Cellular and molecular basis of heavy metal-induced stress in ciliates

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Globally, heavy metals are the major pollutants present in both terrestrial and aquatic ecosystems. Increase in their concentration due to various anthropogenic activities is a matter of concern. Higher concentration of these metals is known to be toxic due to their non-biodegradable nature. Eukaryotic microorganisms, ciliates can be used as cellular tools to assess and study the various mechanisms to overcome heavy metal toxicity. Here we discuss, at cellular level, the effect of heavy metal toxicity on growth rate, behavioural and morphological changes of ciliates. At the molecular level, changes in stress genes like *hsp70*, metallothionein and expression of various antioxidant enzymes (superoxide dismutase, glutathione peroxidase) adopted by ciliates have also been deliberated. It is also being argued that ciliates can be used as biosensor/cellular tools for detecting heavy metal pollution.

Keywords: Ciliates, heat shock protein, heavy metal stress, metallothionein, superoxide dismutase.

HEAVY metals are those metallic elements whose density is relatively higher than water[1]. Some of the essential heavy metals (such as cobalt, copper, chromium, iron, magnesium, manganese, molybdenum, nickel, selenium and zinc) act as important elements in the living systems, as most of them act as cofactors for the enzymes involved in various biochemical and physiological functions required for cellular metabolism and growth[1–3]. Higher concentrations of these metals are known to be toxic due to their non-biodegradable nature[2,4]. The other non-essential heavy metals (viz. aluminium, arsenic, cadmium, lead, mercury, nickel, platinum, titanium and uranium) show toxicity even at low concentrations[1]. Heavy metals cause detrimental effects on all living organisms[5], leading to major ecological disturbances[2].

Since the concentration of heavy metals is increasing day by day in the environment due to domestic, agricultural, medical and technological applications, there is an urgent need to take strict measures to improve the prevailing environmental condition and prevent contamination[1]. The most effective method for detecting pollutants is the use of biological components known as biosensors or bioreceptors. In many reports, yeast, microalgae and protozoa (including ciliates) are used as eukaryotic biosensors to detect metals[2]. Since ciliates have widespread distribution and do not have a cell wall, they can react quickly to environmental stresses[4–7]. In addition, ciliates show a faster growth, can be cultured and maintained under laboratory conditions with ease[8]. These characteristics make them more suitable to be used as cellular tools in ecotoxicological studies[8], and to study physiological and molecular processes involved in metal toxicity[7,9,10].

In this article, the effect of heavy metal toxicity on ciliates along with the mechanisms involved in their defence against heavy metals have been discussed. Further, whether ciliates can be used as whole-cell biosensors/cellular tools to assess heavy metal pollution has also been explored.

Effect of heavy metals on ciliates

Cell survivability

Several studies indicate that heavy metals have adverse effect on the growth rate of ciliates. It has also been shown that per cent survivability of ciliates decreases significantly with increasing heavy metal concentration[5,11–17]. Also, LC_{50} values of various heavy metals, viz. cadmium, copper, chromium, lead, mercury and zinc on different ciliate species have been reported, and it has been found that Cd and Cu are more toxic to most of the ciliate species whereas Zn is least toxic[13]. In addition, it has also been reported that ciliates are highly sensitive to various heavy metals compared to other eukaryotic organisms[4,7,10,14,16,18–21].

Morphological changes

In many studies, it has been suggested that heavy metals induce membrane alteration and inhibit the organization of actin and tubulin, the two main components of cytoskeletons[5,22]. Ciliates when treated with heavy metals lose their morphological integrity and become rounded[5],

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and their movement is also slowed down[15]. In many other cases ciliates have shown intracellular vacuolization, morphological deformities and cellular rupture after the accumulation of metals by the cells[5,20].

