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Original Article

Mechanism of Sodium Selenite-Induced Cataract Through Autophagy in Wistar (Rattus norvegicus) Rats

Cicih Komariah[1,2], Jusak Nugraha[3,*], Hidayat Sujuti[4]

[1]*Doctoral Program of Medical Science, Faculty of Medicine, Airlangga University, Surabaya, Indonesia*
[2]*Departement of Pharmacology, Faculty of Medicine, Jember University, Jember, Indonesia*
[3]*Department of Clinical Pathology, dr. Soetomo Hospital, Airlangga University, Surabaya, Indonesia*
[4]*Department of Ophthalmology, Faculty of Medicine, dr. Saiful Anwar Hospital, Brawijaya University, Malang, Indonesia*

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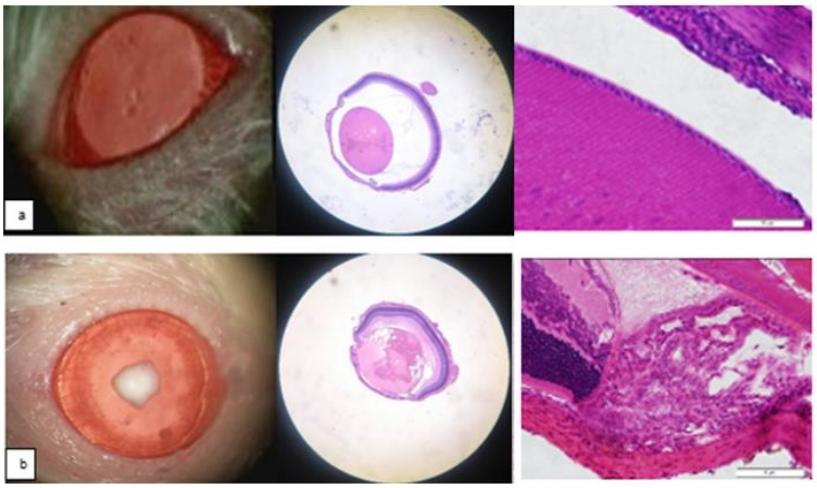
ABSTRACT

Cataracts are characterized by the clouding of the eye lens, resulting from changes in lens metabolism that can lead to lens hydration (fluid accumulation), denaturation of lens proteins, or both. Autophagy is a subcellular process involving the recycling of damaged proteins or organelles by lysosomes. Inhibition of autophagy in lens cells can hinder cell differentiation, contributing to cataract formation. Understanding the functional role of autophagy in lens tissue is essential for considering it as a potential therapeutic strategy for cataract treatment. This study involved several statistical analysis tests, including descriptive statistics to calculate the mean and standard deviation (SD), data normality tests to assess data distribution, data homogeneity tests to examine data diversity, and the Homogeneity of Variance test. A t-test was conducted to assess significant differences between the two independent test groups. Statistical analysis was performed using SPSS version 20.0. This study explored the role of the autophagy pathway in cataracts induced by oxidative stress, focusing on the expression of ROS, SOD, mTOR, LC3-II, p-62, IL-1β, Caspase-3, and lens tissue histology. Cataract formation was clinically examined in the eye lens of experimental animals. Immunohistochemical methods were used to analyze ROS, SOD, LC3-II, p-62, IL-1β, and Caspase-3. ROS, SOD, LC3-II, and p62 were collected from the anterior lens capsule, while IL-1β and Caspase-3 were collected from the eye lens. mTOR expression was collected from the lens capsule and determined by Western blot. The expression levels of mTOR, ROS, SOD, LC3-II, and caspase-3 in Wistar rats induced by the sodium selenite cataract model were lower than those in the control group. On the other hand, ROS expression and IL-1β expression in the sodium selenite-induced cataract model of Wistar rats were higher than those in the controls. The mechanism underlying cataract formation in Wistar rats induced by sodium selenite involves autophagy, as evidenced by the expression of ROS, LC3-II, IL-1β, and caspase-3.

