Bletilla striata polysaccharides protect against mercury-induced oxidative damage to *Drosophila* midguts via modulation of sestrin

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Research Article

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Abstract

Oxidative stress was one of the major causes of heavy metal-induced toxicity in organisms including *Drosophila melanogaster*. *Bletilla striata* polysaccharide (BSP) with anti-oxidative property has been recently recognized as a novel player in the management of oxidative stress response in organisms. Here, we took *Drosophila* midgut as a model to evaluate the protective effects of BSP (50 μg/mL) on mercury chloride-induced gastrointestinal adversities. As a result, BSP was found to significantly improve the survival rates and climbing ability of flies exposed to mercury. Further study demonstrated that BSP significantly alleviated the mercury-induced injury to midgut epithelium, at least partly, through increasing antioxidant enzyme activity (glutathione-S-transferase and superoxide dismutase), decreasing reactive oxidative species production, inhibiting cell apoptosis, restoring intestinal epithelial barrier and regulating stem cell-mediated tissue regeneration. Additionally, oxidative stress responsive gene *sestrin* was involved in the protection of BSP against mercury-induced oxidative damage to midguts. This study suggested that BSP has great potential for future application in the treatment and prevention of heavy metal-induced gastrointestinal adversities in mammals.

Introduction

Mercury was a toxic heavy-metal pollutant with diverse industrial applications. The oxidative stress in tissues such as gastrointestine, kidney and neuron was deemed to be one of the most significant events triggering mercuric toxicology, and thus resulted in oxidative damage to the body of vertebrates and invertebrates, including the fruitfly *Drosophila melanogaster* (Xu et al. 2021; Blas-Valdivia et al. 2021; Chen et al. 2021; Zhao et al. 2021; Teixeira et al. 2018). The midgut of *Drosophila* shared numerous anatomical, biochemical and physiological similarities with vertebrates (Miguel-Aliaga et al. 2018). Therefore, the *Drosophila* midgut has recently become an increasingly popular model system to study the mehanism and treatment of mercuric toxicology in gastrointestinal tract. Exposure of *Drosophila to* mercury chloride resulted in inhibition of antioxidant enzyme activity, reactive oxygen species (ROS) overproduction, lipid peroxidation, reduction of fertility and even alteration of somatic and germline cells (Paula et al. 2014; Mojica-Vázquez et al. 2019). Further study suggested that mercury exposure caused severe oxidative damage to midgut epithelium and disrupted the homeostasis of intestinal stem cells (ISCs) in midguts (Chen et al. 2016; Chen et al. 2021). Therefore, the exploration of new, rich sources of natural antioxidants has become the top priority to prevent and treat various oxidative stress-related diseases including hydrargyrism.

Bletilla striata was a traditional Chinese herb which has been widely used in nutraceutical, medical and pharmaceutical fields (Xu et al. 2019; He et al. 2017). Increasing evidences have demonstrated that BSP might be a major bioactive component present in different extracts of *Bletilla striata*, and often responsible for the various pharmacological activities. *In vitro* study showed that BSP exhibited considerable antioxidant activity via superoxide anion radical-scavenging assay, hydroxyl radical scavenging assay and DPPH free-radical scavenging activity assay (Peng et al. 2014). Exposure to BSP inhibited ROS generation induced by angiotensin II in human mesangial cells via NOX4 and TLR2

pathways (Yue et al. 2016). Additionally, BSP fraction exhibited significant gastroprotective effects against ethanol-induced gastric mucosal lesion in rats by decreasing pro-inflammatory cytokines production, relieving oxidative stress and inhibiting MAPK/NF-κB pathways (Zhang et al. 2019). The antioxidant effect was also confirmed that BSP could suppress lipopolysaccharide-induced ROS generation in C2C12 myoblasts (Liang et al. 2021). Interestingly, some evidences showed that BSP had good anti-inflammatory and antioxidant effects to enhance endothelial cell proliferation and wound healing, suggesting its potential role in the recovery of tissue repair (Chen et al. 2020a; Chen et al. 2020b; Lai et al. 2018). In addition, BSP was reported to improve the locomotion behavior and oxidative stress resistance and extend the lifespan of *C. elegans* through the insulin/IGF signaling pathway (Zhang et al. 2015). The above results indicated that BSP had potent antioxidant activity, which were responsible for its reported pharmacological activity. Further *in vivo* evidences were, however, required to confirm the reliability and effectiveness of BSP in organisms.

Although BSP has been widely used in various oxidative stress-related diseases, little information is available on the protective effects of BSP on hydrargyrism in gastrointestine. In this present study, we took *Drosophila* midgut as a model system to investigate the protective effects and underlying mechanism of BSP against mercury-induced intestinal oxidative damage. Our results would pave the way for further exploitation of BSPs in the field of biomedicine and provide valuable insights into more effective antioxidant therapeutic approaches for heavy metal toxicity.

Materials And Methods

Drosophila melanogaster stocks and culture

The following fly lines were used in this work: *esg-Gal4*; *esg-Gal4, UAS-GFP* (*esgGFP*); w^{1118}; *UAS-sestrin*^{d04539} (BL85440); *UAS-sestrin*^{RNAi} (VDRC104365); *Su(H)GBE-lacZ*. Transgenic lines, *UAS-sestrin*^{d04539} and *UAS-sestrin*^{RNAi} were respectively crossed to *esg-Gal4* and *esgGFP* flies to get the flies having *sestrin* overexpression (*esg-GAL4;UAS-sestrin*^{d04539} and *esgGFP > UAS-sestrin*^{d04539}) and *sestrin* knockdown (*esg-Gal4;UAS-sestrin*^{RNAi} and *esgGFP > UAS-sestrin*^{RNAi}) in midguts. *Drosophila* stocks were reared on standard *Drosophila* medium at constant temperature and humidity (25°C, 60% relative humidity) under a 12 h light/12 h dark cycle.

