for 5 min. Subsequently, the slides were washed, dehydrated using graded alcohols, and mounted in synthetic resin. All sections were observed under a widefield microscope at a magnification of x20 (Zeiss Axio Observer, Jena, Germany).

3.5.6 Iron staining

The iron staining kit (ab150674, Cambridge, UK) was acquired from Abcam to detect ferric iron in tissues. The staining procedure followed the instructions provided by the manufacturer. In brief, the slides were incubated in the working iron stain solution for 3 min, followed by a nuclear fast red solution for 5 min. The sections were then rinsed, dehydrated using graded alcohols, and mounted in synthetic resin (Sigma-Aldrich, Poole, UK). All sections were observed under a widefield microscope at a magnification of x20 (Zeiss Axio Observer, Jena, Germany).

3.5.7 OxyIHC™ Oxidative Stress Detection

The OxyIHC™ Oxidative Stress Detection kit (Sigma-Aldrich, S7450, Poole, UK) evaluated oxidative stress in kidney and liver tissues. The sections were incubated in DNPH solution in the dark for 30 min, followed by a 30-min incubation in a blocking buffer at room temperature. Afterwards, the sections were treated with primary antibodies at a

concentration of 1:100 and incubated overnight at 4 °C. Subsequently, biotinylated secondary antibodies were applied and incubated with the sections. The slides were then subjected to a 10-min incubation with 3% H₂O₂ and a 30-min incubation with streptavidin-conjugated HRP. Afterwards, the slides were treated for 5 min with the DAB-A/B mixture and counterstained with haematoxylin. The slides were visualised using a widefield microscope (Zeiss Axio Observer) at a magnification of x20. For each section, 10 random images were captured.

3.5.8 Immunohistochemistry

The tissues or fixed cells were permeabilised by incubating in 0.1% Triton for 10 min. The tissue sections or cells were incubated with 5% donkey serum (Sigma-Aldrich, Poole, UK) in PBS containing 0.3% Triton X-100 (PBS-T) for 30 min at room temperature to block non-specific binding. The primary antibody, diluted in 1% donkey serum in PBS-T, was then added and incubated overnight at 4°C in a humidified chamber. Subsequently, the tissue sections or cells were washed thrice with 1% donkey serum in PBS-T for 5 min each. For immunofluorescence staining, a fluorochrome-conjugated secondary antibody was added and incubated at room temperature for 1 h, followed by three washes with 1% donkey serum PBS-T for 5 min each. Finally, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI) mounting solution (Vector Laboratories, Burlingame, USA) and prepared for examination. A biotin-conjugated secondary antibody was added and incubated overnight at 4°C in a humidified chamber for chromogenic staining. The sections were washed thrice with 1% donkey serum PBS-T for 5 min each. Next, the ABC-HRP reagent was added and incubated at room temperature for 1 h. A working solution of DAB was prepared and applied to the tissue sections. The reaction was monitored as the chromogenic reaction turned the epitope sites

brown. Subsequently, the sections were washed thrice in PBS for 5 min each. Finally, the sections were mounted using a mounting medium (Abcam, Cambridge, UK) and prepared for examination (**Figure 3.5**). The slides were visualised using a widefield microscope (Zeiss Axio Observer) at a magnification of x20, and 10 random images were captured for each section. The antibodies used in the immunohistochemistry were summarised in **Table 3.2**.

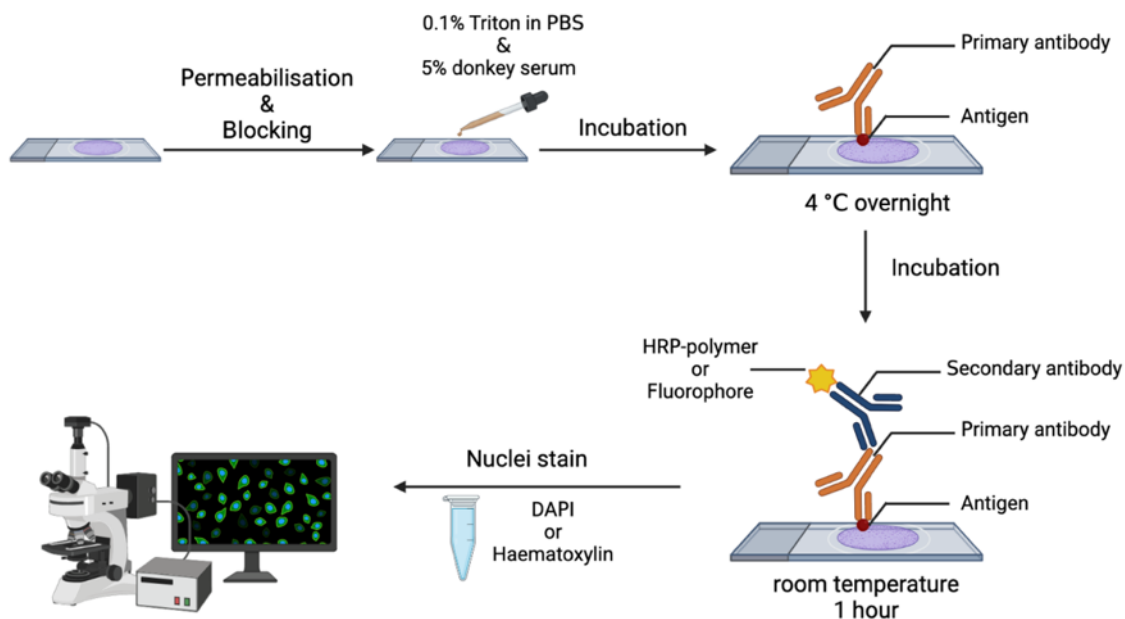


Figure 3.5 Procedure of Immunohistochemistry

Slides were incubated in a permeabilisation buffer for 10 min, followed by 5% donkey serum for 30 min in a humidified chamber to block non-specific antigens. The slides were then incubated overnight at 4°C with the primary antibody, covered with plastic coverslips. Afterwards, they were washed 3 times for 5 min each with the wash buffer. Next, the slides were incubated with either the HRP-polymer secondary antibody or the fluorochrome-conjugated secondary antibody in a humidified chamber for 1 h. Subsequently, they were washed 3 times for 5 min each with a PBS buffer. For nucleus staining, the slides were incubated with haematoxylin or DAPI. Finally, the sections were mounted in synthetic resin, and the cells were visualised using a microscope.

Table 3.2 Immunohistochemistry antibodies

Primary antibodies	Host species	Target species	Concentration	Source
pMLKL	Rabbit	Human, Mouse	1:200	Abcam
Annexin V	Rabbit	Human, Mouse	1:200	Abcam
Annexin V	Mouse	Human, Rabbit	1:200	Abcam
CHMP4B	Rabbit	Human, Mouse	1:200	Abcam
Grp78	Rabbit	Human, Mouse	1:100	Santa Cruz
CHOP	Rabbit	Human, Mouse	1:100	Santa Cruz
LAPM1	Mouse	Human, Rabbit	1:50	Abcam
GSDMD	Rabbit	Human, Mouse	1:200	Abcam
ACSL4	Mouse	Human, Rabbit	1:1000	Abcam
GPX4	Rabbit	Human, Mouse	1:200	Abcam
ASC	Rabbit	Human, Mouse	1:100	Santa Cruz
NLRP3	Mouse	Human, Rabbit	1:200	Abcam
HMGB1	Mouse	Human, Rabbit	1:200	Abcam
TXNIP	Rabbit	Human, Mouse	1:200	Abcam
Secondary	Host species	Target species	Concentration	Source
Donkey anti-Rabbit (Red)	Donkey	Rabbit	1:500	Abcam
Donkey anti-Rabbit (Green)	Donkey	Rabbit	1:500	Abcam
Donkey anti-Mouse (Red)	Donkey	Mouse	1:500	Abcam
Donkey anti-Mouse (Green)	Donkey	Mouse	1:500	Abcam

3.5.9 TUNEL assay

The TUNEL assay kit (Abcam, ab66108, Cambridge, UK) was used to detect DNA fragmentation as a marker of apoptosis. Kidney or liver sections and fixed cells were permeabilised with 20

$\mu\text{g/ml}$ proteinase K for 10 min. Subsequently, an equilibration buffer was applied for 10 min before adding the TdT reaction mix. The samples were then incubated in the dark at 37°C for 1 h. The slices were immersed in the SSC buffer for 15 min to stop the reaction. The slices were then treated with $1\ \mu\text{M}$ To-pro for 20 min, washed, and prepared for visualisation. The slices were visualised using a widefield microscope (Zeiss Axio Observer) at a magnification of x20. ImageJ software was employed for further processing. Apoptosis was quantified by determining the average percentage of TUNEL-positive (TUNEL⁺) cells per field of view. Nuclei were visualised through red To-pro fluorescence (**Figure 3.6**).

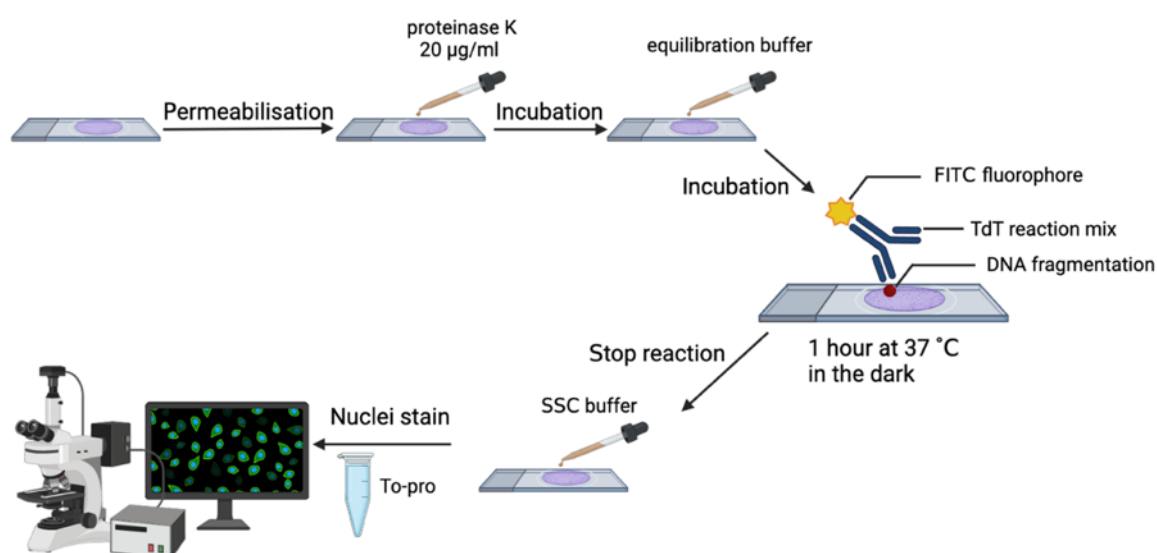


Figure 3.6 Procedure of TUNEL staining

Slides were incubated in permeabilisation buffer ($20\ \mu\text{g/ml}$ proteinase K) for 10 min, followed by incubation in equilibration buffer for 10 min. Then, the sections were incubated with a TdT mixture in a chamber at 37°C for 1 h and then incubated in the SSC buffer to stop the reaction. At last, the slides were stained with $1\ \mu\text{M}$ To-pro for 20 min to stain nuclei and mounted with mounting buffer and visualised the cells using a microscope.

3.6 Intracellular calcium measurement

Fura-2 AM Calcium Indicator (Abcam, ab120873, Cambridge, UK) is a cell-permeable, high affinity Ca^{2+} selective fluorescent dye and was used to detect intracellular Ca^{2+} . The A549 cells were washed and then incubated with $2\ \mu\text{M}$ of the Fura-2 AM in DMSO/HBSS (Gibco, Billings, USA) for 30 min at room temperature in the dark. Following the incubation, the cells were washed 3 times with PBS and re-cultured in the fresh cell culture medium (Gibco, Billings, USA) for up to 300 min to simulate reperfusion. Then, the cells were washed with HBSS (Gibco, Billings, USA) and fixed with 4% paraformaldehyde (Gibco, Billings, USA) at room temperature for 10 min. Subsequently, the cells were examined using a widefield microscope (Zeiss Axio Observer) at a magnification of x20, and 10 random images were captured for each section (Figure 3.7).

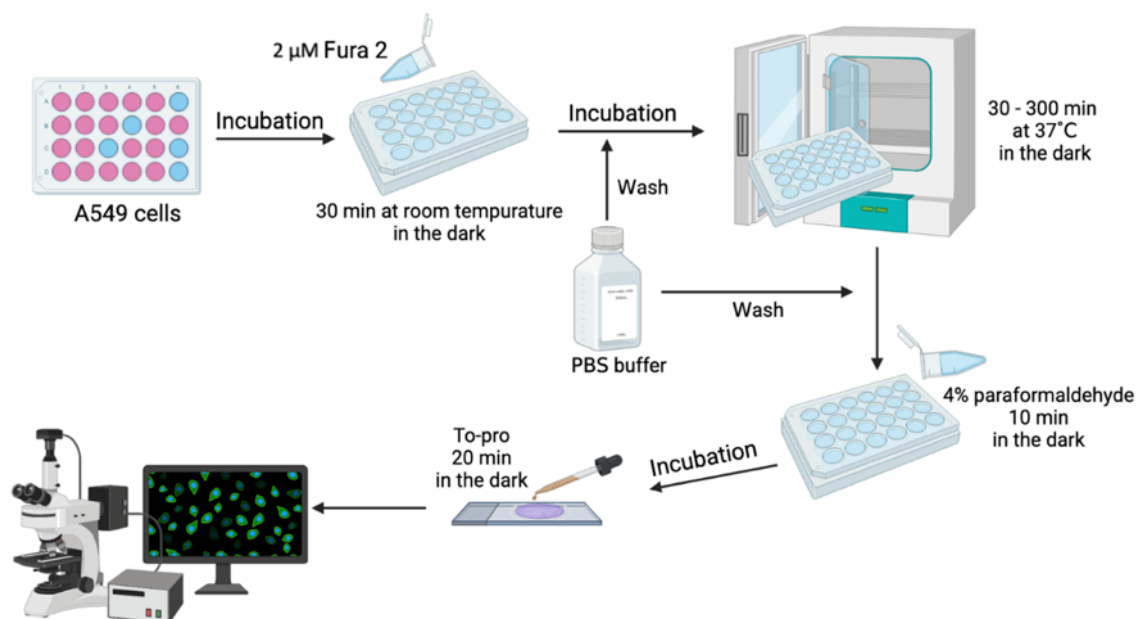


Figure 3.7 Procedure of intracellular calcium measurement

$2\ \mu\text{M}$ calcium indicator Fura-2 AM was added to a 24-well plate containing A549 cells. The plate was then incubated at room temperature in the dark for 30 min. Following the incubation, the cells were washed 3 times with PBS and recultured in the cell culture medium at 37°C for 30 to 300 min. After that, the cells were washed with PBS and fixed with 4% paraformaldehyde. The slides were treated

with 1 μM To-pro for 20 min to stain the nuclei. Finally, the slides were mounted using the mounting buffer, and the cells were visualised using a microscope.

3.7 Membrane lipid labelling

The Dil stain kit was purchased from Invitrogen (D3911, Paisley, UK). The staining medium was prepared by adding five μl of the Dil to 1 mL of cell culture medium (Gibco, Billings, USA). The cells were incubated with the staining medium in the dark for 20 min at 37°C. After being washed with PBS, cells were then fixed with 4% paraformaldehyde (Gibco, Billings, USA) for 10 min and examined using a Zeiss Axio Observer 7 (Zeiss, Watford, UK) microscope under constant exposure time (**Figure 3.8**).

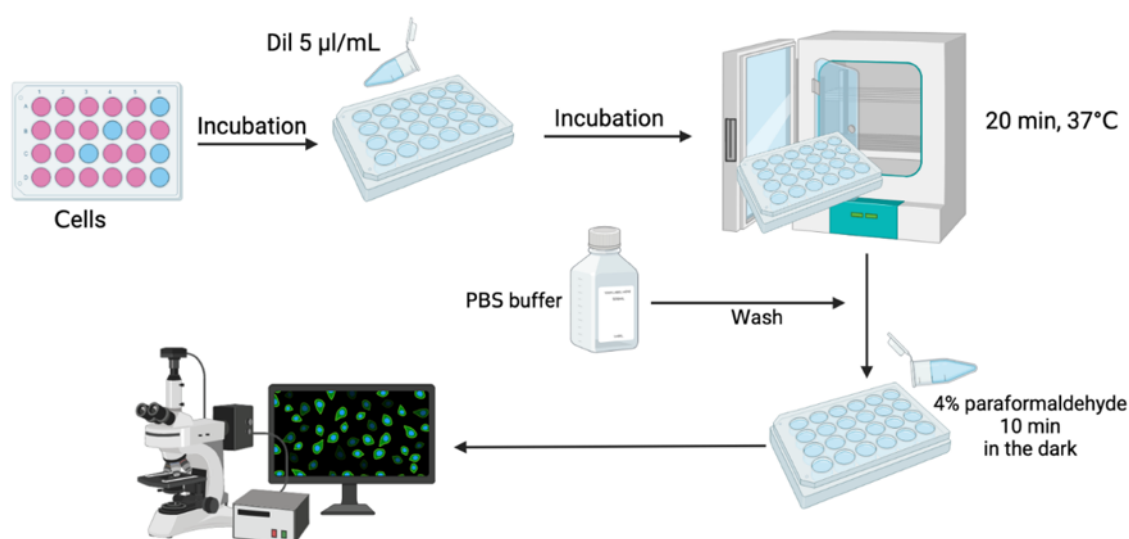


Figure 3.8 Procedure of membrane lipid labelling

25 $\mu\text{l}/\text{mL}$ Dil was added to the cell culture medium and incubated the cells for 20 min at 37°C in the dark. The cells were washed 3 times using PBS, fixed with 4% paraformaldehyde, and then washed. Visualised the cells using a microscope.

3.8 Western Blot

3.8.1 Protein extraction

Cell cultures were washed with cold PBS and then immersed in cell lysis buffer (Invitrogen, Paisley, UK) containing phosphatase inhibitor (Roche Holding AG, Basel, Switzerland) and protease inhibitor (Roche Holding AG, Basel, Switzerland) for 30 min on ice. The cells were transferred into microcentrifuge tubes (Invitrogen, Paisley, UK) by scraping and homogenising the cells with an ultrasonic probe (Soniprep 150, Measuring and Scientific Equipment, Heathfield, UK). The tubes were centrifuged at 10,000 g for 20 min at 4 °C to remove cell debris (**Figure 3.9**).

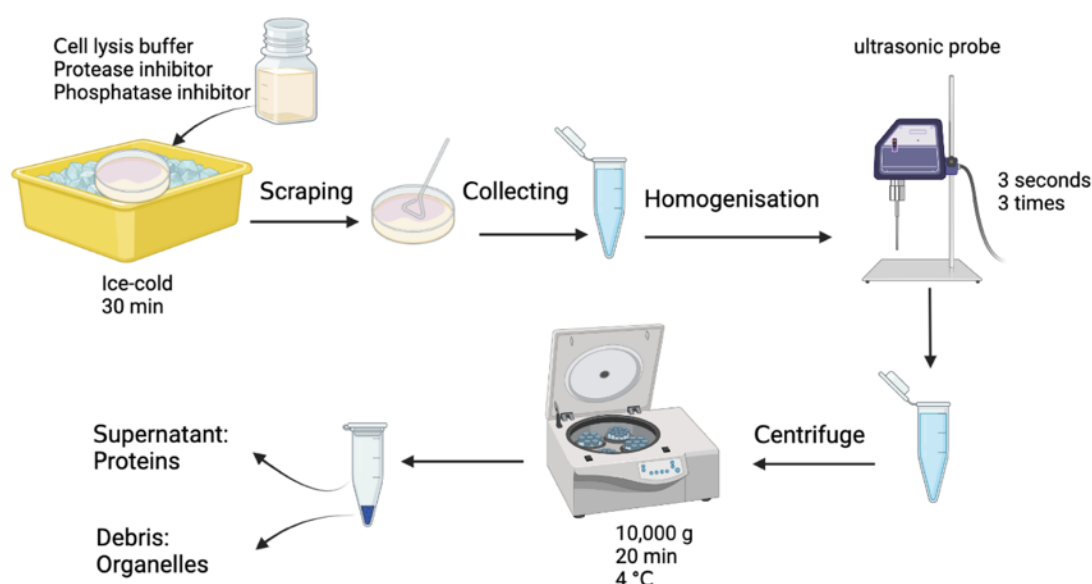


Figure 3.9 Procedure of protein extraction

Scrapped cells were immersed in an ice-cold cell lysis solution composed of cell lysis buffer, protease inhibitors, and phosphatase inhibitors. Then, the cells were homogenised using an ultrasonic probe and centrifuged with 10,000g at 4 °C for 20 min. The supernatant was then collected for blotting.

3.8.2 Protein blotting

After being heated with NuPAGE lithium dodecyl sulphate (LDS) sample buffer (Invitrogen, Paisley, UK) at 98 °C for 10 min, the protein extracts (40 µg/sample) were loaded onto a

NuPAGE 4 to 12% Bis-Tris gel (Invitrogen, Paisley, UK) for electrophoresis. Transferred the gel to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Paisley, UK) and blocked with 5% BSA (Invitrogen, Paisley, UK) in TBS-T for 1 h at room temperature. Membranes were probed with 1:1000 primary antibodies in TBS-T overnight at 4 °C, followed by HRP-conjugated secondary antibody for 1 h. The loading control was the constitutively expressed protein, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or Smooth Muscle Actin (SMA). The blots were visualised using improved chemiluminescence (Santa Cruz, Dallas, USA). GeneSnap (Syngene, Cambridge, UK) was used to identify bands with the G:BOX ichemi image analyser, whilst ImageJ was used to assess band density. The expression of protein bands compared to non-cold-stored control samples was normalised to GAPDH expression. Additional adjustments were made to non-phosphorylated protein bands for phosphorylated protein bands.

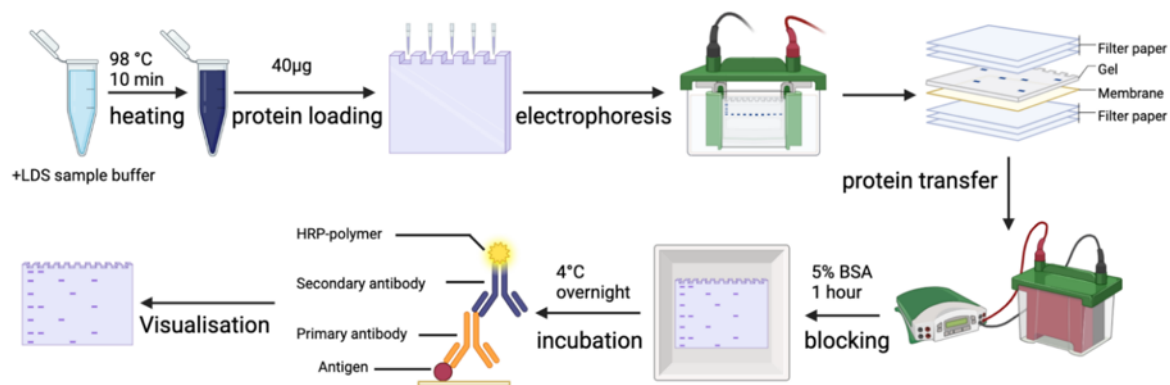


Figure 3.10 Procedure of protein blotting

Protein samples were heated with LDS sample buffer at 98°C for 10 min and then loaded 40 µg onto a NuPAGE gel for electrophoresis. Proteins were then transferred onto a PVDF membrane. After being blocked with 5% BSA for 1 h, the membrane was incubated with a primary antibody at 4°C overnight, followed by incubating a secondary antibody for 1 h. The membrane was then developed in luminol agent and visualised.

LDS: lithium dodecyl sulphate; PVDF: polyvinylidene difluoride; BSA: Bovine Serum Albumin.

Table 3.3 Western blot antibodies

Primary	Host	Target species	Concentration	Source
p-Rip3	Rabbit	Human,	1:1000	CST
Rip3	Rabbit	Human,	1:1000	CST
p-Rip	Rabbit	Human,	1:1000	CST
Rip	Rabbit	Human,	1:1000	CST
pMLKL	Rabbit	Human,	1:1000	CST
MLKL	Rabbit	Human,	1:1000	CST
Annexin V	Rabbit	Human,	1:1000	Abcam
CHMP4B	Rabbit	Human,	1:1000	Abcam
Grp78	Rabbit	Human,	1:1000	Santa Cruz
GSDMD	Rabbit	Human,	1:1000	Abcam
ACSL4	Mouse	Human, Rabbit	1:1000	Abcam
Caspase-1	Rabbit	Human,	1:1000	CST
Caspase-3	Rabbit	Human,	1:1000	CST
Cleaved-caspase-	Rabbit	Human,	1:1000	CST
Caspase-4	Rabbit	Human,	1:1000	CST
Caspase-5	Rabbit	Human,	1:1000	CST
Caspase-8	Rabbit	Human,	1:1000	CST
GPX4	Rabbit	Human,	1:1000	Abcam
ACSL4	Rabbit	Human,	1:1000	Abcam
ASC	Rabbit	Human,	1:1000	Santa Cruz
NLRP3	Mouse	Human, Rabbit	1:1000	Abcam
HMGB1	Mouse	Human, Rabbit	1:1000	Abcam
TXNIP	Rabbit	Human,	1:1000	Abcam
catalase	Rabbit	Human,	1:1000	CST
thioredoxin	Rabbit	Human,	1:1000	CST
SOD1	Rabbit	Human,	1:1000	CST
GAPDH	Rabbit	Human,	1:100000	Invitrogen
SMA	Rabbit	Human,	1:1000	CST

Secondary	Host	Target species	Concentration	Source
Donkey anti-Rabbit	Donkey	Rabbit	1:2000	CST
Donkey anti-Mouse	Donkey	Mouse	1:2000	CST

3.9 Crystal Violet staining

The cells were placed on ice and washed with PBS. Then, the cells were fixed with ice-cold 100% methanol (Sigma-Aldrich, Poole, UK). 0.5% crystal violet (Sigma-Aldrich, Poole, UK) solution in 25% methanol was added and incubated the fixed cells for 10 min. At last, the cells were washed with PBS and then dried.

3.10 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF- α , IL-1 β , IL-6, and HMGB-1 in cell culture medium were measured by ELISA (Abcam, Cambridge, UK). The cell culture medium or serial dilutions of standards were added in 96-well microtiter plates, coated with target protein-specific capture antibodies (1 μ g/mL) overnight at 4°C. A biotinylated antibody was added and incubated at 20°C for 90 min after washing. The plates were read at 492 nm following additional incubation with peroxidase-coupled streptavidin and revelation with orthophenylenediamine.

Chapter FOUR

**Dexmedetomidine elevates plasma membrane resealing function in
necroptotic lung epithelial cells**

4.1 Introduction

Lung transplantation is an effective treatment for patients with progressive respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis [183]. Despite advancements in surgical techniques, perioperative care, and immunosuppressive drugs, lung transplantation outcomes remain the worst compared to other organ transplantations. It is reported that the median survival rate for lung transplant patients is only 5.8 years [184]. Additionally, there has been a 42.2% growth in the waiting list for lung transplants in America over the previous decade, indicating a persistent gap between the demand for lung grafts and the number of available donors [185]. In the UK, only 64% of patients received a lung transplant after 5 years of joining the waiting list, which is the lowest in all solid organ transplantations [186]. Furthermore, COVID-19-related life-limiting fibrosis and respiratory failure that require advanced life-supporting treatments or lung transplantation have provided concerns for exacerbating the shortage of lung donors [187].

IR injury is the primary reason for the low utilisation of lung grafts due to the high risk of developing primary graft dysfunction (PGD) or acute rejection after lung transplantation [188]. PGD is a significant cause of transplant failure during the early postoperative period, affecting approximately 20% to 35% of cases [189]. Effective preservation strategies to reduce lung graft IR injury will improve graft function before transplantation while expanding the lung donor pool [190].

Lung epithelial cells are particularly vulnerable to toxic metabolites and inflammatory mediators due to their high metabolic demands when reperfusion occurs. This vulnerability

leads to various lung injuries, including acute respiratory distress syndrome (ARDS) and other pulmonary complications [191]. IR injury induces activation of several cell death pathways, including necrosis and regulated cell death, such as necroptosis and ferroptosis [192, 193]. Plasma membrane rupture is a crucial characteristic of regulated cell death. Following plasma membrane disruption, cells may undergo a de novo production of damage-associated molecular patterns (DAMPs) and immunological and proinflammatory molecules for a prolonged period. Consequently, these molecules initiate an inflammatory response and trigger an immunological reaction inside the body [87, 194]. Therefore, exploring the underlying molecular mechanisms of regulated cell death may contribute to developing treatments for improving patient outcomes.

The potent and selective α_2 -adrenoceptor agonist Dex, with sedative and analgesic effects, has been used in clinical settings for anaesthesia, analgesia and sedation [195, 196]. In addition, Dex exerts both anti-apoptotic and anti-inflammatory properties [143, 144]. Furthermore, many studies have shown that Dex had protective effects against IR injury in vital organs like the brain [197], lung [177], heart [198], liver [199], kidney [200] and intestine [201]. The underlying mechanism may be due to its parasympathetic excitation, resulting in a dampened inflammatory response and anti-apoptosis and oxidative stress [140, 141]. Therefore, this chapter investigated whether hypothermic hypoxia-reoxygenation leads to necroptosis and plasma membrane disruption in human lung epithelial cells (A549 cell line). And whether Dex modulates membrane fusion protein ESCRT-III recruitment, helping repair the wounded plasma membrane and further reducing necroptosis-induced membrane injury.

4.2 Experimental design

4.2.1 Investigating the protective effect of Dex on human lung tissues

Lung tissues from patients with lung tumours undergoing lobectomy were cold-stored in the UW solutions supplemented with 0.1 nM Dex for 16 h. H&E staining was conducted to evaluate tissue morphology. Apoptosis and necroptosis were assessed by immunofluorescence. Organelles in lung epithelial cells and endothelial cells were examined by transmission electrical microscope.

4.2.2 Investigating the time course of hypothermic hypoxia-reoxygenation-induced necroptosis and membrane damage in A549 cells

A549 cells were preserved in the preservation solution at 4°C for 24 h and then cultured at 37°C in fresh medium for 6, 12, 18, and 24 h. (hypothermic hypoxia-reoxygenation). Meanwhile, the cell cultures were stimulated with standard necroptosis inducer TLQ (150 ng/mL TNF- α , 40 μ M Q-VD-Oph, and 10 μ M LCL161) for 5, 15, 30, 60, and 120 min served as the positive controls. Immunofluorescence staining of p-MLKL and DAPI was conducted to detect necroptosis activation. Western blot was performed to evaluate necroptosis activation by detecting pRip3 and pMLKL. Immunofluorescence staining of Annexin V and DAPI was conducted to detect phosphatidylserine exposure to assess plasma membrane damage. Immunofluorescence staining of CHMP4B and DAPI was conducted to detect CHMP4B accumulation in the plasma membrane.

4.2.3 Investigating the time course of hypothermic hypoxia-reoxygenation-induced NLRP3 activation

A549 cells were preserved in the preservation solution at 4°C for 24 h and then cultured in the fresh warm medium at 37°C for 6, 12, 18, and 24 h. Western blot was conducted to evaluate NLRP3 expression.

4.2.4 Investigating the time course of Ca²⁺ concentration during hypothermic hypoxia-reoxygenation in A549 cells

A549 cells were preserved in the preservation solution at 4°C for 24 h. 2 µM calcium indicator, Fura-2 AM, was added into the UW solution to incubate the cells in the dark for 30 min. Afterwards, the cells were cultured in the warm medium at 37 °C for 5 h. Finally, the fluorescence of cells was examined using a microscope under constant exposure levels.

4.2.5 Investigating the effects of Dex on endoplasmic reticulum (ER) stress and lysosome structure in A549 cells exposed to hypothermic hypoxia-reoxygenation

A549 cells were preserved in the preservation solution supplemented with 0.1 nM Dex or/and 1 nM α_2 -adrenoceptor antagonist atipamezole (Atip) at 0-4°C for 24 h. The cells were cultured in the cell culture medium at 37°C for 6 h. ER stress was evaluated with immunofluorescence by staining Grp78 and CHOP, and lysosome structure was observed by staining LAMP1.

4.2.6 Investigating the effects of Dex on PANoptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation

A549 cells were preserved in the preservation solution supplemented with 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h. After washing with PBS, the cells were cultured in the cell culture medium at 37°C for 6 h. Western blot was conducted to evaluate pyroptosis by detecting GSDMD, caspase-1, and caspase-5. Apoptosis was evaluated by detecting caspase-3, caspase-8, and Annexin V. Necroptosis was evaluated by detecting pRip and pMLKL.

4.2.7 Investigating the effects of Dex on NLRP3 expression in A549 cells exposed to hypothermic hypoxia-reoxygenation

A549 cells were preserved in the preservation solution supplemented with Dex or/and 1 nM Atip at 0-4°C for 24 h and then cultured in the cell culture medium at 37°C for 6 h. Western blot was conducted to evaluate NLRP3 expression.

4.2.8 Investigating the effects of Dex on plasma membrane integrity in A549 cells exposed to hypothermic hypoxia-reoxygenation

A549 cells were stained with DiLC18 (1:200 dilution) for 15 min. After washing with PBS, the cells were preserved in the preservation solution at 4°C for 24 h. The cells were cultured in the cell culture medium at 37°C for 6 h. The cell plasma membrane was observed using microscopy under constant exposure levels.

4.3 Results

4.3.1 Dex protected human lung tissue during cold storage

The supplementation of Dex into the UW solution significantly enhanced the morphology of the human lungs (Figure 4.1A) and reduced the lung injury score (Figure 4.1B, $p < 0.01$). Dex also decreased the number of TUNEL⁺ apoptotic cells (Figure 4.1C and D, $p < 0.05$) and the expression of the necroptosis biomarker p-MLKL during cold storage (Figure 4.1E and F, $p < 0.05$). Furthermore, cold storage resulted in the marginalisation of nuclear chromatin and the expansion of the endoplasmic reticulum (ER) in lung epithelial cells (Figure 4.1G) and endothelial cells (Figure 4.1H). There was also an observed increase in the production of mitophagosomes and phagosomes. The apical surface had a significant reduction in the presence of microvilli, whereas the type II epithelial cells showed a decrease in the observable lamellar bodies (LB). These injuries were mitigated when the UW solution was supplemented with Dex (Figure 4.1 G and H).

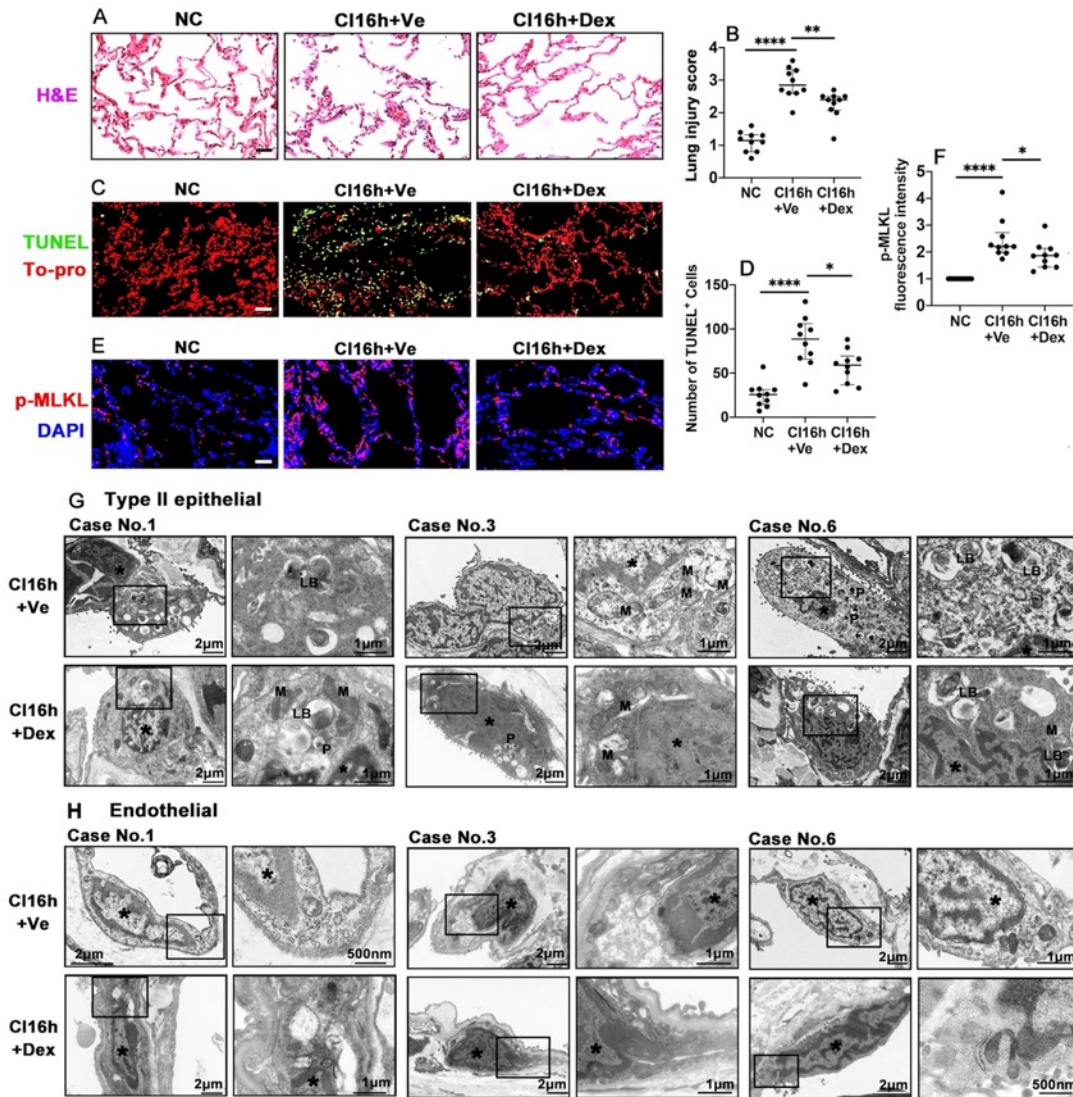


Figure 4.1 Dex supplementation protects human lung tissue during cold storage.

