Journal of Biosciences and Medicines, 2024, 12, 79-88
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Cholesterol and Sericin as First Aid for Damaged Cells

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How to cite this paper: Aksoy, D., Turan, D.N. and Bayraktar, Z.B. (2024) Cholesterol and Sericin as First Aid for Damaged Cells. *Journal of Biosciences and Medicines*, **12**, 79-88.
https://doi.org/10.4236/jbm.2024.124008

Received: January 25, 2024
Accepted: April 7, 2024
Published: April 10, 2024

Abstract

Cells are surrounded by a double-layered phospholipid cell membrane responsible for the isolation of intracellular contents, active regulation of uptake from the extracellular environment, and intercellular connection and communication. These cell membranes must be intact and functionally active for cell survival and biological functioning. Compromised damage repair mechanisms usually result in impaired cellular homeostasis, leading to early or late problems. Chronic myopathies, certain myocardial diseases, aging, and acute or chronic neurodegenerative diseases (like Parkinson and Alzheimer) are directly related to cell membrane damage. This study examined the effect of a cholesterol-loaded nanoparticle (methyl-beta cyclodextrin) or the silk protein sericin on cell membrane and DNA integrity and cell viability in an in vitro cell damage model (frozen-thawed rabbit sperm cells). The cells were stored in liquid nitrogen (−196˚C), thawed in small batches, and treated with cholesterol-loaded cyclodextrin or sericin before incubation at 35˚C for 4 hours. Cell membrane integrity, DNA damage, and viability rates were assessed immediately after thawing and after the incubation period. The administration of sericin and cholesterol in a cell damage model increased cell survival and reduced DNA damage over a 4-hour post-thaw incubation period, suggesting their potential use as a "first aid" intervention at the cellular level.

Keywords

Cell Membrane, DNA, Survival, Damage, Cholesterol, Sericin, Spermatozoa

1. Introduction

The cell membrane is double-layered structure consisting mainly of phospholi-

pids and cholesterol. The ratio of those components in the cell membrane varies with species and cell type. Cell membrane damage can occur for various reasons. Different types of muscular dystrophies (e.g., laminopathy, titinopathy, dystrophinopathy, dysferlinopathy) may occur as a result of mutations in genes controlling cell membrane formation (weak membranes) or because of impaired membrane repair (1). Altered membrane permeability is also observed in the early stages of some muscular dystrophies, such as Duchenne, Becker, tibial, and limb-girdle muscular dystrophy [1].

Membrane damage in internal organs might occur during events that cause ischemia/reperfusion, such as heart attack and stroke. Such ischemia-reperfusion events cause increased oxidative stress, resulting in a high rate of cell membrane damage in the affected tissue that shapes the later stages of the process [2] [3].

Cell membrane damage is also associated with the intracellular entry of toxins produced by bacteria with normal or high inflammatory potential. As these toxins cannot pass through intact cell membranes, they reach the cell interior by creating pores or holes. Depending on which bacteria's toxin enters which cells, different diseases such as pneumonia, meningitis, and septicemia occur [4] [5].

Structural damage or disruption of cell membrane repair mechanisms in nerve cells during the natural aging process contributes to age-related cognitive decline, while its occurrence earlier in life may cause acute or chronic degenerative diseases (such as Alzheimer and Parkinson disease) [6].

When storing cells and tissues for future use, freezing or cooling at sub-zero temperatures also causes damage to many cellular structures, especially the cell membrane [7]. Therefore, the survival time and survival rates of frozen-thawed cells are lower than those of fresh, never-frozen cell cultures [8].

1.1. Repair of Cell Membrane Damage

Cell membrane damage is repaired through various mechanisms. The repair mechanism utilized is generally related to the extent of the damage. Small cell membrane injuries (<1 nanometer) can be self-healing. However, the calcium-dependent exocytosis pathway is used to repair injuries that are several nanometers in size. Stimulation by calcium entering the cell through the injury initiates the main mechanism. In this mechanism, double-layer vesicle-like cellular structures within the cell open to a single-layer, moving toward and patching the injury site in the cell membrane [9] [10].

1.2. Cholesterol and Cyclodextrins

A combination of cholesterol and phospholipids form the basic structure of many cell membranes. As cholesterol is fat and insoluble in water, it is difficult to add to aqueous in vitro biological systems. Therefore, including cholesterol in many environments in which living cells are found, such as cell culture media, causes concern that it will not form a homogeneous mixture. Even if cholesterol can be dissolved in the presence of chemical solvents such as alcohol, the effects of these solvents on cells cannot be ignored [11].