BSP treatment

According to previous studies, 50 μg/mL BSP and 400 μM $HgCl_2$ were used in the present study (Zhang et al. 2015; Yue et al. 2016; Chen et al. 2016). Both $HgCl_2$ (Sigma Aldrich) and BSP (XI'AN ZeiLan) was diluted with 5% sucrose. Flies (1- to 3-day old) were transferred to plastic vials containing $HgCl_2$ and BSP-treated filter paper (3 cm × 2 cm). Filter papers were changed three times a day. In survival assessment, daily mortality was recorded (Fig. 1).

Locomotion assay

After 3 d of $HgCl_2$ and BSP exposure, locomotion assay was carried out to assess locomotor ability of the flies. The test was performed as previously described (Chen et al. 2021).

Measurement of apoptosis and ROS

After 3 d of $HgCl_2$ and BSP exposure, the TdT-mediated dUTP nick end labeling (TUNEL) (Millipore, S7165) and 2, 7-dichloro-dihydro-fluorescein diacetate (DCF-DA) (Beyotime, S0033) staining were respectively performed following the manufacturer's instructions to test the level of cell apoptosis and ROS production in w^{1118}, *sestrin* knockdown and *sestrin* overexpression midguts, according to the previously described protocol (Chen et al. 2016).

Enzyme Assays

The midguts of control, *sestrin* knockdown or *sestrin* overexpression flies treated with $HgCl_2$ and BSP for 3 d were homogenized in 0.2 mL of cold phosphate buffer (0.1 M, pH 7.0), and centrifuged (1,000×g, 5 min). The isolated supernatant was then used to determine the activity of antioxidant enzyme lutathione-S-transferase (GST) and superoxide dismutase (SOD), according to the previously described protocol (Chen et al. 2016).

Immunostaining and image capture

Immunostaining and image capture were performed as previously described (Chen et al. 2021). The primary antibodies used for immunostaining were listed as Table 1. Fluorescent secondary antibodies (1:500) and DAPI (0.5 μg/mL) used were purchased from Invitrogen.

Table 1
The primary antibodies used for immunostaining in this study

Antibody name	Brand name	Dilution ratio
mouse anti-Delta	DSHB	1:50
mouse monoclonal anti-Prospero	DSHB	1:50
rabbit polyclonal anti-GFP	Invitrogen	1:500
mouse anti-Armadillo	DSHB	1:200
rabbit anti-phospho-histone H3	CST	1:1000
mouse anti-LacZ	Promega	1:500

RT-qPCR assay

Total RNA was extracted from midguts treated with $HgCl_2$ and BSP for 3 d using TRIzol reagent (Takara). The RT-qPCR for *sestrin* and *armadillo* was performed using SYBR® Premix Ex Taq™ (Takara). The primers used for RT-qPCR were listed as Table 2. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and *rp49* was used as a normalization control.

Table 2
Primers used for RT-qPCR in this study.

Primer name	Primer sequences
Rp49-Fwd	5'-ACGTTGTGCACCAGGAACTT-3'
Rp49-Rev	5'-CCAGTCGGATCGATATGCTAA-3'
Armadillo-Fwd	5'-TTCGGAACCGTCACAAATGC-3'
Armadillo-Rev	5'-ATCCTCATCGTTCAGCAGCC-3'
Sestrin-Fwd	5'-TACCCCATTGAGCACGGTAT-3'
Sestrin-Rev	5'-GGGTCATCTTCTCACGGTTG-3'

Quantification analysis

The number of TUNEL-, Dl-, esgGFP- and Su(H)GBE-lacZ-positive cells and the fluorescence intensity of DCF-DA in midgut epithelium were tested as previously described (Chen et al. 2021). All quantification data were expressed as Mean ± standard deviation (SD). A Student's t-test was used to determine statistical significance. Statistical significance was shown as *P < 0.05.

Results

BSP improved the survival rates and climbing ability of flies exposed to mercury

We first investigated the effects of BSP on survival and climbing ability of wild-type w^{1118} flies (WT) exposed to mercury chloride. The adult wild-type flies (1- to 3-day old) were reared on food spiked with $HgCl_2$ and/or BSP for 7 d, and the survival rates and climbing ability of flies were then detected. After 3 d post-treatment, survival rates of flies fed with mixture of mercury and BSP were observed to be significantly increased compared with that of mercury-reared flies (Fig. 2A). Meanwhile, exposure of flies to BSP for 3 d significantly alleviated mercury-induced decrease of the climbing ability, indicating the potential protective effect of BSP on mercury-induced toxicity in *Drosophila* (Fig. 2B).

BSP alleviated the mercury-induced oxidative stress and apoptosis in midguts

To determine the effects of BSP on $HgCl_2$-induced oxidative stress, we fed wild-type flies with $HgCl_2$ and BSP for 3 d, and then employed DCF-DA staining and antioxidant enzyme activity assay to monitor the status of oxidative stress in midguts. As shown in Fig. 3B-C, BSP exposure significantly increased the activity of antioxidant enzyme GST and SOD in mercury-exposed wild-type midguts. The staining of DCF-DA showed that the ROS level in midguts exposed to the mixture of BSP and $HgCl_2$ was significantly

decreased compared with the $HgCl_2$-fed wild-type flies (Fig. 3E-F, N). Additionally, TUNEL assay revealed that the number of apoptotic cells in BSP-treated midgut epithelium was significantly decreased compared to the $HgCl_2$-exposed control (Fig. 3J-K, O). The results implied that suppression of oxidative damage by BSP might contribute to midgut protection under $HgCl_2$-induced oxidative stress conditions.