Ciliary pattern formation

Heavy metals have also been shown to affect the ciliary pattern and development of oral primordia[23,24]. Lithium induces cortical changes in *Stlonychia lemnae*, where reduction in fronto-ventral-transverse (FVT) cirri on the ventral surface and increase in dorsal kineties on the dorsal surface have been observed; the adoral zone of membranelles (AZM) also get fragmented[24].

Ultrastructural changes

Both cytoplasmic and nuclear aberrations in ciliates have been observed after heavy metal exposure. The cytoplasmic alterations involve partial mitochondrial degradation and mitochondrial disorganization, cytoplasmic vacuolization, accumulation of membranous debris/electron dense bodies and finally formation of autophagosomes[25–28]. These changes have been observed in *Tetrahymena* sp.[25], *Uronema marinum*[26], *Euplotes vannus*[27] and *Stylonychia mytilus*[28].

Mitochondrial degeneration and/or disintegration of cristae are the most important modifications in vegetative cells of ciliates, especially those treated with higher concentration of non-essential heavy metals like cadmium[27], they have more ability to generate reactive oxygen species (ROS)[29].

Cytoplasmic vacuolization is another major modification that is noticed in heavy metal-treated ciliates. Some studies have indicated the presence of electron-dense particles inside the cytoplasmic vacuoles[28,30]. These particles could be due to the accumulation of metallic free cations resulting in compartmentalization of metal deposits. This is one of the well-known detoxification mechanisms in ciliates involved in sequestration, neutralization and immobilization of toxic heavy metals to produce non-toxic compounds. These cytoplasmic, metallic dense granules finally fuse with digestive vacuoles and are eliminated by defecation[30].

The major changes at the nuclear level involve alteration in the nucleolar organization where nucleolar fusion is observed in macronucleus[28,30]. Fusion of nucleoli might be a good indication for interruption in rRNA synthesis, maturation and transcriptional activity of the cell[30]. Nucleoli are the membrane-less nuclear organelles in which rRNA transcription, maturation and processing occur[31]. Assembly of new ribosomal proteins in the nucleolus is controlled by the nuclear ubiquitin-proteasome pathway (UPP)[32]. It has been reported that oxidative stress impairs the function of UPP[33]. Since, UPP is impaired, the availability of ubiquitin decreases thereby increasing the chances of accumulation and immobilization of nucleoli with defective RNAs and multiple misfolded proteins[34]. This disrupts the normal ribosome biogenesis and affects transcriptional activity[32]. Also, condensation of macronuclear chromatin has been observed[26,27,30]. Micronuclear membrane too undergoes some modifications, including convolution of micronuclear surface and chromatin retraction from the nuclear envelope[28,30].

Generation of reactive oxygen species

Heavy metals induce oxidative stress in the ciliated protozoa by generating increased levels of ROS. Metals with redox activity like Cu, generate ROS directly by autooxidation and metals without redox activity like Cd generate ROS indirectly by affecting the antioxidant defence mechanism[8]. ROS can be detected by fluorescence probes[35] like 2,7-dichlorofluorescein diacetate (DCFH) – specific for hydrogen peroxide, nitrogen oxide, peroxylnitrite, hydroxyl ion and peroxyl radicals; hydroethidine or dihydroethidium (HE) – specific for only superoxide anion[10,36], and dihydrorhodamine 123 (DHR) – specific for hydrogen peroxides or peroxynitrite[10,35]. These fluorophores get oxidized and are converted to their active form in the presence of ROS, namely 2,7-dichlorofluorescein[10], ethidium[10,36] and rhodamine 123 (ref. 10) respectively, and release fluorescence at specific wavelengths; thus helping in ROS detection. Increase in the intensity of fluorescence in metal-treated cells compared to the control shows the generation of ROS in the former[10].

Defence mechanisms in ciliates

To combat the heavy metal toxicity, ciliates have developed many defence mechanisms like increased production of several antioxidant enzymes and stress-induced genes, namely metallothionein (MT) and heat shock proteins (HSPs) for their survival (Figure 1).