Cyclodextrins are used for many purposes, such as solubilizing water-insoluble chemicals and transporting drugs to target tissues in medicine. Cyclodextrins are toroid-shaped molecules produced from starch. There are alpha-, beta-, and gamma-cyclodextrins that differ in the size of the central hole. Methyl-beta cyclodextrin has been used in some studies to transport cholesterol to target cells by making it water soluble [12].

1.3. Sericin

Sericin is a protein produced by the silkworm. It enables the silk fibers to adhere to one another and the cocoon to be shaped. It is included in the "glue-like" protein family [13]. Sericin is rich in arginine and lysine amino acids, so it easily adheres to cell membranes [14]. In addition, it readily dissolves in water and can be added to foodstuffs and cell culture media. Regional applications of 8% sericin are used for wound healing, burn treatment, and inflammation treatment [15].

The examples given above demonstrate that damage to the cell membrane and other cellular structures triggers or contributes to many health problems. Regulating or accelerating the mechanisms involved in the repair of cell membranes and cellular structures may offer treatment alternatives for many diseases.

This study aimed to investigate the possibility of improving the survival time and cell membrane repair capabilities of cells with low survival potential (damage to the cell membrane and other cellular structures such as DNA) by applying two different substances, cholesterol and sericin. This was evaluated by analyzing rates of viability, intact cell membranes, and DNA integrity in cells incubated at 35˚C for 4 hours after thawing.

2. Methods

Since no intervention is made on live animals in our country, ethics committee approval is not required for laboratory studies. Therefore, ethics committee approval was not required for this study. All chemical reagents used (Sigma-Aldrich) were provided by the reproduction and artificial insemination laboratory of the Veterinary Medicine Faculty. All experiments were carried out in the same laboratory with the joint participation of all members of the research team.

2.1. Provision and Thawing of Frozen Cells

Frozen rabbit sperm cells were provided at no cost by the Faculty of Veterinary Medicine of Aydın Adnan Menderes University. The cells were received in 0.25-mL sequins of 5×10^6 cells, frozen in containers of liquid nitrogen at −196˚C. The cells were stored in the laboratory during the study and thawed before each experiment in a 35˚C water bath for 1 minute. Rabbit spermatozoa were selected for this study because of their rich plasma membrane content, as they have a high cell membrane/cytoplasm ratio and are the largest available mammalian spermatozoa. Another reason for selecting this cell model was that

the laboratory analyses for the detection of cell membrane damage, mitochondrial damage, DNA damage, cell viability rate, and acrosomal damage in spermatozoa can be performed quickly and easily. For each experiment, a total of 6 sequins were thawed as described above and combined in a test tube. The pooled samples were then diluted 1:1 using Tris-citric acid-glucose diluent [16]. The diluted samples were homogenized by pipetting and equally divided into 3 equal aliquots. The first aliquot was designated as the control group. Cholesterol-loaded cyclodextrin at a concentration of 2.5 mg/mL was added to the second aliquot, and 0.5% sericin was added to the third aliquot. The samples were then incubated in a 35˚C water bath for 4 hours. Cell viability rates, proportions of cells with intact cell membranes, and DNA integrity ratios were determined immediately after thawing and at the end of the incubation period.

2.2. Loading Cholesterol onto Cyclodextrin

Because cholesterol is not water-soluble, it is loaded onto nanoparticles (methyl-beta cyclodextrin molecules) to enable it to be dissolved in water and delivered to cells. This was done using the loading method previously described by Purdy and Graham [17]. In brief, cyclodextrin dissolved in methanol was mixed with cholesterol dissolved in chloroform. Nitrogen gas was used to evaporate the solvents, then the precipitate was dissolved in Tris/citric acid/glucose solution to make a 50 mg/mL stock solution. The stock solution was sonicated to ensure the cholesterol-loaded cyclodextrin was completely dissolved. The solution was stored at room temperature (20˚C - 22˚C) throughout the study and mixed with a vortex mixer immediately before use.

2.3. Viability Analysis

Supravital staining with the eosin-nigrosine staining technique described by Björndahl *et al.* [18] was used to determine the proportions of nonviable and viable cells. In this method, cells that uptake the dye have lost the selective permeability of the cell membrane and are considered dead. Cells showing dye exclusion (no or partial staining) still have selective permeability and are classified as viable (**Figure 1**). A total of 200 cells were counted from each sample and the ratios of viable cells were determined. A differential interference contrast (DIC) microscope (Olympus BX53) with the integrated camera was used to examine the samples.