BSP-induced sestrin expression protected against mercury-induced oxidative stress and cell apoptosis in *Drosophila* midguts

Since *sestrin* was reported to be involved in combating toxic chemicals including mercury, we then investigated its role in the protection of BSP against mercury-induced oxidative injury in midguts (Chen et al. 2021). As shown in Fig. 3A, RT-qPCR indicated that the transcriptional levels of *sestrin* were significantly upregulated in BSP-exposed midguts. Subsequently, the levels of oxidative stress and apoptosis in *sestrin* knockdown (*esg-Gal;UAS-sestrin*^{RNAi}) and *sestrin* overexpression (*esg-Gal;UAS-sestrin*^{d04539}) flies treated with mercury and BSP mixture were further determined. Consequently, significant decrease in the activity of antioxidant enzyme GST and SOD (Fig. 3B-C) and increase in the level of ROS (Fig. 3F-G, N) and the number of apoptotic cells (Fig. 3K-L, O) were observed in *sestrin* knockdown midguts when compared to the control midguts treated with mercury and BSP. However, there seemed to be no significant difference between the control and *sestrin* overexpression flies treated with mercury and BSP in oxidative stress and apoptosis (Fig. 3F, H, K, M, N-O). Overall, these results indicated that *sestrin* was required for BSP to protect against mercury-induced oxidative damage to *Drosophila* midguts.

BSP-induced sestrin expression alleviated mercury-induced intestinal epithelial barrier disruption

The integrity of intestinal epithelial barrier was known to play an important role in maintaining homeostasis in digestive tract of *Drosophila*. Armadillo (Arm) was a critical component of adherens junction. RT-qPCR suggested that exposure to BSP significantly increased *arm* expression in mercury-treated midguts (Fig. 4A). Arm staining showed that the mercury-treated midgut epithelium were less organized compared with the wild-type (Fig. 4B-C), BSP feeding, however, alleviated mercury-induced disorganization of midgut epithelium (Fig. 4C-D). Additionally, inhibiting *sestrin* expression caused the overall arrangement of midguts more disorganized compared with the wild-type exposed to mercury and BSP (Fig. 4D-E). However, *sestrin* overexpression did not further alleviate mercury-induced disruption of midgut epithelium (Fig. 4D, F). Based on these data, we concluded that BSP alleviated mercury-induced intestinal epithelial barrier disruption, at least partly, through via modulation of *sestrin* expression.

BSP exposure alleviated mercury-induced midgut regeneration

Exposure to mercury has been known to lead to oxidative damage to midgut epithelium, which in turn stimulated ISCs to divide and differentiate faster into different types of cells in midguts to maintain intestinal homeostasis (Chen et al. 2016). Therefore, the ISC metabolism could be deemed to be an important indicator for intestinal injury. We speculated that if BSP had a protective effect against intestinal injury, the division of ISCs stimulated by mercury might be significantly inhibited. Then, we used genetic method to mark the progeny cells of ISCs in midguts (*esgGFP*) by GFP. As expected, BSP significantly decreased the mercury-induced increase in the number of GFP-positive cells in midguts (Fig. 5B-C, P). Meanwhile, the staining of phosphorylated histone 3 (PH3, a marker for mitosis) further showed that there was a significant reduction in pH3-immunoreactive cell nuclei in BSP-treated midguts compared with mercury-treated flies (Fig. 5R). All these results strongly demonstrated that BSP exposure significantly alleviated mercury-induced midgut regeneration, indicating its protective effect against mercury-induced tissue injury.

Sestrin was required for BSP-induced midgut regeneration in response to mercury exposure

Next, we explored whether *sestrin* was involved in the BSP-induced midgut regeneration in response to mercury exposure. We found that after BSP exposure, the number of GFP-positive cells in *sestrin* knockdown midguts (*esgGFP > UAS-sestrin*^{RNAi}) was decreased significantly compared to that of the control flies (Fig. 5C-D, P). Consistently, a significant decrease of the PH3-positive cells was observed in *sestrin* knockdown midguts when compared to the control treated with $HgCl_2$ and BSP (Fig. 5R). But *sestrin* overexpression (*esgGFP > UAS-sestrin*^{d04539}) seemed not to impact the process of BSP-mediated midgut regeneration (Fig. 5C, E, P, R). Subsequently, we conducted immunohistochemistry for Suppressor of Hairless [Su(H)] and Delta (Dl) which marked the transient enteroblasts (EBs) and ISCs, respectively, to evaluate the effects of BSP on the cell fate specification of ISCs in midguts. Further analysis showed that no significant difference was observed in the number of Dl-positive ISCs and the percentage of Su(H)-positive cells in GFP-positive cells between the control, *sestrin* overexpression and *sestrin* knockdown group treated with BSP, indicating that BSP did not impact the cell fate specification of ISCs in midguts (Fig. 5P-Q). Together, these results suggested that *sestri*n was required for BSP-induced intestinal regeneration in response to mercury exposure.

Discussion

The BSP, a main active substance of *Bletilla striata*, attracted worldwide attention in the medical and pharmaceutical field due to its biological properties. However, there were few reports about the application of BSP in the treatment of heavy metal-induced toxicology. In this work, we investigated the defensive role and underlying mechanism of BSP against mercury-induced gastrointestinal adverse in *Drosophila*. We demonstrated that exposure to BSP significantly increased antioxidant enzyme activity (GST and SOD), decreased mercury-induced ROS production, inhibited cell apoptosis, restored intestinal epithelial barrier, and alleviated midgut regeneration via modulation of *sestrin* expression in midguts.

In the past decades, a broad range of bio-functional components of traditional Chinese medicine *Bletilla striata* have been identified, among which BSP with strong antioxidant activities was quite a representative one (He et al. 2017; Xu et al. 2019). Increasing evidences revealed that BSP had strong gastrointestinal protection both in vitro and in vivo (Wang et al. 2020; Zhang et al. 2019; Luo et al. 2018; Luo et al. 2019). The mechanism of gastrointestinal protection of BSP might be probably associated with its antioxidant properties. In mice and rat, BSP protected against ethanol-induced gastric oxidative injury by increasing the activities of antioxidant enzymes (SOD, CAT and GSH-Px) and decreasing oxidation products (MDA and LPO) and cell apoptosis. The histological and biochemical analysis revealed that BSP alleviated ethanol-induced gastric damage, inflammatory cell infiltration and disruption of gastric mucosal epithelium (Wang et al. 2020; Zhang et al. 2019). In the present study, we found that BSP not only dramatically decreased the activities of antioxidant enzymes including GST and SOD, but also ameliorated the mercury-induced ROS overproduction and cell apoptosis in midgut epithelium. These results further demonstrated that the gastrointestinal protective effects of BSP might be closely associated with the mitigation of oxidative stress.