* Corresponding author: Jusak Nugraha
✉ E-mail: jusak-n@fk.unair.ac.id

GRAPHICAL ABSTRACT



Introduction

Cataracts are characterized by the clouding of the eye lens, resulting from changes in lens metabolism that can lead to lens hydration (fluid accumulation), denaturation of lens proteins, or both. Cataracts are typically a progressive condition [1]. The incidence of cataracts is 3.9% in individuals aged 55–64 years and increases to 92.6% in those aged 80 years and above [2]. Senile cataracts, which result from age-related changes in lens formation after the age of 40, involve a clinical aging process that begins as early as the fourth decade of life and is characterized by presbyopia [3, 4]. On the other hand, congenital cataracts are opacities in the lens that have been present since birth or acquired within the first year of life. They can also develop in association with disease processes during pregnancy, such as rubella [4]. According to several studies, oxidative stress is strongly suspected as the leading cause of senile cataracts [5]. Oxidative stress arises from an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the biological system's ability to detoxify these reactive products [6]. Oxidative stress has been implicated in various systemic disorders, including cardiovascular disorders, neurodegenerative diseases, and cancer [7]. ROS itself is a metabolic by-product produced by biological systems, consisting of superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) [8, 9]. Excessive accumulation of ROS disrupts intracellular homeostasis, and autophagy, in turn, reduces oxidative damage by engulfing and irreversibly degrading oxidized substances. As a result, the lens's transparency gradually decreases, leading to cataract formation [10].

Several physiological processes in cells, such as protein phosphorylation, activation of transcription factors, apoptosis, immunity, and differentiation, rely heavily on ROS production. Hence, maintaining low levels of ROS in cells is crucial [11].

Elevated ROS production can have harmful effects on essential cellular structures, such as proteins, lipids, and nucleic acids [12]. A substantial body of evidence suggests that oxidative stress may contribute to the onset and/or progression of various diseases, including cancer, diabetes mellitus, metabolic disorders, atherosclerosis, and cardiovascular disease [13]. Mitochondria are the primary source of ROS production during both physiological and pathological conditions.

For instance, $O_2^{\bullet-}$ can form during cellular respiration, as well as via lipoxygenase (LOX) and cyclooxygenase (COX) during arachidonic acid metabolism and from endothelial cells and inflammatory cells [14].

Other non-enzymatic molecules with free radical scavenging properties, such as vitamins, melatonin, and glutathione (GSH) can neutralize ROS [15]. However, when antioxidant defenses fail to effectively neutralize ROS, they persist in the body and oxidize vulnerable biomolecules [16]. This excessive ROS level can lead to damage to cellular proteins, membrane lipids, and nucleic acids, ultimately impairing cellular function [16]. One of the effects of ROS accumulation is the induction of the autophagy process. Autophagy is a subcellular mechanism for the turnover of damaged proteins or organelles by lysosomes [17]. Disturbances in autophagy have been implicated in the pathophysiology of various eye diseases, including glaucoma, diabetic retinopathy, retinoblastoma, orbital thyroid disease, and cataracts [18]. Essentially, autophagy represents a catabolic process in which cells degrade and recycle cellular components within lysosomes [18]. The primary function of autophagy is to act as a form of cellular defense during stress conditions [19]. In this process, macromolecules are broken down into small molecule precursors to support metabolic pathways [20].

While autophagy pathways may play a fundamental role in the cataracts pathogenesis, the exact mechanism of how oxidative stress and autophagy are associated with cataracts has not been fully established. Understanding the functional role of autophagy in lens tissue is essential for considering it as a potential therapeutic strategy for cataract treatment.

For this study, cataracts were induced in 9-day-old rats through intraperitoneal injection of sodium selenite, which can serve as a model of a senile cataract due to its similar characteristics. The induction of sodium selenite in the eye lens leads to a series of oxidation processes, representing oxidative stress [20]. This study aims to explore the role of the autophagy pathway in cataracts induced by oxidative stress, focusing on the expression of ROS, SOD, mTOR, LC3-II, P-62, IL-1β, Caspase-3, and lens tissue histology. It is hoped that this study will offer new insights into cataract pathogenesis by targeting the autophagic pathway for potential clinical applications.