Lung tissue from the patient was cut into 3 pieces, 2 pieces were stored in 4 °C UW solution saturated with Dex (0.1 nM) or PBS (Ve) for 16 h (lung graft cold ischemia CI16h), separately; another piece was served as the naive control (NC). **(A)** Histology (H&E staining) of the lung tissue. **(B)** Lung injury score. The injury criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Scores 0: normal, 1: <25%, 2: 25-50%, 3: 50-75%, 4: > 75% percentage area. **(C)** Lung tissue apoptotic cell death was detected by in situ TUNEL assay. **(D)** The number of TUNEL⁺ cells. Labelling of **(E)** p-MLKL (red) and fluorescent intensity **(F)** p-MLKL in lung tissue. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) or To-pro (red). Scale bar: 50 µm. Data are presented as scatter plots and expressed median with interquartile range (n = 10); *p < 0.05, **p < 0.01 and ****p < 0.0001. Transmission electron microscopy was performed on lung samples from patients No. 1, 3, and 6; **(G)** type II epithelial cells and **(H)** endothelial cells were observed. Asterisk: chromatin condensation; P: phagosome; LB: lamellar body; M: mitochondrial.

4.3.2 Hypothermic hypoxia-reoxygenation activated necroptosis in A549 cells

MLKL phosphorylation is considered a key marker of necroptosis. To investigate whether hypothermic hypoxia-reoxygenation induces necroptosis, A549 cells were preserved in the UW solution at 4°C for 24 h and then cultured in fresh culture medium for 6, 12, 18, and 24 h, respectively. Other A549 cohort cultures were treated with TLQ for 5, 15, 30, 60, and 120 min at 37°C, which served as positive controls. The results showed that p-MLKL accumulated at the plasma membrane at 5, 15, 30, 60, and 120 min after TLQ treatment (Figure 4.2A). Hypothermic hypoxia-reoxygenation also activated MLKL and accumulated p-MLKL in the plasma membrane at 6, 12, and 18 h after reoxygenation (Figure 4.2B)

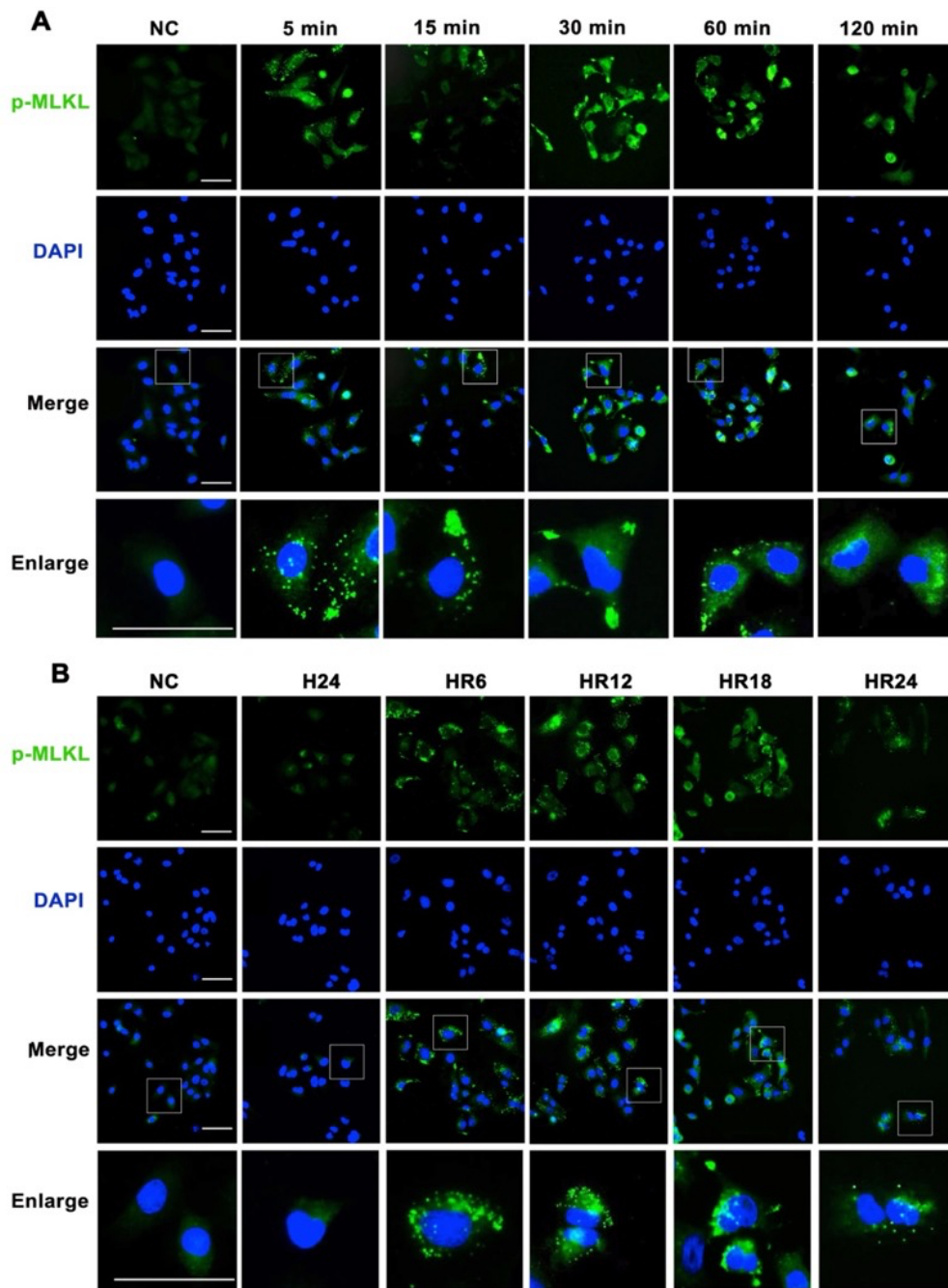


Figure 4.2 Hypothermic hypoxia-reoxygenation activates necroptosis time-dependently in A549

cells. (A) A549 cells were cultured in RPMI 1640 cell culture medium and then treated with TLQ (150 ng/mL TNF- α , 40 μ M Q-VD-Oph, and 10 μ M LCL161) necroptosis inducer for 5, 15, 30, 60, and 120 min. (B) A549 cells were cultured in RPMI 1640 cell culture medium, then preserved in the UW solution at 0-4°C for 24 h and recultured at 37°C in fresh medium for 6, 12, 18, and 24 h. Immunofluorescence staining of p-MLKL (green) and DAPI (blue) was conducted. $n = 5$. Scale bar: 50 μ m.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; DAPI: 4',6-diamidino-2-phenylindole.

4.3.3 Hypothermic hypoxia-reoxygenation damaged the plasma membrane in A549 cells

During necroptosis, the activation of MLKL triggers the scrambling of lipids, resulting in the exposure of phosphatidylserine on the membrane. The exposure to phosphatidylserine reveals the damage to the plasma membrane. During the period of membrane resealing, the cell surface releases small vesicles generated with the injured plasma membrane. These vesicles can be labelled with Annexin V. Therefore, a quantitative assessment of plasma membrane damage and resealing function was performed using immunofluorescent labelling of Annexin V. The results demonstrated that the administration of TLQ resulted in the release of Annexin V⁺ vesicles within 60 min. Furthermore, significant pores were found at 120 min after TLQ treatment. (Figure 4.3A). Under the hypothermic hypoxia-reoxygenation stimuli, the plasma membrane was much more seriously damaged with high phosphatidylserine exposure when compared with TLQ treatment. At the same time, Annexin V⁺ vesicles were also detected within 12 h after reoxygenation. However, no vesicles were found in the negative control (NC) or after hypothermic hypoxia (H24) (Figure 4.3B).

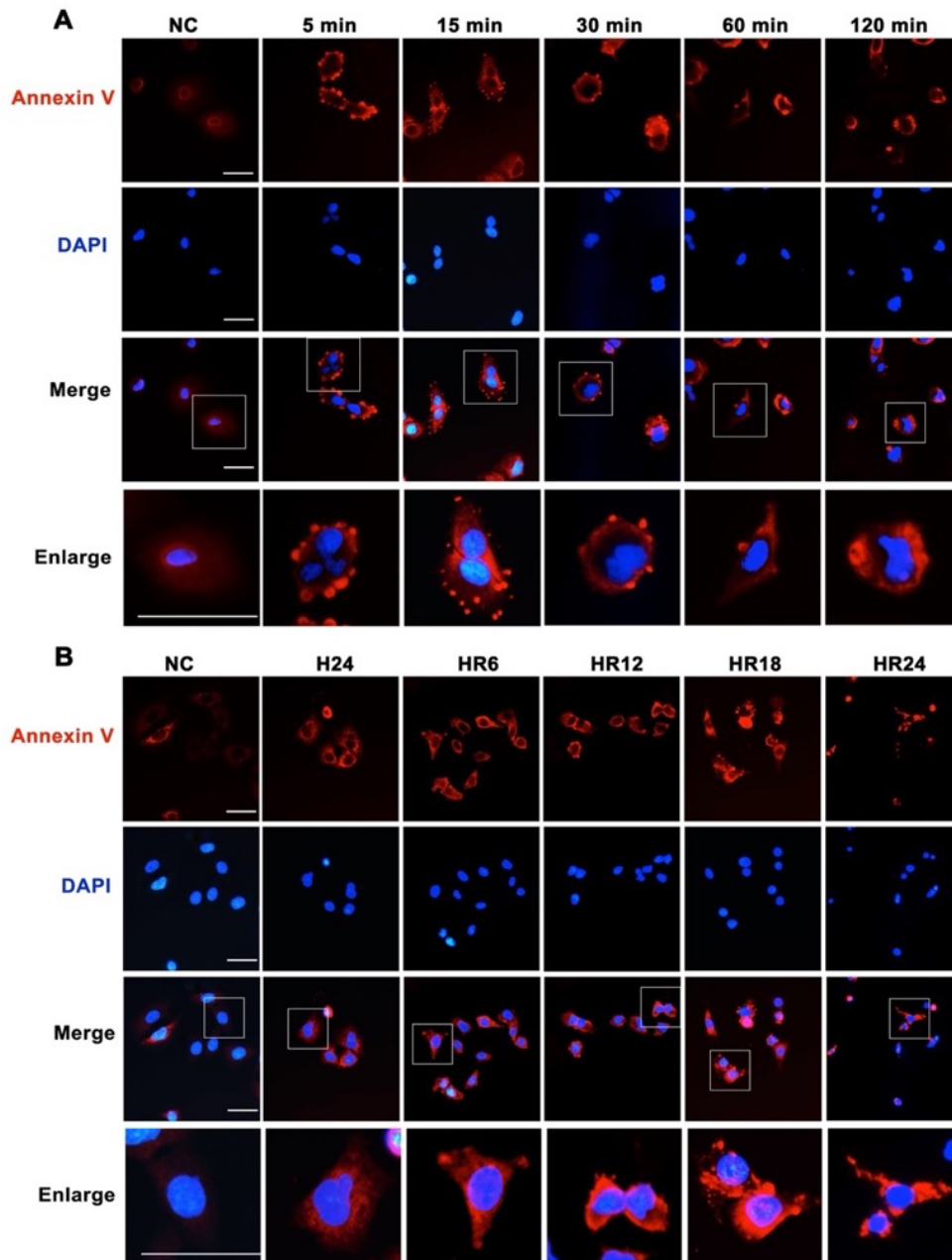


Figure 4.3 Hypothermic hypoxia-reoxygenation damages the plasma membrane in A549 cells. (A)

A549 cells were cultured in RPMI 1640 cell culture medium and then treated with TLQ (150 ng/mL TNF- α , 40 μ M Q-VD-Oph, and 10 μ M LCL161) necroptosis inducer for 5, 15, 30, 60, and 120 min. (B) A549 cells were cultured in RPMI 1640 cell culture medium, then preserved in the UW solution at 0-4°C for 24 h and recultured at 37°C in fresh medium for 6, 12, 18, and 24 h. Immunofluorescence staining of Annexin V (red) and DAPI (blue) was conducted. n = 5. Scale bar: 50 μ m.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia reoxygenation; DAPI: 4',6-diamidino-2-phenylindole.

4.3.4 CHMP4B translocated to the plasma membrane during necroptosis

CHMP4B is a subunit of ESCRT-III that participates in membrane repair by facilitating the formation of membrane vesicles. Thus, the location of CHMP4B was detected by immunofluorescent staining in the present study. The results showed that CHMP4B was recruited to the plasma membrane rather than cytoplasm after TLQ treatment within 30 min, suggesting membrane resealing may occur early (Figure 4.4A). A similar result was found during reoxygenation, where CHMP4B was relocated in the plasma membrane within 18 h, especially at 6 h after reoxygenation. At the same time, no CHMP4B relocation was found during hypothermic hypoxia (H24), which is consistent with the previous result that hypothermic hypoxia did not lead to necroptosis and membrane wound (Figure 4.4B).

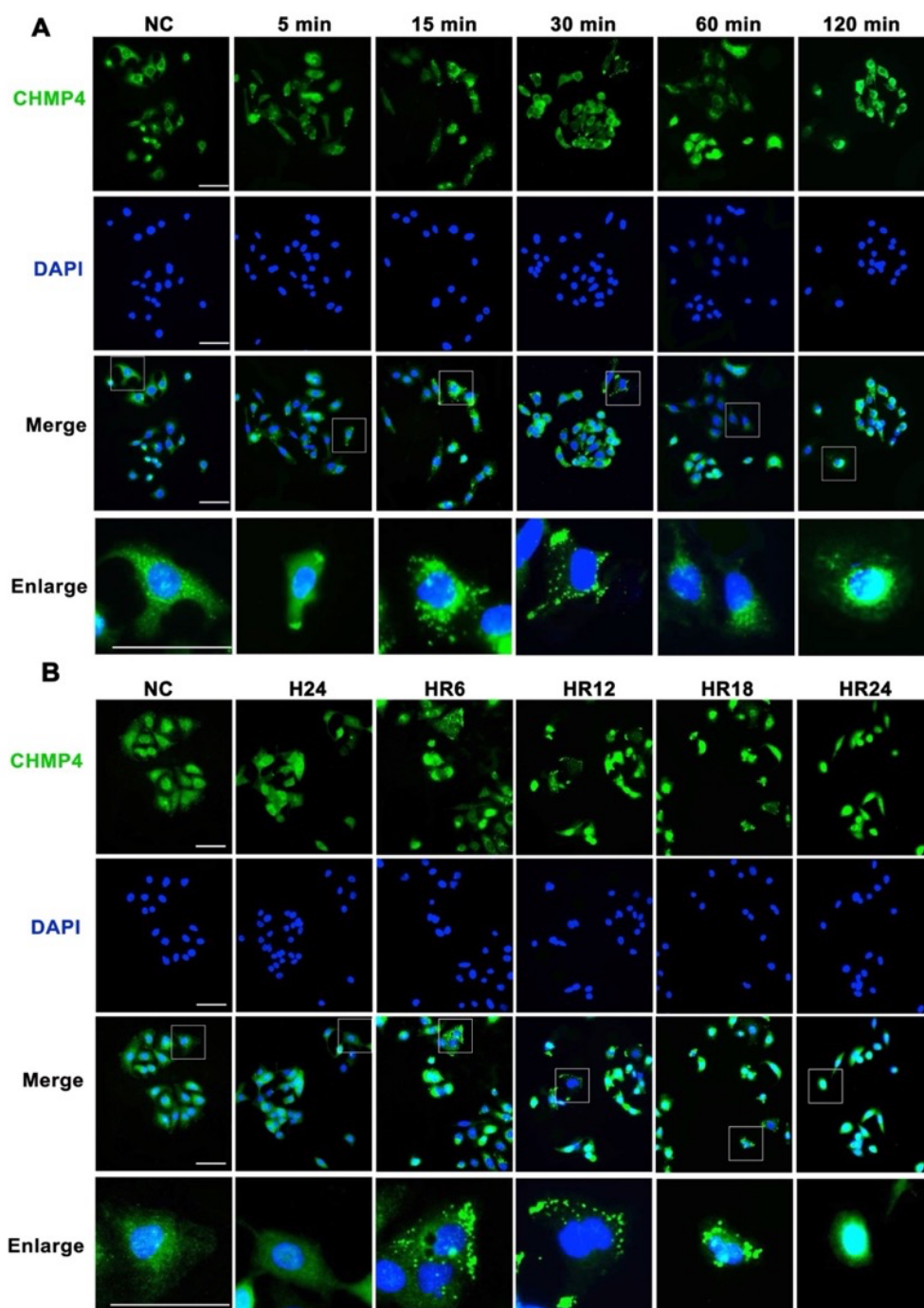


Figure 4.4 CHMP4B translocates to the plasma membrane during necroptosis. (A) A549 cells were cultured in RPMI 1640 cell culture medium and then treated with TLQ (150 ng/mL TNF- α , 40 μ M Q-VD-Oph, and 10 μ M LCL161) necroptosis inducer for 5, 15, 30, 60, and 120 min. (B) A549 cells were cultured in RPMI 1640 cell culture medium, then preserved in the UW solution at 0-4°C for 24 h and recultured at 37°C in fresh medium for 6, 12, 18, and 24 h. CHMP4B (green) and DAPI (blue) immunofluorescence staining were conducted. n = 5. Scale bar: 50 μ m. NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; CHMP4B: Charged multivesicular body protein 4b; DAPI: 4',6-diamidino-2-phenylindole.

4.3.5 Hypothermic hypoxia-reoxygenation induced necroptosis and inflammasome activation in A549 cells

Phosphorylation of Rip3 and MLKL was detected to evaluate necroptosis (Figure 4.5A).

Hypothermic hypoxia-reoxygenation significantly increased the expression of p-Rip3 (Figure 4.5B) and p-MLKL (Figure 4.5C), which both reached a peak at 6 h (HR6, $p < 0.001$) and then declined to the baseline at 24 h (HR24). NLRP3 was evaluated to assess inflammasome activation (Figure 4.5A). Results showed that the expression of NLRP3 was increased at 12 (HR12) and 18 h (HR18) after reoxygenation (Figure 4.5D, $p < 0.01$, separately), which was consistent with the timeline of necroptosis activation. Meanwhile, CHMP4B expression showed no change after reoxygenation (Figure 4.5E).

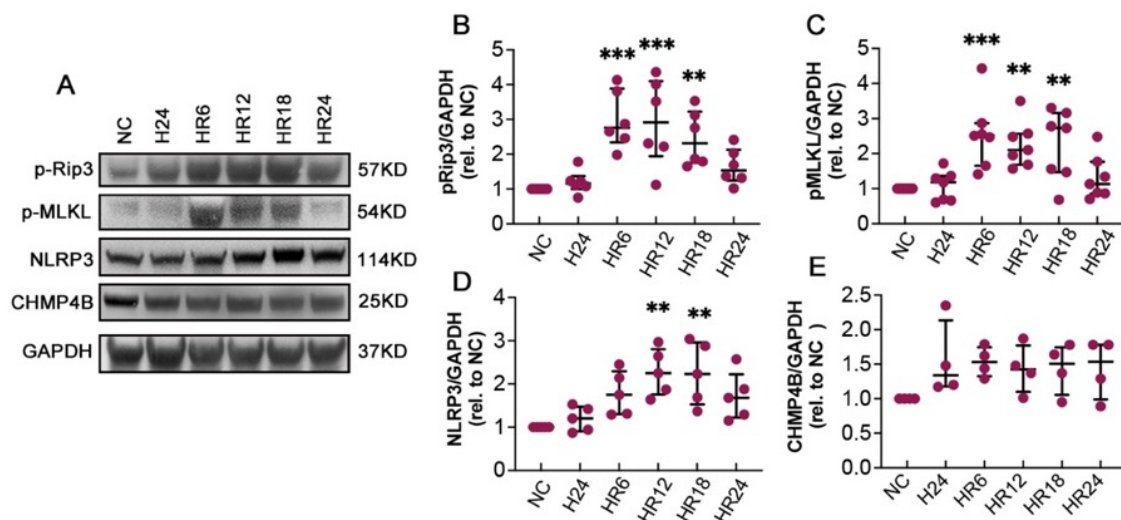


Figure 4.5 Hypothermic hypoxia-reoxygenation induces necroptosis and inflammasome activation in A549 cells. A549 cells were cultured in RPMI 1640 cell culture medium, then preserved in the UW solution at 0-4°C for 24 h and recultured at 37°C in fresh medium for 6, 12, 18, and 24 h. (A) Western blot detected pRip3, pMLKL, NLRP3, and CHMP4B expression. Analysis shows the expression of (B) pRip3, (C) pMLKL, (D) NLRP3, and (E) CHMP4B. DAPDH as the loading control. Data were analysed by unpaired t-test and are presented as scatter plots, median with interquartile range. $n = 4-6$. ** $p < 0.05$ and *** $p < 0.001$.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; pRip3: phosphorylated receptor-interacting protein kinase 3; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; NLRP3: NLR Family Pyrin Domain Containing 3; CHMP4B: Charged multivesicular body protein 4b; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4.3.6 Hypothermic hypoxia-reoxygenation elevated intracellular Ca^{2+}

To investigate the concentration and impact of Ca^{2+} during hypothermic hypoxia-reoxygenation, the Fura-2 AM, a Ca^{2+} indicator that exhibits green fluorescence, was used to evaluate the levels of intracellular Ca^{2+} at various time points. The results indicated an increase in intracellular Ca^{2+} concentration during the first phase of hypothermic hypoxia, and this elevation persisted after reoxygenation. It is noteworthy that two distinct peaks were seen at 30 min (HR30, $p < 0.05$) and 300 min (HR300, $p < 0.001$) after the process of reoxygenation (Figure 4.6).

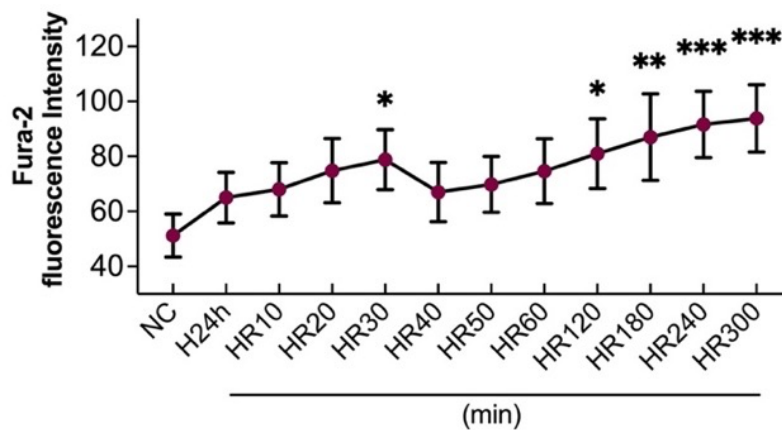


Figure 4.6 Hypothermic hypoxia-reoxygenation causes intracellular Ca^{2+} elevation. A549 cells were preserved in 0-4 °C UW solution for 24 h, then probed with 2 μM calcium indicator Fura-2 AM for 30 min and then cultured in 37 °C medium for 5 h. The fluorescence intensity of Fura-2 AM of 90 cells was calculated in each group. $n = 5$. Data were analysed with unpaired t-test and are presented as mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

NC: naïve control; H24h: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation.

4.3.7 Dex ameliorated necroptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation

To clarify whether Dex can reduce necroptosis in lung A549 cells during hypothermic hypoxia-reoxygenation, Dex with or without its inhibitor, Atip, was added to the UW solution to preserve the cells. Then, the expression of pRip and pMLKL was assessed by western blot (Figure 4.7A). The results showed that compared with negative control (NC), the expression of pRip ($p < 0.0001$, Figure 4.7B) and pMLKL ($p < 0.001$, Figure 4.7C) significantly increased at 6 h after reoxygenation (HR6). Dex saturation (HR6+Dex) significantly reduced the expression of pRip ($p < 0.05$, Figure 4.7B) and pMLKL ($p < 0.01$, Figure 4.7C), demonstrating Dex inhibited hypothermic hypoxia-reoxygenation induced necroptosis. α_2 -adrenoceptor antagonist Atip (HR6+Dex/Atip) significantly inhibited the reduction of pRip ($p < 0.05$) but failed to block the effect of Dex on pMLKL reduction.

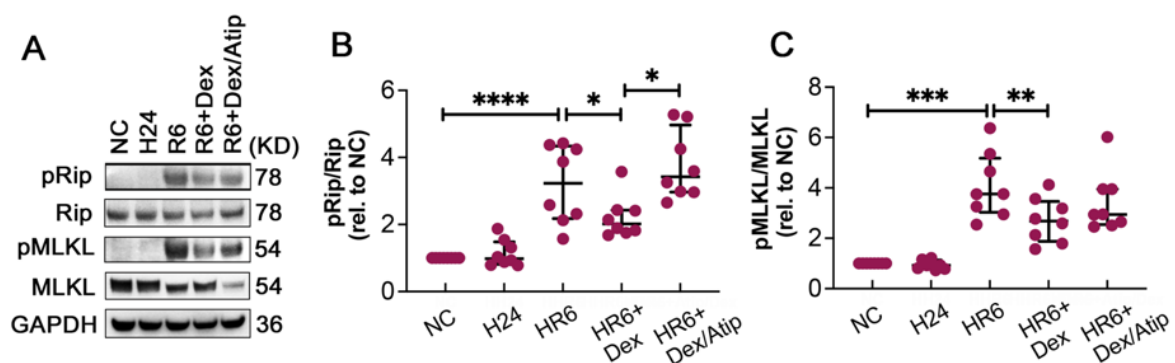


Figure 4.7 Dex ameliorates necroptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation.

A549 cells were preserved in the UW solution with or without 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h, then recultured at 37°C in the cell culture medium for another 6 h. (A) Western blot was conducted to evaluate necroptosis by detecting pRip and pMLKL. (B) D. Western Blot analysis of pRIP activation as a ratio to total RIP. (C) D. Western Blot analysis of pMLKL activation as a ratio to total MLKL. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plots, median with interquartile range. $n = 8$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; Dex: dexmedetomidine, Atip: atipamezole; pRip: phosphorylated receptor-interacting protein kinase; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4.3.8 Dex ameliorated pyroptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation

Pyroptosis was evaluated after hypothermic hypoxia-reoxygenation by detecting GSDMD, caspase-1, and caspase-5 using western blot (Figure 4.8A). The results showed that hypothermic hypoxia-reoxygenation (HR6) significantly activated GSDMD (Figure 4.8B, $p < 0.0001$), caspase-1 (Figure 4.8C, $p < 0.001$), and caspase-5 (Figure 4.8D, $p < 0.0001$). Dex saturation (HR6+Dex) significantly reduced the activation of GSDMD ($p < 0.05$, Figure 4.8B), caspase-1 ($p < 0.01$, Figure 4.8C), and caspase-5 ($p < 0.05$, Figure 4.8D), whilst the reduction of GSDMD ($p < 0.05$, Figure 4.8B) and caspase-5 ($p < 0.01$, Figure 4.7D) was reversed by co-saturation with Atip (HR6+Dex/Atip).

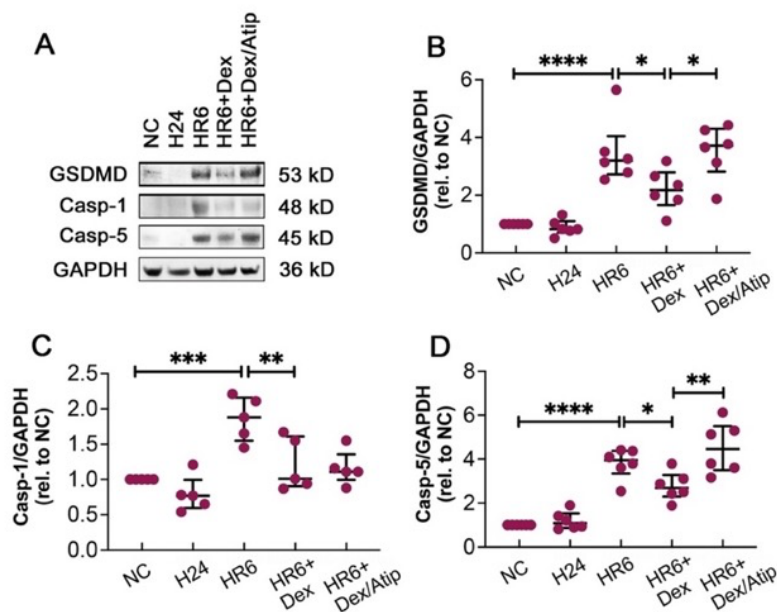


Figure 4.8 Dex ameliorates pyroptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation.

A549 cells were preserved in the UW solution with or without 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h, then recultured at 37°C in the cell culture medium for another 6 h. (A) Western blot was conducted to evaluate pyroptosis by detecting GSDMD, caspase-1, and caspase-5. Analysis shows the expression of (B) GSDMD, (C) caspase-1, and (D) caspase-5. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plots, median with interquartile range. n = 5-6. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia reoxygenation; Dex: dexmedetomidine, Atip: atipamezole; GSDMD: Gasdermin D; caspase-1: caspase-1; caspase-5: caspase-5; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4.3.9 Dex ameliorated apoptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation

Apoptosis was evaluated after hypothermic hypoxia-reoxygenation by detecting caspase-3, caspase-8, and Annexin V (Figure 4.9A). Hypothermic hypoxia (H24) significantly increased the expression of caspase-8 (p < 0.01, Figure 4.9C), demonstrating apoptosis may occur early at the cold storage stage. Meanwhile, reoxygenation (HR6) increased the expression of caspase-3 (p < 0.0001, Figure 4.8B) and caspase-8 (p < 0.01, Figure 4.9C), indicating reoxygenation initiated both Intrinsic and extrinsic apoptotic pathways. Dex saturation (HR6+Dex) significantly reduced the elevation of caspase-3 (p < 0.01), whilst Atip (HR6+Dex/Atip) had no effects on this reduction (Figure 4.9B). In addition, Annexin V had no change under Dex or Atip stimuli during hypothermic hypoxia-reoxygenation.

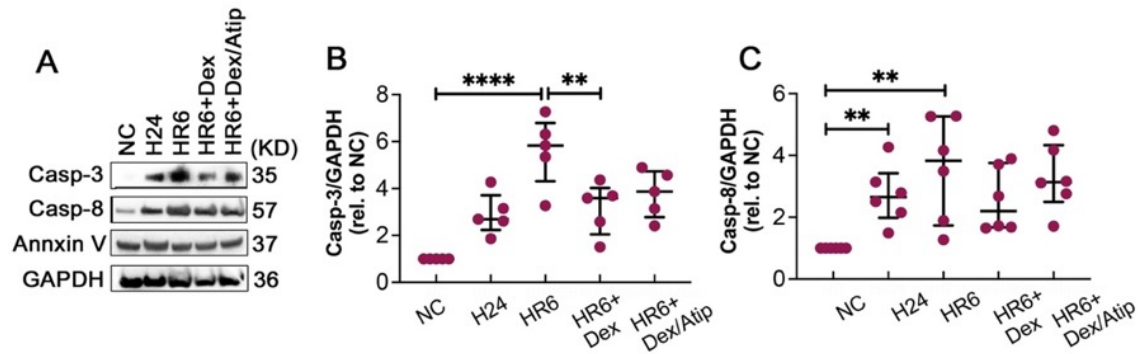


Figure 4.9 Dex ameliorates apoptosis in A549 cells exposed to hypothermic hypoxia-reoxygenation.

A549 cells were preserved in the UW solution with or without 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h, then recultured at 37°C in the cell culture medium for another 6 h. (A) Western blot was conducted to evaluate apoptosis by detecting caspase-3, caspase-8, and Annexin V. Analysis shows the expression of (B) caspase-3, (C) caspase-8, and (D) Annexin V. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plots, median with interquartile range. n = 5-6. ** p < 0.01 and **** p < 0.0001.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia reoxygenation; Dex: dexmedetomidine, Atip: atipamezole; casp-3: caspase-3; casp-8: caspase-8; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4.3.10 Dex ameliorated NLRP3 expression and plasma membrane damage in A549 cells exposed to hypothermic hypoxia-reoxygenation

NLRP3 expression was significantly increased after 6 h of reoxygenation (HR6, p < 0.001, Figure 4.10A and B) and Dex (HR6+Dex) reduced NLRP3 expression (p < 0.01, Figure 4.10B), and this reduction was reversed by co-saturated with Atip (HR6+Dex/Atip, p < 0.05, Figure 4.10B). DiLC 18 was used to stain membrane lipids to evaluate plasma membrane integrity (Figure 4.10C). Hypothermic hypoxia-reoxygenation (HR6) increased the plasma membrane permeability, allowing more dye to enter the cells, thereby staining the intracellular organelle membranes bright yellow. Membrane vesicles labelled by DiLC18 were found when the UW solution was saturated with Dex (HR6+Dex) or Dex combined with Atip (HR6+Dex/Atip).

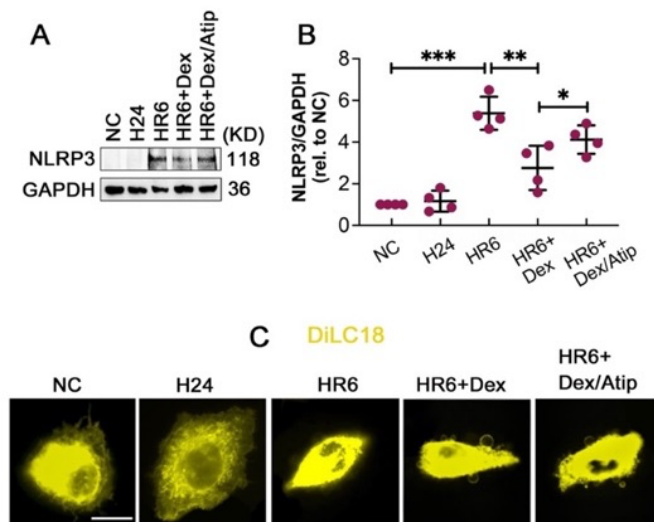


Figure 4.10 Dex ameliorates NLRP3 expression and enhances plasma membrane vesicles release in A549 cells exposed to hypothermic hypoxia-reoxygenation. A549 cells were cultured in RPMI 1640 cell culture medium and then preserved in the UW solution with or without 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h. After washing with PBS, the cells were recultured at 37°C in the cell culture medium for another 6 h. (A) Western blot was conducted to evaluate NLRP3 expression. (B) The analysis shows the expression of NLRP3. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plots, median with interquartile range. n = 4. * p < 0.05, ** p < 0.01, *** p < 0.001. (C) DiLC18 stained the cell plasma membrane, and the plasma membrane vesicles were observed. Scare bar = 20 μm.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; Dex: dexmedetomidine, Atip: atipamezole; NLRP3: NLR Family Pyrin Domain Containing 3; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4.3.11 Dex ameliorated ER stress and lysosomal rupture in A549 cells exposed to hypothermic hypoxia-reoxygenation

Increasing evidence has demonstrated that ER stress is a novel subcellular effector in the IR injury associated with organ transplantation. To further explore the degree of ER stress evaluating the anti-ER stress effect of Dex in lung epithelial cells, ER intraluminal proteins - Grp78 and CHOP, were stained (Figure 4.11). Results showed that the expression of Grp78

and CHOP significantly increased after hypothermic hypoxia-reoxygenation (HR6).

Lysosomal-regulated endoplasmic reticulum (ER) turnover prevents excessive expansion of the ER and reduces misfolded protein accumulation under ER stress. Thus, double staining of LAMP-1 (lysosome-associated membrane protein-1) with either Grp78 or CHOP was conducted (Figure 4.11). The results indicated that following 24 h of hypothermic hypoxia (H24), the lysosomes distributed throughout the cells. The preservation of cells in the UW solution supplemented with Dex demonstrated the prevention of lysosomal rupture under hypothermic hypoxia (H24+Dex). Furthermore, after reoxygenation (HR6), the cells exhibited indications of cellular demise, shown by increased cell volume and a scarcity of lysosomes. The administration of Dex (HR6+Dex) effectively preserved the size and structure of lysosomes in the reoxygenation phase. Atip (HR6+Dex/Atip) inhibited the protective effect of Dex.