Intestinal mechanical barrier was mainly composed of intestinal mucosal epithelial cells and intercellular connection. The intestinal epithelium was deemed to be the first barrier to prevent the absorption of the noxious substances into the body of organisms. Appropriate adhesion between epithelial cells made a great contribution to maintaining intestinal mechanical barrier (Adesanoye et al. 2020; Hollander, 1999). Therefore, maintaining the integrity of the intestinal mechanical barrier should be a promising therapeutic strategy to maintain intestinal health. In the present study, we found that mercury-induced oxidative stress resulted in the severe cell apoptosis in midgut epithelium, indicating damaged intestinal mechanical barrier. Additionally, our data revealed that mercury exposure might impact the intercellular connection in midgut epithelium. Within the epithelial cell membrane of *Drosophila* midgut, Arm, an intracellular anchor protein, was associated with transmembrane protein E-cadherin to form a complex, which mediates adherens junction formation (Oda and Takeichi, 2011). The precise localization of Arm in midgut epithelium was, therefore, of particular importance in maintaining a healthy *Drosophila* gut (Lee et al. 2021). In this study, we found that the localization of Arm in midgut epithelium showed clear disruption in response to mercury feeding, indicating a poorly organized midgut epithelium. However, BSP could not only alleviate mercury-treated cell apoprosis, but also improve the overall arrangement of mercury-treated midgut epithelium, thereby increasing the barrier function of midgut epithelium. These results indicated that BSP might serve as a novel protective agent for the restoration of intestinal epithelial barrier disruption. Consistent with our study, BSP was reported to protect against thioacetamide-induced intestinal epithelial barrier disruption in cirrhosis rats, at least partly, by elevating expression of the the intestinal barrier transmembrane protein occludin and zonula occludens-1 at tight junctions (Luo et al. 2018). Similarly, *in vitro* assay also demonstrated that BSP ameliorated lipopolysaccharide-induced intestinal epithelial barrier disruption in rat intestinal epithelial cell line, and the mechanisms could be related with decreasing the inflammatory cytokine levels and elevating the expression of intestinal barrier transmembrane proteins (Luo et al. 2019). Through the above results, we supposed that maintaining the integrity of the gastrointestinal epithelial cell barrier might be an important

protective mechanism that contributed to the protective effects of BSP against gastrointestinal injury. Interestingly, Arm has been reported to be not only an intracellular anchor protein mediating the formation of adherens junction in gut epithelium, but also an important regulator of tissue regeneration as a member of the Wnt/Wingless signaling pathway in *Drosophila* midguts (Lin et al. 2008; Lee et al. 2021). Therefore, further studies on Arm involved in BSP-protective effect and its molecular mechanisms need to be done.

Stem cell-based tissue regeneration was known to be essential for maintaining the integrity of guts in response to various harmful substance assaults (Jiang et al. 2016; Nászai et al. 2015). Upon intestinal challenged such as mercury, the rate of ISC division increased dramatically to replenish the damaged cells of gut epithelium (Chen et al. 2016). Considering the importance of tissue regeneration in the maintenance of intestinal epithelial homeostasis, we speculated that the protective mechanisms of BSP might be related to ISC-mediated tissue repair. Strikingly, we observed that BSP exposure did not promote, but significantly alleviated ISC-mediated midgut regeneration induced by mercury. Considering the fact that the rate of ISC division was usually closely related with degree of intestinal injury, we proposed that BSP exposure indeed alleviated mercury-induced midgut injury (Lee et al. 2021).

Sestrins were highly evolutionary conserved proteins, while *Drosophila* had only a single orthologue (Lee et al. 2010). Increasing number of studies showed that *sestrin* played an important role in protecting organism against adverse stress. In *Drosophila* muscle and nervous system, s*estrin* was required for normal chill coma recovery after acute exposure to low temperatures (Cobb et al. 2021). The *Sestrin* knock-out impaired the development and shortened the lifespan of *Drosophila* exposed to a low-leucine diet (Valenstein et al. 2022). Meanwhile, *sestrin* was reported to protect *Drosophila* brain cells from chromium-induced adversities by increasing autophagy (Singh and Chowdhuri, 2018). Additionally, *sestrin* protected *Drosophila* midguts from mercury-induced oxidative damage by inhibiting ROS overproduction and stimulating the ISC-mediated intestinal regeneration program (Chen et al. 2021). Similarly, Sestrin 2 activation might played a critical role in hepatic regeneration through the modulating Nrf2 expression (Oliveira et al. 2021). In the present study, we found that *sestrin* was involved in the protection of BSP against mercury-induced oxidative damage to *Drosophila* midguts by increasing antioxidant enzyme activity, decreasing ROS production, inhibiting cell apoptosis, restoring intestinal epithelial barrier and modulating ISC-mediated midgut regeneration (Fig. 6). These studies suggested potential therapeutic implications of *sestrin* against heavy metal-induced adversities in organisms. Taken together, our findings gave a more complete understanding about defensive role and underlying mechanism of BSP mercury-induced against intestinal oxidative damage.

In conclusion, the results of the present study demonstrated that exposure of flies to BSP significantly increased antioxidant enzyme activity (GST and SOD), decreased ROS production, inhibited cell apoptosis, restored intestinal epithelial barrier and alleviated the process of ISC-mediated tissue regeneration induced by mercury via upregulating *sestrin* expression in midguts. The present study gave a more complete understanding about defensive role and underlying mechanism of BSP against mercury-

induced oxidative damage to guts, indicating great potential of BSP for future application in the treatment and prevention of oxidative stress-related diseases in the gastrointestinal tract.