Materials and Methods

This study utilized a True Experimental Design with a post-test-only group. This design emphasizes internal validity, allowing the researchers to control external factors that may influence the experimental results. The study is causal in nature, and evidence was obtained by comparing the experimental group (treated) with the control group (nontreated).

The experimental units in this study included Wistar rats, selected based on specific inclusion criteria: body weight between 9-11 grams, age of 9 days, and exhibiting good physical health, characterized by clear eyes, white fur, agile movements, and normal feces consistency. Rats with pre-existing cataracts or those that fell ill or died during the adaptation period of the treatment were excluded from the study. The sample size was determined using a formula, resulting in n = 11 for each group, requiring a minimum of 11 rats in each group. The study comprised two groups: the control group without cataracts and the treatment group administered 25 μmol/kg body weight of sodium selenite. Each group consisted of 12 rats, totaling 24 Wistar rats.

This study utilized a randomization approach, assigning each sample a number and selecting them through a lottery method. The independent variable in this study was the administration of 25 μmol/kg body weight of sodium selenite. The dependent variables were mTOR expression, ROS expression, SOD expression, LC3-II expression, p-62 expression, IL-1β expression, and caspase-3 expression. Control variables related to cataract induction in 9-day-old rats included administering the injection intraperitoneally, swiftly, and precisely, separating the mother and her offspring during the injection (minimizing the distress of the offspring), performing the injection at 9 a.m. in a dimly lit room, and returning the mice to their mothers after the injection.

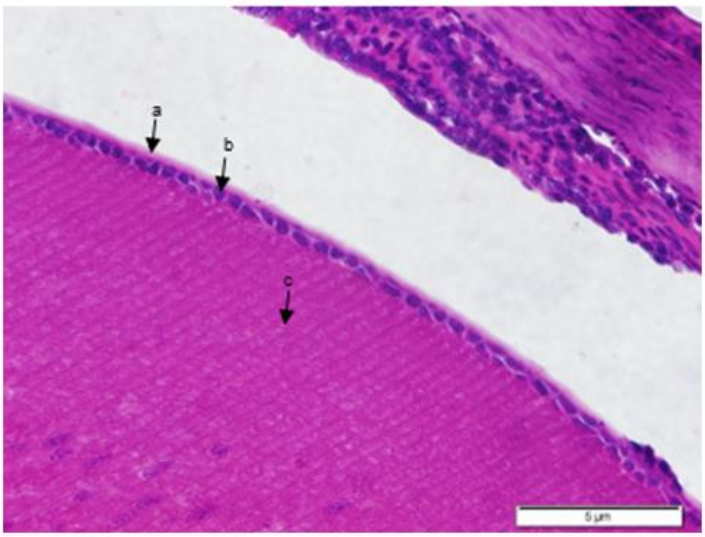


Figure 1: Histology of the eye lens in the control group: (a) Lens capsule; (b) Subcapsular epithelium; and (c) Lens fiber cells (examined using an Olympus BX-53 light microscope, magnification 100x)

Results and Discussion

Lens cloudiness was observed in all sodium selenite (Na_2SeO_3) induced rat groups, while the control group had clear lenses. Changes in lens opacities can be seen in Figure 3. Both eyes of all control group mice maintained clear lenses (Figure 1). Cataract formation was induced by subcutaneous injection of Na_2SeO_3 (25 µmol/kg BW) on day 10, becoming evident when the rats opened their eyes. Slit lamp examination of rat eye lenses revealed cataracts in all animals injected with Na_2SeO_3. The cataracts observed in the sodium selenite treatment group were primarily nuclear cataracts. These findings demonstrate that a single subcutaneous dose of Na_2SeO_3 (25 µmol/kg BW) successfully induced cataract formation.