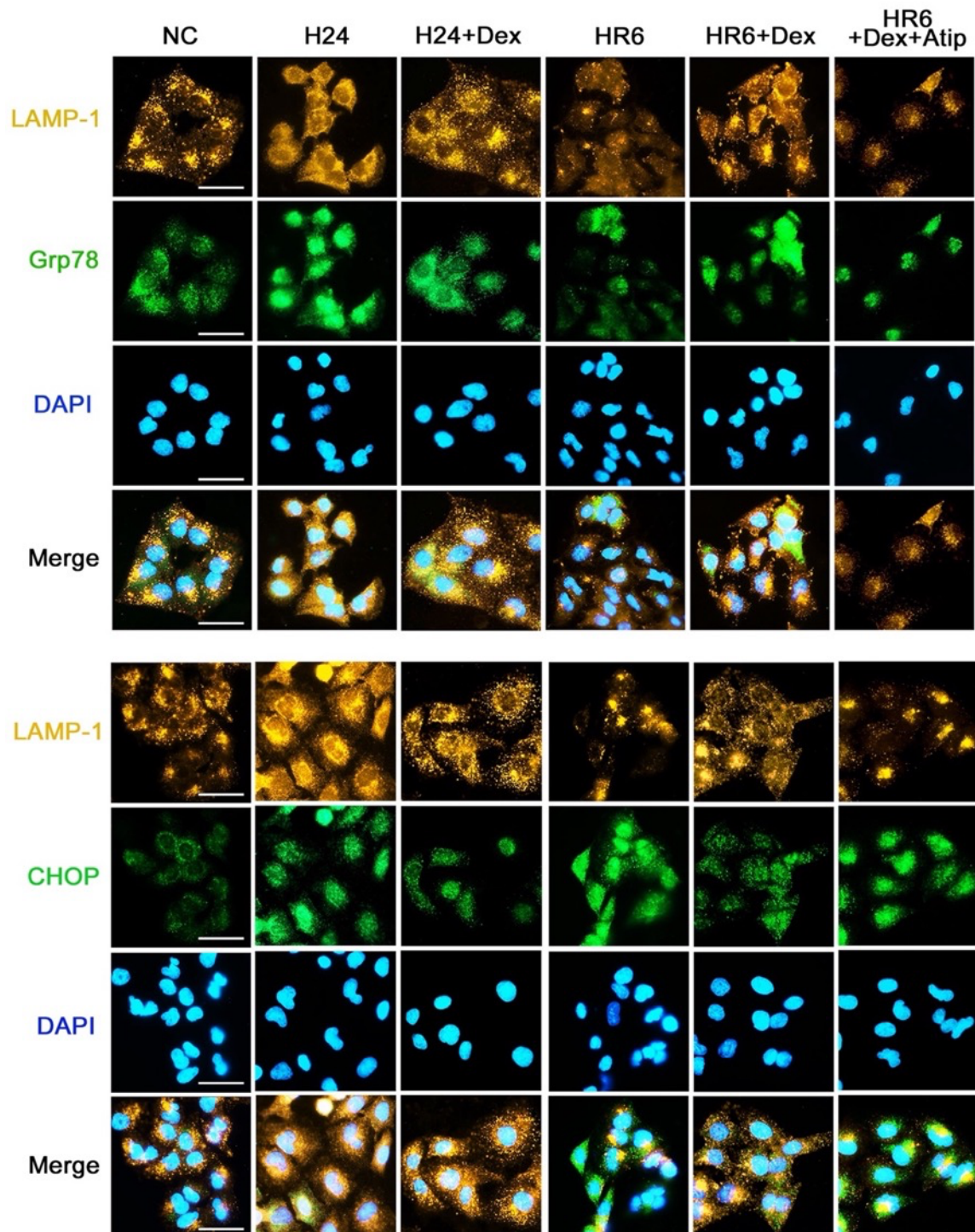


Figure 4.11 Dex alleviates ER stress and lysosomal rupture in A549 cells exposed to hypothermic hypoxia-reoxygenation. A549 cells were preserved in the UW solution with or without 0.1 nM Dex or/and 1 nM Atip at 0-4°C for 24 h, then recultured at 37°C in the cell culture medium for another 6 h. Expression of Grp78 (green), CHOP (green), and LAMP-1 (yellow) was assessed by immunofluorescence staining. n = 5. Scale bar = 50 μ m. n = 5.

NC: naïve control; H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia reoxygenation; Dex: dexmedetomidine, Atip: atipamezole; Grp78: 78-kDa glucose-regulated protein; CHOP: The C/EBP Homologous Protein; LAMP1: Lysosomal-associated membrane protein 1; DAPI: 4',6-diamidino-2-phenylindole.

4.4 Discussion

The present study indicated that necroptosis was involved in hypothermic hypoxia-reoxygenation, leading to plasma membrane disruption, Ca^{2+} influx, ER stress, inflammasome activation, and lysosomal rupture in human lung epithelial A549 cells. The membrane repair mediated by CHMP4B translocation was observed during reoxygenation. Meanwhile, Dex, when saturated in the UW solution, reduced necroptosis and inflammasome activation and maintained the volume and structure integrity of lysosomes. This is the first study to investigate the protective effects of Dex on necroptosis-induced membrane disruption and its membrane self-resealing function during hypothermic hypoxia-reoxygenation. The results could provide scientific insights into further understanding of the pathogenesis of IR and provide molecular bases to develop novel therapeutic strategies for protection against graft IR injury.

The TNF- α receptor is a well-known receptor leading to necroptosis, but the activation of TNF- α also frequently triggers apoptosis. LCL161 is a small molecule mimetic of Smac, which can inhibit apoptosis by effectively inhibiting cIAPs and is often combined use with TNF- α to induce necroptosis [202]. Quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone (Q-VD-Oph) is a true pan-caspase inhibitor that can inhibit caspase 8, leading to Rip1 ubiquitylation and collaborates with Rip3 to form necrosome, further recruits and phosphorylates MLKL to induce necroptosis [203]. Therefore, TNF- α , LCL161, and Q-VD-Oph

(TLQ) were used together to stimulate A549 cells to induce necroptosis as the positive control in the present study.

It is well known that necrosis is a feature of IR. However, regulated necrosis, such as necroptosis, has not been fully explored. Therefore, we simulated a lung transplantation procedure using a cell culture model with human lung epithelial A549 cells. The A549 cells were preserved in the UW solution at 4 °C for 24 h to simulate cold ischaemia, and then the cells were incubated in a cell culture medium at 37°C to mimic reperfusion. Taken together, we used the standard *in vitro* TLQ induced-necroptosis model to evaluate the level of necroptosis and membrane rupture and repair process during hypothermic hypoxia-reoxygenation.

Different from the better-understanding apoptosis, necroptosis is a newly discovered regulated cell death with special characteristics at both the mechanistic and phenotypic levels [204, 205]. Necroptosis is mainly controlled by protein kinases Rip1, Rip3, and MLKL [206]. Phenotypically, a cell undergoing necroptosis features cell swelling and plasma membrane pores formation. Pathologically, the leak of cellular contents after membrane rupture cause tissue inflammation and immunogenicity [207]. Therefore, necroptosis has been linked to various inflammatory diseases and immune responses, which may contribute to PGD or acute rejection (AR) after transplantation [208, 209].

In the present study, the immunofluorescence results showed that pMLKL accumulated at the plasma membrane but not in the cytosol after reoxygenation. Western blot results also demonstrated that the level of pMLKL increased to peak at 6 h and then reduced to the

basic line at 24 h after reoxygenation. These results support our hypothesis that necroptosis may be involved in lung IR, which is a potential reason for poor outcomes after lung transplantation *per se*. Moreover, pMLKL accumulation was observed in reoxygenation but not in the cold storage phase, suggesting that the initialisation of necroptosis may be energy and enzyme-dependent [210].

A similar result was found in Rip3 phosphorylation. Rip3 is an upstream protein of MLKL. When caspase 8 activation is inhibited, Rip1 and Rip3 bind to each other to phosphorylate MLKL and activate the necroptotic pathway. A previous study has indicated necroptosis is a highly energy-consuming process [210]. Rip3 is a metabolism regulator that can switch TNF-induced cell death from apoptosis to necroptosis [211]. After activation, Rip3 interacted with metabolic enzymes like glycogen phosphorylase, glutamate-ammonia ligase, and glutamate dehydrogenase 1, contributing to necroptosis by mediating the use of glycogen, glutamine and glutamate as energy substrates [211]. This process is inhibited during cold ischaemia because energy metabolism is suspended. After reoxygenation, mitochondrial oxidative metabolism recovers, and Rip3 initiates necroptosis by activating MLKL, resulting in necroptosis.

After 24 h of reoxygenation, the expression of pRip3 and pMLKL decreased to the baseline in the present study. Meanwhile, we noticed nearly half of the A549 cells were dead and detached from the bottom of Petri dishes, which may partly explain this reduction. However, a previous study showed that after losing plasma membrane integrity for several hours, necroptotic cells continue *de novo* synthesis of cytokines and chemokines [211]. Therefore, in clinical practice, those necroptotic-cellular corpses and continuing synthesised cytokines

and chemokines may be up-taken by innate immune cells and promote adaptive immune responses in the body, increasing the risk of acute rejection and severe inflammatory responses.

Plasma membrane resealing is essential for injured cells to maintain plasma membrane integrity. Different mechanisms for plasma membrane resealing have been described, including patching with endo-membranes, endocytosis, and extracellular budding [130, 131]. However, whether these resealing pathways exist in hypothermic hypoxia reoxygenation-induced necroptosis and whether this process has a similar mechanism or regulating proteins compared with other types of cell death remains unexplored. In addition, phosphatidylserine (PS) localises the inner leaflet of the plasma membrane. When cells undergo regulated cell death, PS is exposed on the cell surface before plasma membrane disruption, which can be labelled by Annexin V [133]. Meanwhile, when cells undergo membrane resealing, small “bubbles” formed with wounded plasma membrane are released from the cell surface that also can be labelled by Annexin V [212]. In the present study, PS exposure was found during cold storage. At the same time, membrane “bubbles” were observed after reoxygenation, suggesting that the damage in the plasma membrane started at cold preservation and can be partly recovered during reoxygenation. The results indicated that the membrane resealing pathway participated in necroptotic cell resuscitation as a self-repair strategy.

ESCRT participates in numerous membrane fission events, including cytokinesis and viruses budding [213, 214]. Recent studies also demonstrated that ESCRT was required to rapidly close small wounds at the plasma membrane [215-217]. ESCRT-III activity maintains the

plasma membrane integrity following MLKL phosphorylation, delaying the onset of membrane injury and cell death. Following an injury to the plasma membrane, the ESCRT-III machinery relocates from the cytosol to the injury sites, co-localising with the MLKL N-terminal domain and contributing to membrane resealing by releasing membrane vesicles. CHMP4B is a component of the ESCRT-III. In the present study, we found that the translocation of CHMP4B occurred during the reoxygenation phase between 6 to 18 h, which was consistent with the timing of MLKL phosphorylation and membrane “bubbles” appearance, suggesting that CHMP4B may regulate membrane repair. Therefore, any intervention that can maintain the function of CHMP4B would be enormously beneficial.

Minor wounds (nanometer diameter) in the plasma membrane can be resealed using calcium-dependent and calcium-independent pathways [218]. In our experiments, Fura-2 AM was applied to detect intracellular Ca^{2+} concentration during hypothermic hypoxia-reoxygenation. The results showed that the intracellular Ca^{2+} concentration increased from cold storage to reoxygenation. Interestingly, we found 2 peaks at 30 min and 300 min after reoxygenation, indicating that the cells may capture Ca^{2+} from the extracellular medium and release Ca^{2+} from the intracellular ER storage. It has been suggested that chelated intracellular Ca^{2+} abolished necroptosis, indicating an essential role of intracellular Ca^{2+} in necroptosis. However, further studies also found that extracellular Ca^{2+} removal had no significant effects on necroptosis [219]. Taken together, in the early stage (~ 30 min after reperfusion), Ca^{2+} rises due to intracellular calcium released may play an important role in initiating necroptosis.

Previous studies suggested lysosomes may combine with ESCRT-III, participating in plasma membrane repair through exocytosis [220]. Therefore, immunofluorescent staining of LAMP-1 was conducted in the present study. Surprisingly, we found that in the normal A549 cells, small granules of lysosomes were clustered around the nuclei. By contrast, diffusing cytoplasmic LAMP-1 immunostaining was observed in the cells undergoing cold ischaemia, suggesting that LAMP-1 spilt into the cytoplasm due to lysosomal rupture. The lysosomal rupture is a lethal event because excessive lysosomal proteases could activate hydrolases and digest vital proteins in the cytosol, inducing cytosolic acidification and consequently leading to cell death [221]. However, whether the lysosomal rupture in our study contributes to necroptosis during reoxygenation is still unknown and needs further exploration. Meanwhile, LAMP-1 nearly disappeared after 6 h of reoxygenation; combining with our previous data that the cells lost plasma membrane integrity due to necroptosis, we speculate that part of LAMP-1 was also leaked out of the cells with other DAMPs and proteases. In addition, we also found that the treatment of Dex in cold-sored cells maintained the morphology and integrity of lysosomes. This suggested that Dex may provide a protective effect at an early stage, which may also contribute to inhibiting necroptosis in lung grafts.

In summary, the present study indicated that cold storage leads to necroptosis in the lung epithelial cells by increasing the phosphorylation of Rip3 and MLKL. This, in turn, results in lung graft dysfunction and causes adaptive immune responses and inflammatory reactions after lung transplantation. As a self-repair mechanism, membrane resealing can be used as a potential strategy for rescuing the necroptotic cells in lung grafts.

Chapter FIVE

Hypothermic hypoxia-reoxygenation activates several regulated cell death pathways in human HK2 cells

5.1 Introduction

IR injury is one of the most important concerns in kidney transplantation. The most important consequences are a higher risk of DGF, acute rejection, longer hospital stays, and poor outcomes in recipients [30, 222, 223]. Due to the high cellular metabolic rate at higher temperatures and organ oxygen reduction, the organ must be preserved in a cold preservation solution immediately after the stop of blood supply in the donor [224]. Cold ischaemic time is restricted to 24 h; however, donated kidneys that are cold preserved longer than 16 h frequently lead to an increased risk of IR injury [225, 226]. Reperfusion injury occurs in restoring blood circulation to the graft in the recipient and is usually exacerbated by prolonged cold ischaemic time.

Alterations in cellular metabolic processes primarily cause IR injury in response to hypoxia and the consequent production of ROS [16]. During ischaemia, oxygen reduction to mitochondrial oxidative phosphorylation means the kidney graft cannot maintain the ATP level to keep cell functions. Reduced ATP production also causes F_0F_1 -ATP synthase to act in reverse in the mitochondrial membrane, hydrolysing ATP generated by glycolysis to pump protons, thereby increasing the acidity [16]. In addition, anaerobic glycolysis produces ATP instead, accumulating lactate in cells [16]. In normal conditions, the monocarboxylate transporter pumps H^+ and lactate out of the cells to prevent acidosis; however, in the absence of blood flow, they cannot be eliminated from the extracellular. Moreover, the accumulation of electrons at coenzyme Q in mitochondria leads to the accumulation of succinate in the electron transport chain, which has been recognised as a universal marker of ischaemia [16, 227]. These contribute to ischaemia injury, disrupt cell function, or even lead to cell death. Succinate is swiftly oxidised upon reperfusion, producing ROS through the

mitochondrial respiratory chain. ROS causes oxidative damage and induces mitochondrial permeability transition pores (MTPT), leading to cell death. Then, DAMPs such as HMGB1 and mtDNA are released from dying cells, leading to the recruitment of immune cells to the kidney graft, contributing to organ injury and rejection [227].

Following retrieval, kidney grafts are usually washed and preserved in ice-cold preservation solutions to maintain excised graft viability before transplantation [55]. Over the past decade, several types of cold storage solutions, effective cold-preserved kidneys and other organs, have entered the market. Cold-preserved grafts in the UW solution provide an extracellular oncotic force to minimise cellular swelling, while this solution also contains electrolytes to maintain ion gradients. Therefore, it is the current gold standard in clinics for renal preservation to reduce IR injury. Cold preservation has been demonstrated to improve glomerular filtration, reduce delayed graft function, and increase graft survival; however, these effects were reduced with storage times longer than 10 h [228, 229]. Therefore, new strategies are required to store kidneys for extended periods.

IR injury induces multiple forms of cell death, such as necrosis and regulated cell death [230, 231]. In kidney transplantation, apoptosis is the most common form, while necroptosis and ferroptosis have recently been discovered [90, 91]. Necroptosis is initiated by activating several cell surface death receptors, such as the TNFR, resulting in the phosphorylation of MLKL [206]. Ferroptosis is an iron-dependent, non-receptor-mediated cell death induced by lipid peroxidation [232]. The significance of ferroptosis and necroptosis to IR injury is membrane rupture, which produces damage-associated molecular patterns (DAMPs) and immunogenic patterns, impairing patient outcomes in the transplantation [208, 233]. While

the role of ferroptosis and necroptosis in IR is well known, the molecular mechanisms of inflammatory and immunology responses in organ transplantation have not been thoroughly explored.

In this chapter, using human kidney tubular cells (HK2 cell line), we investigated the impacts of prolonged cold ischaemic time (24 h) and the following reperfusion on the activation of multiple regulated cell death pathways, including apoptosis, necroptosis, ferroptosis, and pyroptosis. The inflammation during the reperfusion stage was also evaluated.

5.2 Experimental design

5.2.1 Investigating the impact of hypothermic hypoxia-reoxygenation on the activation of regulated cell death pathways in HK2 cells

HK2 cells were preserved in the UW solution at 4°C for 24 h and then cultured in fresh medium at 37°C for 3, 6, 9, and 12 h (hypothermic hypoxia-reoxygenation). Apoptosis was evaluated using a TUNEL assay, and the expression of caspase-3 and caspase-8 was determined using a western blot. Necroptosis was evaluated by pMLKL and pRip using immunofluorescence staining and western blot. Pyroptosis was evaluated by detecting GSDMD, caspase-4, and caspase-5 using immunofluorescence staining and western blot. Ferroptosis was evaluated by detecting GPX4 and ACSL4 using immunofluorescence staining and western blot.

5.2.2 Investigating the impact of hypothermic hypoxia-reoxygenation on inflammation in HK2 cells

HK2 cells were preserved in the UW solution at 4°C for 24 h and then cultured in fresh medium

at 37°C for 3, 6, 9, and 12 h (hypothermic hypoxia-reoxygenation). Inflammasome activation was evaluated by detecting NLRP3 and ASC using immunofluorescence staining and western blot. Immunofluorescence staining of HMGB1 to evaluate cytoplasm HMGB1 level. HMGB1, TNF- α , IL-1 β , and IL-6 in the cell culture medium were assessed by ELISA.

5.3 Results

5.3.1 *In vitro* hypothermic hypoxia-reoxygenation induced apoptosis of HK2 cells

Apoptosis in the HK2 cell cultures was evaluated with TUNEL staining, a sensitive technique to detect DNA fragmentation during apoptosis (Figure 5.1A). Intrinsic and extrinsic apoptotic pathways were assessed using western blot to analyse caspase-8 and caspase-3 expression (Figure 5.1B). Compared to the control (NC), only a few TUNEL⁺ apoptotic cells were observed after the cells were cold-preserved for 24 h (H24). At the same time, there were massive TUNEL⁺ apoptotic cells after 24 h of cold preservation following 12 h of reoxygenation (HR12). However, western blot (Figure 5.1B) showed that there was no significant increase in the expression of either caspase-3 (Figure 5.1C) or caspase-8 (Figure 5.1D) after cold preservation (H24), whilst reoxygenation time-dependently increased both intrinsic and extrinsic apoptosis as evidenced by continuing increased c-caspase-3 p19 and p17 fragments (Figure 5.1C), total caspase-8 and its p10 fragment (Figure 5.1D).

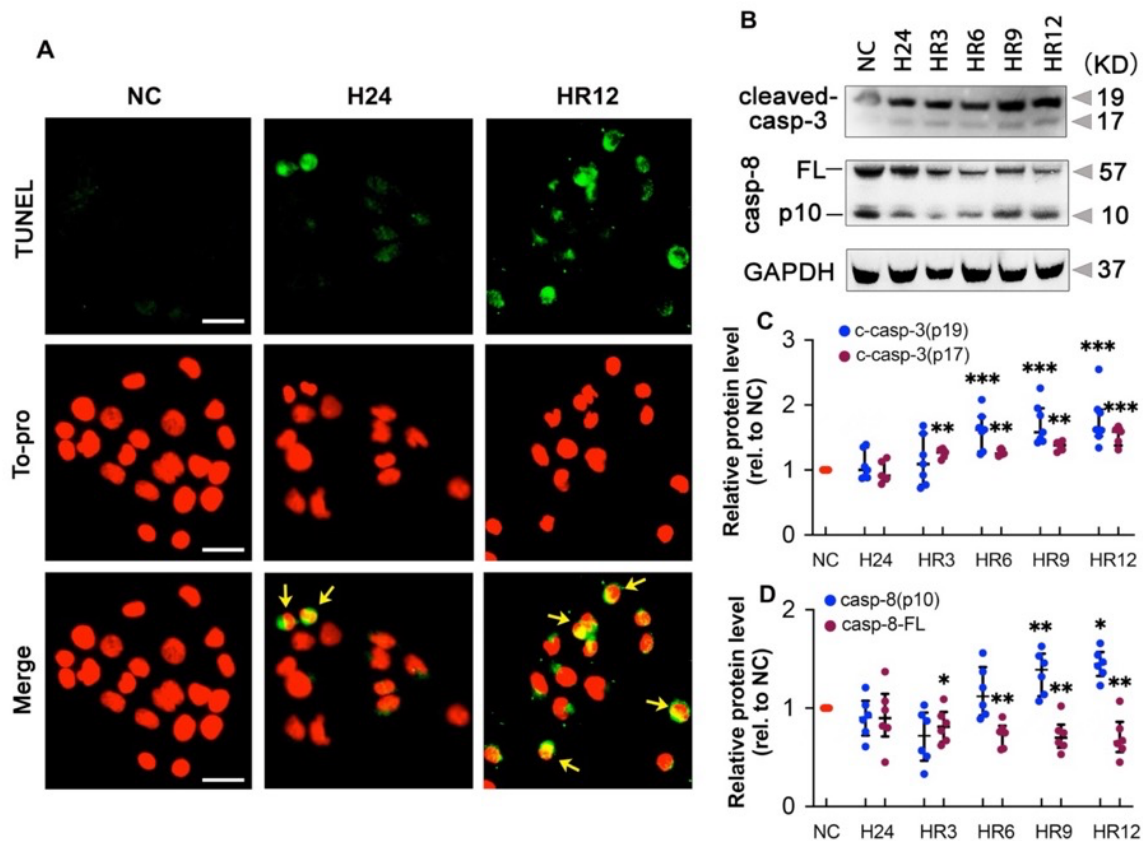


Figure 5.1 In vitro hypothermic hypoxia-reoxygenation induces apoptosis of HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischemic cold storage (hypothermic hypoxia) following cultured with RPMI 1640 medium at 37°C for 3, 6, 9 or 12 h (reoxygenation). **A.** In situ, apoptosis was stained with TUNEL (green) assay, and nuclei were stained with To-pro (red). Scale bar = 50 μ m. Yellow arrows indicate TUNEL⁺ apoptotic cells. **B.** Intracellular apoptotic proteins expression of cleaved-caspase-3 (c-caspase-3) p19 and p17 fragments, and caspase-8 (caspase-8) and its p10 fragment was evaluated using western blot. **C.** Western Blot analysis of c-caspase-3 p19 subunit (p19) and p17 subunit (p17). **D.** Western Blot analysis of caspase-8 (FL) and its p10 subunit (p10). GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 6-7. *p < 0.05, ** p < 0.01, *** p < 0.001.

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.3.2 *In vitro* hypothermic hypoxia-reoxygenation induced necroptosis of HK2 cells

Immunofluorescence results (Figure 5.2A) showed that increased pMLKL was observed to accumulate at the plasma membrane, particularly at cell-to-cell junctions, after 12 h of reoxygenation (HR12). Western blot (Figure 5.2B) demonstrated significant increases of MLKL and RIP phosphorylation following 3 ($p < 0.05$, $p < 0.01$), 6 ($p < 0.001$, $p < 0.0001$), 9 ($p < 0.001$, respectively), and 12 h ($p < 0.001$, respectively) of reoxygenation as compared with control cells (Figure 5.2C and D), indicating an exacerbation of necroptosis. Our results showed that necroptosis mainly occurred at the reoxygenation stage.

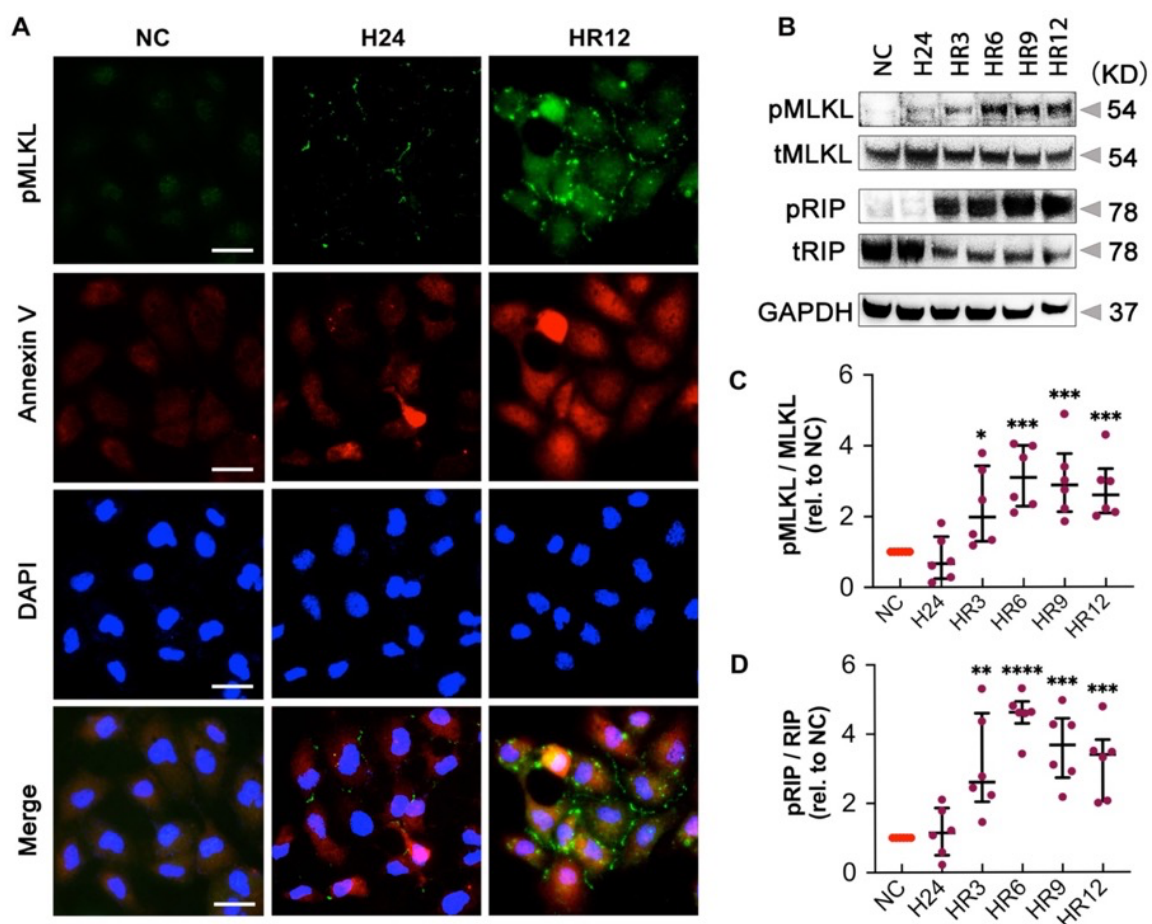


Figure 5.2 In vitro hypothermic hypoxia reoxygenation induces necroptosis of HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischemic cold storage (hypothermic hypoxia) following cultured with RPMI 1640 medium at 37°C for 3, 6, 9 or 12 h (reoxygenation). **A.** Necroptosis was stained with pMLKL (green) and cell plasma

membrane was stained with Annexin V (red). Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. **B.** Necroptosis biomarkers pMLKL, MLKL, pRIP, and RIP were evaluated using western blot. **C.** Western Blot analysis of pMLKL activation as a ratio to total MLKL. **D.** Western Blot analysis of pRIP activation as a ratio to total RIP. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 6. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; MLKL: Mixed Lineage Kinase domain-like protein; pRIP: phosphorylated Receptor Interacting Protein; RIP: Receptor Interacting Protein; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.3.3 *In vitro* hypothermic hypoxia-reoxygenation induced pyroptosis of HK2 cells

IR-induced PANoptosis contains molecules critical for pyroptosis, apoptosis, and necroptosis. Since our previous data suggested that apoptosis and necroptosis were significantly increased after hypothermic hypoxia-reoxygenation, executioner proteins of pyroptosis were also investigated. Immunofluorescence results (Figure 5.3A) showed that a high level of pyroptosis biomarker gasdermin D (GSDMD) dotted or linearly aggregated localised to the apical surface of most cells after reoxygenation (HR12) when compared with control (NC) or cold preserved cells (H24). Western blot (Figure 5.3B) also demonstrated that hypothermic hypoxia-reoxygenation significantly increased the expression of total GSDMD at 3 h of reoxygenation ($p < 0.05$), and GSDMD 30 kDa N-terminal fragment exhibited a trend towards an increase from 3 h until 12 h of reoxygenation (Figure 5.3C, $p < 0.05$, separately). Because GSDMD is activated by caspase-1 cleavage, we also evaluated total caspase-1 and cleaved caspase-1 (p20) (Figure 5.3B). We observed a trend towards an increase in both total caspase-1 ($p < 0.05$) and c-caspase-1 ($p < 0.01$) from 3 h to 12 h of reoxygenation (Figure 5.3D), demonstrating caspase-1 activation. In addition, similar to GSDMD and caspase-1, the

expression of caspase-4 also increased in a time-dependent manner at 6 ($p < 0.01$), 9 ($p < 0.05$), and 12 h ($p < 0.05$) of reoxygenation (Figure 5.3E). Another pyroptosis executioner protein, caspase-5, exhibited a trend towards an increase until 6 h ($p < 0.01$), followed by a trend towards a decrease until 12 h of reoxygenation (Figure 5.3F). Our results indicated that pyroptosis mainly occurred at the reoxygenation stage, and PANoptosis may occur in HK2 cells that experienced hypothermic hypoxia-reoxygenation.

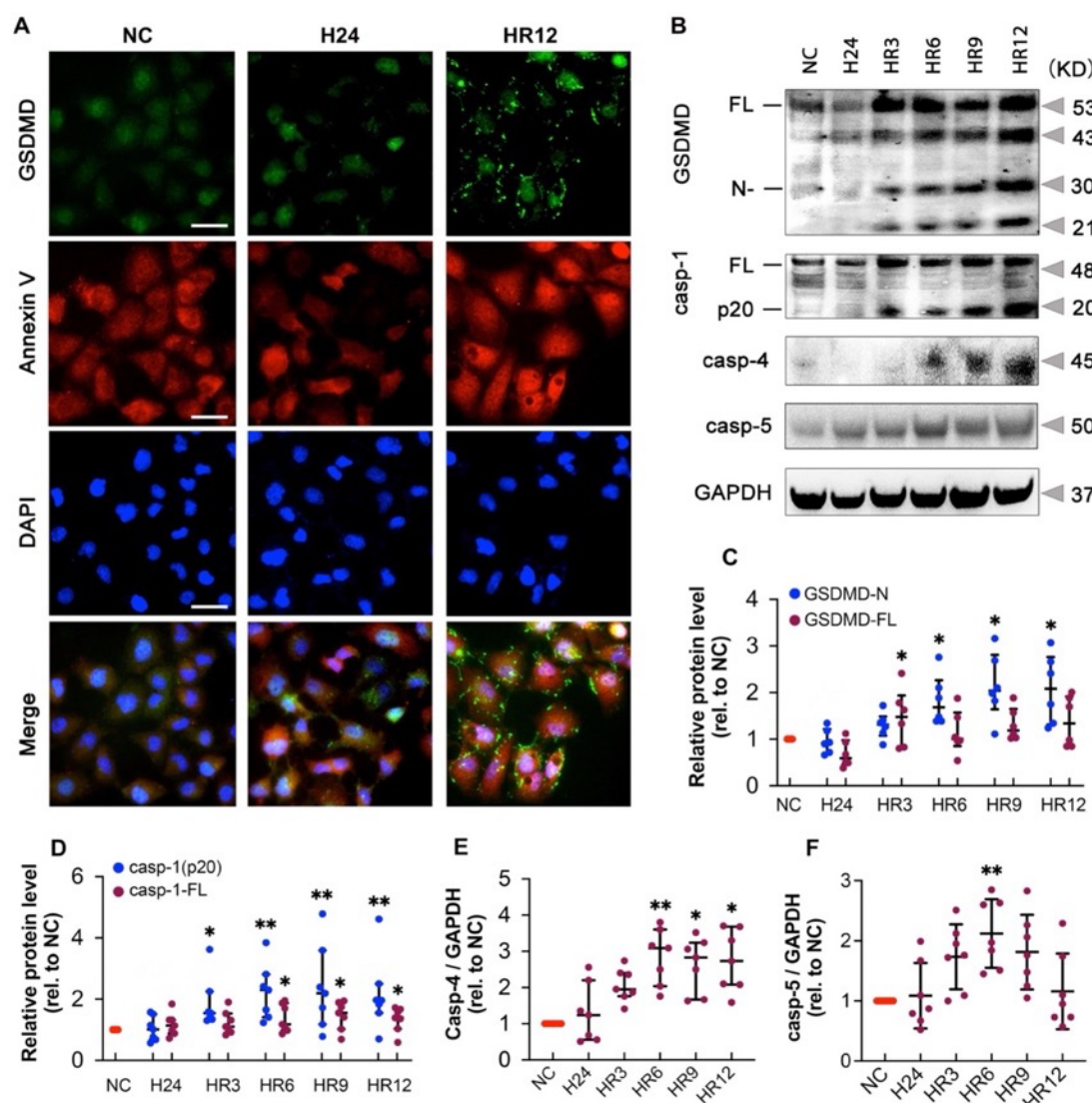


Figure 5.3 In vitro hypothermic hypoxia-reoxygenation induces pyroptosis of HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischemic cold storage (hypothermic hypoxia) following cultured with RPMI 1640 medium at 37°C

for 3, 6, 9 or 12 h (reoxygenation). **A.** Pyroptosis was stained with GasderminD (GSDMD, green) and the cell plasma membrane was stained with Annexin V (red). Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. **B.** Intracellular pyroptotic proteins expression of GSDMD and its N- terminal, caspase-1 (caspase-1) and its p20 subunit (p20), caspase-4 (caspase-4), and caspase-5 (caspase-5) was evaluated using western blot. **C.** Western Blot analysis of GSDMD (FL) and its N-terminal. **D.** Western Blot analysis of caspase-1 (FL) and its p20 subunit (p20). **E.** Western Blot analysis of caspase-4. **F.** Western Blot analysis of caspase-5. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 6-7. *p < 0.05, ** p < 0.01, *** p < 0.001.

DAPI: 4',6-diamidino-2-phenylindole; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.3.4 *In vitro* hypothermic hypoxia-reoxygenation induced ferroptosis of HK2 cells

The ACSL4 expression was evaluated as a ferroptosis biomarker, and the expression of an antioxidant enzyme GPX4 was assessed as a protective molecular against ferroptosis.

Immunofluorescence results (Figure 5.4A) indicated that ACSL4 expression increased, while GPX4 expression decreased in the cytosol at 12 h of reoxygenation, indicating ferroptosis occurred (Figure 5.4A). Additionally, western blot (Figure 5.4B) demonstrated a significant decrease in GPX4 expression at 24 h of cold preservation (H24) relative to control cells (p < 0.01), indicating that hypothermic hypoxia significantly reduced the capacity of antioxidant stress of the cells. This trend of decrease continued following reoxygenation at 3 (p < 0.01), 6 (p < 0.01), 9 (p < 0.0001), and 12 h (p < 0.0001) (Figure 5.4C). Meanwhile, ACSL4 expression exhibited a trend towards an increase after reoxygenation and reached the peak at 12 h (Figure 5.4D, p < 0.001), consistent with immunofluorescence results.