Declarations

Ethical Approval

not applicable.

Authors' contributions

Z.C. performed the experiments, analyzed the data and wrote the main manuscript text. D.W. designed experiments, reviewed and edited the manuscript.

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Availability of data

All data generated or analyzed during this study is available from the corresponding author on reasonable request.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figures

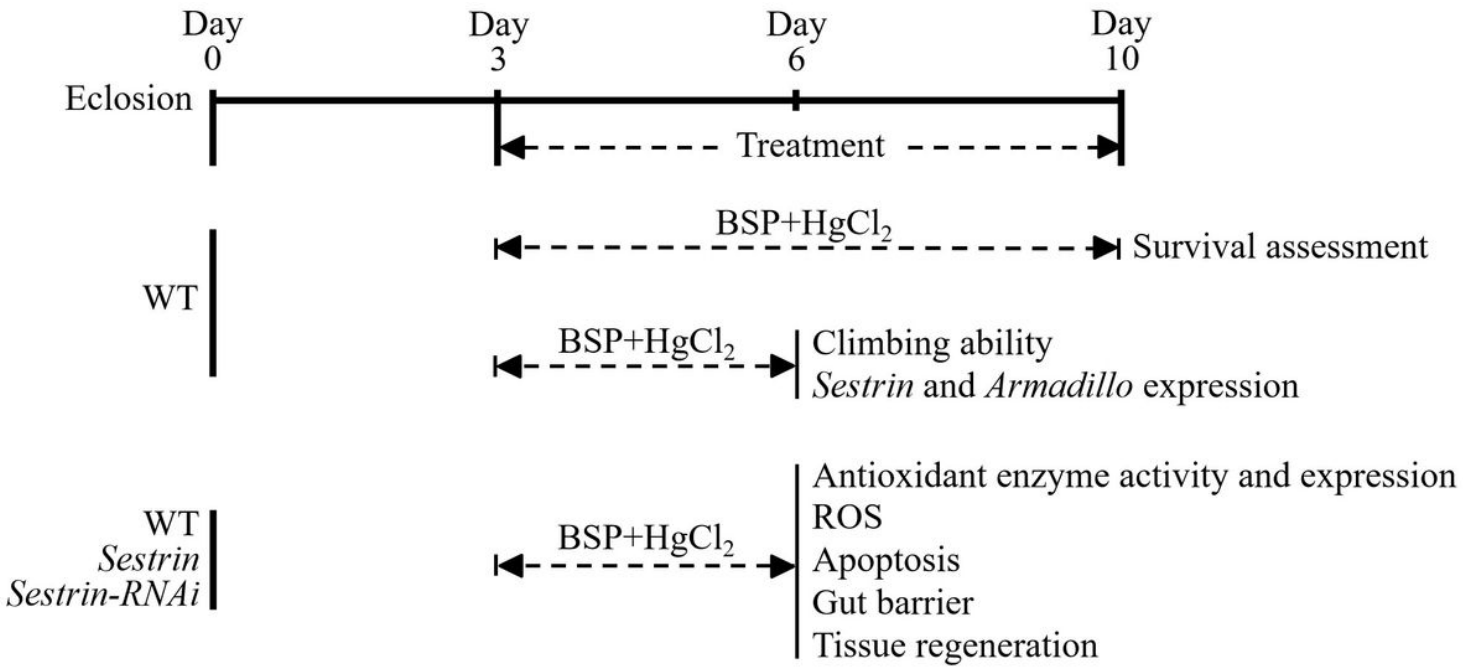

Figure 1

Schematic diagram of the experimental treatment protocol

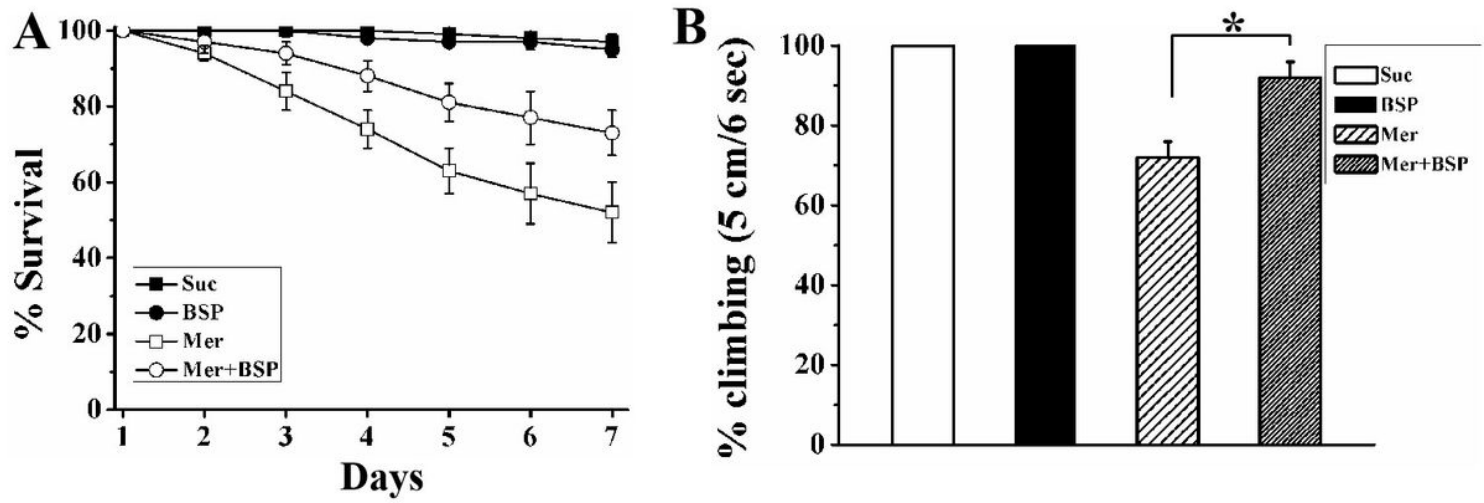

Figure 2

BSP exposure alleviated the decrease of survival rates and climbing ability of flies exposed to mercury. (A) Survival rates of w^{1118} flies treated with $HgCl_2$ and/or BSP for 7 d (n = 40). (B) Climbing ability of w^{1118} flies treated with $HgCl_2$ and/or BSP for 3 d (n = 40). *$p<0.05$ in comparison to the $HgCl_2$-treated midguts