The lens in the cataract experimental animals exhibited several abnormal changes in its histological tissue, as demonstrated in Figure 2. Cataracts were characterized by lens fibers with various abnormal features, including a thickened and wrinkled lens capsule (Figure 2a), disorganized lens fibers (Figure 2b), lens cell proliferation (Figure 2c), and lens fiber edema (Figure 2d).

The differences in the results of slit lamp examination, histology, and histopathology in the eyes of normal and treated experimental animals are illustrated in Figure 3 (from left to right). a) The results of slit lamp examination in experimental animals without sodium selenite injection, along with histological images of normal eyes at 40x magnification and 100x magnification and b) the results of slit lamp examination in experimental animals injected with 25 µmol/kg body weight of sodium selenite, with histological images of the eye at 40x magnification and 100x magnification (examined using an Olympus BX-53 light microscope).

Regarding the descriptive data of the sample, the study assessed several variables, namely the levels of Reactive Oxygen Species (ROS), p62, Interleukin (IL)-1β, Superoxide Dismutase (SOD), LC3-II, caspase-3, and mTOR. The average values of ROS, p62, and IL-1β were higher in the cataract-induced group compared to the control group. Meanwhile, the levels of SOD, LC3-II, caspase-3, and mTOR were lower in the cataract-induced group compared to the control group. The normality test and homogeneity analysis were conducted to determine the distribution and diversity of research data and to guide further analysis. The Shapiro-Wilk test was used for the normality analysis due to the sample size ($n < 50$), while the Homogeneity of Variance test was employed for the homogeneity analysis. IL-1β and LC3-II were found to have non-normal distributions, whereas the other variables had normal distributions. Moreover, the homogeneity test results for IL-1β, SOD, and LC3-II levels showed inhomogeneous distribution values.

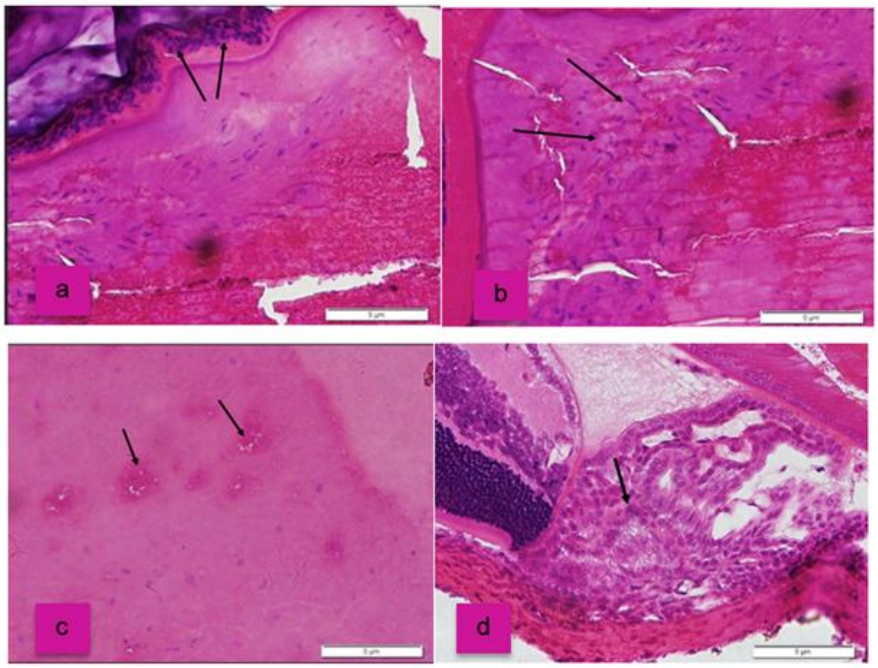


Figure 2: Histopathology of the eye lens in the cataract group indicated by the black arrow: (a) Thickened and wrinkled lens capsule; (b) Disorganized lens fibers; (c) Lens cell proliferation; and (d) Lens edema (examined using an Olympus BX-53 light microscope, total magnification 100x)