2.4. Evaluation of Cell Membrane Integrity

Cell membrane integrity was assessed with the hypoosmotic swelling test especially developed for spermatozoa. In this test, cells are exposed to a hypoosmotic medium and those with an intact cell membrane exhibit swelling and tail curling (**Figure 2**). Fructose solution adjusted to an osmotic pressure of 80 mOsm was used in the test, which was performed as recommended by Ramu and Jeyendran [19]. For each sample, a total of 200 cells were counted and the proportion with intact cell membranes was determined. The same DIC microscope (Olympus BX53) with the mounted camera was used to examine the samples.

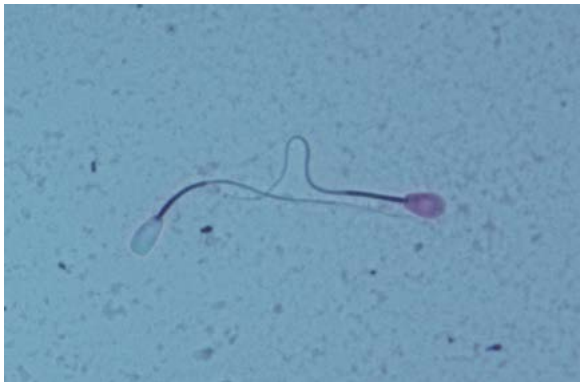

Figure 1. Image of a sperm cell that is not stained, indicating viability (left), and a stained cell that is nonviable (right) (400x magnification).

Figure 2. An image showing two intact cells. The cell on the left shows no swelling or tail curling, indicating impaired cell membrane integrity, whereas the cell on the right shows tail curling due to swelling, indicating an intact cell membrane (400x magnification).

2.5. Detection of DNA Damage

The extent of DNA damage in the cells was determined using single-cell gel electrophoresis (comet assay) performed as described by Küçük *et al.* [20]. In brief, the cells were incubated in lysis solution containing proteinase K to release the DNA masses in the head section of the cells. The released DNA was migrated by electrophoresis and the images obtained were analyzed using an image analysis program (Tri-Tek-Corp). In the comet assay images, the ratio of intact DNA in the head to the mass of fragmented DNA (comet tail) was determined (**Figure 3**). The same DIC microscope with the mounted camera (Olympus BX53) was used to examine the samples, and the obtained images were analyzed using computer software.

2.6. Statistical Analysis

At least 5 replicates of the experiments were performed per group. The data are presented as means and standard error of the mean. ANOVA (analysis of variance) was used for statistical comparisons of data between the groups. The SPSS statistical analysis package was used to calculate P values, with $P < 0.05$ accepted as statistically significant.

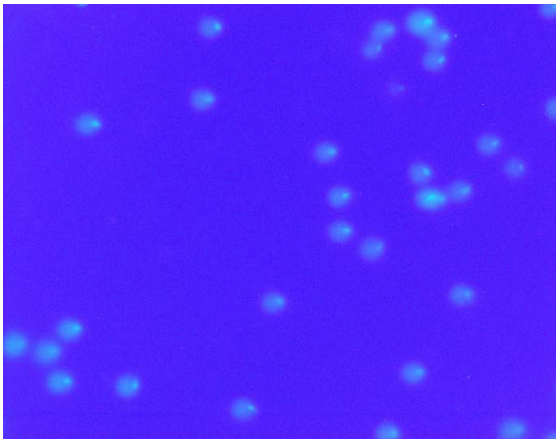

Figure 3. Image of DNA masses that migrated in the electrical field during the comet test, before computer-aided image analysis (100x magnification).

3. Results

Four-hour incubation at 35˚C immediately after thawing and aliquoting the cells resulted in a significant decrease in all vitality parameters (Tables 1-3). The addition of sericin and cholesterol resulted in no significant increase in the proportion of cells with intact cell membranes after incubation ($P > 0.05$; Table 1).

However, the addition of cholesterol and sericin to the diluent before incubation significantly increased the viability rates of the cells after 4 hours ($P < 0.01$; Table 2).

In addition, both cholesterol and sericin added to the diluent before incubation significantly increased the ratio of intact DNA in the cells after 4 hours of incubation ($P < 0.01$; Table 3).