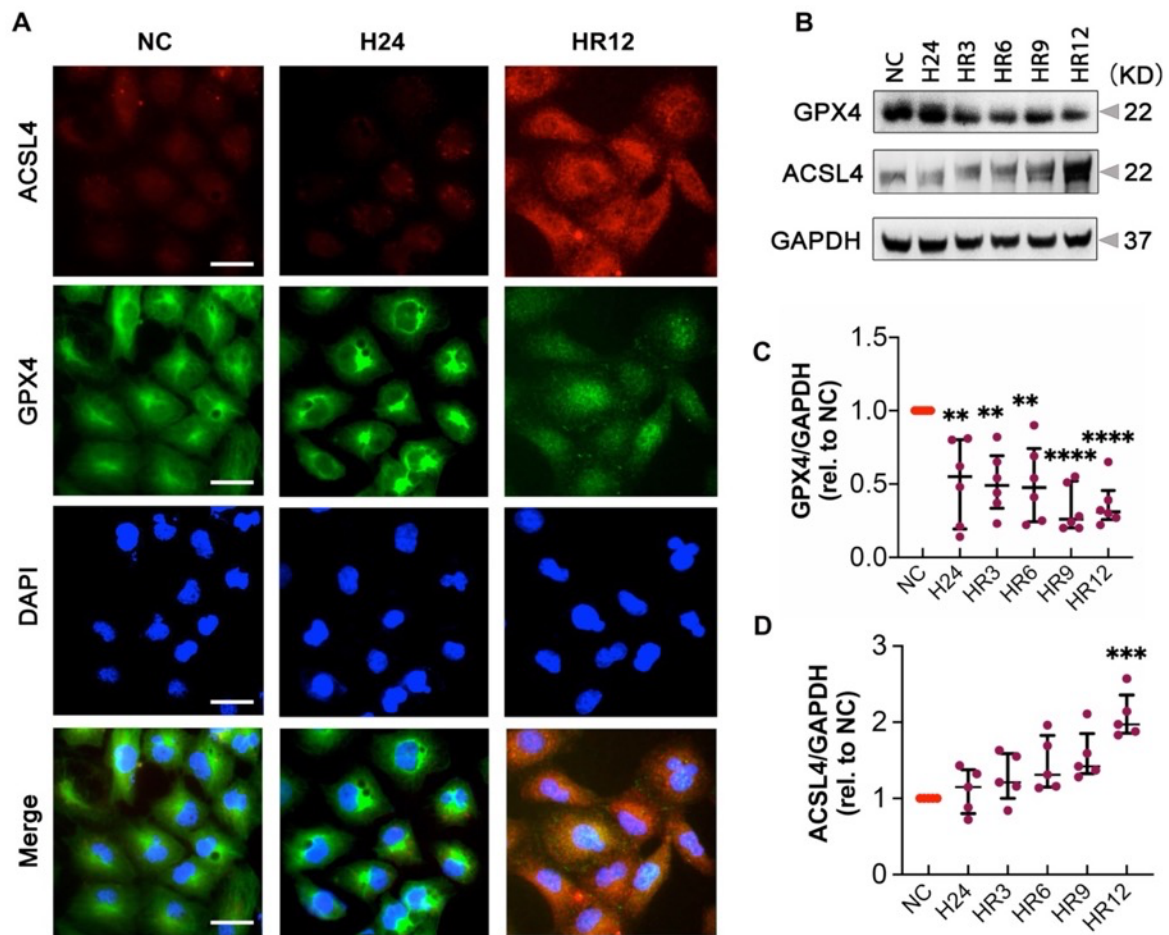


Figure 5.4 In vitro hypothermic hypoxia-reoxygenation induces ferroptosis of HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischemic cold storage (hypothermic hypoxia) following cultured with RPMI 1640 medium at 37°C for 3, 6, 9 or 12 h (reoxygenation). **A.** Ferroptosis was stained with GPX4 (green) and ACSL4 (red). Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. **B.** Ferroptosis biomarkers GPX4 and ACSL4 were evaluated using western blot. **C.** Western Blot analysis of GPX4 expression. **D.** Western Blot analysis of ACSL4 expression. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 5-6. ** p < 0.01, *** p < 0.001, **** p < 0.0001.

GPX4: Glutathione peroxidase 4; ACSL4: Acyl-CoA Synthetase Long Chain Family Member 4; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.3.5 *In vitro* hypothermic hypoxia-reoxygenation activated Inflammasome in HK2 cells

NLRP3 and ASC were detected to evaluate inflammasome activation. The results (Figure 5.5A) showed that compared to control cells (NC), the expression of NLRP3 and ASC was significantly increased after 12 h of reoxygenation (HR12). Meanwhile, western blot results (Figure 5.5B) demonstrated that both NLRP3 and ASC expression exhibited a trend towards an increase during reoxygenation and reached the peak at 12 h (Figure 5.5C and D; $p < 0.001$ and $p < 0.01$, separately). Our results indicated that hypothermic hypoxia-reoxygenation induced inflammasome activation mainly at the reoxygenation stage.

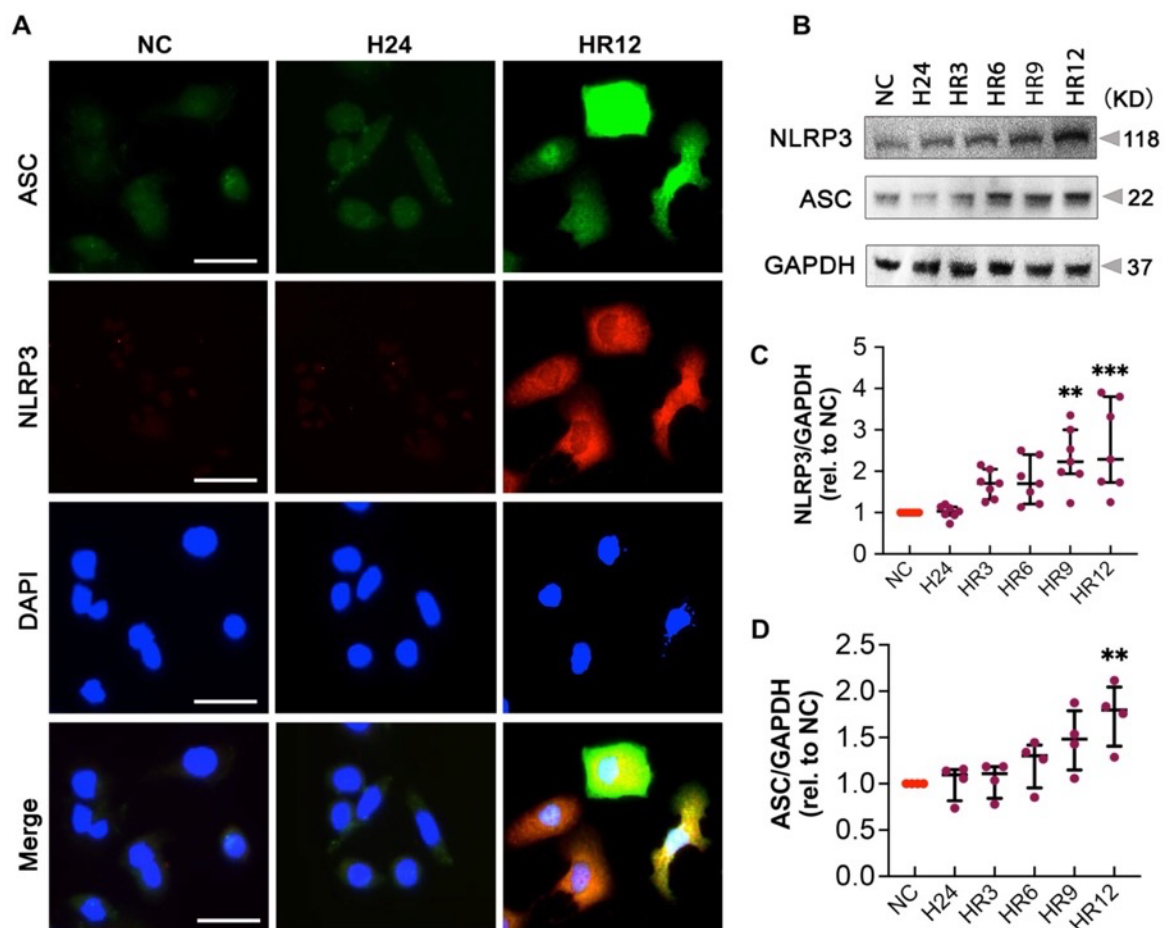


Figure 5.5 *In vitro* hypothermic hypoxia-reoxygenation activates Inflammasome in HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischemic cold storage (hypothermic hypoxia) following cultured with RPMI 1640 medium at 37°C for 3, 6, 9 or 12 h (reoxygenation). **A**. inflammasome complexes were stained with ASC (green) and NLRP3 (red). Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. **B**. Inflammasome complexes of ASC and

NLRP3 were evaluated using western blot. **C.** Western Blot analysis of NLRP3 expression. **D.** Western Blot analysis of ASC expression. GAPDH as the loading control. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 4-7. ** p < 0.01, *** p < 0.001.

ASC: apoptosis-associated speck-like protein containing CARD; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.3.6 *In vitro* hypothermic hypoxia-reoxygenation induced inflammatory cytokines release in HK2 cells

HMGB1, which belongs to DAMPs, can be released by cells undergoing regulated cell death and activating inflammatory responses. The results (Figure 5.6A) showed that HMGB1 significantly increased in the nuclei after 24 h of cold preservation (H24) and then translocated to the cytosol after reoxygenation for 12 h (HR12). In addition, ELISA results suggested that the levels of HMGB1 (Figure 5.6B, p < 0.001), TNF- α (Figure 5.6C, p < 0.0001), IL-1 β (Figure 5.6D, p < 0.0001), and IL-6 (Figure 5.6C, p < 0.0001) were significantly increased in the cell culture medium when compared with control cells after 12 h of reoxygenation.

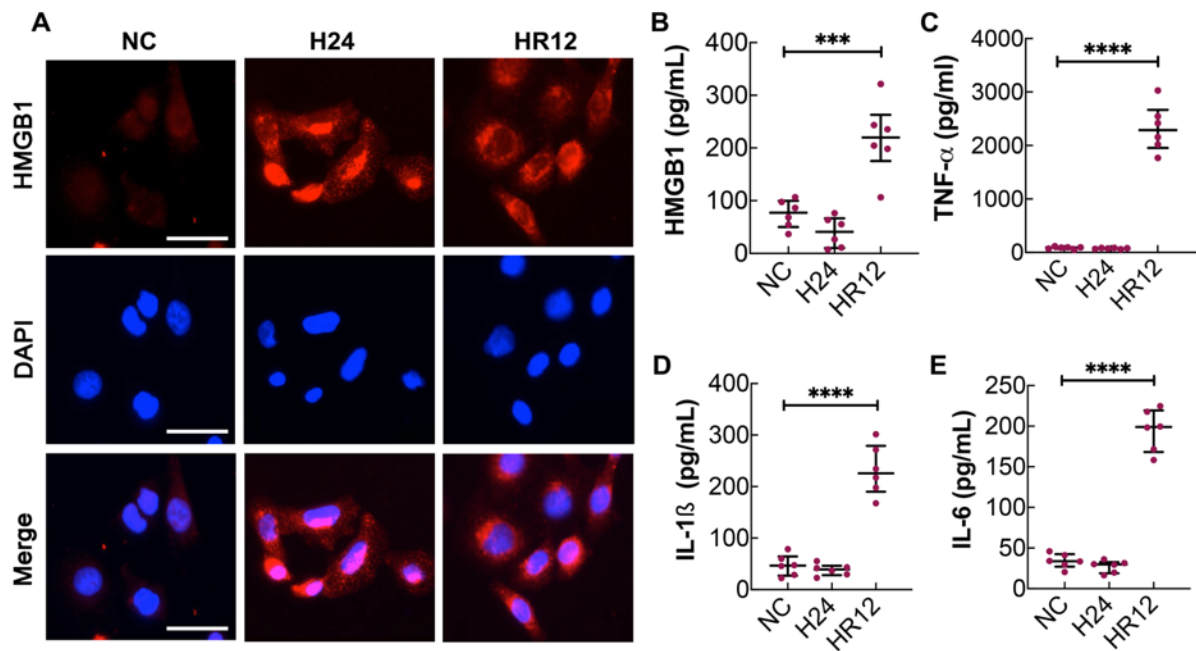


Figure 5.6 In vitro hypothermic hypoxia-reoxygenation induces inflammatory cytokines release in HK2 cells. Human Kidney 2 (HK2) proximal tubular cells were cold preserved in the UW preservation solution at 4°C for 24 h to simulate ischaemic cold storage (hypothermic hypoxia) before being cultured with RPMI 1640 medium at 37°C for 3, 6, 9 or 12 h (reoxygenation). **A.** Location of HMGB1 (red) was stained using immunofluorescence. Nuclei were stained with DAPI (blue). Scale bar = 50 µm. The concentration of **B.** HMGB1, **C.** TNF-α, **D.** IL-1β, and **E.** IL-6 in the cell culture medium was assessed by ELISA. Data were analysed by unpaired t-test, and are presented as scatter plot, median with interquartile range. n = 6. *** p < 0.001, **** p < 0.0001.

HMGB1: High mobility group box 1; TNF-α: Tumour Necrosis Factor alpha; IL: Interleukin; DAPI: 4',6-diamidino-2-phenylindole; NC: Negative Control; H24: hypothermic hypoxia for 24 h; HR3, 6, 9, 12: hypothermic hypoxia for 24 h following reoxygenation for 3, 6, 9, 12 h.

5.4 Discussion

This chapter demonstrated that hypothermic hypoxia-reoxygenation induced activation of pyroptosis, apoptosis, necroptosis (PANoptosis), and ferroptosis in kidney tubular HK2 cells.

In a time-dependent manner, these inflammatory cell deaths activated the NLRP3

inflammasome and increased the production and release of HMGB1, TNF- α , IL-1 β , and IL-6, resulting in significant cell damage.

IR injury starts early in the transplant at an early hypothermic hypoxia stage [234]. Although cold perfusion and cold storage are basic techniques for slowing cell metabolism and reducing oxygen and ATP consumption, hypothermic hypoxia has been shown to induce metabolic and functional cell injury by impacting ionic homeostasis and mitochondrial and enzyme functions [235]. Nevertheless, a previous study suggested that in cultured renal tubular epithelial cells, mitochondrial changes, ROS generation, direct DNA damage, and activation of proteasomes and serine proteases start as early as two hours after cold exposure [234]. Mitochondria contains all the necessary elements to initiate cell death, which are released into the cytosol after a cold-induced elevation in mitochondrial permeability transition and swelling [236-238]. Therefore, mitochondrial alterations may be critical components of cold injury and play a main role in triggering regulated cell death pathways after reperfusion, resulting in a high rate of cell death.

Our *in vitro* findings indicated that both intrinsic and extrinsic apoptosis pathways are involved in IR injury. It has been reported that pro-apoptotic molecules, such as cytochrome c, are released into the cytoplasm when mitochondria are damaged from IR injury. The interaction between cytochrome c and apoptotic protease activating factor 1 (Apaf-1) results in the formation of the apoptosome complex. The apoptosome complex is responsible for the recruitment and activation of caspase-9, which then triggers the activation of executioner caspase-3, leading to the execution of apoptosis [239]. External death ligands, such as TNF- and FasL, also bind to their respective death receptors on the cell surface,

resulting in the recruitment and assembly of adaptor proteins, such as caspase-8, to initiate extrinsic apoptosis [240]. While the intrinsic pathway is generally considered the primary contributor to IR-induced apoptosis due to mitochondrial dysfunction, our data indicated that both the intrinsic and extrinsic pathways might participate in graft injury by intricately interconnecting and cross-regulating each other. In addition, the kidney tubular epithelial cells are especially susceptible to necroptosis and ferroptosis [241]. The necroptosis or ferroptosis of these cells results in the disruption of the tubular architecture, the loss of renal function, and the impairment of the viability of the kidney graft [241]. It has been reported that necroptosis and ferroptosis can occur simultaneously and interact with each other or other cell death mechanisms, such as apoptosis, during IR injury [242]. The contribution and interaction of these cell death pathways may vary based on the ischaemic time, graft quality, and patients. Therefore, understanding and targeting these pathways can facilitate the development of therapeutic interventions that protect kidney grafts from IR injury and enhance transplant outcomes.

Since our *in vitro* data suggested that apoptosis and necroptosis were significantly increased after hypothermic hypoxia-reoxygenation, executioner proteins of pyroptosis were also investigated to evaluate PANoptosis. PANoptosis is carried out by the PANoptosome, a molecular complex composed of caspase-1, caspase-8, Rip1, NLRP3, and other components that are capable of simultaneously activating three important forms of regulated cell death, which includes pyroptosis, apoptosis, and necroptosis [243]. PANoptosis has been associated with numerous diseases, including neurological disorders [244], cancers [245, 246], microbial infections [247-249], and metabolic disorders [250]. Although PANoptosis has been demonstrated to occur during IR injury [251, 252], it has not been studied as

thoroughly in transplant settings. Therefore, our findings indicated that PANoptosis might exist in HK2 cells after hypothermic hypoxia-reoxygenation, which may represent a novel therapeutic target for the protection of donor organs in transplantation. However, based on prior research that developed a strategy for analysing PANoptosis [253], we only validated the existence of PANoptosis-like cell death (the simultaneous occurrence of pyroptosis, apoptosis, and necroptosis), which is the first and most fundamental step. Further studies are warranted to investigate the PANoptosome that simultaneously regulates these three regulated cell deaths in the transplantation setting.

PANoptosis is associated with activating the NLRP3–ASC–caspase-1 protein complex, also known as the NLRP3 inflammasome [124]. NLRP3 inflammasome activation can trigger the self-cleavage and activation of pro-caspase-1, producing proinflammatory cytokines [254]. In addition, active caspase-1 cleaves GSDMD transported to the cell membrane and creates membrane pores, mediating the release of downstream cytokines [255]. Those cytokines, such as TNF- α , IL-1 β , and DAMPs, act as alarmins to initiate and amplify the inflammatory response [255, 256]. Our *in vitro* data demonstrated that NLRP3 inflammasome complex, including NLRP3, ASC, and caspase-1, significantly increased in a time-dependent manner after reoxygenation, which is in accordance with the increase of TNF- α , IL-1 β , IL-6, and HMGB1 released into the cell culture medium, suggesting that regulated cell death induced by hypothermic hypoxia-reoxygenation is closely related to NLRP3 inflammasome activation. Taken together, our findings show a molecular link between the inflammatory-regulated cell death system and the NLRP3 inflammasome, which may identify therapeutic targets for future research on kidney transplantation.

Of note, *in vitro* data showed that HMGB1 significantly increased in the nuclei after 24 h of hypothermic hypoxia, then translocated to the cytosol and released into the cell culture medium after reoxygenation. This is in accordance with a previous study showing that HMGB1 was increased in the liver graft after 16 h of cold preservation [257]. HMGB1 is an essential DAMPs molecule that can be released into the extracellular and interact with certain pattern recognition receptors, such as Toll-like receptors (TLRs) and the receptor for advanced glycation end-products (RAGE), leading to the production of pro-inflammatory cytokines [258]. Therefore, it can be concluded that even though regulated cell death or NLRP3 inflammasome is less likely to occur under hypothermic hypoxia conditions, the important organelles damage and DAMPs formation happened during cold storage and may play an important mediating role in reperfusion injury. Moreover, these early injuries will represent crucial intervention targets for protecting renal grafts against IR injury.

In conclusion, our results suggest the contribution of various regulated cell death pathways in an *in vitro* model, consequently inducing inflammatory factors. Further, *in vivo* kidney transplant research is required to validate these targets. In addition, these cell death pathways may interact with and promote each other and might lead to further kidney graft injury. Additional research is warranted to explore effective strategies to reduce these regulated cell death pathways and the following inflammatory responses in the context of IR injury.

Chapter SIX

**Dexmedetomidine and noble gases protect kidney grafts from
donation after circulatory death**

6.1 Introduction

Chronic kidney disease (CKD) is characterised by a continuing decline in glomerular filtration rate (GFR) and is always related to increased morbidity and mortality [259]. In England, it is reported that CKD stages 1-5 affected 12.6% of the population aged 16 years and older in the year of 2009/2010 and 13.9% in the year of 2016 [260]. Worldwide, it is estimated that nearly 5.439 million people will have to receive kidney replacement therapy by 2030 because of the progression from CKD to end-stage renal disease (ESRD) [261]. By 2040, ESRD will be the fifth largest cause of death in the world, bringing substantial healthcare budgets for individuals, families, and nations [262].

As the gold standard of therapy for ESRD patients, kidney transplantation significantly increases patient survival rate, reduces post-surgical complications, and improves the health-related quality of life compared with dialysis [263, 264]. In the recent 2 decades, the collaboration of NHS Blood and Transplant with appropriate professional and patient partners as part of a national strategy has increased organ donation and transplantation in the UK [265]. However, donor organ demand still exceeds supply, which forces many ESRD patients to continue dialysis [265]. In 2019, there were only 2,280 patients who had a kidney transplant out of almost 5,000 individuals on the transplant waiting list in the UK [266]. It is estimated that the average wait for a kidney graft is over 3 years, and nearly 12% of patients die while awaiting a kidney transplant or are removed from the transplant list owing to disease progression before getting a kidney [267]. Meanwhile, transplanted kidneys usually survive 12-20 years, after which recipients require a second transplant or dialysis [268]. Therefore, it is urgent to enhance the longevity and efficacy of transplants and facilitate the availability of more organs for transplant.

Although most recipients obtain kidneys from DBD donors, there has been a significant increase in the number of transplants using kidneys from DCD donors, such as in the UK, Netherlands, and Belgium [30]. In the UK, DCD donation has increased significantly over the last decade from 335 donors in 2009/2010 to 634 in 2019/2020, accounting for 36% of all deceased donor kidney transplants [266]. However, most countries worldwide have no DCD donation programmes because DCD kidneys are more susceptible to IR injury, and the outcomes are inferior compared to DBD kidneys, especially when the donors are elderly or have comorbidities [269, 270].

Cold storage with a preservation solution is the most common method to reduce IR injury and maintain graft viability before implantation [55]. Therefore, new strategies are required to preserve kidneys for extended periods or to preserve kidneys obtained from extended criteria or DCD donors. In addition, current cold preservation procedures are based on immersion in a cold storage solution under air [60]. Research on cold storage solutions modifying the gas composition of the solution for improving preservation should be considered [172, 271]. Noble gases, such as Ar and Xe, have been used in food preservation and clinical settings by limiting oxidation before their development for graft preservation [272-274]. In addition, our previous studies have demonstrated that Dex exerted potential protective effects against IR injury in kidneys [140, 200, 275, 276]. Therefore, in the present study, we aim to investigate whether combining different concentrations of noble gases and Dex in the UW solution could provide greater protection against IR injury and regulate cell death to improve graft preservation from a porcine DCD donation.

6.2 experimental design

6.2.1 investigating the effects of cold storage on the morphology of porcine DCD kidney grafts

kidneys from experimental mini-pigs were procured 30 min after circulatory death and then preserved at 4 °C in the UW solution for 24 or 72 h. Negative control tissues were fixed with 4% paraformaldehyde immediately after procurement without cold storage. Fixed grafts were sectioned into 5 µm slices, and then Hematoxylin and eosin (H&E) staining and Periodic Acid Schiff (PAS) staining were conducted to evaluate graft morphology.

6.2.2 Investigating the effects of cold storage on the oxidative stress of porcine DCD kidney grafts

kidneys from experimental mini-pigs were procured 30 min after circulatory death and then preserved at 4 °C in the UW solution for 24 or 72 h. Negative control tissues were fixed with 4% paraformaldehyde immediately after procurement without cold storage. Fixed grafts were sectioned into 5 µm slices, and then oxidative stress was evaluated using OxyIHC oxidative stress assay.

6.2.3 Investigating the effects of Dex, Xe, or Ar on regulated cell death of porcine DCD kidney grafts cold stored for 24 or 72 h

Kidneys from experimental mini-pigs were procured 30 min after circulatory death and then stored at 4 °C in the UW solution with 0.1 nM Dex plus different concentrations of Xe and Ar for 24 or 72 h. Positive control tissues were fixed after cold storage for 24 or 72 h, supplemented with 50% N₂, 45% O₂, and 5% CO₂. Fixed grafts were sectioned into 5 µm slices, and multiple

regulated cell death, including apoptosis, necroptosis, and ferroptosis, were measured by detecting TUNEL⁺ cells, pMLKL, and GPX4 expression using immunofluorescence staining.

6.3 Results

6.3.1 Cold preservation impaired kidney morphology and increased renal oxidative stress

To explore the impacts of cold ischaemia on DCD kidney grafts, we established an *ex vivo* model of DCD kidney preservation. Kidney injury was evaluated using H&E and PAS staining, and the oxidative stress was examined using an OxyIHC assay. H&E staining results (Figure 6.1A) showed that compared to non-cold preserved kidneys (NC), kidneys cold-stored for 24 h (CI24h) or 72 h (CI72h) had darker purple-coloured cytoplasm and deep blue nuclei, indicating that hypertonic UW solution caused renal tissue shrinkage. At 24 h of cold storage, moderate cell dissociation and loss of nuclei staining in both tubular and glomerular cells were observed. Whilst at 72 h of cold storage, renal tissue showed loss of tubule architecture, most cells were necrotic and dissolved, the nuclei were broken down, and loss of staining, and the glomerular contracture shrank due to the extensive necrosis of glomeruli. PAS staining (Figure 5.1B) also demonstrated that cold storage significantly damaged renal structure by causing cell necrosis and dissociation in both tubular and glomerular. Immunohistochemical staining with OxyIHC (Figure 6.1C) showed that cold storage slightly increased oxidative stress in kidney grafts cold-stored for 24 h with increased yellow to brown cells and induced severe oxidative damage in kidney grafts cold stored for 72 h with massive yellow to brown cells.

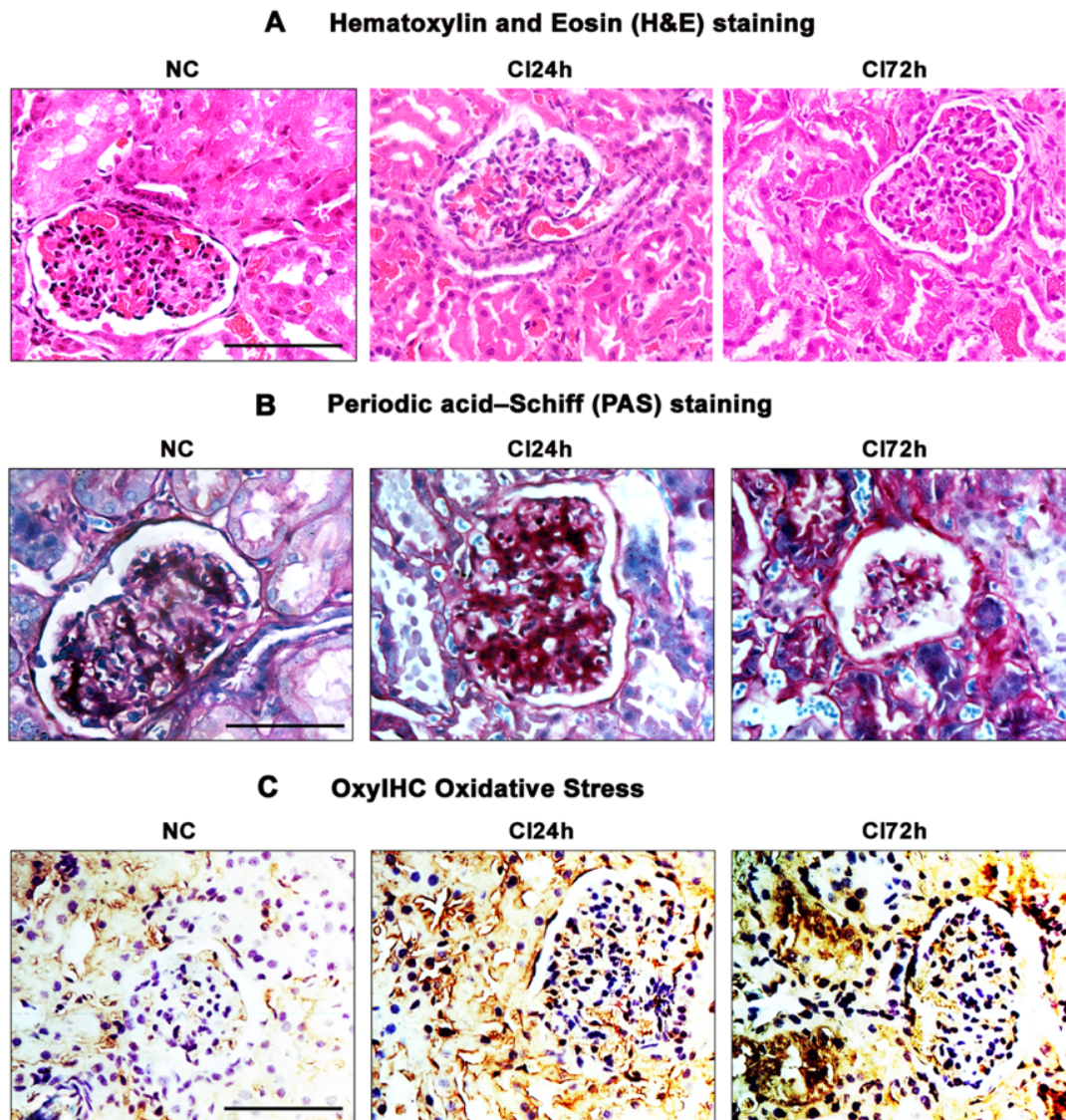


Figure 6.1 Cold preservation damages DCD kidney morphology and increases renal oxidative stress. Experimental mini-pig kidneys were harvested 30 min after circulatory death and then stored at 0-4 °C in the UW solution (cold ischaemia) for 24 h (CI24h) or 72 h (CI72h). Negative control (NC) tissues were fixed upon procurement without cold storage. **A.** H&E staining of kidneys cold-preserved for 24- or 72-h. **B.** PAS staining of kidneys cold-preserved for 24- or 72-h. **C.** Oxidative stress of kidney tissues was assessed with immunochemistry. n = 5. Scale bar = 200 µm.

CI: H&E: Haematoxylin & Eosin; PAS: Periodic acid-Schiff; CI: cold ischaemia.

6.3.2 Dex and noble gases protected DCD kidney morphology cold-stored for 24 h

According to the H&E staining results (Figure 6.2A), cold storage (CI24h) significantly reduced the average tubular cell health rating from 89.0% to 50.1% ($p < 0.001$, Figure 6.2B) and reduced the average glomerular cell health rating from 89.3% to 65.2% ($p < 0.001$, Figure 6.2C), as well as increased kidney injury score from 1.07 ± 0.58 to 3.16 ± 0.44 ($p < 0.0001$, Figure 6.2D). All solutions apart from Dex alone (CI24h+Dex) significantly increased tubular cell health rating, especially the solutions supplemented with 30% Ar (CI24h+Dex+30%Ar), 50% Ar (CI24h+Dex+50%Ar), and 50% Xe (CI24h+Dex+50%Xe) ($p < 0.01$, separately) preserved most healthy cells (Figure 6.2B). Meanwhile, the glomerular cell health rating was significantly increased by 50% Xe (CI24h+Dex+50%Xe), 50% Ar (CI24h+Dex+50%Ar), or 25% Xe plus 25% Ar solutions (CI24h+Dex+25%Xe+25%Ar) ($p < 0.01$, separately; Figure 6.2C). In total, 15% Xe and 15% Ar (CI24h+Dex+15%Xe+15%Ar, $p < 0.05$), 25% Xe and 25% Ar (CI24h+Dex+25%Xe+25%Ar, $p < 0.01$), as well as 50% Xe (CI24h+Dex+50%Xe, $p < 0.05$), and 50% Ar (CI24h+Dex+50%Ar, $p < 0.01$) significantly reduced kidney injury score induced by cold storage (Figure 6.2D).

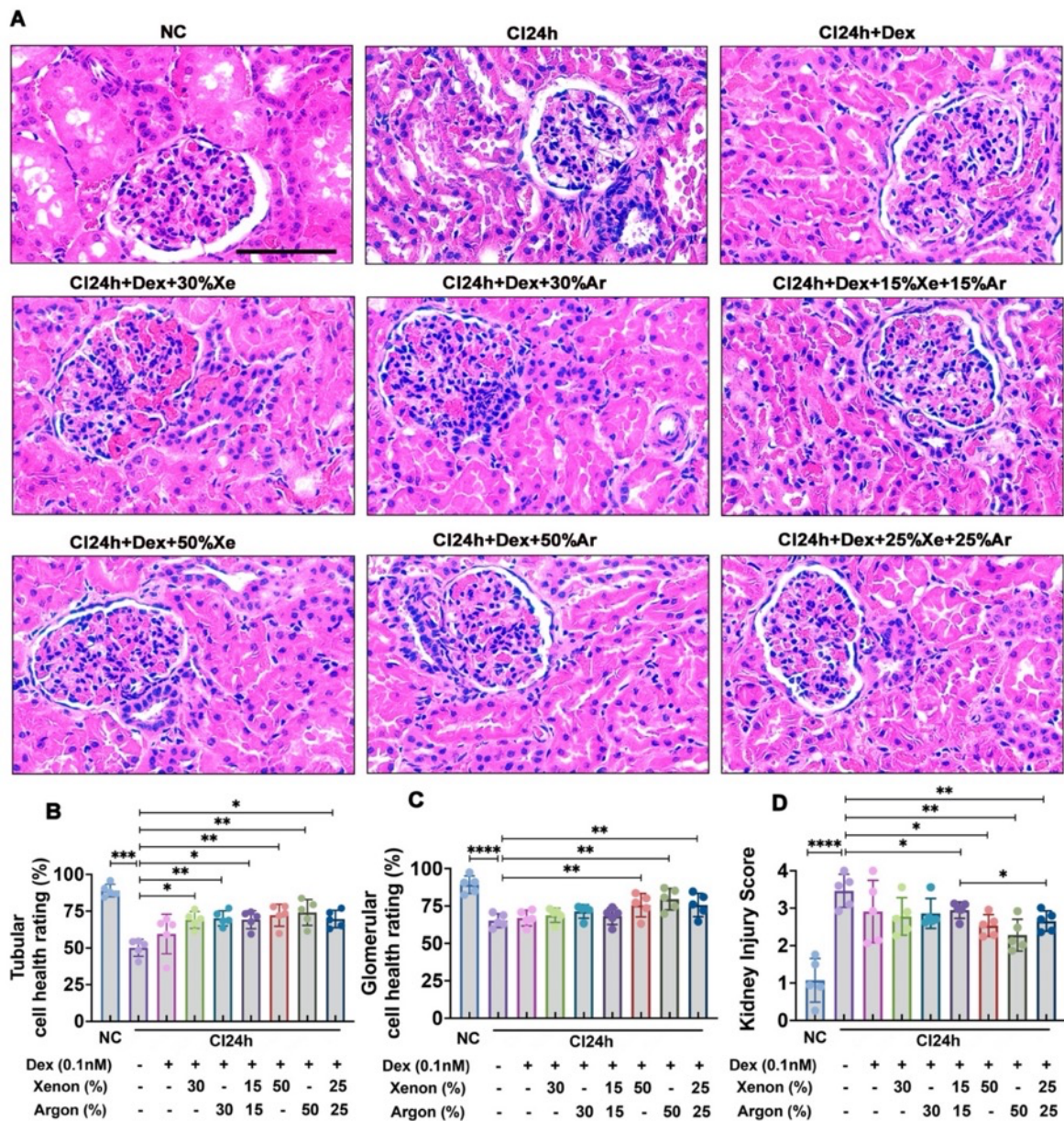


Figure 6.2 Dex and noble gases protect DCD kidney morphology after cold stored for 24 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death. Then they were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** H&E staining of the kidney cold stored for 24 h. **B.** Tubule and **C.** glomerular cell health rating per section. **D.** Kidney injury score. The injury criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Scores 0: normal, 1: <25%, 2: 25-50%, 3: 50-75%, 4: > 75% percentage area. Data were analysed with one-way ANOVA or unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 200 μ m. *p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

6.3.3 Dex and noble gases protected DCD kidney morphology cold-stored for 72 h

Kidney injury was also evaluated after cold storage in the UW solutions for 72 h (Figure 6.3A). The kidneys had severe injury after long-term cold storage (CI72h). The average healthy tubular cells rating was reduced from 78.6% to 31.3% ($p < 0.001$, Figure 6.3B), the average healthy glomerular cells rating was decreased from 91.6% to 41.1% ($p < 0.0001$, Figure 6.3C), and the kidney injury score was significantly increased from 1.07 ± 0.29 to 3.50 ± 0.34 ($p < 0.001$, Figure 6.3D). The solutions supplemented with Dex plus 50% Xe (CI72h+Dex+50%Xe), 50% Ar (CI72h+Dex+50%Ar), or 25% Xe plus 25% Ar (CI72h+Dex+25%Xe+25%Ar), significantly increased tubular cell health rating ($p < 0.05$, separately). The 50% Ar solution (CI72h+Dex+50%Ar) gave better protection to tubule cells than the 30% Ar solution (CI72h+Dex+30%Ar, $p < 0.05$, Figure 6.3B). In addition, all solutions except for Dex alone or 30% Xe significantly increased glomerular cell health rating, especially the solution supplemented with Dex plus 50% Ar (CI72h+Dex+50%Ar) preserved most healthy tubule cells ($p < 0.001$, Figure 6.3C). In total, Dex plus 50% Xe (CI72h+Dex+50%Xe, $p < 0.05$), 50% Ar (CI72h+Dex+50%Ar, $p < 0.01$), and 25% Xe plus 25% Ar (CI72h+Dex+25%Xe+25%Ar, $p < 0.05$) significantly reduced kidney injury score induced by long-term cold storage (Figure 6.3D).