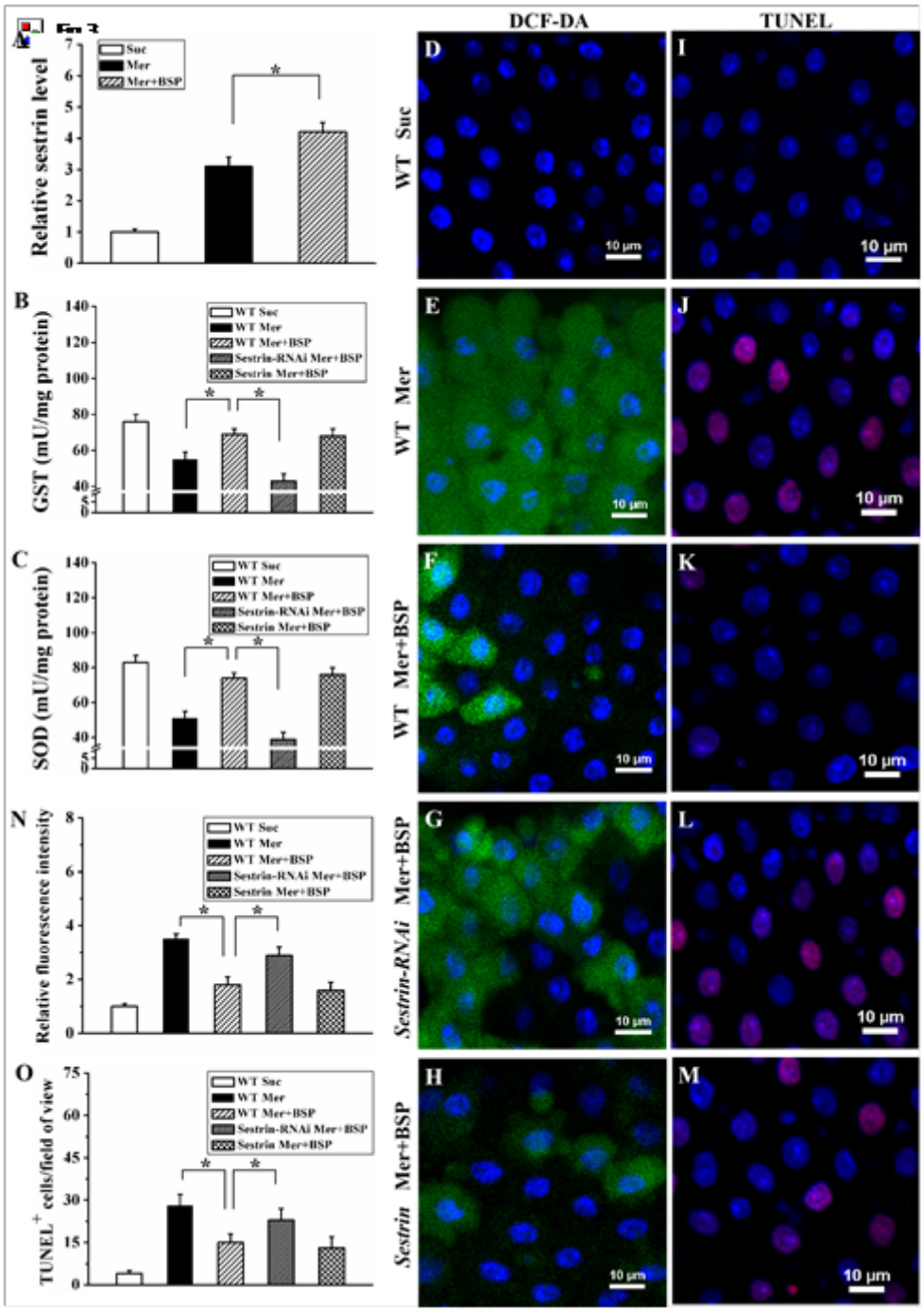

Figure 3

BSP protected against mercury-induced oxidative damage to *Drosophila* midguts by modulating *sestrin* expression. (A) Transcriptional level of *sestrin* in midguts treated with $HgCl_2$ and/or BSP for 3 d (n = 15). *p<0.05 in comparison to $HgCl_2$-treated control. (B-C) Graphical representation of GST- (B) and SOD-activity (B) in midguts exposed with $HgCl_2$ and BSP (n = 60). *p<0.05 in comparison to $HgCl_2$-treated control. (D-H) DCF-DA stainings (green) of $HgCl_2$-fed control, *sestrin* knockdown midguts and *sestrin* overexpression midguts treated with or without BSP to monitor the ROS level. (I-M) TUNEL stainings (red) of $HgCl_2$-fed control, *sestrin* knockdown midguts and *sestrin* overexpression midguts treated with or without BSP to monitor the apoptosis level. (N-O) Quantification of the DCF-DA fluorescence intensity (N)

and TUNEL assays (O) in control, *sestrin* overexpression and *sestrin* knockdown flies (n = 15). *p<0.05 in comparison to control treated with BSP and $HgCl_2$