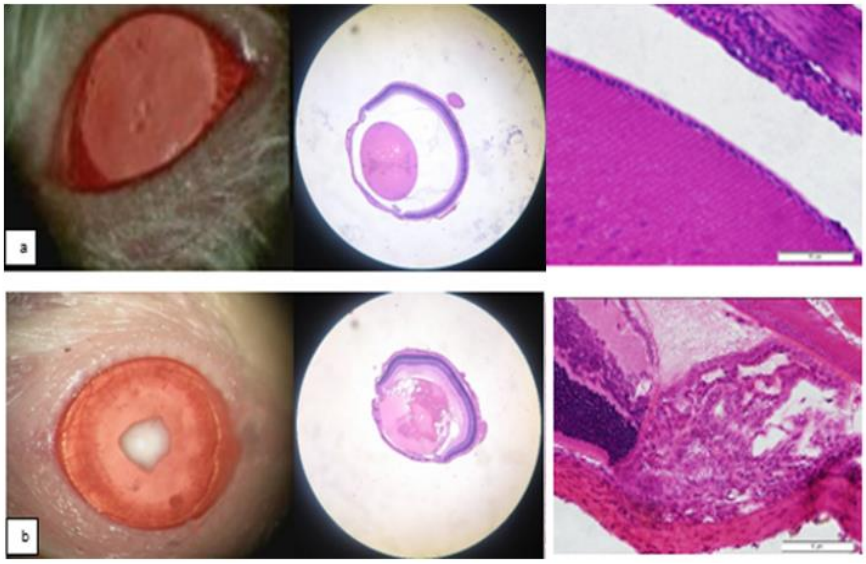


Figure 3: Results of slit lamp examination and histological images of the study: Figure a represents the lens group without sodium selenite injection, while image b represents the lens group injected with sodium selenite

The comparative test, using an independent T-test (parametric), revealed no significant difference between the control and treatment groups in the variable p62 levels. However, statistically significant differences were observed between the control and treatment groups for the other variables. The examination of mTOR levels demonstrated an average of 32.05 ± 4.02 in the control group and 21.95 ± 2.73 in the cataract group, showing a statistically significant difference (p-value = 0.001). In addition, the examination of ROS levels revealed an average of 27.40 ± 9.72 in the control group and 38.14 ± 8.31 in the cataract group, with a statistically significant difference (p-value = 0.008).

The analysis of SOD levels indicated an average of 27.90 ± 1.29 in the control group and 19.44 ± 4.52 in the cataract group, showing a statistically