4. Discussion

Stimulating or accelerating the repair activities of cells that are damaged and have impaired function as a result of artificial or natural causes in order to extend their lifespan and reduce the extent of injury is an important biotechnology that can be used both in vitro and *in vivo*. We have not come across any publication in the literature investigating the effectiveness of both cholesterol and serine on cell membrane, DNA damage. The method of using cholesterol-loaded cyclodextrin nanoparticles to deliver cholesterol to damaged cells used in this study was previously shown to artificially increase the cholesterol levels of cells by up to 40% and increase their resistance to the damage caused by freezing at −196˚C and thawing, resulting in higher viability rates [21]. It was also shown that cells supplemented with cholesterol by the same method may be more resistant to oxidative stress caused by hydrogen peroxide [22]. These examples demonstrate the positive effects of previous structural preparation or chemical modification of cells against certain foreseen conditions. In the current study, we determined that the cholesterol added to the diluent did not cause a statistically significant decrease in cell membrane damage at the end of a 4-hour incubation period at

Table 1. Intact cell membrane ratios immediately after thawing and after the 4-hour incubation.

Group	Cells with intact cell membrane (%)
Sericin	11.6 ± 0.91
Cholesterol	13.4 ± 0.89
Control	10.4 ± 0.32
After thawing	14.7 ± 0.77

Table 2. Cell viability rates immediately after thawing and after the 4-hour incubation.

Group	Cell viability rate (%)
Sericin	36.0 ± 1.58 [a]
Cholesterol	40.4 ± 1.91 [a]
Control	25.8 ± 0.92 [b]
After thawing	49.6 ± 2.31

Different letters in the same row indicate a statistical difference ($P < 0.01$).

Table 3. Intact DNA ratios immediately after thawing and after the 4-hour incubation.

Group	Ratio of intact DNA (%)
Sericin	40.3 ± 1.82 [a]
Cholesterol	38.5 ± 1.90 [a]
Control	28.8 ± 1.91 [b]
After thawing	42.1 ± 2.11

Different letters in the same row indicate a statistical difference ($P < 0.01$).

35˚C ($P > 0.05$), but only produced a numerical improvement (10.4% vs. 13.4%). However, both cholesterol and sericin were associated with significantly increased cell viability rates after incubation ($P < 0.01$). This demonstrates the cell survival-enhancing effect of both sericin and cholesterol at the doses used in our study.

Considering the molecular size of sericin, its multi-amino acid protein structure [14] suggests there is no or very limited entry through the cell membrane. Therefore, the effect observed in this study may be due to its glue-like properties [13], by which it closes holes and injuries in the cell membrane and prevents loss of intracellular contents. Cholesterol transfer is thought to exert effects both in the cell membrane and within the cell due to the small molecular structure of the cholesterol being delivered.

DNA damage can occur at any time, even in healthy cells. It has long been known that DNA damage activates the mechanisms of apoptosis, also known as programmed cell death [23]. The mechanisms required for the cell to detect and halt DNA damage (DNA repair, cell division arrest, or apoptosis) differ between cells. Similarly, different cells also have varying thresholds for initiating apopto-

sis or susceptibility to DNA damage [24]. We did not find any publication similar to our study in the literature. Therefore, we think that the results of our study are very important. In this study, the inhibiting or mitigating effect of both cholesterol and sericin on DNA damage in sperm is important in terms of preventing the initiation of apoptotic mechanisms. This is explanation for the higher cell survival rate observed at the end of the incubation period.

A limitation of this study is that the results are based solely on frozen-thawed spermatozoa. It is clear that these findings must be confirmed or tested in other cell types.

In conclusion, the administration of sericin and cholesterol in a cell damage model increased cell survival and reduced DNA damage over a 4-hour post-thaw incubation period, suggesting their potential use as a "first aid" intervention at the cellular level.

5. Recommendations

It would be beneficial to repeat this study with different cells, especially human somatic cell lines and even frozen human stem cells. In addition, further investigations can be performed by identifying a group of repair-related target genes and determining alterations in the mRNA levels of these genes in damaged cells compared to a control group.

The results of this study for both cholesterol and sericin have implications for their potential use after the thawing of many commercially valuable human cells frozen in vitro (e.g., stem cells, somatic and cancer cell lines, embryos, oocytes, and sperm cells) and even during the storage of donor tissues and organs to enhance and sustain viability until transplantation. In addition, trials of these agents (especially sericin) can be conducted using *in vivo* systems (directly in living organisms) to evaluate their effects on cell damage in diabetes mellitus and intestinal mucosal damage following long-term antibiotic treatment.

Considering that the series is a waste product of the silk production industry, its use for human health purposes also provides additional benefits such as reducing waste and protecting the environment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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