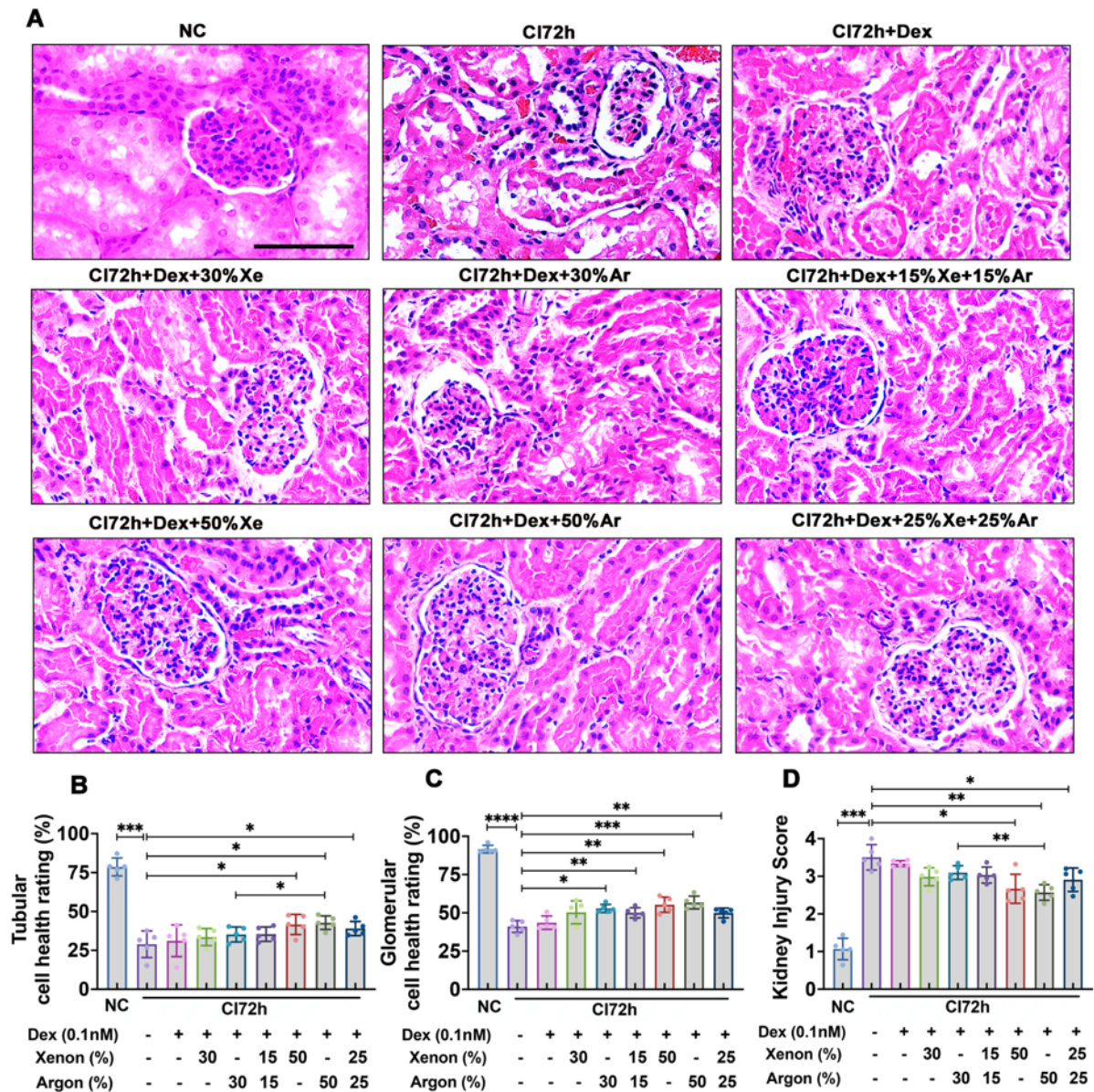


Figure 6.3 Dex and noble gases protect DCD kidney morphology after cold stored for 72 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death. Then they were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** H&E staining of the kidney cold stored for 72 h. **B.** Tubule and **C.** glomerular cell health rating per section. **D.** Kidney injury score. The injury criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Scores 0: normal, 1: <25%, 2: 25-50%, 3: 50-75%, 4: > 75% percentage area. Data were analysed with unpaired t-test and presented as scatter plots with bar, mean $\pm$ SD. $n = 5$. Scale bar = 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

6.3.4 Dex and noble gases reduced apoptosis in DCD kidneys cold-stored for 24 h

Kidney sections were stained with TUNEL (green) and To-pro (red) to visualise and quantify apoptosis after 24 h of cold storage (Figure 6.4A). Compared to the non-cold storage DCD kidney grafts (NC), the average number of TUNEL⁺ apoptotic cells was significantly increased from 3.80 ± 1.48 to 31.6 ± 3.91 cells per field after 24 h of cold storage (CI24h, $p < 0.001$, Figure 6.4B). There was a significant decrease in TUNEL⁺ cells at 24 h in all cold storage solutions supplemented with Dex and varying concentrations of Xe and/or Ar, especially the solution supplemented with Dex plus 50% Ar (CI24h+Dex+50%Ar) and Dex plus 25% Xe and 25% Ar (CI24h+Dex+25%Xe+25%Ar), which reduced TUNEL⁺ cells by nearly 70% to 6.80 ± 1.92 cells per field ($p < 0.0001$, separately, Figure 6.4B). Therefore, it can be concluded that Dex and noble gases strongly diminished apoptosis in cold-stored kidney grafts.

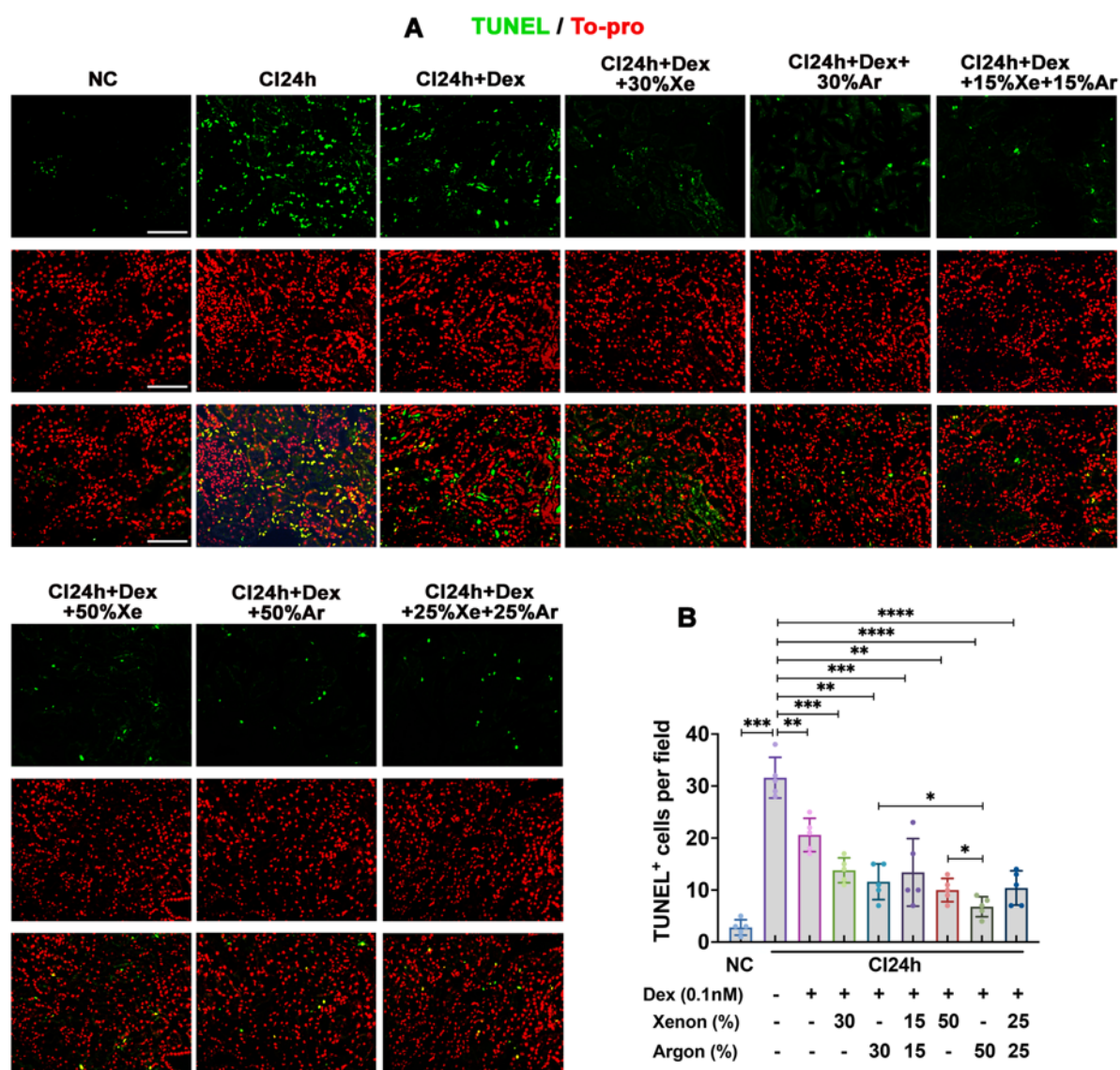


Figure 6.4 Dex and noble gases reduce apoptosis in DCD kidneys cold stored for 24 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death. Then they were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** TUNEL (green) and To-Pro (red) staining in the kidney upon 24 h cold storage. **B.** Average number of TUNEL⁺ apoptotic cells per field of view upon 24 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling.

6.3.5 Dex and noble gases reduced apoptosis in DCD kidneys cold-stored for 72 h

Kidney sections were stained by TUNEL (green) and To-pro (red) to visualise and quantify apoptosis after 72 h of cold storage (Figure 6.5A). Compared to non-cold storage DCD kidney grafts (NC), the average number of TUNEL⁺ apoptotic cells was significantly increased after 72 h of cold storage (CI72h) from 3.20 ± 1.32 to 50.80 ± 8.53 ($p < 0.0001$, Figure 6.5B). All cold storage solutions supplemented with Dex and varying concentrations of Xe and/or Ar significantly reduced the average number of TUNEL⁺ apoptotic cells induced by long-term cold storage, and higher concentrations of noble gas appeared to maximise the reduction in apoptosis.

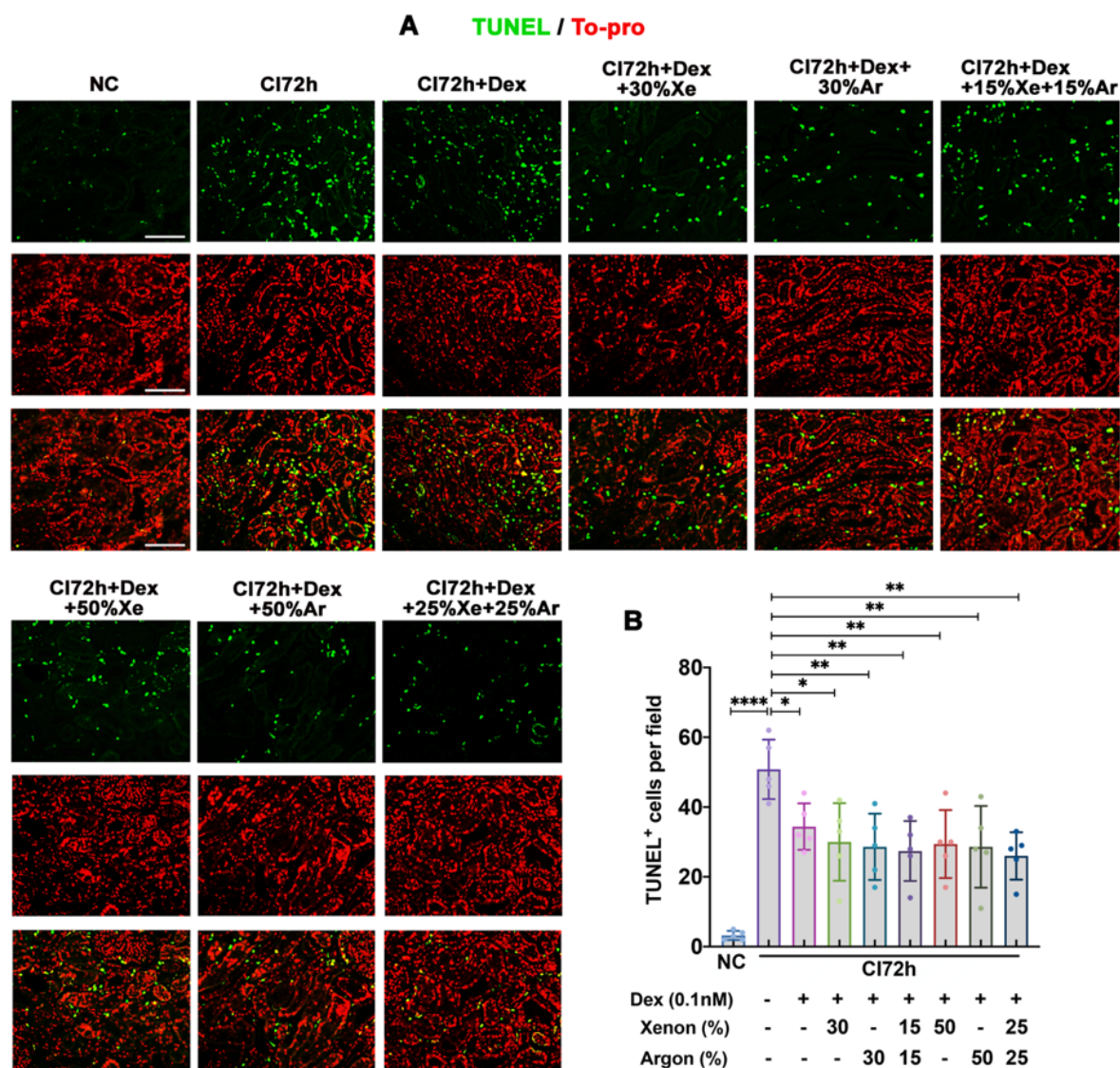


Figure 6.5 Dex and noble gases reduce apoptosis in DCD kidneys cold stored for 72 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death. Then they were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** TUNEL (green) and To-Pro (red) staining in the kidney upon 72 h cold storage. **B.** The average number of TUNEL⁺ apoptotic cells per field of view upon 24 h of cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling.

6.3.6 Dex and noble gases reduced necroptosis in DCD kidneys cold-stored for 24 h

Then, we quantified the presence of pMLKL, a mediator of necroptosis, in cold-stored renal cortex slices (Figure 6.6A). Results showed that cold storage significantly increased the expression of pMLKL at 24 h (CI24h) when compared to non-cold storage control ($p < 0.001$, Figure 6.6B), indicating necroptosis occurred. Notably, pMLKL was mainly located on renal tubular cells but not glomerular cells, suggesting that renal tubular cells were more susceptible to necroptosis. Additionally, all groups supplemented with Dex and/or noble gases showed a trend toward decreased pMLKL expression. At the same time, high concentrations of 50% Xe (CI24h+Dex+50%Xe) or 50% Ar (CI24h+Dex+50%Ar) reduced pMLKL expression by more than 30% separately ($p < 0.01$, separately; Figure 6.6B).

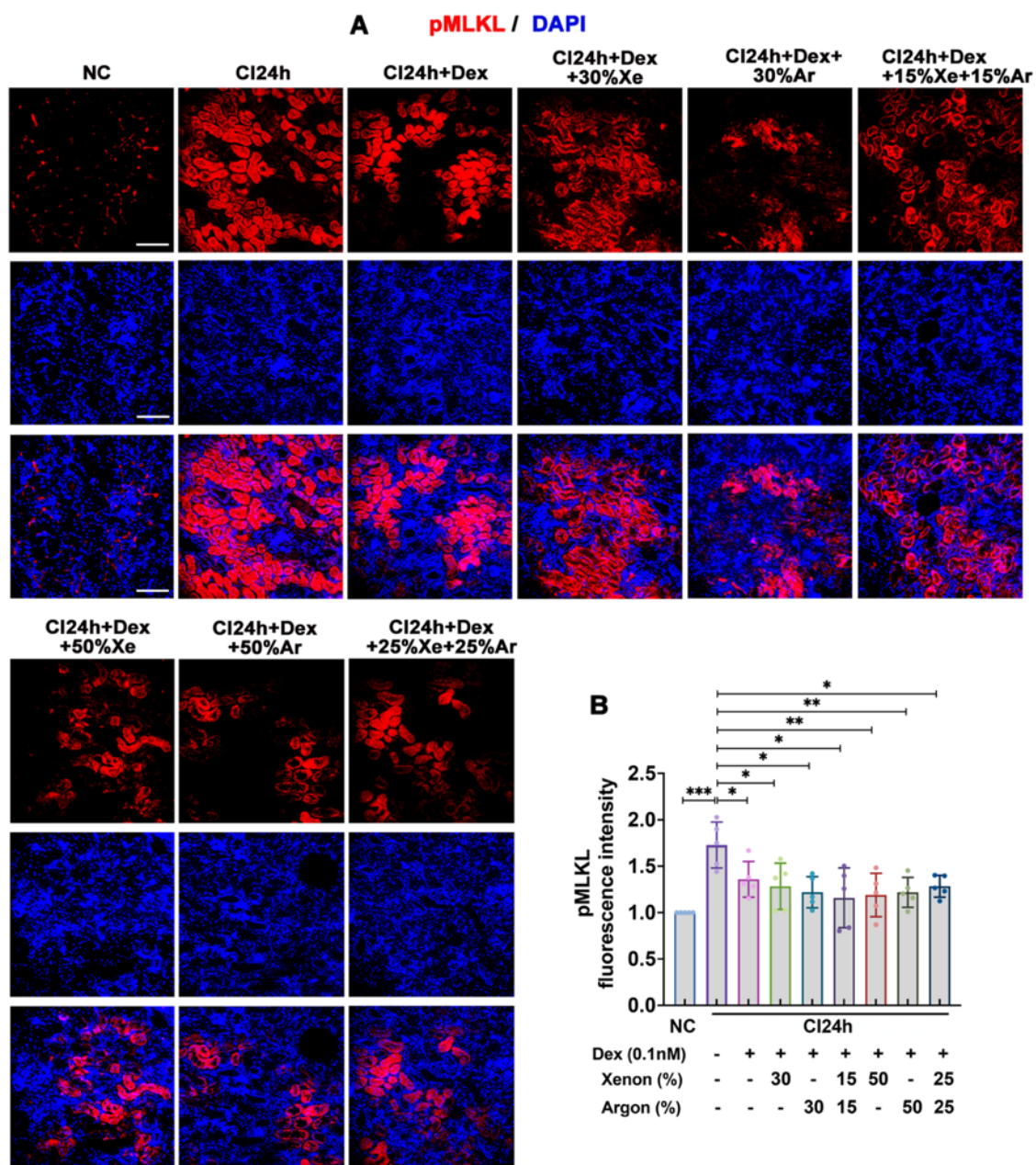


Figure 6.6 Dex and noble gases reduce necroptosis in DCD kidneys cold stored for 24 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** pMLKL (red) and DAPI (blue) staining in the kidney upon 24 h cold storage. **B.** Average pMLKL fluorescence intensity per field of view upon 24 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; DAPI: 4',6-diamidino-2-phenylindole.

6.3.7 Dex and noble gases did not affect necroptosis in DCD kidneys cold-stored for 72 h

After being cold stored for 72 h (CI72h), pMLKL expression was identified in around 70% of renal tubules, demonstrating massive necroptosis (Figure 6.7A and B, $p < 0.0001$). However, there was no significant decrease in pMLKL expression when solutions were supplemented with Dex or noble gases.

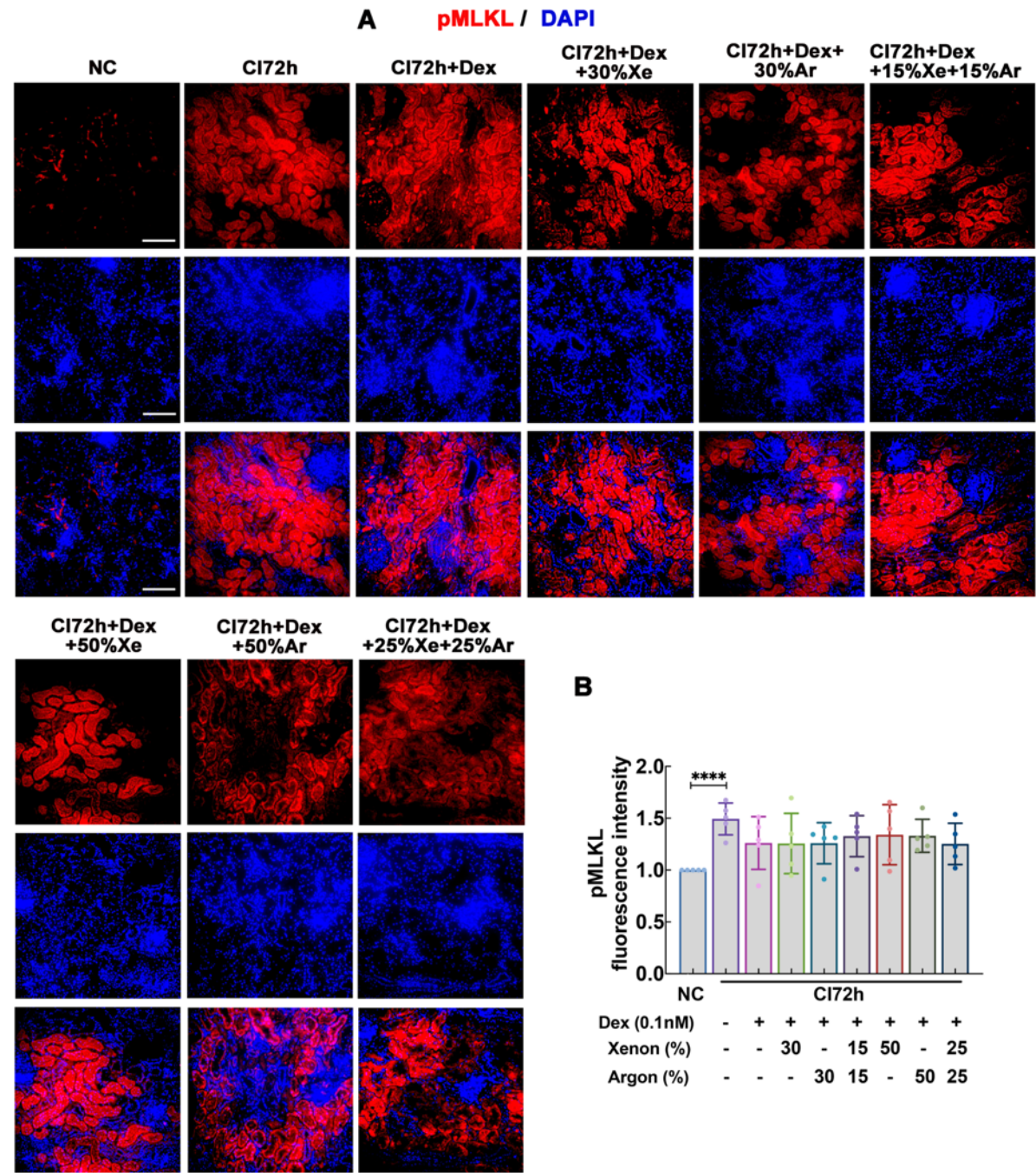


Figure 6.7 Dex and noble gases do not affect necroptosis in DCD kidneys cold stored for 72 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** pMLKL (red) and DAPI (blue) staining in the kidney upon 72 h cold storage. **B.** Average pMLKL fluorescence intensity per field of view upon 72 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 200 μ m. **** p < 0.0001.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; DAPI: 4',6-diamidino-2-phenylindole.

6.3.8 Dex and noble gases did not change GPX4 expression in DCD kidneys cold-stored for 24 h

The expression of GPX4, an antioxidant enzyme that acts as a protective factor against ferroptosis, was detected in porcine DCD kidney grafts after 24 h of cold storage (Figure 6.8A). Compared to non-cold storage kidneys (CI24h), cold storage (CI24h) significantly increased GPX4 expression (p < 0.01, Figure 6.8B), indicating that GPX4 may increase against lipid peroxidation of renal tissue caused by cold ischaemia. Additionally, the solution supplemented with Dex plus 25% Xe and 25% Ar (CI24h+Dex+25%Xe+25%Ar, p < 0.001) further increased GPX4 expression compared with non-cold storage kidneys (NC), indicating that Dex and noble gases may enhance the antioxidant capability of kidney grafts. However, there were no significant changes between the different solutions.

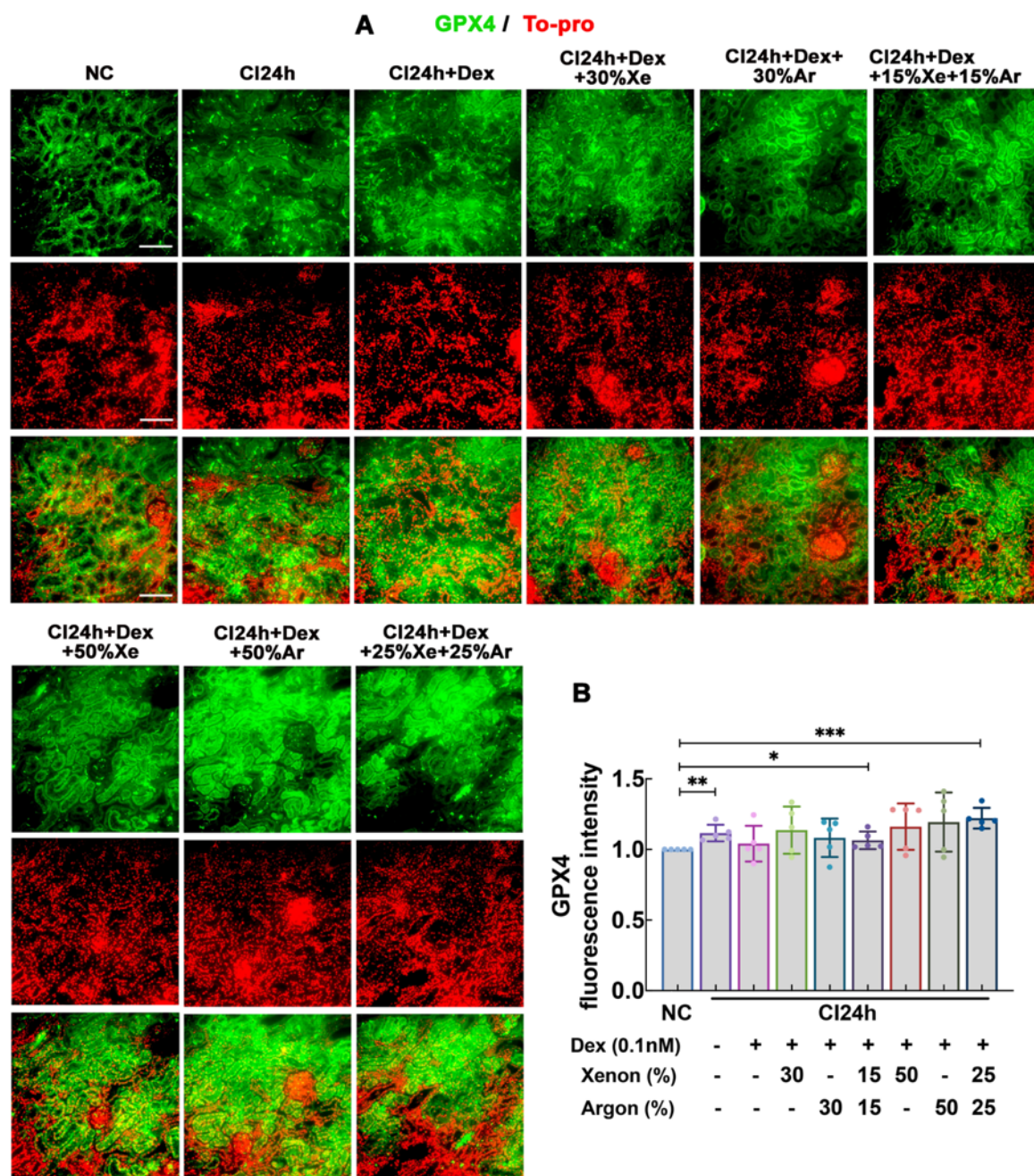


Figure 6.8 Dex and noble gases do not affect GPX4 expression in DCD kidneys cold stored for 24 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** GPX4 (green) and To-pro (red) staining in the kidney upon 24 h cold storage. **B.** Average GPX4 fluorescence intensity per field of view upon 24 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 200 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; GPX4: Glutathione peroxidase 4.

6.3.9 Dex and noble gases did not change GPX4 e in DCD kidneys cold-stored for 72 h

After being cold stored for 72 h (CI72h), GPX4 expression was significantly increased compared to non-cold storage kidneys (Figure 6.9A and B, $p < 0.01$), whilst there was no significant difference in GPX4 expression between all solutions.

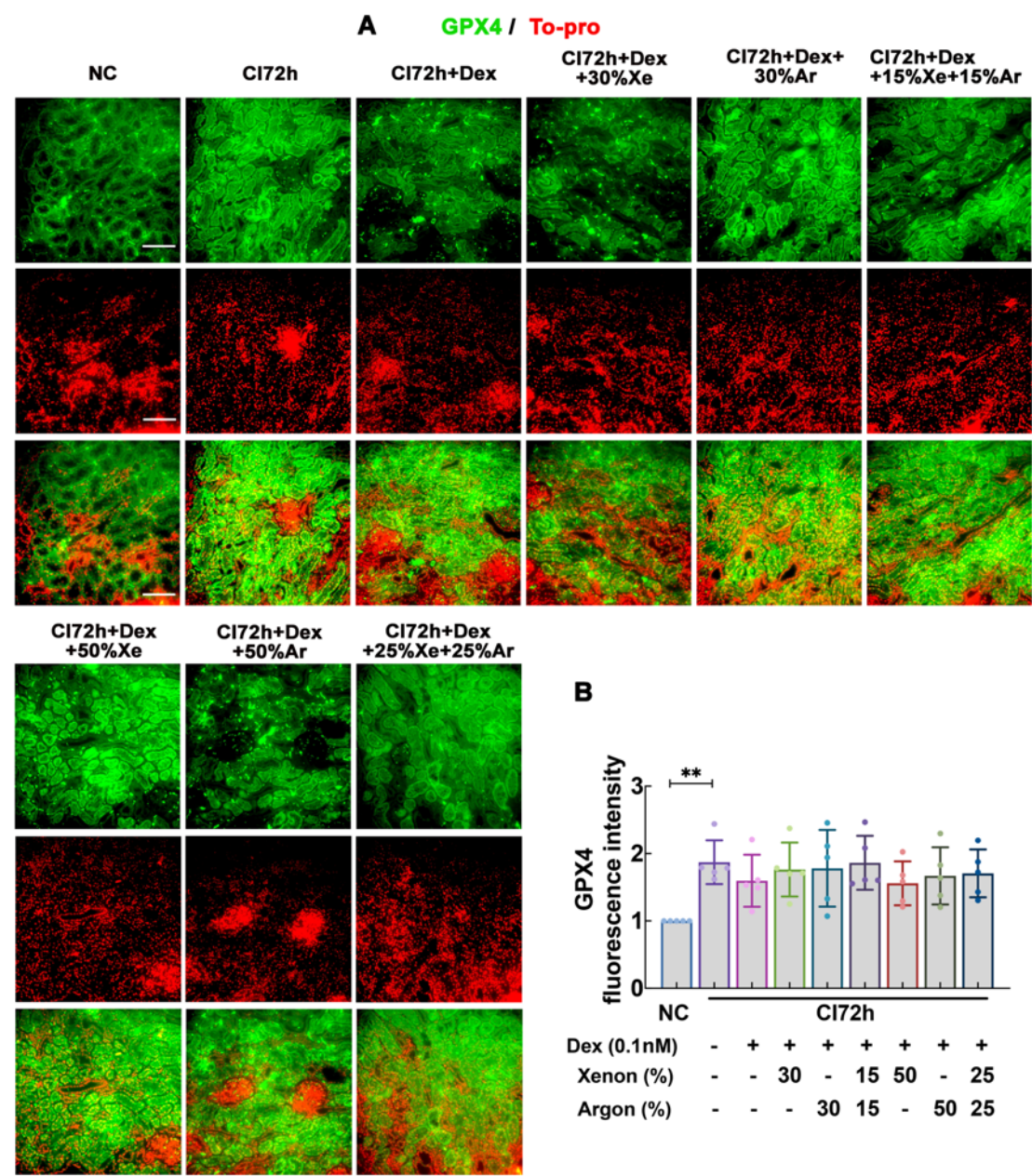


Figure 6.9 Dex and noble gases do not affect GPX4 in DCD kidneys cold stored for 72 h. Experimental mini-pig kidneys were harvested 30 min after circulatory death. Then they were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** GPX4 (green) and To-pro (red) staining in the kidney upon 72 h cold storage. **B.** Average GPX4 fluorescence intensity per field of view upon 72 h cold storage. Data were analysed by One-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 200 μ m. * p < 0.05, ** p < 0.01.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; GPX4: Glutathione peroxidase 4.

6.4 Discussion

The use of kidneys from DCD donation has become widespread to expand the donor pool. Therapeutic techniques that improve the long-term survival of DCD kidney grafts during cold storage before transplantation increase the safe use of kidneys [277, 278]. This study suggested that warm ischaemia followed by prolonged cold storage for 24 or 72 h significantly impaired the kidney architecture in DCD porcine kidney grafts, increased renal tissue oxidative stress, and activated several regulated cell death pathways, such as apoptosis and necroptosis. These inflammatory cell death forms may significantly shorten the long-term survival of DCD kidneys. Furthermore, UW storage solutions supplemented with Dex and the noble gases Xe and Ar significantly improved the quality of cold-stored DCD kidneys by attenuating the activation of apoptotic and necroptotic execution proteins and upregulating the antioxidant enzyme GPX4. The UW solution containing Dex plus 50%Ar would be the best option for maintaining graft quality during cold storage.

This study used a porcine model to mimic clinic DCD donation because it closely reflects humans in size, physiology, anatomy, development, and immunology [279, 280]. In most

clinical cases, grafts are usually cold-preserved no longer than 16 h before implantation [225]. This study referred to extreme cases with longer cold ischaemic time, as 24 h and 72 h of cold storage models were conducted, respectively. Due to morphological damage being the gold standard criteria for IR injury [281, 282], we found that cold storage caused a variant degree of structural damage in kidney grafts from the DCD porcine, including loss of tubule architecture, cell death and dissolve, nuclei break down and glomerular contracture shrank. These disruptions to renal architecture may cause uncontrolled paracellular diffusion of water, ions, and macromolecules, resulting in a decreased glomerular filtration rate (GFR). These changes are associated with delayed graft function, chronic graft dysfunction, and graft rejection [283, 284]. Local pro-inflammatory cytokines leak through the bloodstream, which may also cause systemic inflammatory response syndrome (SIRS) and multiorgan failure syndrome (MODS) [285]. Because of these pre-existing damages before transplantation, post-surgical complications may be more severe in DCD grafts. Therefore, finding effective therapies to improve kidney graft quality and function in the early cold storage period is urgent.

One of the most interesting results in our investigation is that unlike *ex vivo* porcine kidneys that presented significant apoptosis and necroptosis after cold storage, in Chapter Five, HK2 cells were less likely to undergo apoptosis or necroptosis under hypothermic hypoxia conditions. This may be because regulated cell death processes are largely dependent on energy and enzymes [286-288]; these types of cell death may not occur due to insufficient energy production and enzyme inactivation at a low temperature *in vitro*. In contrast, in DCD donors in this Chapter, kidney grafts were excised after 30 min of cardiac arrest, and the hypoxia and hypotension associated with cardiac arrest lead to systemic hemodynamic disturbances and metabolic abnormalities that may result in cell damage during the warm

ischaemia period [289]. Moreover, the cold perfusion and storage may further aggravate kidney injury induced by warm ischaemia due to cold tolerance being finite in tissues; the ATP consumption continues at low temperatures [16]. Therefore, our results indicated that all possible measures should be taken in clinical practice to reduce the warm ischaemia injury.