significant difference (p-value = 0.043). However, the examination of p62 levels displayed an average of 27.92 ± 5.85 in the control group and 31.38 ± 6.22 in the cataract group, with no statistical significance (p-value = 0.174). The analysis of LC3-II levels showed an average of 62.77 ± 13.56 in the control group and 18.82 ± 3.87 in the cataract group, demonstrating a statistically significant difference (p-value = 0.001). Moreover, the examination of IL-1β levels showed an average of 17.30 ± 3.48 in the control group and 56.91 ± 18.30 in the cataract group, indicating a statistically significant difference (p-value = 0.001). Finally, the examination of caspase-3 levels revealed an average of 45.89 ± 13.74 in the control group and 21.32 ± 14.20 in the cataract group, demonstrating a statistically significant difference (p-value = 0.001). The sodium selenite cataract model is typically induced by a single subcutaneous injection of 19-30 μM/kg sodium selenite (Na_2SeO_3) into lactating rats aged 10-14 days, during the critical period of lens maturation around 16 days of age [21]. The results of this study demonstrate that a single subcutaneous injection of Na_2SeO_3 (25 μmol/kg BW) successfully induced cataract formation, while the control group showed no lens opacification. The autophagy process is tightly regulated through signaling pathways involving mTOR and AMPK, which monitor the nutritional status of cells [22]. mTOR, a serine/threonine kinase highly conserved in mammalian cells, forms two distinct protein complexes: mTORC1 and mTORC2. mTORC1 regulates cell cycle progression and protein synthesis through activation of ribosomal kinase S6 (S6K1 and S6K2) and eukaryotic initiation factor 4E (eIF4E). At the same time, mTORC2 modulates cell differentiation, proliferation, invasion, and glucose metabolism by phosphorylating protein kinase B (PKB, AKT) at serine 473 [24]. The results of this study indicate that the difference in mTOR levels between the control and treatment groups is statistically significant (p-value = 0.001), indicating a significant effect of the treatment variable on mTOR. Furthermore, the study found that the difference in ROS levels between the control and treatment groups was statistically significant (p-value = 0.008), showing that the treatment variable had a significant effect on ROS. The path coefficient is positive (0.527), indicating that sodium selenite injection had a positive or increasing effect on the ROS variable. About 90% of intracellular ROS is generated by the electron transport chain in the inner mitochondrial membrane and includes H2O2, superoxide (O2-), and hydroxyl radicals (OH-). ROS can cause oxidation of organelles, nucleic acids, proteins, and lipids, resulting in cellular damage. ROS play a dual role in autophagy regulation: they not only trigger autophagy pathways to maintain redox homeostasis and remove oxidized organelles and components, but they can also inhibit autophagy, possibly by directly oxidizing ATG proteins (ATG7 and ATG10) or inactivating autophagy modulators (TFEB and PTEN). In the cataractogenesis context, ROS-induced damage to lens cells can lead to protein oxidation, DNA damage, and lipid peroxidation. Notably, in advanced cataracts, over 90% of cysteine and half of methionine residues are oxidized [25]. In patients with type 2 diabetes, ROS was shown to decrease the LC3II/I ratio in endothelial cells. Although ROS can regulate autophagy through various signaling pathways, the exact mechanism requires further investigation. Superoxide Dismutase (SOD) is an enzyme responsible for catalyzing the superoxide conversion into oxygen and hydrogen peroxide (H_2O_2). Through their activity, SOD enzymes control the levels of various ROS and reactive nitrogen species, thereby limiting the potential toxicity of these molecules and controlling broad aspects of cellular life regulated by their signaling functions [26]. The ROS accumulation in cells activates autophagy processes associated with decreased or impaired SOD as an antioxidant. Mutations in the SOD1 gene modulate autophagy, and in transgenic mice expressing SOD1 mutations, there is an increase in autophagy activity. In the present study, SOD1G93A transgenic mice showed inhibition of mTOR and accumulation of LC3-II. Autophagy is considered as a system of random degradation, but certain substrates, such as p62, are preferentially degraded by autophagy. p62 is a stress-induced scaffold protein [9] and a ubiquitin-binding protein that co-localizes with