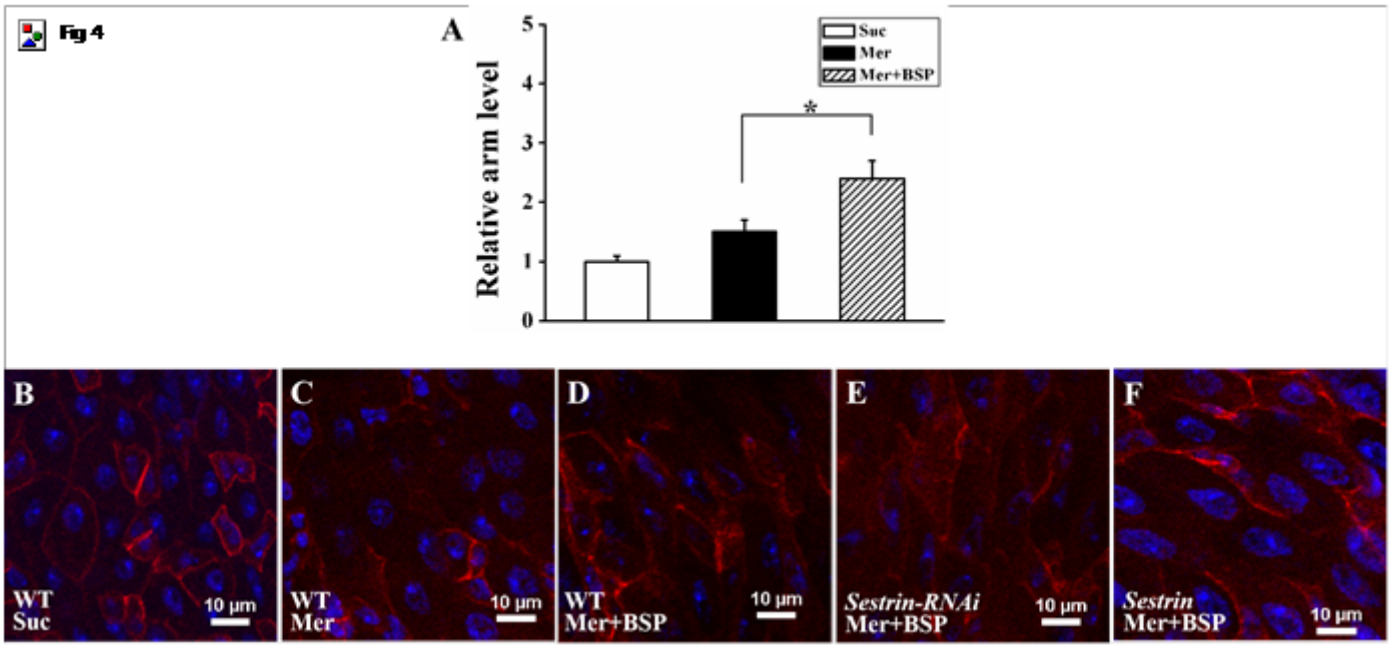

Figure 4

BSP alleviated mercury-induced midgut epithelial barrier disruption through regulating *sestrin* expression. Quantification of *arm* mRNA expression in w^{1118} midguts treated with BPA and mercury for 3 d (n = 10). (B-F) Midguts were co-stained for Arm (red) and DAPI (blue). *p<0.05 in comparison to mercury-treated control