Interestingly, our *ex vivo* results showed that GPX4 expression was significantly increased during cold storage, which was different from *in vitro* data shown in Chapter Five, in which GPX4 was significantly decreased in HK2 cells that experienced hypothermic hypoxia-reoxygenation. GPX4 is an essential anti-ferroptosis enzyme with the capacity to neutralise lipid peroxidation, while cold storage may trigger lipid peroxidation in liver sinusoidal endothelial cells [290]. We hypothesise that this difference may be caused by the absence of a warm ischaemia period in the *in vitro* study or that ferroptosis in kidneys may be largely influenced by other cells, such as macrophages, which are crucial for maintaining tissue homeostasis by regulating inflammation and iron, lipid, and amino acid metabolisms through their special functions like ROS production [291-293]. In addition, GPX4 is only an indicator of sensitive ferroptosis but does not demonstrate the occurrence of ferroptosis; thus, further investigations about quantifying lipid peroxides [294, 295] and other ferroptosis mediators [296, 297] are required to validate the *ex vivo* data further. To date, most of the research has focused on the contributions and molecular mechanisms of ferroptosis to warm IR [298-301]; little research has been conducted on how ferroptosis affects cold ischaemia injury of transplantation, particularly for marginal or DCD donation. Due to the complex contribution of ferroptosis to cold IR injury, more in-depth studies are warranted.

In the present study, we found that all cold storage solutions but Dex alone significantly increased tubular cell death rating, while Dex combined with 50% Xe or 50% Ar significantly increased glomerular injury at 24 h; this is likely because tubule cells are more sensitive to injuries, such as IR injury [302]. Meanwhile, all solutions significantly reduced both TUNEL⁺ and necroptotic cells at 24 h of cold storage, suggesting an organoprotective effect of Dex and noble gases in reducing apoptosis and necroptosis. In contrast, high concentrations of noble gases, especially 50% Ar, showed better effects.

There has been significant interest in the renoprotective properties of Xe and Ar. A previous study revealed that pre-conditioning with 70% Xe significantly increases renal function with earlier creatinine normalisation and prolonged survival time in kidney transplanted mice [167]. This renal protective effect of Xe may be mediated by upregulating HIF-1 α in the mammalian target of the rapamycin (mTOR) pathway, leading to an increase in hypoxia-tolerating factors erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [168]. Additionally, in our study, the UW solution containing 50% Ar reduced morphological damage, apoptosis and necroptosis better than solutions containing 50% Xe. This is consistent with a previous study that showed that an Ar-saturated cold storage solution significantly improved creatinine clearance and decreased urinary albumin post-transplantation compared to groups with storage solutions saturated with air or Xe [171]. Evidence of an Ar-saturated storage solution in protection against IR injury in the setting of renal transplantation is less established compared to Xe. Nonetheless, the present study suggested that Ar treatment of the graft storage solution could also lead to good early functional recovery, graft quality and survival.

In conclusion, our results provide evidence supporting the clinical potential of Dex and noble gases supplemented cold storage for transplant graft preservation, while Dex combined with a high concentration of noble gas, especially 50% Ar, showed the best benefits to improve kidney graft morphology and reduce regulated cell death during cold storage. Clinical translation of both components may significantly expand the donor pool by including more renal grafts, shorting waiting times, and improving patient survival and quality of life.

Chapter SEVEN

Dexmedetomidine and noble gases in combination against ferroptosis through Txnip-mediated anti-oxidative stress in liver transplantation

7.1 Introduction

Liver transplantation is a life-saving therapy for patients with end-stage liver diseases. In 2019, more than 34,500 liver transplants were performed worldwide [303]. Due to advancements in surgical techniques, immunosuppressive drugs, and post-operative care, the success rate of liver transplantation has significantly increased [304]. However, this field has several challenges, and the most important challenge is the organ donor shortage [305, 306]. In 2019, approximately 118,000 patients were waiting for an organ transplant globally, with the majority of them awaiting a kidney or liver [303].

One strategy to expand the availability of liver donor organs is to increase deceased organ donation, such as increasing DCD donors to expand the donor pool [85]. DCD liver transplantation is currently associated with several challenges, including an increased risk of IR injury, which can result in graft dysfunction and failure [85, 307, 308]. In addition, it has been reported that, in the UK, graft loss and recipient mortality were approximately twice as high with DCD livers compared to DBD livers [309]. In addition, graft failure progressed continuously over 180 days in DCD livers as opposed to 20 days in DBD livers, significantly limiting the use of DCD livers [310]. Several strategies have been proposed to mitigate IR injury to improve the quality of DCD liver transplantation, such as using pharmacological agents, like anti-inflammatory agents [311] and antioxidant treatments [312], to reduce inflammation and oxidative stress during transplantation.

It has been well explored how mitochondrial metabolism change contributes to IR injury and poor outcomes of liver transplantation. Due to the imbalance between the overproduction of ROS and antioxidant mechanisms within the body, mitochondrial oxidative stress is a

significant issue in liver transplantation ^[16]. Meanwhile, oxidative stress can initiate a signalling cascade that activates regulated cell death pathways. For instance, oxidative stress activates the p53 pathway, resulting in apoptosis or ferroptosis [88]. By interfering with cellular redox homeostasis, oxidative stress also induces necroptosis [313]. These types of regulated cell death trigger the release of inflammatory cytokines, such as TNF- α , IL-1, and IL-6, and have been implicated in the damage to liver grafts [92, 115, 314]. Recently, thioredoxin interacting protein (Txnip), a glucose and oxidative stress sensor that can regulate mitochondrial redox homeostasis, has been found to play a critical role in the oxidative stress [315]. It has been shown that Txnip may translocate from the cytoplasm to the nucleus or mitochondria, depending on the circumstances [316]. During IR injury, Txnip transfers to mitochondria to bind and inhibit the activity of thioredoxin, an essential enzyme that reduces oxidised proteins, preventing its antioxidant function and leading to an increased ROS overproduction in cells [317]. Therefore, antioxidant therapy targeting Txnip expression and translocation may be a suitable strategy to reduce oxidative stress and related regulated cell death processes in liver transplant patients.

The supplementation of noble gases such as Xe or Ar during cold storage has been considered a strategy to reduce the IR injury [318, 319]. Dex has been reported to mitigate IR injury by reducing oxidative stress and inflammatory responses [320, 321]. On this basis, we hypothesised that supplementing Dex and noble gases to cold storage solutions may protect liver transplants from IR injury during the cold ischemia period by suppressing oxidative stress. We also explored whether this protection is mediated by Txnip expression or translocation. In addition, because Ar, Xe, and Dex each have unique organoprotective properties, we investigated whether combining these agents in cold storage solutions could

provide increased protection against ischemia injury for enhanced organ preservation, such as against structural damage, inhibit the activation of regulated cell death, and reduce oxidative stress in cold storage.

7.2 Experimental design

7.2.1 Investigating the effects of Dex, Xe, or Ar on the morphology of porcine DCD liver grafts cold-stored for 24 or 72 h

Livers from experimental mini-pigs were procured 30 min after circulatory death and then stored at 4 °C in the UW solution with 0.1 nM Dex combined with different concentrations of Xe and Ar for 24 or 72 h. Negative control tissues were fixed with 4% paraformaldehyde immediately after procurement without cold storage. Positive control tissues were fixed after cold storage for 24 or 72 h in a cold storage solution supplemented with 50% N₂, 45% O₂, and 5% CO₂. Fixed liver grafts were sectioned into 5 µm slices, and then H&E staining was conducted to evaluate liver graft morphology.

7.2.2 Investigating the effects of Dex, Xe, or Ar on regulated cell death of porcine DCD liver grafts cold-stored for 24 or 72 h

Livers from experimental mini-pigs were procured 30 min after circulatory death and then stored at 4 °C in the UW solution with 0.1 nM Dex combined with different concentrations of Xe and Ar for 24 or 72 h. Levels of regulated cell death, including apoptosis, necroptosis, and ferroptosis, were measured by detecting TUNEL⁺ cells, pMLKL and GPX4 expression, separately.

7.2.3 Investigating the effects of high concentration Xe, or Ar on glycogen, reticulin framework, and iron deposition in porcine DCD liver grafts cold-stored for 24 or 72 h

Livers from experimental mini-pigs were procured 30 min after circulatory death and then stored at 4 °C in the UW solution with 0.1 nM Dex combined with different concentrations of Xe and Ar for 24 or 72 h. Glycogen content in the liver grafts was measured by PAS staining, reticulin framework was detected by reticulin staining, and iron staining detected iron deposition.

7.2.4 Investigating the effects of high concentration Xe or Ar on oxidative stress level and Txnip expression

Livers from experimental mini-pigs were procured 30 min after circulatory death and then stored at 4 °C in the UW solution with 0.1 nM Dex combined with different concentrations of Xe and Ar for 24 or 72 h. Oxidative stress was detected by OxyIHC oxidative stress assay, and Txnip was detected by immunochemistry.

7.2.5 Investigating the time course of hypothermic hypoxia-reoxygenation on ferroptosis activation in human liver HepG2 cells

Human liver HepG2 cells were preserved in 4 °C UW solution for 24 h (H24h) to simulate hypothermic hypoxia condition, then cultured in fresh DMEM medium at 37 °C for 6 (HR6), 24 (HR24), and 48 h (HR48) to simulate reoxygenation. Ferroptosis was evaluated by detecting GPX4 and ACSL4 expression using western blot. Endothelial reticulum stress was evaluated by detecting Grp78 expression, and intracellular HMGB1 was evaluated using western blot.

7.2.6 Investigating the effects of Dex and Ar in combination on ferroptosis in human liver

HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Human liver HepG2 cells were preserved in 4 °C UW solution for 24 h (H24h) and then cultured in fresh DMEM medium or DMEM medium saturated with 0.1 nM Dex combined with 50% Ar at 37 °C for 48 h (HR48). Ferroptosis was evaluated by detecting GPX4 and ACSL4 expression using western blot. The viability of cell cultures was detected by crystal violet staining.

7.2.7 Investigating the effects of Dex and Ar in combination on the expression of antioxidant proteins in human liver HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Human liver HepG2 cells were preserved in 4 °C UW solution for 24 h (H24h) and then cultured in fresh DMEM medium or DMEM medium saturated with 0.1 nM Dex combined with 50% Ar at 37 °C for 24 h (HR24). Antioxidant proteins catalase, thioredoxin, and SOD1 were detected by western blot.

7.2.8 Investigating the effects of Dex and Ar in Txnip expression and translocation in human liver HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Human liver HepG2 cells were preserved in 4 °C UW solution for 24 h (H24h) and then cultured in fresh DMEM medium or DMEM medium saturated with 0.1 nM Dex or 50% Ar at 37 °C for 4 h (HR4). Txnip expression was detected by western blot. Txnip translocation was detected by immunofluorescence.

7.2.9 Investigating the effects of Dex and Ar in plasma membrane integrity in human liver

HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Human liver HepG2 cells were stained with DiLC18 for 15 min. After washing with PBS, the cells were preserved in 4 °C UW solution for 24 h (H24h) and then cultured in fresh DMEM medium or DMEM medium saturated with 0.1 nM Dex or 50% Ar at 37 °C for 4 (HR4) and 24 h (HR24). The cell plasma membrane was observed using microscopy.

7.3 Results

7.3.1 Dex and noble gases protected DCD liver morphology after cold storage for 24 h

A morphological evaluation of the samples obtained from porcine was performed before preservation and after 24 h of cold preservation in the UW solution supplemented with Dex, Xe, and/or Ar (Figure 7.1A). As expected, the liver morphology obtained by H&E staining revealed a significant difference between livers from negative control tissues (NC) and cold stored tissues (CI24h). Normal tissues showed well-preserved hepatic architecture, while the liver morphology of cold-stored livers (CI24h) exhibited several morphological changes, including cellular swelling, plasma membrane rupture, nuclei broken down and loss of staining, interstitial oedema, as well as denudation of the sinusoidal lining cells. The liver injury score was also increased to 3.21 ± 0.84 after cold storage compared to negative control tissues (1.27 ± 0.34 , $p < 0.01$, Figure 7.1B). Both low and high concentrations of Ar significantly preserved hepatocyte plasma membrane integrity, reduced hepatocyte necrosis, and decreased liver injury score to 1.96 ± 0.43 (CI24h + Dex + 30%Ar, $p < 0.05$) and 1.93 ± 0.43 (CI24h + Dex + 50%Ar, $p < 0.05$, Figure 7.1B), separately. Additionally, 50% Ar (CI24h + Dex + 50%Ar) exhibited a better protective effect than 30% Xe, as evidenced by a significantly reduced liver injury score ($p < 0.05$, Figure 7.1B).

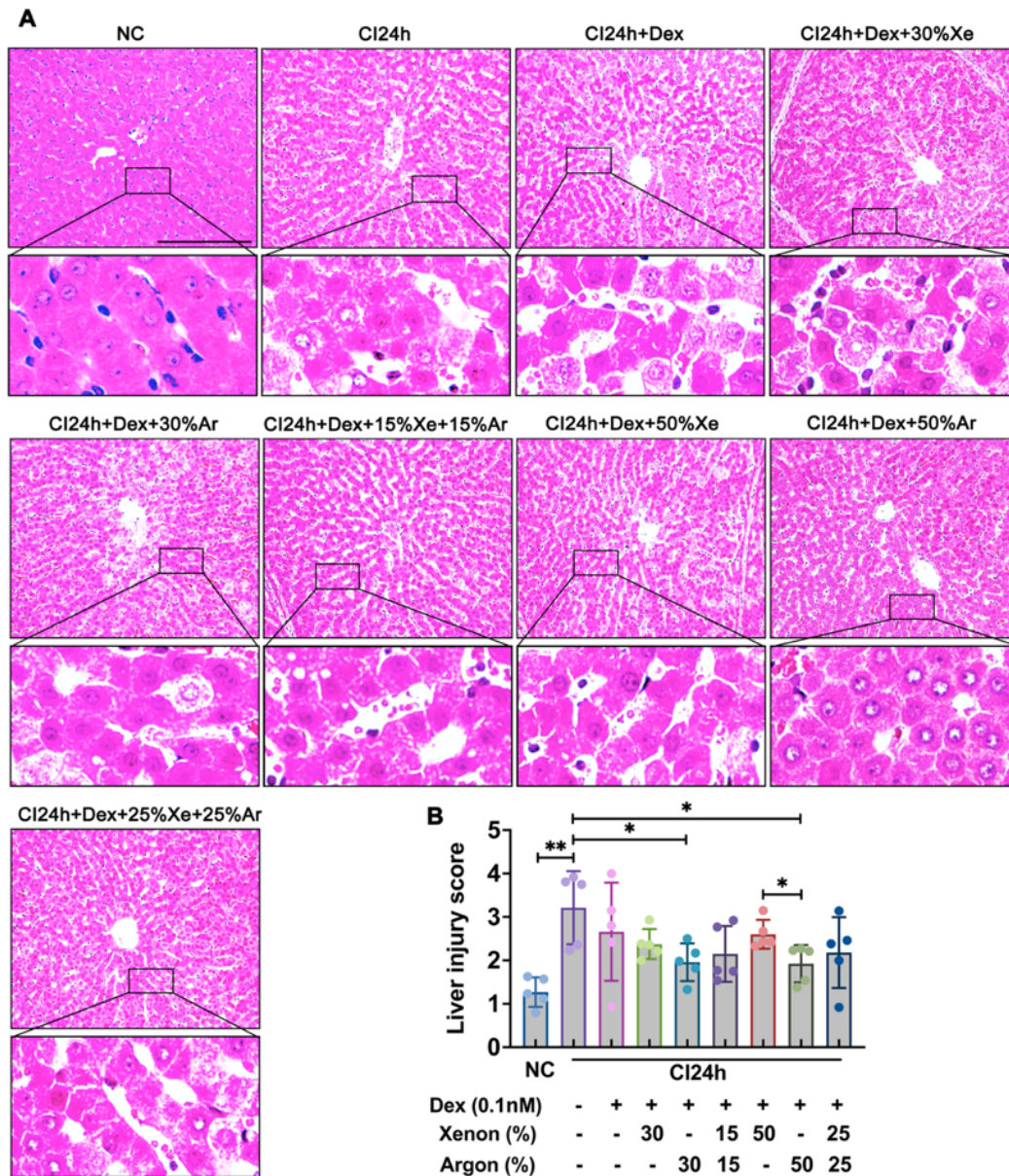


Figure 7.1 Dex and noble gases protect DCD liver morphology after cold stored for 24 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** H&E staining of the liver cold stored for 24 h with Dex or noble gases Xe or/and Ar. **B.** Liver injury score. The injury criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Scores 0: normal, 1: <25%, 2: 25-50%, 3: 50-75%, 4: > 75% percentage area. Data were analysed with unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 200 μ m. *p < 0.05, ** p < 0.01.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

7.3.2 Dex and noble gases protected DCD liver morphology after cold storage for 72 h

A morphological evaluation of the samples obtained from porcine was also performed after 72 h of cold storage. Results showed that cold storage for 72 h (CI72h) resulted in severe liver injury as evidenced by cell dissociation and nuclei loss (Figure 7.2A) and an increased liver injury score (3.23 ± 0.52 , $p < 0.001$) when compared to non-cold storage DCD liver grafts (1.33 ± 0.38 , Figure 7.2B). Only Dex combined with 50% Ar (CI72h + Dex + 50%Ar) significantly well-preserved hepatocytes and reduced liver injury score (2.32 ± 0.53 , $p < 0.05$) in all solutions. These results demonstrated that warm and cold ischaemia extensively injured DCD liver grafts, and the cold storage solution supplemented with Dex combined with 50% Ar (CI72h + Dex + 50%Ar) may be a protective preservation strategy to keep liver graft quality.

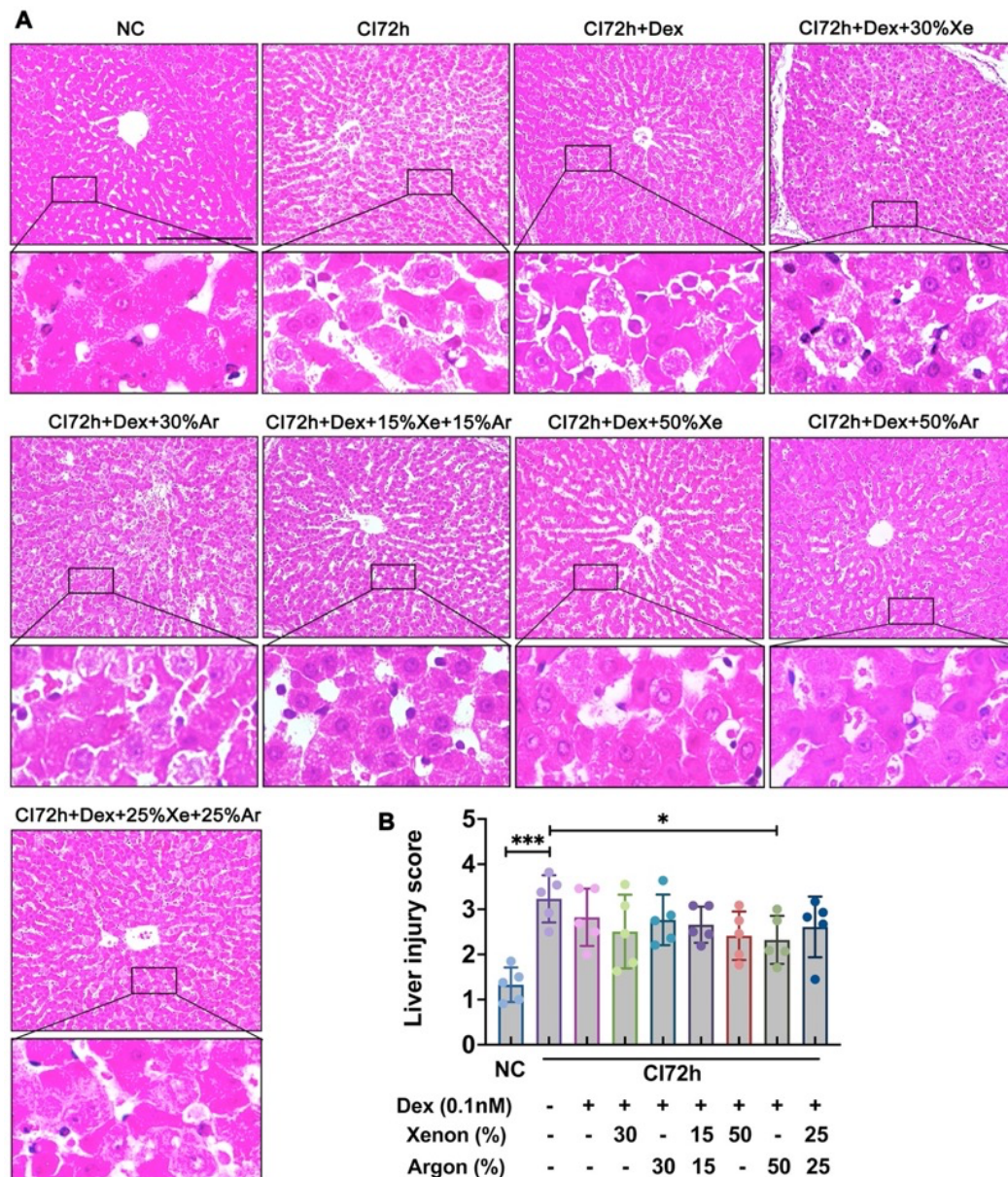


Figure 7.2 Dex and noble gases protect DCD liver morphology after cold stored for 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** H&E staining of the liver cold stored for 72 h with Dex or noble gases Xe or/and Ar. **B.** Liver injury score. The injury criteria were evaluated with cell disruption, nuclear loss, and tissue structure damage. Scores 0: normal, 1: <25%, 2: 25-50%, 3: 50-75%, 4: > 75% percentage area. Data were analysed with unpaired t-test and are presented as scatter plot with bar, mean ± SD. n = 5. Scale bar = 200 µm. *p < 0.05, *** p < 0.001.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

7.3.3 Dex and noble gases reduced apoptosis in DCD livers cold storage for 24 h

Liver sections were stained with TUNEL (green) and To-pro (red) to visualise and quantify apoptosis after 24 h of cold storage (Figure 7.3A). Compared to the non-cold storage DCD liver grafts (NC), the average number of TUNEL⁺ apoptotic cells significantly increased from 33.04 ± 7.43 to 229.41 ± 65.28 cells per field after 24 h of cold storage (CI24h, $p < 0.0001$, Figure 7.3B). There was a significant decrease in TUNEL⁺ cells at 24 h in cold storage solutions supplemented with Dex and varying concentrations of Xe and/or Ar, especially the solution supplemented with Dex combined with 50% Xe (CI24h + Dex + 50%Xe, 96.74 ± 13.84 , $p < 0.0001$), Dex combined with 50% Ar (CI24h + Dex + 50%Ar, 96.92 ± 25.73 , $p < 0.0001$), and Dex combined with 25% Xe and 25% Ar (CI24h + Dex + 25%Xe + 25%Ar, 96.92 ± 23.56 , $p < 0.0001$).

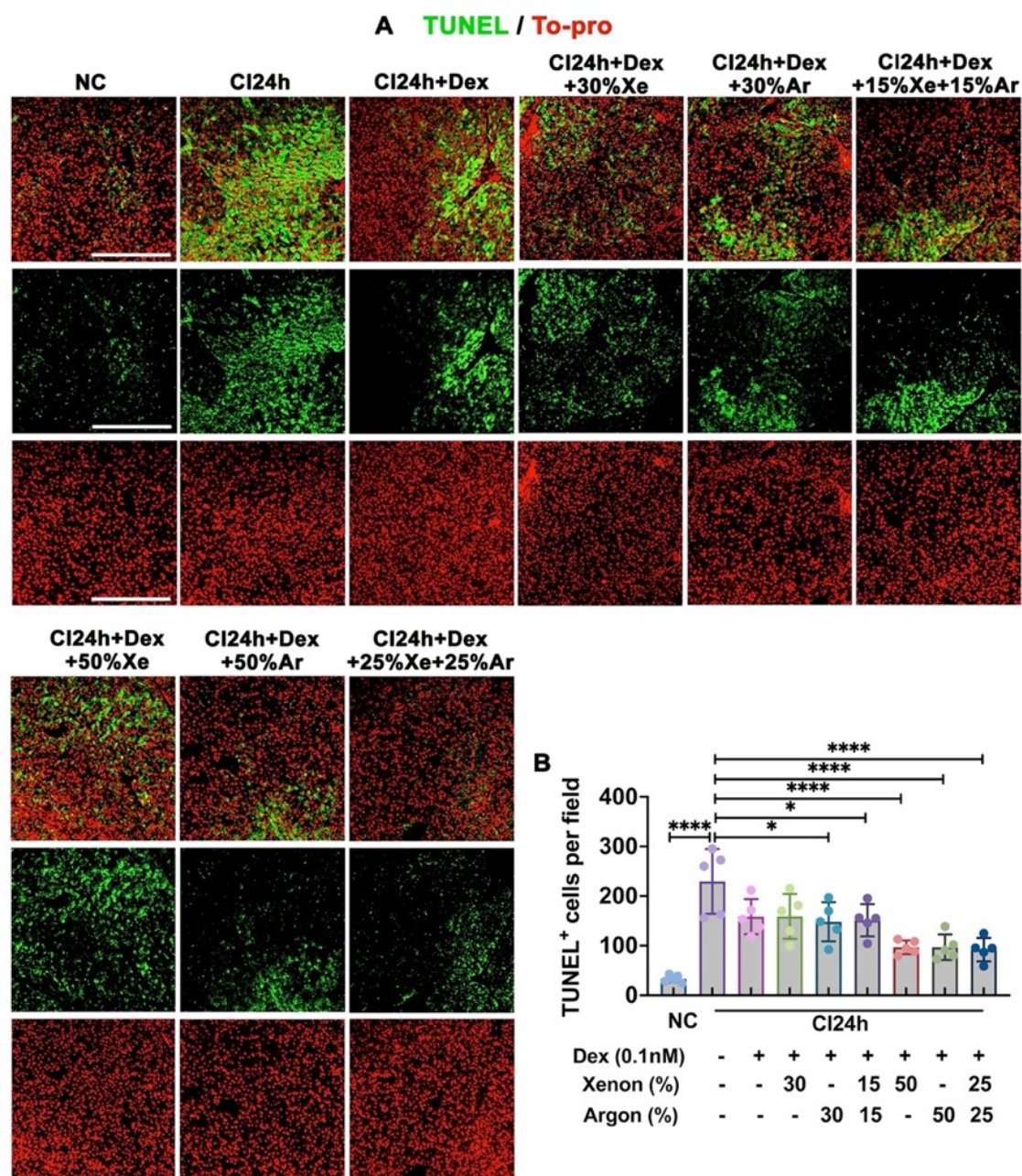


Figure 7.3 Dex and noble gases reduce apoptosis in DCD livers cold stored for 24 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** TUNEL (green) and To-Pro (red) staining in the liver upon 24 h cold storage. **B.** Average number of TUNEL⁺ apoptotic cells per field of view upon 24 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 500 μ m. * $p < 0.05$, **** $p < 0.0001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling.

7.3.4 Dex and noble gases reduced apoptosis in DCD livers cold storage for 72 h

Liver sections were stained by TUNEL (green) and To-pro (red) to visualise and quantify apoptosis after 72 h of cold storage (Figure 7.4A). Compared to non-cold storage DCD liver grafts (NC), the average number of TUNEL⁺ apoptotic cells significantly increased after 72 h of cold storage from 51.22 ± 10.58 to 515.84 ± 159.71 (CI72h, $p < 0.0001$, Figure 7.4B). Cold storage solutions supplemented with Dex and high concentrations of Xe and/or Ar significantly reduced the average number of TUNEL⁺ apoptotic cells to 188.48 ± 50.33 (CI72h + Dex + 50%Xe, $p < 0.01$), 175.86 ± 61.77 (CI72h + Dex + 50%Ar, $p < 0.001$), 200.66 ± 84.01 (CI72h + Dex + 25%Xe + 25%Ar, $p < 0.01$). In addition, the high concentration of Ar supplementation (CI72h + Dex + 50%Ar) more effectively reduced the number of TUNEL⁺ apoptotic cells than the low concentration of Ar supplementation (CI72h + Dex + 30%Ar, 322.08 ± 81.96 , $p < 0.01$). Therefore, it can be concluded that Dex and noble gases strongly diminished apoptosis in cold-stored liver grafts.

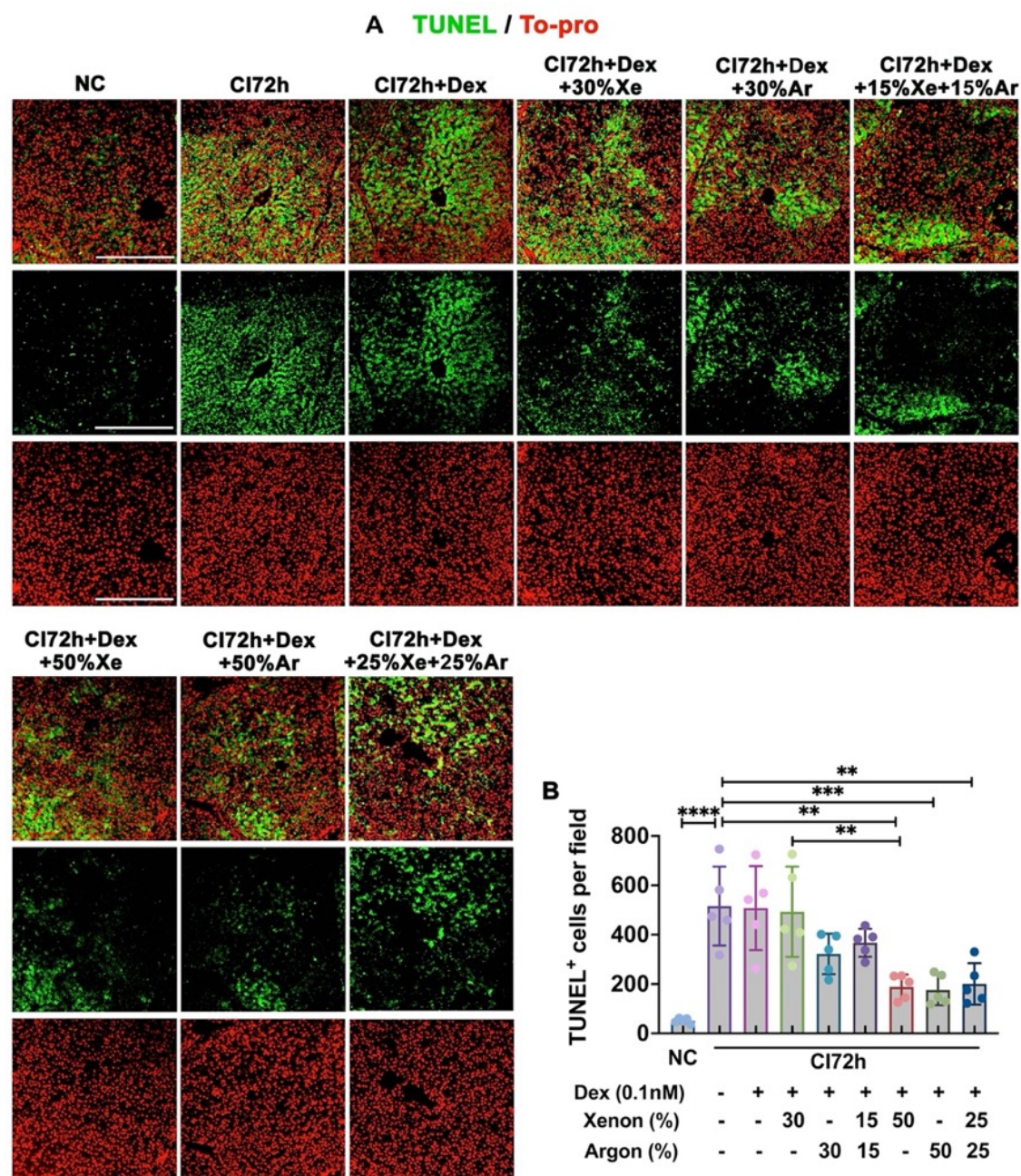


Figure 7.4 Dex and noble gases reduce apoptosis in DCD livers cold stored for 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** TUNEL (green) and To-Pro (red) staining in the liver upon 72 h cold storage. **B.** Average number of TUNEL⁺ apoptotic cells per field of view upon 72 h cold storage. Data were analysed by one-way ANOVA following Tukey multiple comparisons and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 500 μ m. ** p < 0.01, *** p < 0.001, ****p < 0.0001.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling.

7.3.5 Dex and noble gases reduced necroptosis in DCD livers cold-stored for 24 h

Then, we explored whether cold storage leads to necroptosis by detecting a necroptosis mediator, pMLKL, in DCD porcine livers (Figure 7.5A). Results showed that cold storage significantly increased the expression of pMLKL at 24 h (CI24h) by 51.6% when compared to non-cold storage control (NC, $p < 0.0001$, Figure 7.5B), indicating necroptosis occurred. Solutions supplemented with Dex combined with both low (CI24h + Dex + 30%Ar, $p < 0.05$) and high (CI24h + Dex + 50%Ar, $p < 0.01$) concentrations of Ar, as well as Dex combined with 25% Xe and 25% Ar (CI24h + Dex + 25%Xe + 25%Ar, $p < 0.05$) showed a trend toward decreased pMLKL expression.

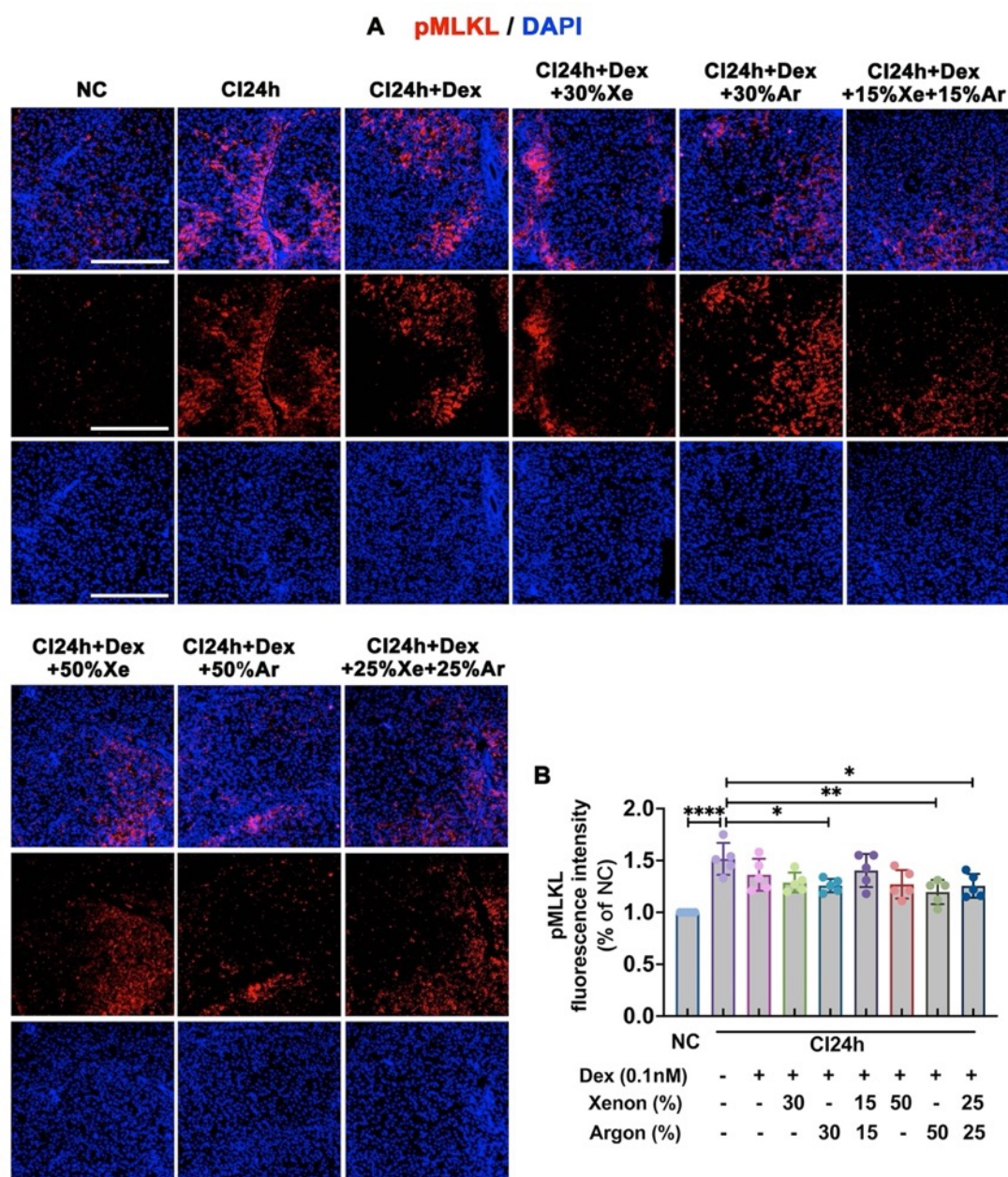


Figure 7.5 Dex and noble gases reduce necroptosis in DCD livers cold stored for 24 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** pMLKL (red) and DAPI (blue) staining in the liver upon 24 h cold storage. **B.** Average pMLKL fluorescence intensity per field of view upon 24 h cold storage. Data were analysed by unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. n = 5. Scale bar = 500 μ m. *p < 0.05, ** p < 0.01, **** p < 0.0001.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; DAPI: 4',6-diamidino-2-phenylindole.