ubiquitinated protein aggregates [27]. Accumulation of p62 in mouse cells and tissues indicates defects in autophagy [28-30]. It has been used as a marker for autophagy inhibition and defects in autophagy degradation [31]. P62 is a multifunctional scaffold protein that interacts with various proteins to regulate processes, including apoptosis, necroptosis, and redox states, through the regulation of the KEAP1-NRF2 pathway. The homeostatic regulation of these processes in the body can affect p62 levels independently of autophagy. Unlike LC3-II, an increase in p62 is not always indicative of autophagy inhibition [32]. To date, microtubule-associated protein light chain 3 (LC3), the mammalian homolog of yeast Atg8, is the only known protein to exist on autophagosomes. Therefore, this protein serves as a widely used marker for autophagosomes [33]. Changes in LC3-II levels have been observed depending on the tissue and cell environment [34]. LC3-II, during phagophore formation, interacts with adapter proteins to engulf and process cellular components in autophagolysosomes. Thus, the LC3-II level correlates with the number of autophagosomes and is considered as an indicator of autophagosome formation [35]. Analysis of LC3 levels has been shown to be an efficient way to monitor autophagic activity. In this study, the immunohistochemical method was used to measure LC3-II expression in the eye lens capsule of Wistar rats induced with a single dose of 25 μmol/kg sodium selenite. The results of a significant decrease in LC3-II compared to the control group indicate that LC3-II plays an important role in the autophagy mechanism related to cataracts induced by oxidative stress, namely sodium selenite. Autophagy function can be influenced by several cytokines, immunoglobulins, and immune-related cells, where TGF-β, interferon (IFN)-γ, interleukin (IL)-1, IL-2, and IL-12 act as inducers of autophagy, while IL-4, IL-10, and IL-13 act as inhibitors of autophagy. After macrophages are exposed to Toll-like receptors (TLR), pro-IL-1β is specifically sequestered into autophagosomes, and autophagosomes activation with rapamycin induces the degradation of pro-IL-1β [36]. Deficiency of autophagy due to the loss of Atg5 in dendritic cells (DC) interferes with the secretion of cytokines, IL-2, IFN-α by CD4+ T cells, and independently IL-1β. In addition, p62/SQSTM1 levels can modulate the intensity of IL-1β signaling through IL-1β receptors, and increased autophagic degradation of p62/SQSTM1 has an anti-inflammatory effect. This effect is primarily mediated by Atg16 and E3 binding to Cullin-3 [37]. The results of this study indicate a statistically significant difference in IL-1β levels between the control and treatment groups (p-value = 0.001), signifying that the treatment variable has a significant effect on IL-1β. The positive path coefficient (0.843) indicates that the treatment resulted in a positive or increasing influence on the IL-1β variable. IL-1β has been shown to promote lens epithelial cell proliferation and collagen synthesis, which contributes to cataract formation. Moreover, IL-1β induces IL-6 expression and secretion via the p38 MAPK/NF-κB pathway, potentially initiating posterior capsule opacification (PCO) formation, where IL-1 action may be mediated through IL-6 production [38]. Caspase-3, along with caspases 6 and 7, is a major effector caspase whose role in the regulation of autophagy has been extensively studied. Previous research suggested a role for mammalian caspase-3 in autophagy using tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in HeLa cells, where Beclin-1 is cleaved in a TNF-dependent manner by caspase. This cleavage of Beclin-1 by caspase-3 significantly impairs autophagic flux when apoptotic stimuli are present [39]. In murine bone marrow cells subjected to prolonged starvation, the initial autophagic response shifts toward apoptosis. During this transition, Beclin-1 is cleaved by caspase-3, resulting in a C-terminal fragment that promotes cell death through the mechanisms mentioned above [40]. The results of this study reveal a statistically significant difference in caspase-3 levels between the control and treatment groups (p-value = 0.001), indicating that the treatment variable has a significant effect on caspase-3. The negative path coefficient (-0.676) suggests that oxidative stress induced by sodium selenite has a negative or decreasing effect on caspase-3 variables. Overall, data from various studies demonstrate the involvement of