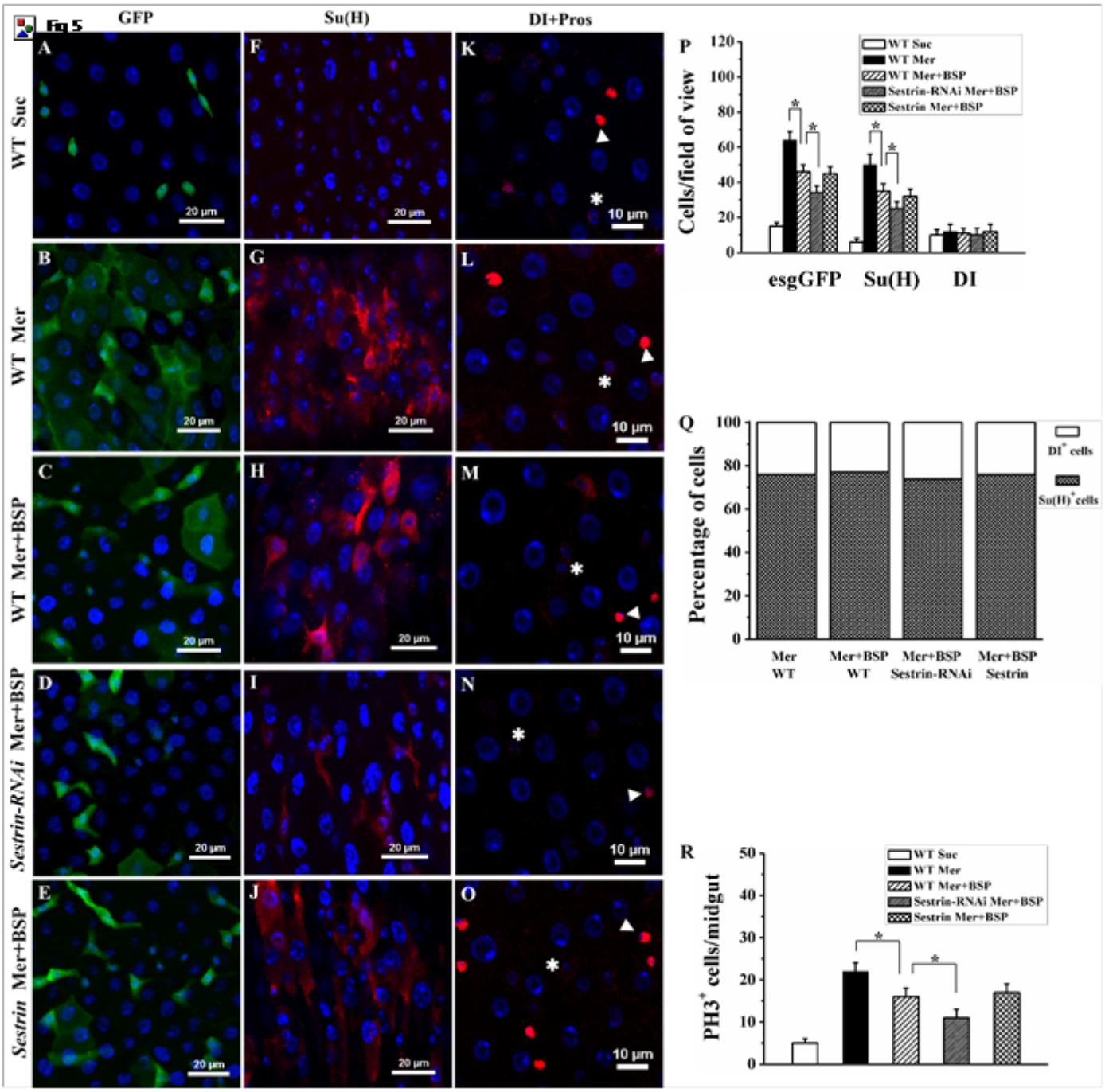

Figure 5

Sestrin-modulated intestinal regeneration was required for BSP to protect against mercury toxicity in*Drosophila* midguts. (A-O) Immunofluorescent stainings of GFP (green) (A-E) , Su(H)GBE-lacZ [Su(H)] (red) (F-J), and Dl + Pros (red) (K-O) in control, *sestrin* knockdown midguts and *sestri*n overexpression midguts. Asterisks and arrowheads in K to O indicated ISCs and EEs, respectively. (P) Quantification of various types of cells (n = 20 view fields). (Q) Percentage of Su(H)-positive and Dl-positive cells in GFP-positive cells. (R) Quantification of PH3-positive cells (n = 15). *p<0.05 in comparison to the control treated with mercury and BSP

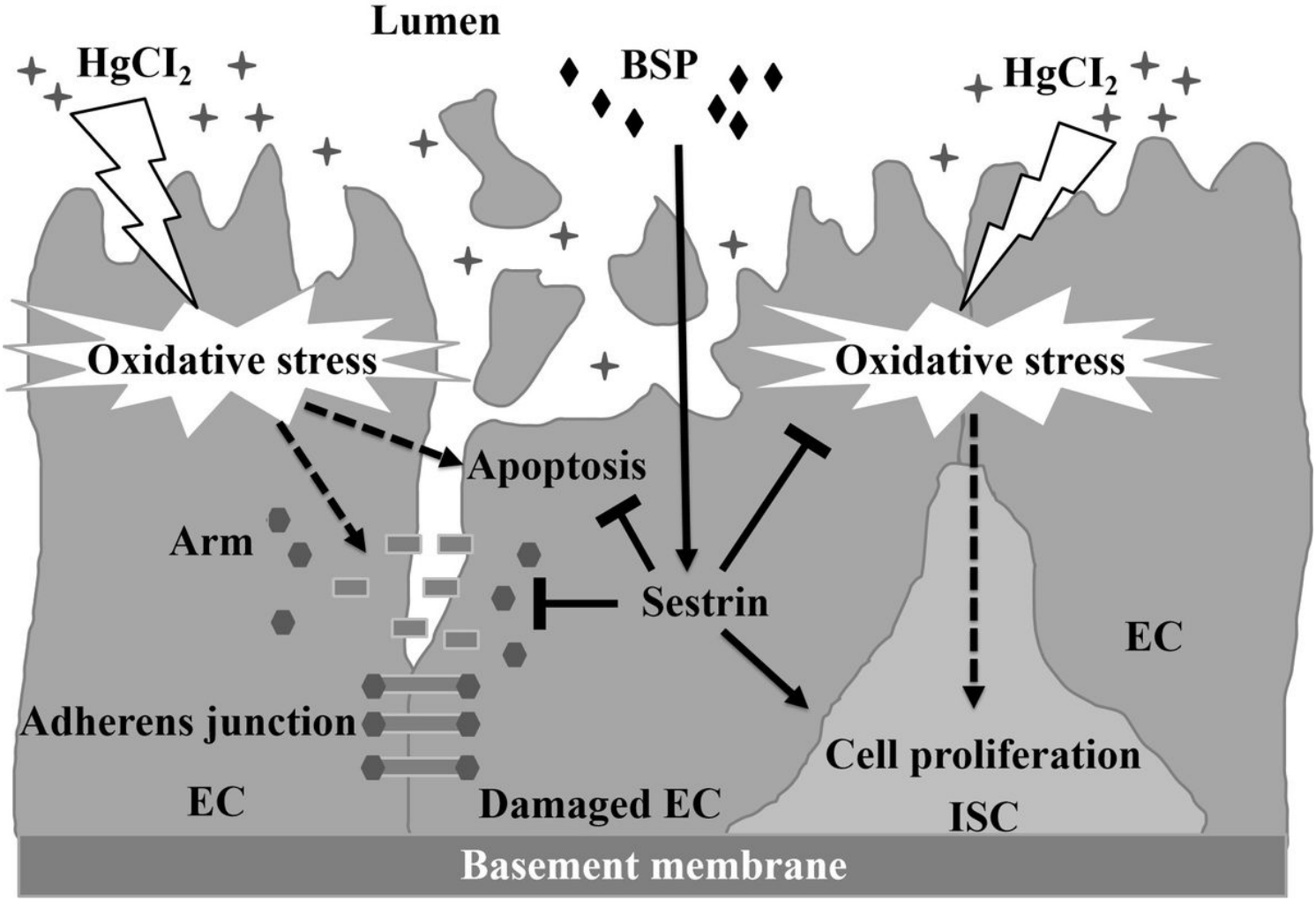

Figure 6

Ameliorative mechanism of BSP in mercury-induced gastrointestinal toxicity in *Drosophila*midguts