7.3.6 Dex and noble gases reduced necroptosis in DCD livers cold stored for 72 h

After being cold stored for 72 h (CI72h), pMLKL expression was significantly increased 2.1 times in the porcine livers than in non-cold stored livers (NC, $p < 0.0001$, Figure 7.6A and B), demonstrating massive necroptosis. However, there was no significant decrease in pMLKL expression when solutions were supplemented with Dex or noble gases. High concentrations of Xe (CI72h + Dex + 50%Xe) or Ar supplementation (CI72h + Dex + 50%Ar) significantly reduced pMLKL expression induced by cold storage ($p < 0.05$, separately, Figure 7.6B). The results demonstrated that DCD liver grafts suffered extensive damage, which may be related to necroptosis activation, while cold storage solution saturated with Dex and the high concentration of Xe (CI72h + Dex + 50%Xe) or Ar (CI72h + Dex + 50%Ar) may improve liver graft quality through decreasing necroptosis.

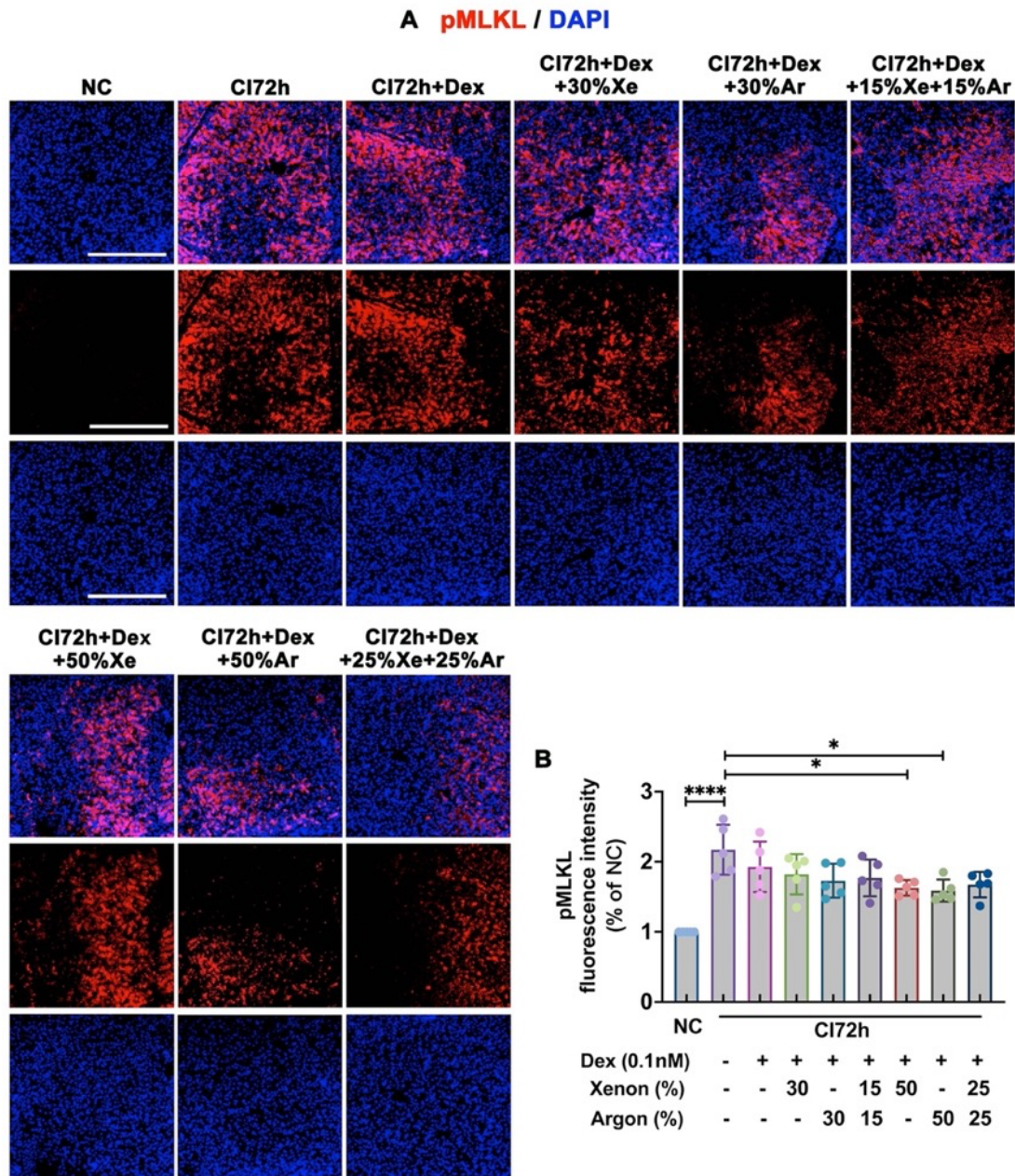


Figure 7.6 Dex and noble gases reduce necroptosis in DCD livers cold stored for 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** pMLKL (red) and DAPI (blue) staining in the liver upon 72 h cold storage. **B.** Average pMLKL fluorescence intensity per field of view upon 72 h cold storage. Data were analysed by unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 500 μ m. * $p < 0.05$, **** $p < 0.0001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; pMLKL: phosphorylated Mixed Lineage Kinase domain-like protein; DAPI: 4',6-diamidino-2-phenylindole.

7.3.7 DCD livers cold-stored for 24 h increased GPX4 expression

The expression of GPX4, an antioxidant enzyme that acts as a protective factor against ferroptosis, was detected in porcine DCD livers after 24 h of cold storage (Figure 7.5A). The results showed that cold storage (CI24h) significantly increased GPX4 expression ($p < 0.001$, Fig.5B), indicating that GPX4 may increase against lipid peroxidation induced by cold ischaemia. Additionally, the GPX4 expression in livers was significantly higher in the solution supplemented with Dex combined with 50% Ar (CI24h+Dex+50%Ar) than in the solution supplemented with Dex combined with 50% Xe (CI24h+Dex+50%Xe) ($p < 0.05$, Figure 7.5B), indicating that Ar may give better protection to liver grafts through increasing antioxidant capability.

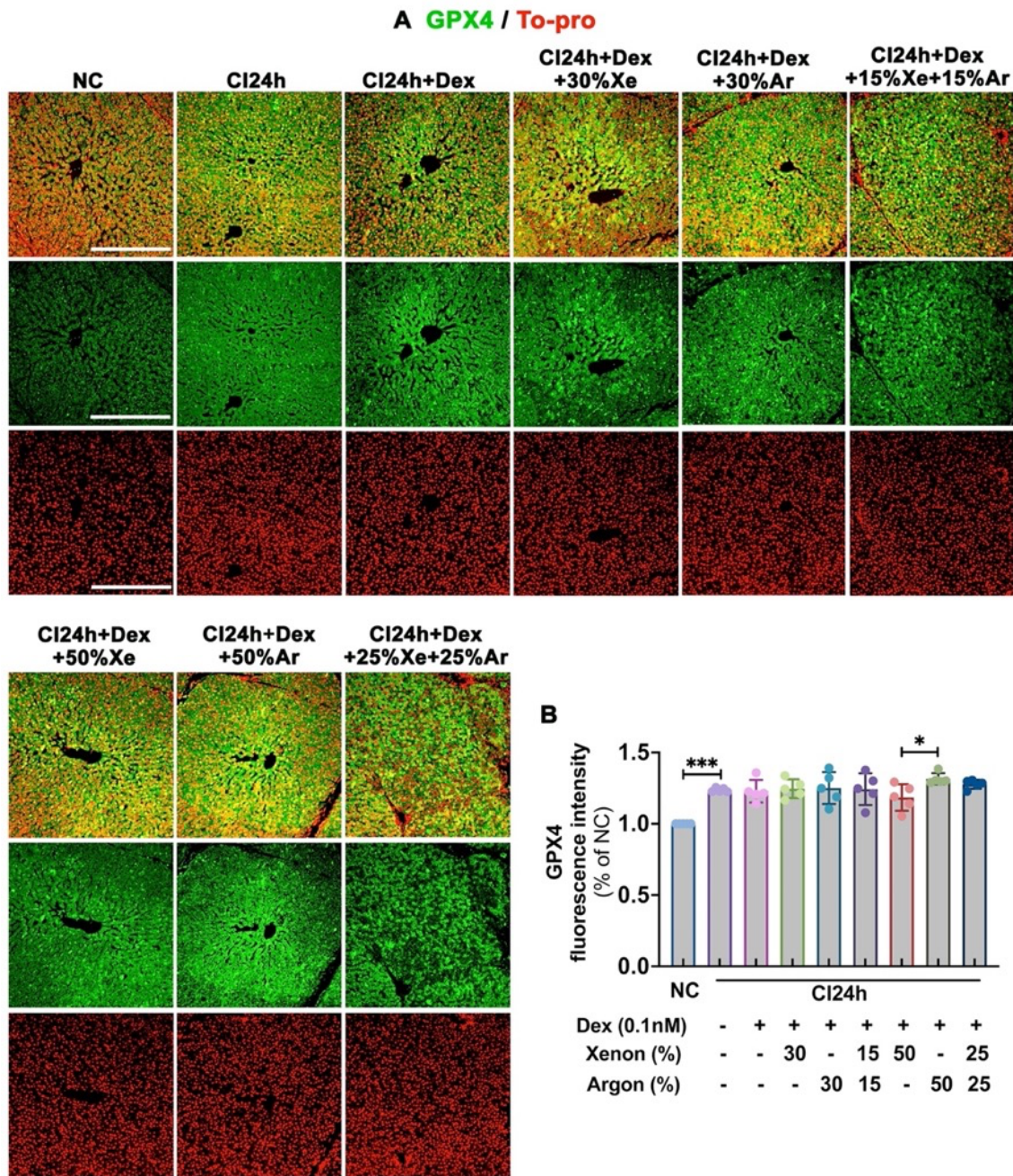


Figure 7.7 DCD livers cold stored for 24 h increases GPX4 expression. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 24 h (CI24h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** GPX4 (green) and To-pro (red) staining in the liver upon 24 h cold storage. **B.** Average GPX4 fluorescence intensity per field of view upon 24 h cold storage. Data were analysed by unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 500 μm . * $p < 0.05$, *** $p < 0.001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; GPX4: Glutathione peroxidase 4.

7.3.8 Dex and noble gases reduced ferroptosis in DCD livers cold-stored for 72 h

After being cold stored for 72 h (CI72h), GPX4 expression was significantly decreased compared to non-cold storage kidneys (NC, Figure 7.8A and B, $p < 0.05$), showing ferroptosis may occur due to prolonged cold storage. High concentration of Ar (CI72h + Dex + 50%Ar, $p < 0.05$) and Dex combined with 25% Xe and 25% Ar (CI72h + Dex + 25%Xe + 25%Ar, $p < 0.05$) significantly reduced ferroptosis induced by cold storage by increasing GPX4 expression (Figure 7.8B).

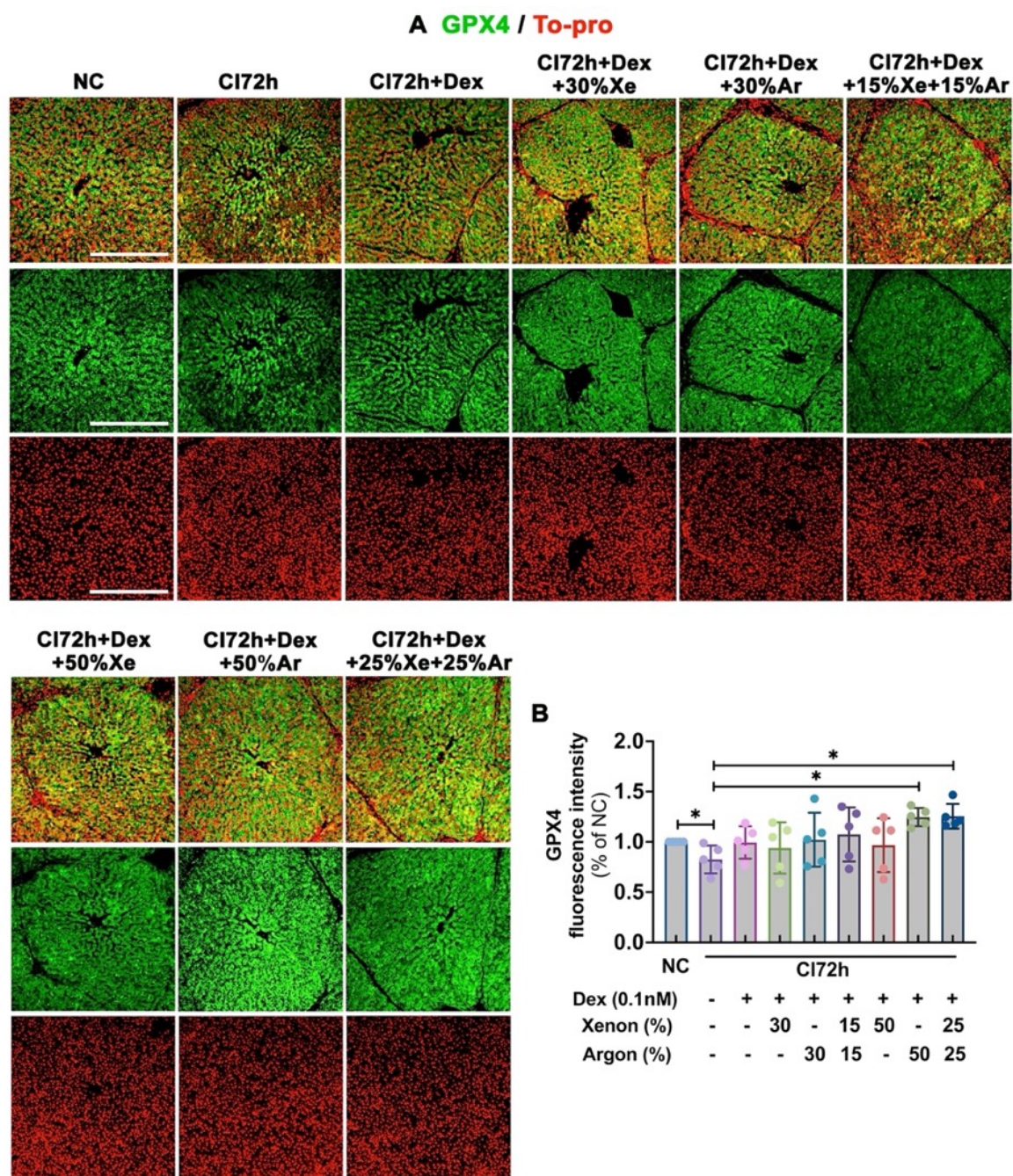


Figure 7.8 DCD livers cold stored for 72 h decrease whereas Dex and noble gases increase GPX4 expression. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus varying concentrations of Xe and Ar for 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** GPX4 (green) and To-pro (red) staining in the liver upon 72 h cold storage. **B.** Average GPX4 fluorescence intensity per field of view upon 72 h cold storage. Data were analysed by unpaired t-test and are presented as scatter plot with bar, mean $\pm$ SD. $n = 5$. Scale bar = 500 μ m. * $p < 0.05$, *** $p < 0.001$.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; GPX4: Glutathione peroxidase 4.

7.3.9 Dex and noble gases attenuated glycogen depletion in cold-stored DCD livers

PAS staining was conducted to evaluate glycogen deposits in cold-stored porcine livers (Figure 7.9). Results showed that the glycogen in cell cytosol was stained dark purple, and the nuclei were stained deep blue. After cold storage for 24 h (CI24h), there was moderate glycogen depletion, as evidenced by an increased number of hepatocytes with lighter purple-coloured cytoplasm. However, there was no change in glycogen content when solutions were supplemented with Dex and high concentrations of noble gases (CI24h + Dex + 50%Xe and CI24h + Dex + 50%Ar). In addition, after being cold stored for 72 h, most hepatocytes had lighter purple-coloured cytoplasm, indicating severe glycogen depletion. Dex combined with 50% Xe or 50% Ar (CI72h + Dex + 50%Xe and CI72h + Dex + 50%Ar) attenuated this depletion.

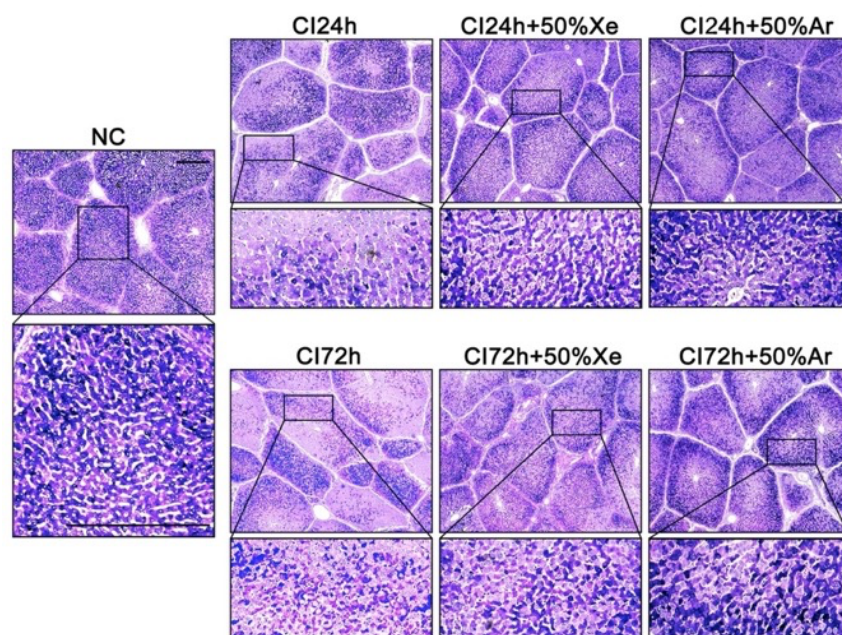


Figure 7.9 Dex and noble gases restore glycogen content in DCD livers cold stored for 24 or 72 h.

Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus 50% of Xe or Ar for 24 (CI24h) or 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. PAS staining was conducted in the liver upon 24 or 72 h of cold storage. Scale bar = 500 µm.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; PAS: periodic acid-Schiff.

7.3.10 Dex and noble gases reduced reticulin framework collapses in cold-stored DCD livers

The reticulum staining was conducted to detect reticular fibre, which is part of the extracellular matrix and helps assess the architecture of the hepatic plates (Figure 7.10). Results showed that the collapse of reticular fibre occurred after both 24 (CI24h) and 72 h (CI72h) cold storage and was more serious in the latter, showing severe reticulin framework collapse with massive hepatocyte necrosis. The solutions supplemented with Dex and high concentration of noble gases alleviated the reduction of reticular fibre, especially to the livers cold stored for 72 h.

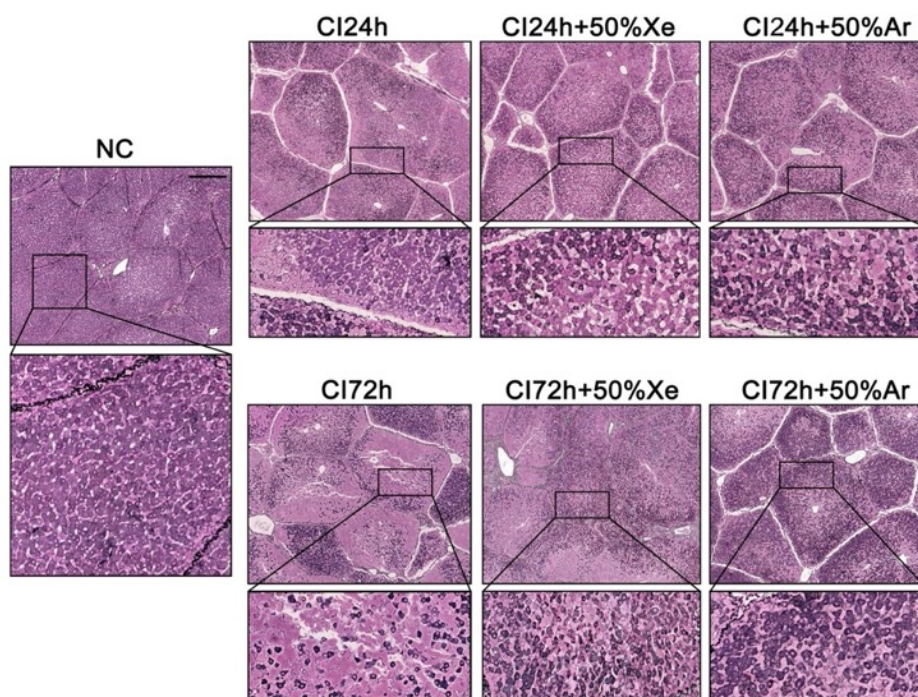


Figure 7.10 Dex and noble gases reduce reticulin framework collapses in DCD livers cold stored for 24 or 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus 50% of Xe or Ar for 24 (CI24h) or 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. Reticulin staining was conducted in the liver upon 24 or 72 h of cold storage. Scale bar = 500 µm.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

7.3.11 Dex and noble gases reduced iron deposition in cold-stored DCD livers

Ferroptosis is an iron-dependent regulated cell death; thus, we examined if the iron content varied after cold storage in porcine DCD livers (Figure 7.11). No iron deposition was observed in non-cold stored (NC) or 24 h cold-stored livers (CI24h). However, a large quantity of iron was present in hepatocytes and was distributed throughout the liver tissue after 72 h of cold storage (CI72). At the same time, the solutions supplemented with Dex and high concentrations of noble gases reduced iron deposition.

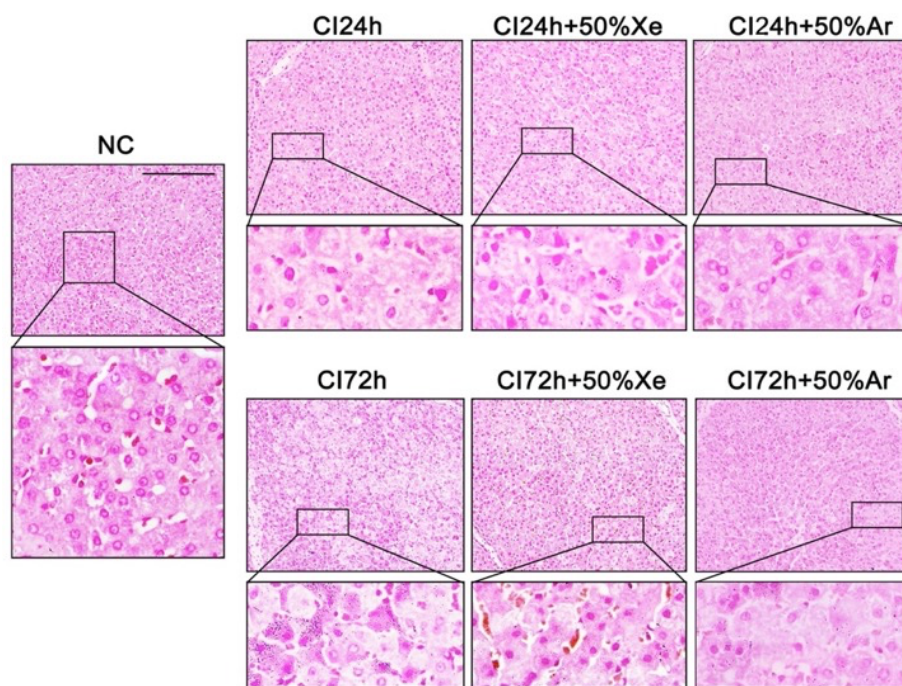


Figure 7.11 Dex and noble gases reduce iron deposition in DCD livers cold stored for 24 or 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus 50% of Xe or Ar for 24 (CI24h) or 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. Iron staining was conducted in the liver upon 24 or 72 h of cold storage. Scale bar = 500 µm.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control.

7.3.12 Dex and noble gases reduced oxidative stress and Txnip expression in DCD livers

cold storage leads to oxidative stress that may trigger ferroptosis, while TXNIP is a thioredoxin-binding protein that mediates oxidative stress by regulating intracellular ROS. Therefore, we detected oxidative stress and Txnip expression in porcine DCD livers cold-stored for 24 or 72 h (Figure 7.12). The results showed that the oxidative stress was increased after both 24 (CI24h) and 72 h (CI72h) of cold storage and ameliorated by Dex and 50% Ar or Xe (Figure 7.12A and C). Meanwhile, the expression of Txnip also increased after cold storage for both 24 and 72 h, which was reduced when the solution was supplemented with Dex and 50% Xe or Ar (Figure 7.12B and D). Surprisingly, we found that the distribution of Txnip expression throughout the liver section significantly overlapped with the location of oxidative stress, indicating the strong relationship between oxidative stress and Txnip expression.

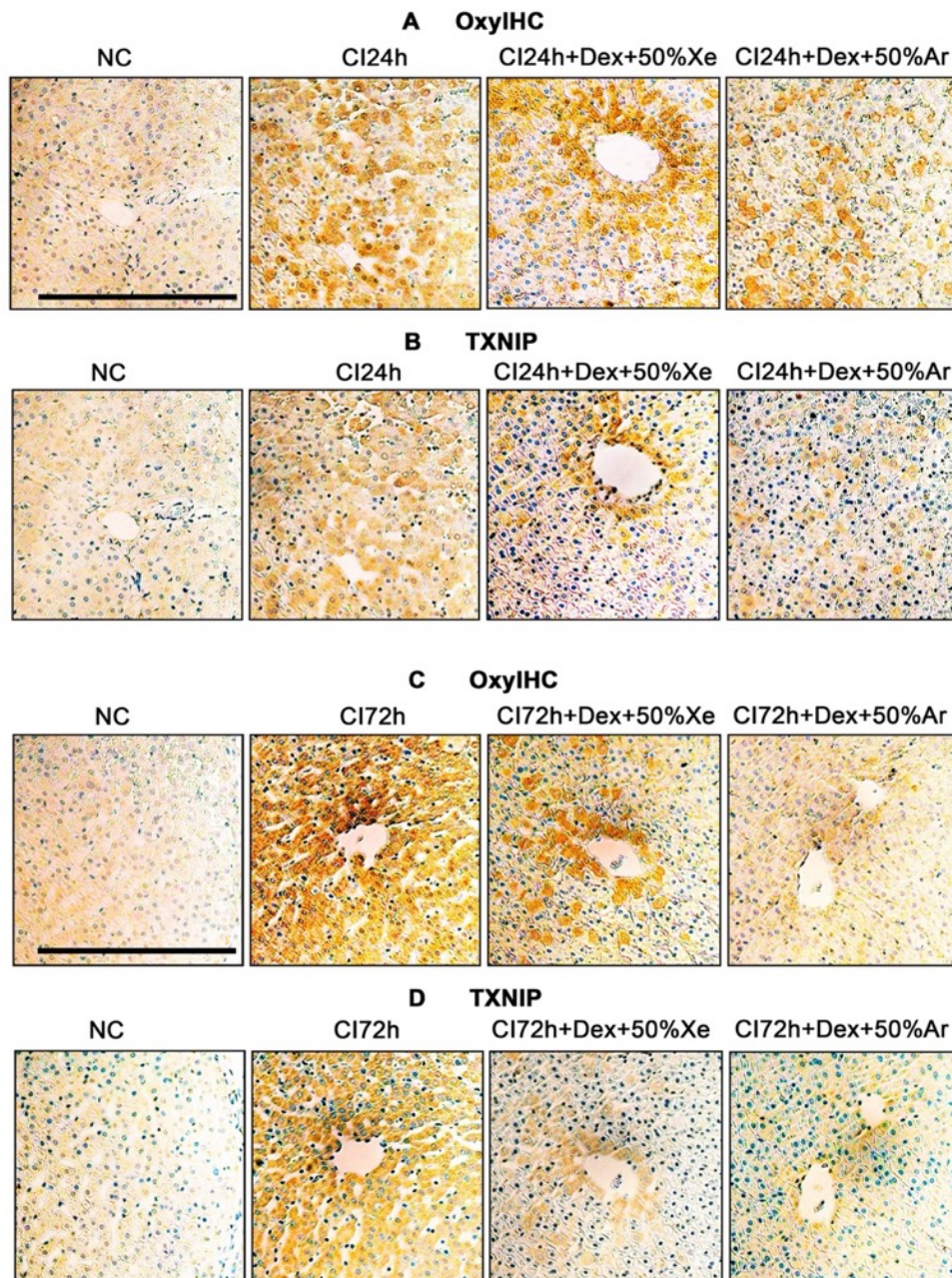


Figure 7.12 Dex and noble gases reduce oxidative stress and TXNIP expression in DCD livers cold stored for 24 or 72 h. Experimental mini-pig livers were harvested 30 min after circulatory death and then were stored at 0-4 °C in the UW solution with 0.1 nM Dex plus 50% of Xe and Ar for 24 (CI24h) or 72 h (CI72h). Control tissues (NC) were fixed upon procurement without cold storage. **A.** Oxidative damage in DCD livers cold stored for 24 h was evaluated by OxyIHC oxidative stress detecting assay. **B.** TXNIP expression in DCD livers cold stored for 24 h was evaluated by immunochemistry. **C.** Oxidative damage in DCD livers cold stored for 72 h was evaluated by OxyIHC oxidative stress detecting assay. **D.** TXNIP expression in DCD livers cold stored for 72 h was evaluated by immunochemistry. n = 5. Scale bar = 500 μ m.

DCD: Donation after circulatory Death; Dex: Dexmedetomidine; Xe: Xenon; Ar: Argon; CI: cold ischaemia; NC: negative control; TXNIP: thioredoxin-interacting protein.

7.3.13 Hypothermic hypoxia-reoxygenation activated ferroptosis, ER stress, and increased intracellular HMGB1 in human HepG2 cells

Our previous results indicated that cold ischaemia injury induced oxidative stress and activated oxidative stress-related ferroptosis in DCD liver tissues. Using a human HepG2 hypothermic hypoxia-reoxygenation model to simulate IR, we evaluated ferroptosis activation and cell ER injury and inflammation *in vitro*. The results indicated that hypothermic hypoxia-reoxygenation time-dependent increased GPX4 expression until 48 h of the reoxygenation ($p < 0.05$, Figure 7.13A and B) when compared with hypothermic hypoxia group (H24). The expression of ACSL4 also significantly increased at 24 ($p < 0.05$) and 48 h ($p < 0.0001$) after reoxygenation, indicating the occurrence of ferroptosis (Figure 7.13A and C). In addition, the expression of Grp78 significantly increased time-dependent after reoxygenation for 6, 24, and 48 h ($p < 0.05$, separately) when compared with negative control cultures, indicating ER stress started early during the reperfusion stage (Figure 7.13A and D). As a result, the intracellular HMGB1, which has a prototypical alarmin function activating innate immunity, also increased accordingly after reoxygenation at 24 ($p < 0.01$) and 48 h ($p < 0.001$).

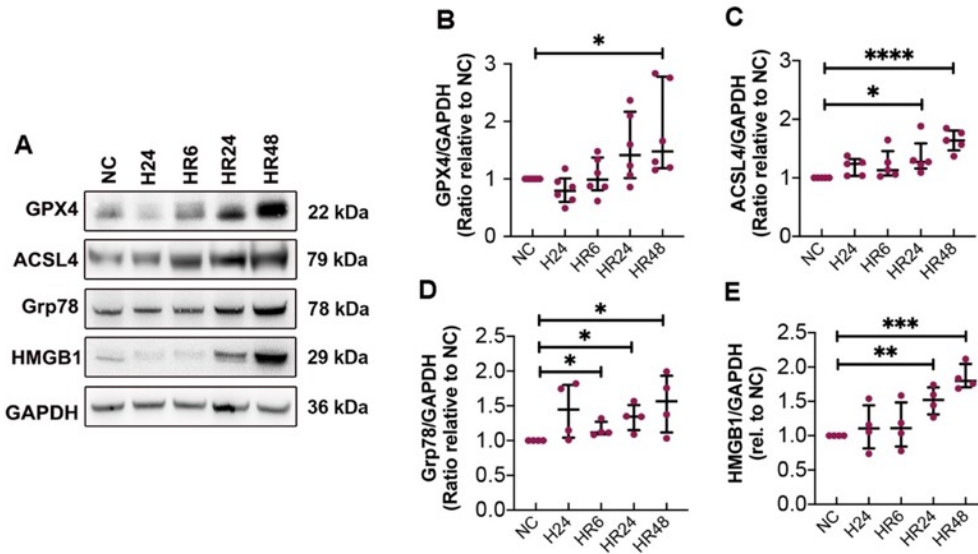


Figure 7.13 Hypothermic hypoxia-reoxygenation activated ferroptosis, induced ER stress, and increased intracellular HMGB1 in human HepG2 cells. Human liver HepG2 cells were preserved in 0-4 °C UW solution for 24 h (H24h) to simulate hypothermic hypoxia condition, then re-cultured in 37 °C fresh DMEM media for 6 (HR6), 24 (HR24), and 48 h (HR48) to simulate reoxygenation. (A) Ferroptosis was evaluated by detecting GPX4 and ACSL4 expression, endothelial reticulum stress was evaluated by detecting Grp78 expression, and Intracellular HMGB1 was evaluated using Western blot. Analysis shows the expression of (B) GPX4, (C) ACSL4, (D) Grp78, and (E) intracellular HMGB1. GAPDH as a loading control. Data were analysed by unpaired t-test and are presented as scatter plots, median with interquartile range. n = 4 - 6. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; NC: negative control; GPX4: Glutathione peroxidase 4; ACSL4: Acyl-CoA Synthetase Long-Chain Family Member 4; Grp78: Glucose-Regulated Protein 78; HMGB1: High mobility group box 1. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

7.3.14 Dex and Ar reduced ferroptosis during hypothermic hypoxia-reoxygenation in human

Hep G2 cells

We evaluated whether saturated Dex combined with 50% Ar in the UW solution inhibits ferroptosis following reoxygenation. After 48 h of reoxygenation (HR48), the expression of GPX4 (P < 0.001, Figure 7.14A and B) and ACSL4 (p < 0.0001, Figure 7.14A and C) increased

significantly. In contrast, Dex and 50% Ar significantly decreased ACSL4 expression ($p < 0.05$, Figure 7.14C), indicating that Dex and 50% Ar inhibited ferroptosis. In addition, crystal violet staining (Figure 7.14D) demonstrated that hypothermic hypoxia (H24) significantly decreased cell viability, whereas Dex combined with 50% Ar (H24 + DA) significantly inhibited cell death. Meanwhile, Dex combined with 50% Ar significantly increased cell viability (HR48 + DA) after 48 h of reoxygenation.

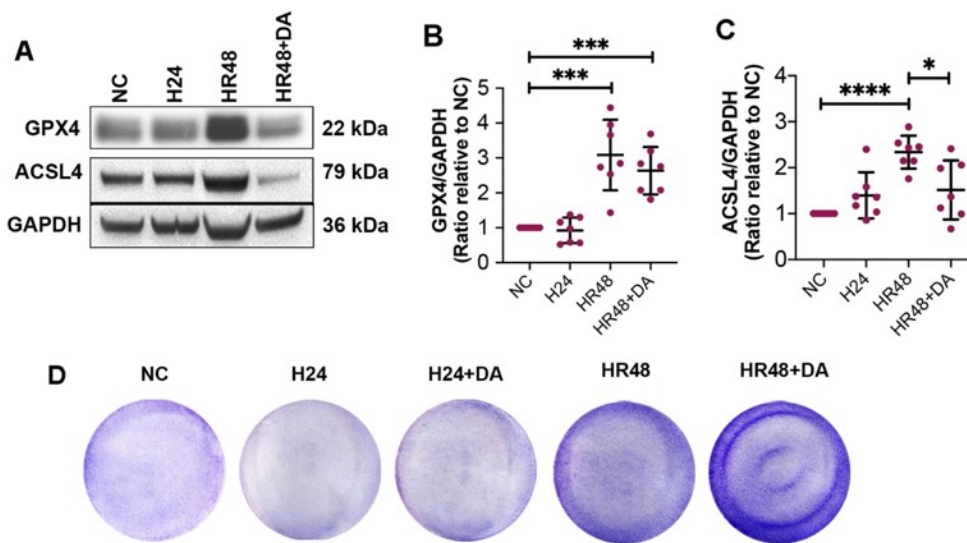


Figure 7.14 Dex and Ar reduced ferroptosis during hypothermic hypoxia-reoxygenation in human Hep G2 cells. Human liver HepG2 cells were preserved in 0-4 °C UW solution for 24 h (H24h) to simulate hypothermic hypoxia condition, then re-cultured in 37 °C fresh DMEM media or DMEM media saturated with 0.1 nM Dex combined with 50% Ar for 48 h (HR48 and HR48 + DA) to simulate reoxygenation. (A) Ferroptosis was evaluated by detecting GPX4 and ACSL4 expression. Analysis shows the expression of (B) GPX4, (C) ACSL4, and (D) Cell cultures viability was evaluated by crystal violet staining. GAPDH as a loading control. Data were analysed by unpaired t-test and are presented as scatter plots, median with interquartile range. $n = 7$. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

H24: hypothermic hypoxia for 24 h; HR: hypothermic hypoxia-reoxygenation; DA: Dex + Ar; NC: negative control; GPX4: Glutathione peroxidase 4; ACSL4: Acyl-CoA Synthetase Long-Chain Family Member 4; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

7.3.15 Dex and Ar, in combination, increased the expression of antioxidant proteins in human liver HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Catalase, thioredoxin, and SOD-1 were antioxidant proteins; thus, they were detected to assess the effects of Dex and Ar on early-oxidative stress at 4 h and late-oxidative stress at 24 h of reoxygenation (Figure 7.15). At the early stage, there were no significant differences in the expression of all antioxidant proteins, regardless of whether the cells were preserved in Dex- and Ar-supplemented solutions (Figure 7. 15A, B, C, and D). In contrast, at the late-oxidative stress stage, Dex and 50% Ar significantly increased the expression of catalase and thioredoxin ($p < 0.05$, separately; Figure 7.15B and C) rather than SOD-1 (Figure 7.15D), indicating that Dex and 50% Ar may increase the antioxidant capability during hypothermic hypoxia-reoxygenation.