caspase-3 in the mechanism of autophagy. Evidence suggests ROS can induce autophagy through an mTOR-dependent pathway [33, 41]. ROS production activates hypoxia-inducible factor-1 (HIF-1), erythroid nuclear factor 2–related factor 2 (NRF2), p53, and forkhead box O-3 (FoxO3) [42]. The p53 induction by ROS can be associated with DNA damage due to ROS, which, in turn, induces p53 transcription. ROS increases AMPK phosphorylation, leading to mTORC1 inhibition, and influences the regulation of PI3K-AKT signaling to reduce mTORC1 activity, resulting in autophagy induction. Thus, ROS can initiate autophagy through AMPK activation and mTORC1 inactivation [43, 44]. The effect of ROS on mTOR, although statistically weak, is significant due to ROS involvement in various signaling cascades related to oxidative stress. Furthermore, mTOR itself can be induced or inhibited by various cellular stimuli. The effect of ROS on LC3-II levels showed significant results, indicating an effect between ROS and LC3-II levels, albeit weak, according to the study's results. LC3-II is present in the internal and external compartments of autophagosomes, and during phagophore formation, LC3-II interacts with adapter proteins to engulf and process cellular components in autophagolysosomes. The role of autophagy in regulating IL-1β secretion may be time and context-dependent, where in the absence of inflammasome activating signals, autophagy can remove pro-IL-1β and inflammasome components from cells, whereas in their presence, autophagy can act as a secretory pathway for IL-1β release. Caspase-3 plays a critical role as the main effector of caspase-8, which is activated during both the extrinsic and intrinsic apoptotic pathways. Caspase-3 inhibits cellular function by cleaving numerous protein substrates during programmed cell death. The study indicates that an increase in p62 leads to a decrease in caspase-3 levels, although the effect is weak. This effect occurs through a series of processes that ultimately activate caspase-8. Once activated, caspase-8 triggers a cascade of processes, leading to the activation of effector caspases, including caspase-3 [45]. Pro-IL-1β has been further studied as a target of rapamycin-induced autophagy in macrophages [46]. This study demonstrates that IL-1β has a significant effect on the incidence of cataracts induced by oxidative stress through the process of autophagy, with an increase in IL-1β levels contributing strongly to cataract formation. The development of autophagy is regulated by the interaction of several Atg with specific regulators, including caspases [47]. While the effect may be statistically moderate, the research provides evidence of the significant role of caspase-3. LC3-II levels are closely associated with the number of autophagosomes and are considered as a reliable indicator of autophagosome formation [48]. To date, LC3 is the only mammalian homolog of yeast Atg8 known to be present in autophagosomes, making it a widely used marker for autophagosomes [33]. The results of this study successfully demonstrate that LC3-II plays a vital role in the mechanism of oxidative stress-induced cataracts through autophagy, with a significant and robust effect.

While not all variables showed significant results or a strong influence in the analysis of the effect between variables, this study has the potential to provide robust initial *in vitro* data to support further evaluation *in vivo*. The study revealed that oxidative stress resulted in decreased expression of p-mTOR, increased expression of ROS, decreased expression of SOD, increased expression of p62, decreased expression of LC3-II, increased expression of IL-1β, and decreased expression of caspase-3. These results align with previous studies and further establish a potential link between cataracts, oxidative stress, and the process of autophagy. A novel finding from this study is that ROS, IL-1β, caspase-3, and LC3-II may be significant factors related to the autophagy process in cataracts induced by oxidative stress, given their fairly strong influence on this mechanism. The study contributes to the overall understanding of cataract pathophysiology, and the insights gained may inform future therapeutic approaches for experimentally induced cataracts. This study employed a sodium selenite-induced cataract model in mice, which may not entirely replicate the complex and multifactorial nature of cataracts in humans. However, this limits the

applicability of the findings to human cataract patients. Furthermore, this article predominantly focuses on oxidative stress in the context of cataract formation, but does not explore specific sources or mechanisms of oxidative stress beyond ROS. A more comprehensive analysis of oxidative stress factors would be advantageous.

Conclusion

According to the path analysis results, it can be concluded that there are significant influence relationships between ROS > LC3-II; mTOR > IL-1β; IL-1β > Cataract; SOD > Cataract; Caspase-3 > Cataract; and LC3-II > Cataract. However, no significant effects were found between ROS > mTOR; ROS > p62; p62 > SOD; p62 > Caspase-3; and p62 > IL-1β. The study demonstrates that in sodium selenite-induced cataracts, the expression of mTOR, SOD, LC3-II, and Caspase-3 is lower than in the control group, while the expression of ROS, p62, and IL-1β is higher. ROS, IL-1β, Caspase-3, and LC3-II may play a significant role in the autophagy process during oxidative stress-induced cataracts. Further research using various autophagy markers is needed to enhance the understanding of the variables that influence this mechanism. In addition, conducting research with autophagy inhibitors would help further evaluate the relationship between oxidative stress and autophagy.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the article and agreed to be responsible for all aspects of this work.

ORCID

Cicih Komariah
https://orcid.org/0009-0004-0721-3620
Jusak Nugraha
https://orcid.org/0000-0001-6700-9921
Hidayat Sujuti
https://orcid.org/0000-0001-6568-3584

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