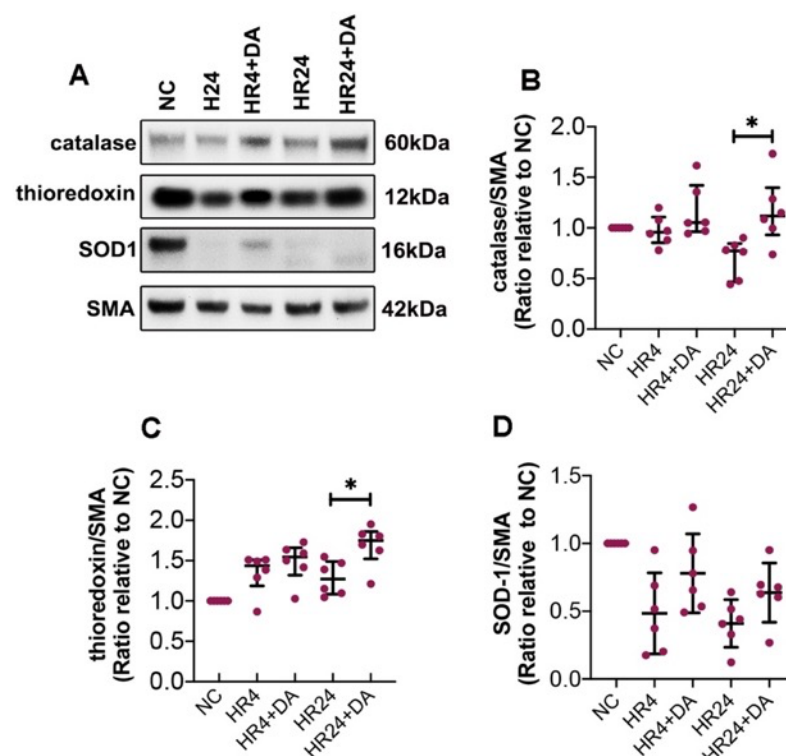


Figure 7.15 Dex and Ar in combination increased the expression of antioxidant proteins in human liver HepG2 cells. Human liver HepG2 cells were preserved in 0-4 °C UW solution or UW solution for

24 h (H24h) and then re-cultured in 37 °C fresh DMEM media or DMEM media saturated with 0.1 nM Dex combined with 50% Ar for 4 (HR4 and HR4 + DA) or 24 h (HR24 and HR24 +DA). (A) Antioxidant proteins catalase, thioredoxin, and SOD1 were detected by Western blot. Analysis shows the expression of (B)catalase, (C) thioredoxin, and (D) SOD-1. SMA as a loading control. Data were analysed by unpaired t-test and are presented as scatter plots, median with interquartile range. n = 6. * p < 0.05. HR: hypothermic hypoxia-reoxygenation; DA: Dex + Ar; NC: negative control; SOD-1: superoxide dismutase 1; SMA: Smooth muscle actin.

7.3.16 Dex and Ar in combination reduced Txnip expression and translocation in human liver HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Txnip is a natural inhibitor of thioredoxin, and its translocation to mitochondria can bind to and directly inhibit thioredoxin, resulting in increased ROS production in cells. The immunofluorescence result (Figure 7.16A) showed that, in HepG2 cells, Txnip was located in nuclei and cytoplasm (NC). After 4 h of hypothermic hypoxia-reoxygenation (HR4), Txnip was transferred substantially to the mitochondrial location. Meanwhile, most Txnip was localised in the nucleus when the UW solution was saturated with Dex (HR4 + D) or 50% Ar (HR4 + A). This indicates that Dex and Ar may influence oxidative stress by modulating Txnip translocation. In addition, the expression of Txnip was also significantly increased after 4 h of hypothermic hypoxia-reoxygenation ($p < 0.0001$), and 50% Ar reduced this increase significantly ($p < 0.05$, Figure 7.16B and C).

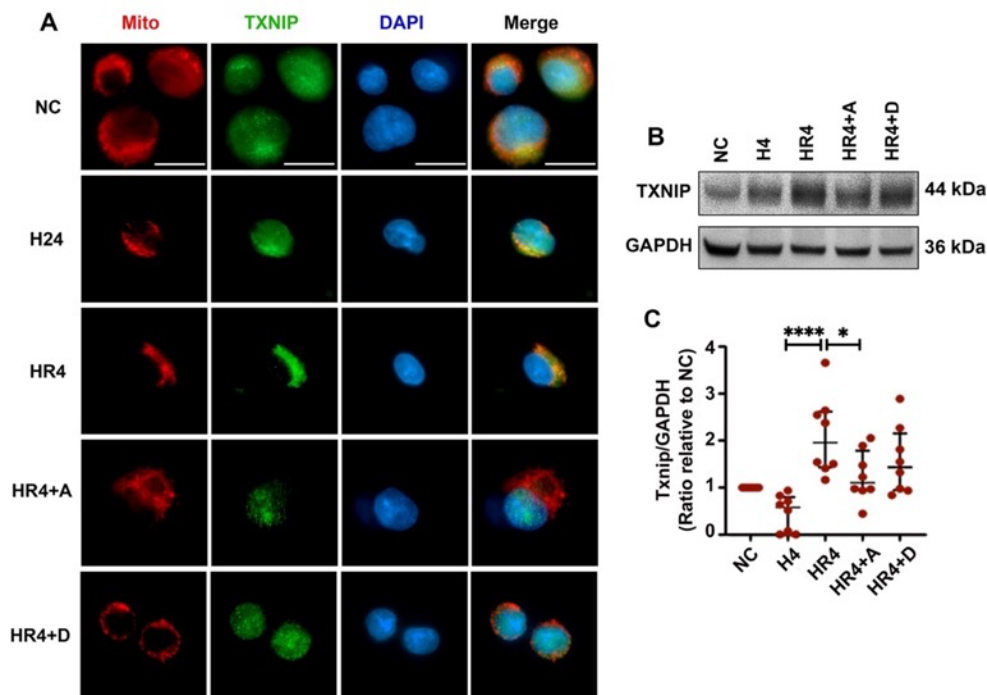


Figure 7.16 Dex and Ar in combination reduced Txnip expression and translocation in human liver HepG2 cells. Human liver HepG2 cells were preserved in 0-4 °C UW solution or UW solution for 24 h (H24h) and then re-cultured in 37 °C fresh DMEM media or DMEM media saturated with 0.1 nM Dex (HR + D) or 50% Ar (HR + A) for 4 h. (A) Mitochondrial (Red) and Txnip (Green) were observed by immunofluorescence. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 20 µm. (B) The expression of Txnip was detected by Western blot. Analysis shows the expression of (C) Txnip. GAPDH as a loading control. Data were analysed by unpaired t-test and are presented as scatter plots, median with interquartile range. n = 8. * p < 0.05, **** p < 0.0001.

H4: hypothermic hypoxia for 4 h; HR: hypothermic hypoxia reoxygenation; D: Dex; A: Ar; NC: negative control; Txnip: thioredoxin-interacting protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

7.3.17 Dex and Ar evaluated plasma membrane resealing function in human liver HepG2 cells exposed to hypothermic hypoxia-reoxygenation

Plasma membrane resealing is an essential mechanism that allows cells to maintain cellular integrity and survival; therefore, DiLC 18 dye was used to stain membrane lipids to evaluate

plasma membrane integrity. The results showed that the cell membrane was stained in bright yellow in the normal HepG2 cells (NC), and almost no DiLC18 dye entered the cells due to the plasma membrane integrity. Both hypothermic hypoxia (H24) and reoxygenation (HR4 and HR24) injury may increase the permeability of the cell membrane and allow more DiLC18 to enter the cells, thereby staining the intracellular organelle membranes bright yellow. Membrane vesicles labelled by DiLC18 were found when the UW solution was saturated with Dex (HR4 + D and HR24 + D) or 50% Ar (HR4 + A and HR24 + A), demonstrating that Dex and Ar may repair the damaged cell membrane through the formation of membrane microvesicles (Figure 7.17).

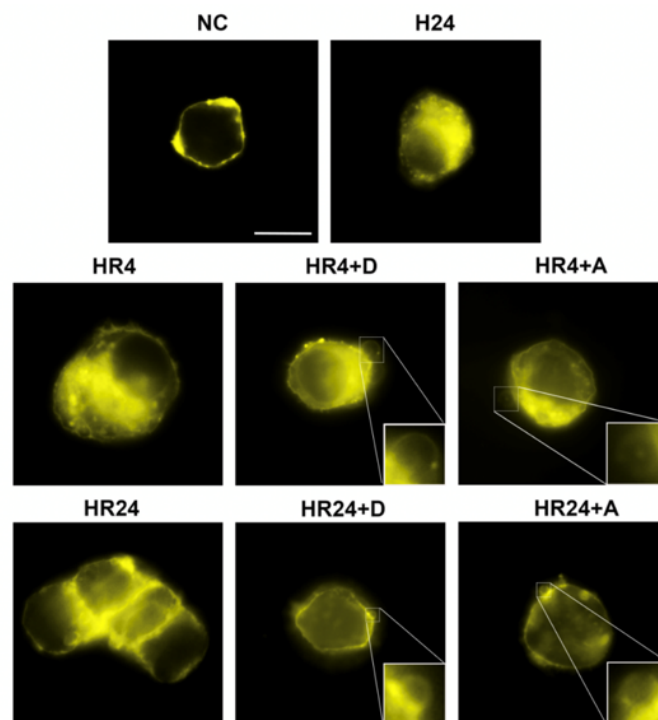


Figure 7.17 Dex and Ar evaluate plasma membrane resealing function in human liver HepG2 cells. Human liver HepG2 cells were preserved in 0-4 °C UW solution (NC) or UW solution for 24 h (H24h) and then re-cultured in 37 °C fresh DMEM media or DMEM media saturated with 0.1 nM Dex or 50% Ar for 4 h or 24 h. DiLC18 stained plasma membranes, and the membrane vesicles were captured under an immunofluorescence microscope. Membrane vesicles were framed and enlarged in the lower right corner of the image. Scale bar: 20 μ m.

H24: hypothermic hypoxia for 24 hs; HR: hypothermic hypoxia-reoxygenation; D: Dex; A: Ar; NC: negative control.

7.4 Discussion

DCD has emerged as a potential strategy for addressing the shortage of donor organs; however, it is associated with several challenges, including an increased risk of IR injury, which contributes to liver dysfunction and failure. In the present study, using a DCD porcine model, we demonstrated that liver grafts suffered a severe injury after long cold storage, such as severe liver architecture damage, the activation of apoptosis, necroptosis, and ferroptosis, as well as hepatocyte glycogen depletion, reticulin framework collapse, iron deposition, and oxidative stress. Additionally, we found that supplemented Dex and noble gases in the UW cold storage solution significantly reduced the activation of apoptosis, necroptosis, and ferroptosis induced by long cold storage, as well as attenuated glycogen depletion, reticulin framework collapse, iron deposition, and oxidative stress. Moreover, the UW solution containing Dex combined with 50% Ar would be the best option for maintaining liver graft quality during cold storage. *In vitro*, we found that HepG2 cells experienced hypothermic hypoxia-reoxygenation, leading to massive damage by initiating ferroptosis. Dex and 50% Ar saturated in the UW solution significantly increased the expression of anti-oxidative stress proteins catalase and thioredoxin, inhibiting ferroptosis and maintaining cell viability. Moreover, our results found that Txnip, a thioredoxin partner, increased and transferred to the mitochondria, which may participate in suppressing oxidative stress. Dex and Ar significantly inhibited Txnip translocation, which may help to reduce oxidative stress and ferroptosis. Moreover, we also found that hypothermic hypoxia-reoxygenation increased plasma membrane permeability, and Dex and Ar may repair the damaged cell membrane by

forming membrane vesicles. In total, this study suggested ferroptosis as a potential therapeutic target in DCD liver transplantation, and we identified 0.1 nM Dex combined with 50% Ar as a potential novel treatment to reduce ferroptosis by suppressing oxidative stress.

Static cold storage to preserve liver grafts has a long history of success in liver transplantation; however, cold storage itself can diminish the viability of the liver grafts and cause hepatocyte injury [322, 323]. The liver is a highly metabolic organ that requires constant oxygen and nutrients to keep functions [55]. During cold storage, the liver lacks oxygen and nutrients, damaging the electron transport chain in mitochondria and potentially reducing ATP production. Furthermore, cold storage induces mitochondria enlargement and membrane damage, accumulating ROS that leads to oxidative damage [324]. Several studies have found that oxidative stress was elevated in patients undergoing liver transplantation, which was associated with an increased risk of complications, such as graft failure and infection [325, 326]. Reperfusion contributes to graft dysfunction and failure after transplantation by causing additional cellular injury and inflammation [323, 327]. Dex has been shown to have organ protection in the liver against IR injury by reducing cellular oxidative stress. This is achieved by reducing the activity of enzymes that produce ROS, such as NADPH oxidase and xanthine oxidase, as well as increasing the activity of antioxidant enzymes, such as SOD and catalase, which help to neutralise ROS [328-330]. Meanwhile, though the protective molecular mechanisms of Xe and Ar against IR injury remain largely undefined, it has been proposed that noble gases might affect the opening of the mitochondrial ATP-sensitive K⁺ (mitoKATP) channels, thus increasing mitochondrial function and reducing ROS production [173]. This study showed stronger liver protection in lowering

oxidative stress when Dex was combined with noble gases Xe or Ar, reducing regulated cell death during cold storage.

Our *ex vivo* results indicated that the distribution of Txnip expression throughout the liver section significantly overlapped with the location of oxidative stress, and hypothermic hypoxia-reoxygenation significantly increased Txnip expression in HepG2 cells, indicating Txnip may regulate oxidative stress at an early stage (during cold storage). In addition, saturated Dex and Ar in the UW solution significantly reduced Txnip expression and its translocation to mitochondria and increased thioredoxin expression. This may explain how Dex and Ar enhanced the antioxidant function of HepG2 cells and protected the cells from hypothermic hypoxia-reoxygenation injury. Txnip and thioredoxin are two proteins closely related to each other in regulating cellular redox homeostasis [331]. Thioredoxin is a ubiquitous oxidoreductase that is critical in maintaining cellular redox balance. Thioredoxin catalyses the reduction of disulfide bonds in proteins, leading to the formation of thiols and the reduction of ROS levels in cells [332]. In contrast, Txnip is shuttled into mitochondria from the nucleus under oxidative stress. Txnip inhibits thioredoxin redox, leading to a hydrogen peroxide increase in mitochondria. Hydrogen peroxide is a potent oxidising agent that can cause damage to proteins, lipids, and DNA, leading to cell death [333]. Therefore, transferring Txnip into the mitochondria under oxidative stress significantly contributes to cellular survival.

Despite the significant impact of regulated cell death on liver transplantation outcomes, further research is still needed to determine the optimal treatment against regulated cell death to improve the success of liver transplantation. Recently, efforts have been made to develop strategies to prevent or inhibit regulated cell death in liver transplantation,

including using drugs that can inhibit specific regulated cell death pathways, such as caspase inhibitors [101, 334] or ferroptosis inhibitors [335]. Additionally, machine perfusion, a technique that involves pumping a preservation solution through the liver graft before transplantation, has reduced regulated cell death and improved outcomes in the transplantation [336]. Our study showed that different types of regulated cell death may occur time-dependently according to cold ischaemic time. Apoptosis and necroptosis may happen at the early stage during cold storage, while ferroptosis occurs late until 72 h of cold storage. Therefore, anti-regulated cell death pharmacological agents that specific apoptosis and necroptosis should be highly considered in the clinical setting [337]. In addition, since both Dex and noble gases Xe and Ar could provide strong anti-regulated cell death properties, they can be considered used in machine perfusion that may significantly reduce IR injury in liver transplantation.

Notably, in this study, as an iron-dependent regulated cell death, ferroptosis occurred after 72 h of cold storage, consistent with the result that a large quantity of iron was present in hepatocytes and distributed throughout the liver tissue after 72 h of cold storage. It also can be concluded that Dex and noble gases may inhibit ferroptosis by reducing iron deposition. Moreover, we found that as an anti-ferroptosis enzyme, the expression of GPX4 increased significantly after 24 h of cold storage and decreased after 72 h, indicating that the antioxidant function of the liver may, to some extent, prevent the occurrence of ferroptosis. In addition to containing antioxidant enzymes that can neutralise ROS, the liver produces and stores antioxidants, including vitamins E and C that may directly eliminate ROS [338]. Additionally, the liver synthesises glutathione, a tripeptide antioxidant that protects cells from oxidative injury [339]. These are potential ferroptosis inhibitors that may keep GPX4

highly expressed to inhibit ferroptosis. However, how these antioxidant factors affect GPX4 expression and how they modulate ferroptosis need further research.

The plasma membrane integrity is essential for proper cell functions [134]. Plasma membrane injury and membrane pores have been linked to several regulated cell death pathways. For instance, plasma membrane damage leads to the release of iron, promoting lipid peroxide accumulation and the initiation of ferroptosis [340]. In addition, when undergoing necroptosis, ubiquitination of pMLKL inserts into the plasma membrane, followed by the rapid influx of Ca^{2+} into cells, leading to phosphatidylserine exposure and plasma membrane disruption [132]. Our results found that hypothermic hypoxia for 4 h may increase the permeability of the cell membrane and allow more DiLC18 dye to enter the cells, thereby staining the intracellular organelle membranes bright yellow, indicating plasma membrane hyperpermeability or disruption occurred at a very early stage during hypothermic hypoxia. Conversely, lipid peroxidation can also damage the plasma membrane, activating plasma membrane repair mechanisms [341]. Cell plasma membrane repair is a self-defence mechanism that maintains membrane integrity and prevents cell death. In this study, after reoxygenation, we observed small yellow membrane vesicles stained by DiLC18 were released from injured cells when the UW solution saturated with Dex and Ar, suggesting Dex and Ar may promote plasma membrane resealing function, which may help to reduce regulated cell death and decrease intracellular cytokines and immunoregulatory molecules release during IR injury.

Our research also revealed that Dex and the noble gases Xe or Ar significantly reduced glycogen depletion and hepatocyte reticulin framework collapse. It has been reported that

the lack of oxygen during cold storage of livers for transplantation activates glycolysis [342]. Energy derived from glycolysis is essential for cell survival, and regulated cell death may occur if glycolysis is inhibited in the liver due to glycogen deficiency [343]. Since most donors are cadaveric, optimising their nutritional status before a transplant through pretreatment is practically impossible. Therefore, it is important to reduce glycogen depletion to keep hepatocytes surviving during cold storage. Although preservation solutions may help restore energy, primary graft dysfunction and non-function remain problematic in the early postoperative period following liver transplantation [342]. To date, no definitive reasons have been identified for the pathogenesis of this complication, and the nutritional status of the liver may play a significant factor. Our results showed that supplemented Dex and noble gases significantly reduced hepatocyte glycogen depletion during cold storage, which may keep the nutritional status of the liver and contribute to energy restoration after reperfusion.

We also observed that cold storage significantly damaged the liver reticulin framework. The reticulin fibres are made up of collagen and glycoprotein, and they form a delicate framework around the liver cells. The reticulin framework provides structural support to the liver cells and helps maintain the normal architecture of the liver [344]. Our results suggested that cold storage may cause the collagen fibres to contract, disrupting the reticulin framework, leading to a loss of liver function and increasing the risk of complications after transplantation. Although our study observed that Dex and noble gases significantly alleviated the reduction of reticular fibres, it is still important to deeply explore the molecular mechanism. This may provide new insight into strategies to improve the

quality of liver grafts and treat diseases that cause hepatocyte loss or regeneration, such as acute liver necrosis.

In conclusion, our results provide evidence supporting the clinical potential of Dex and noble gases supplemented in cold preservation solution for transplant graft preservation, while Dex combined with a high concentration of noble gas, especially 50% Ar, show the best benefits to improve liver graft morphology and reduce regulated cell death during cold storage. Clinical translation of both components may significantly expand the donor pool by including more liver grafts, shorting waiting times, and improving patient survival and quality of life.

Chapter EIGHT

Final Discussion

The cold preservation strategy introduced and evaluated in this PhD study may yield enhanced protection and improve the use of DCD grafts, prolonging the life span in patients with end-stage function or failure of organs.

8.1 Rationale of the Study

The DCD porcine model was chosen for this study because of its morphological, physiological, and genetic similarities to humans. Pigs have a genetic makeup that is similar to humans. This genetic similarity lays the foundation for the applicability of study results to humans and facilitates a better understanding of disease mechanisms and responses to treatment in humans [345]. Additionally, the similarity between the immune systems of pigs and humans is essential for future research in transplantation immunology and the evaluation of immunomodulatory treatments [346]. Therefore, all these make grafts from pigs to be suitable for testing and evaluating the translational applicability of our novel organ preservation strategies.

Despite a noticeable rise in the number of individuals willing to donate organs, the demand for organ transplantation far exceeds the supply of organ donors. To enlarge the donor pool, not only elderly donors but also DCD donors have been increasingly used clinically. As a result, there is an increasing need to improve the preservation quality of grafts to increase the number of viable organs for transplantation. Although the existing preservation solutions are relatively safe and effective for organ preservation, IR injury still adversely affects graft quality. A more in-depth exploration of the composition of preservation solutions is needed to improve the anti-ischaemic capacity and the effectiveness of cold preservation.

In addition, to improve organ utilisation, donor organs must be transported remotely from the donor hospital to the recipient hospital under particular circumstances. Prolonged ischaemia exacerbates IR injury, which causes damage to the graft and compromises its function after transplantation [43]. Organ preservation solution is crucial for maintaining the viability and

function of grafts during this preservation period. Studying the composition and properties of preservation solutions, including investigating the appropriate balance of electrolytes, osmotic pressure, energy substrates, antioxidants, and other additives to create an optimal environment for graft preservation, is crucial for mitigating IR injury and enhancing graft survival.

Existing preservation solutions can be differentiated based on K^+ levels, types of macromolecular colloids, and the presence of antioxidants for preventing IR injury [347]. Current research mainly focuses on modifying the original components and adding new elements to the UW solution. Some new preservation solutions and components have been developed, and their preservation effects have been preliminarily validated through experiments [348-350]. Moreover, due to relatively saturated research on the composition of preservation solutions, the latest research has focused on adding gases and hormones, which have become popular in recent years [350].

My PhD project presented here provides evidence to support the clinical potential of using Dex in combination with noble gases (Xe and Ar) for graft preservation. Specifically, we found that 0.1 nM Dex combined with a high concentration of noble gas, particularly 50% Ar, provided the most significant benefits in improving the morphology of kidney and liver grafts and reducing regulated cell death during cold storage. If successfully translated to clinical practice, the combined use of Dex and noble gases may significantly impact the pool of available kidney and liver grafts, reducing waiting times for transplantation and ultimately improving patient survival rates and quality of life *per se*.

8.2 Summary of the Study

This PhD project study examined the protective effects of Dex combined with noble gases (Xe and Ar) on various DCD donor organs, including human lung tissues, pig kidneys and livers. Dex supplemented into the UW preservation solution improved lung morphology and reduced apoptosis and necroptosis in lung tissues during cold storage. *In vitro*, hypothermic hypoxia-reoxygenation experiments showed that Dex mitigated necroptosis, inhibited inflammation, and improved cellular plasma membrane resealing function in lung epithelial cells. Meanwhile, UW preservation solutions supplemented with Dex, Xe, and Ar improved the quality of pig DCD kidney and liver grafts by reducing regulated cell death (apoptosis, necroptosis, and pyroptosis) and oxidative stress. Dex, Xe, and Ar alone or in combination showed protective effects in reducing IR injury and maintaining the quality of DCD donor organs. Specifically, combining Dex and 50% Ar was most effective in preserving kidney and liver graft quality.

8.3 Translational potential of this study

The porcine model used in this study closely mimics the physiological and immunological aspects of human organ transplantation and increases the likelihood that the findings apply to human transplantation scenarios. In addition, the molecular mechanisms of regulated cell death, including processes like ferroptosis and necroptosis, are conserved between pigs and humans [286]. Therefore, insights gained from studying regulated cell death in my project will likely have direct implications for understanding similar processes in humans. Lastly, given the increasing reliance on marginal donors to meet the growing demand for transplantation, it is vital to refine graft preservation strategies as the marginal organs are

more susceptible to IR injury. We identified that Dex combined with 50% Ar supplemented in an organ preservation solution is an effective treatment that may increase the utilisation rate of marginal donor organs and may be more likely to translate successfully to humans.

8.4 Limitations of this study

Firstly, in the *ex vivo* cold preservation study, livers and kidneys were cut into slices (0.5 x 1 cm) and then preserved in the cold UW solution supplemented with Dex and noble gases, which is different from cold perfusion in clinical setting that flush the whole organ with preservation solution. This study may not accurately reflect the *in vivo* conditions where the preservation solution drugs interact with the entire organ environment and may not fully represent the heterogeneous drug distribution in intact organs. However, tissue slices allow for quicker exposure of drugs to the tissue and offer more uniform drug exposure across the tissue, enabling better control in experimental conditions. Therefore, the findings in this study need to be validated in whole organs or *in vivo* studies for clinical relevance.

Secondly, cell lines lack the complexity of whole organs, such as diverse cell types, extracellular matrix, and physiological conditions found in intact tissues and organs, which only partially replicate the dynamic and constantly changing conditions within living organisms. This may limit the comprehension of drug responses to a more complex biological context. Despite this limitation, the *in vitro* experiments in this study are still helpful settings for conducting mechanistic investigations and gaining insights into fundamental biological processes. In addition, this research established *in vitro* and *ex vivo* models to complement each other to further support the treatment effectiveness. However, these findings must be validated in future large animal *in vivo* transplantation experiments.

Cell lines (A549, HK2, HepG2 cell line) were used to establish the hypothermic hypoxia-reoxygenation model to mimic the clinical scenario of IR injury. A549 cells are adenocarcinoma human alveolar basal epithelial cells, and HepG 2 cells are derived from human hepatocellular carcinoma. Cancer cell lines often deviate significantly from the normal physiological characteristics of the original tissue. This can limit the relevance of findings, especially when studying transplantation processes that involve non-cancerous tissues. In addition, cancer cells often harbour mutations that lead to aberrant abnormal signalling activations [351]. This can impact the interpretation of results, as the responses observed in cancer cells may not accurately represent normal cellular responses in transplantation scenarios. However, A549 cells maintain characteristics of type II alveolar epithelial cells and still can be used to model the alveolar microenvironment *in vitro*. Since lung transplantation involves the transplantation of alveolar-capillary units, using a cell line derived from the alveolar epithelium is highly relevant to study the cellular aspects of lung transplantation. HK-2 and HepG 2 cells can also serve as models for the renal tubular and liver microenvironment. Studying how these cells respond to various conditions, such as oxidative stress, IR injury, and inflammation, helps to provide insights into cellular processes relevant to kidney and liver transplantation, including graft dysfunction and rejection.

Thirdly, the underlying molecular mechanisms of Dex in protecting hypothermic hypoxia-reoxygenation injury and the concentration of Dex used in this study still need to be fully assessed. Dex is a potent α_2 adrenergic agonist that interacts with imidazoline receptors. Dex activates α_2 receptors resulting in protein inhibition and the nitric oxide-cGMP pathway. Our previous studies showed that cold preservation solution supplemented with 0.1 nM Dex

conferred significant protection against lung graft ischaemic damage by attenuating regulated cell death and the subsequent inflammatory response through activating α_2 AR [352]. Meanwhile, Dex also protected organs by inhibiting the release of inflammatory cytokines, such as TNF- α , IL-1, IL-6, IL-8, and HMGB1, by activating the PI3K/Akt [147], ERK1/2 and PKC [148] signalling pathways. Whether the protective effect of Dex found in this study is related to active α_2 receptors or other signalling pathways is unknown and needs to be further explored. In addition, determining its dose-response curve vs effects is important in translating to clinical use in transplant settings.

8.5 Clinical implications of this study

The quality of organ transplantation and patient outcomes are closely related to graft damage during the warm and cold ischaemia stage. When a significant number of parenchymal cells in the graft die, it negatively impacts graft function. Moreover, cell death influences immune responses related to IR injury and immune rejection due to the release of various cellular contents [353]. The interplay among innate and adaptive immunity and inflammation on organ function, rejection, and survival is increasingly recognised. However, advancements that target only recipient immune responses have not significantly improved long-term graft survival [354]. Therefore, it is crucial to explore graft damage and how newly discovered regulated cell death regulates inflammation and immune response in the transplanted organs.

Based on our preliminary findings, organs procured from DCD rat donors were more susceptible to necroptosis due to prolonged warm ischaemia than organs procured from living donors [352]. Upon initiation of necroptosis caused by warm ischaemia, the organs exhibited higher susceptibility to cold ischaemia injury. Hence, cold preservation after warm ischaemia

accelerates the progression of necroptosis and inflammation, resulting in increased severity of organ damage. The present study demonstrated the occurrence of necroptosis and apoptosis in DCD lungs, kidneys, and livers during cold preservation, whereas the reperfusion facilitated a high propensity for ferroptosis and pyroptosis. This indicated that it is crucial to thoroughly evaluate the consequences of necroptosis throughout the ischaemia period, including evaluating graft functions before transplantation and addressing inflammation and immune response after transplantation. Furthermore, engaging in a comprehensive investigation of necroptosis and necroptosis inhibitors is essential, which may enhance the overall quality of transplanted organs, particularly in the context of using organs procured from the elderly, marginal, or DCD donors. Following transplantation surgery, it is important to consider the implications of ferroptosis and pyroptosis on organ functions and patient outcomes. These two distinct types of regulated cell death can potentially significantly influence post-transplant immunological rejection and infections.

Research on improving organ preservation solutions mainly focuses on adding protective drugs to the UW solution. The main additives being studied are: (1) anti-ischaemic drugs: studies have shown that anti-ischaemic drugs such as trimetazidine have antioxidant properties. Using UW solution containing trimetazidine for cold preservation of mouse liver improved liver microcirculation, alleviated oxidative stress, protected mitochondrial structure, and improved mitochondrial function [355]. (2) Proteasome inhibitors: the ubiquitin-proteasome system (UPS) is an energy-dependent system that reduces misfolded proteins and regulates various cellular activities [356]. A previous study reported that cold preservation significantly decreased the ATP level in ischaemic organs, causing pathological and physiological organ damage. Adding proteasome inhibitors to the UW solution helped

maintain the integrity of cell ultrastructure during the cold storage [357]. (3) Hormones: melatonin is a hormone secreted periodically by the pineal gland. Studies found that adding melatonin to preservation solutions enhanced the anti-ischaemic properties in liver grafts. This is mainly due to the increased expression of eNOS and tissue heme oxygenase, the alleviation of mitochondrial damage, and the reduction of oxidative stress [358, 359]. (4) Statins: Statins are currently commonly used to treat lipid abnormalities and have varying anti-inflammatory, antioxidant, and immune-regulatory effects. They protect the cell plasma membrane by activating eNOS to release NO. Studies have shown that adding statins to UW solution significantly reduced endothelial cell damage [360, 361].

In the present study, we used Dex as a protective drug for organ preservation solutions because our preliminary research has fully demonstrated the protective effects of Dex in the organ IR injury [140, 352, 362, 363]. These protective effects are achieved by activating α_2 AR to regulate various signalling pathways. Moreover, clinical studies have also found the benefits of Dex in reducing ischaemia injury during surgery and inhibiting postoperative inflammatory responses [364-366]. Although our experimental data showed that supplemented 0.1 nM Dex to UW solution did not significantly alleviate cold ischaemia injury to the kidney and liver grafts, *in vitro* experiments have shown that Dex significantly reduced regulated cell death and inflammation responses after hypothermic hypoxia-reoxygenation. Therefore, we speculate that the protective effects of Dex may mainly manifest during the reperfusion phase.

More research is needed on the protective effects of Xe and Ar on organs, especially in the field of transplantation. However, the protective effects of Xe and Ar on ischaemia injury have been validated, particularly regarding neuroprotection following cardiac arrest resuscitation.

In experimental settings, the protective effects of Xe and Ar have been most reported in *in vitro* studies and animal models of cardiac IR injury [367-369]. Besides, the safety and protective effects of Xe have been reported in clinical trials. Preliminary Phase 2 and 3 clinical trials showed that Xe significantly reduced hypoxia-induced brain white matter ischaemia injury in patients with out-of-hospital cardiac arrest. However, it did not significantly impact brain neurologic function and survival rate at 6 months [370]. Another study has shown that inhalation of Xe alleviated myocardial injury in patients with out-of-hospital cardiac arrest [371]. On the other hand, clinical research on Ar is still lacking, but its organoprotective effects have been demonstrated in *in vitro* and animal studies [175, 372-375]. In fact, Ar has been tested in various ischaemic injury models, with concentrations ranging from 20% to 80% in oxygen. In our study, we found the protective effects of Xe and Ar on grafts were in a concentration-dependent manner, but the combination of these two gases did not enhance this protective effect. Although the specific molecular mechanism is still unclear, considering that Ar is much cheaper than Xe, using Ar for clinical use may yield encouraging results.

In conclusion, our results provide evidence supporting the clinical potential of Dex and noble gases Xe and Ar saturated cold storage for transplant graft preservation, while Dex combined with a high concentration of noble gas, especially 50% Ar, showed the best benefits to maintain graft morphology and reduce regulated cell death during cold storage. Clinical translation of Dex and Ar in combination may be beneficial in expanding the donor pool by including more grafts, shorting waiting times, and improving the quality of life. However, further validation is needed to assess the outcomes after engraftment with the strategy to preserve grafts in clinical settings.

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Resume and Publication list during my PhD study

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Education

Master in Anaesthetics	Army Medical University, Chongqing, China	2012-2015
Bachelor in Anaesthetics	Central South University, Hunan, China	2006-2011
PhD Candidate	Imperial College London	2018?...present

Research Grants

2019-2021

The National Natural Science Foundation, P.R. China.

ESCRT-III membrane recruitment reduces lung epithelial necroptosis induced by renal cold ischemia/reperfusion injury.

As an anesthesiologist and a PhD student dedicated to organ transplantation, my PhD work is a pivotal bridge between my clinical experience and academic pursuits. My PhD project delves into the intricate dynamics of organ transplantation, aiming to enhance our understanding of organ donation, graft cold storage, ischaemia reperfusion injury, and transplant outcomes. This research not only refines my clinical thinking but also equips me with a deeper understanding of the complexities inherent in organ transplantation. The insights gained from my PhD study encourage me to pursue my career development by fostering innovation in

surgical techniques, improving the quality of organ transplantation, and paving the way for future advancements in transplant research.

Publication list during my PhD study

1. **Qian Chen**, Xiangfeng Liu, Zhiheng Liu, et al. Tackling regulated cell death yields enhanced protection in lung grafts. *Theranostics*. 2023,13(13):4376-4390.
2. Chang E, Wang Y, Zhu R, Wu L, Yang Y, Zeng S, Li N, Ruan X, Sun M, Zhang W, Zhou J, Miao M, Zhi H, Zhao H, **Chen Q**, Sun Q, Chang E, Chang A, Zhang T, He X, Liu K, Ma S, Zhu W, Zhang Y, Magnani L, Ma D, Zhang J. General anaesthetic action profile on the human prefrontal cortex cells through comprehensive single-cell RNA-seq analysis. *iScience*. 2023 Mar 31;26(4):106534.
3. **Qian Chen**, Zhigang Qin, Yibing Sun, et al. Dexmedetomidine Activates Akt, STAT6 and IRF4 Modulating Cytoprotection and Macrophage Anti-Inflammatory Phenotype Against Acute Lung Injury in vivo and in vitro. *Journal of Inflammation Research*. 2022,15: 2707-2720.
4. Sunshan Ku, Xingtong Chen, **Qian Chen**, et al. Effects of dexmedetomidine on the function of distal organs and oxidative stress after lower limb ischaemia-reperfusion in elderly patients undergoing unilateral knee arthroplasty. *Br J Clin Pharmacol*. 2021 Nov;87(11):4212-4220.
5. Lin Chen, Alam Azeem, **Qian Chen**, et al. Pretreatment with valproic acid alleviates pulmonary fibrosis through epithelial–mesenchymal transition inhibition in vitro and in vivo. *Lab Invest* 101, 1166–1175 (2021).
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