Antioxidant enzymes

ROS (such as superoxide radicals, hydrogen peroxides and hydroxyl ions) are involved in various pathological, processes, including lipid peroxidation, oxidation of proteins and damage to nucleic acids. These radicals are believed to cause much of the tissue damage resulting from inflammation and can eventually induce cell death by apoptosis/necrosis[37].

Antioxidant enzymes (such as superoxide dismutase (SOD), ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin) are known to be involved in ROS detoxification and in protecting the cells from oxidative stress[8,38] (Figure 2).

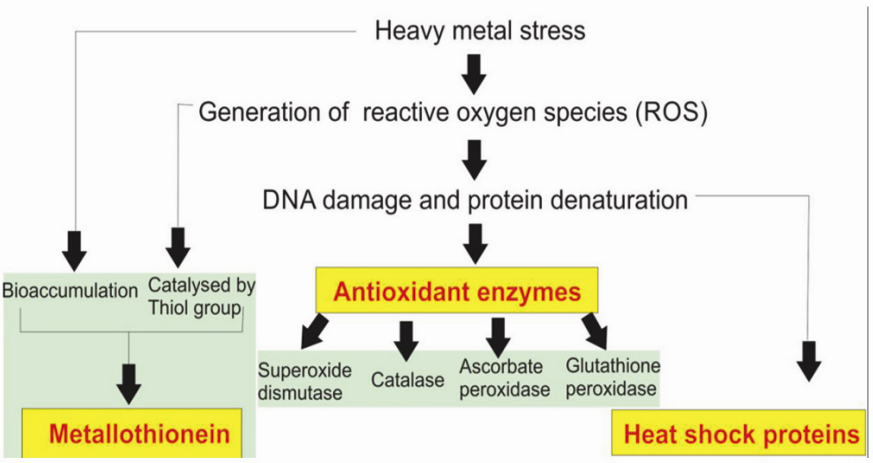

Figure 1. Defence mechanisms in ciliates against heavy metal stress. Antioxidant enzymes, metallothionein and heat shock protein genes are majorly activated during metal stress.

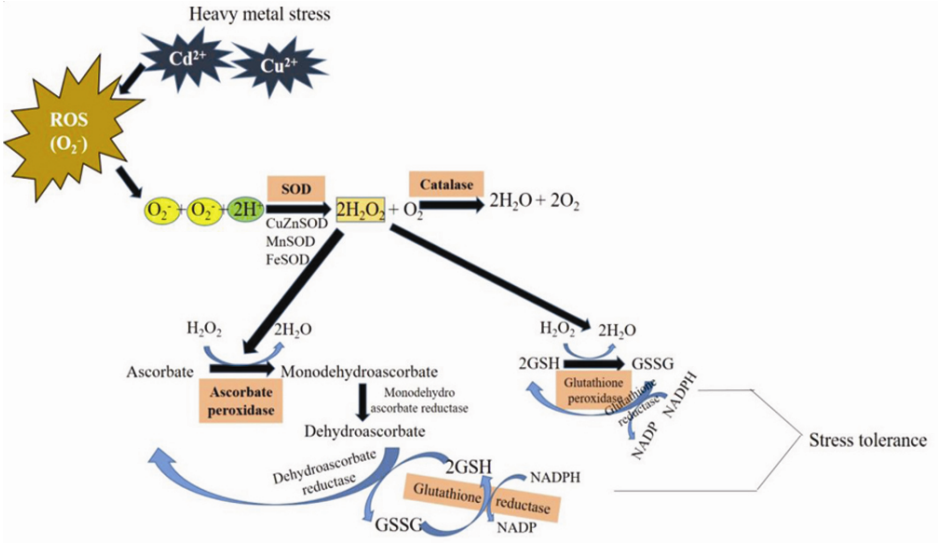

Figure 2. Flow chart showing mechanism of important antioxidant enzymes – superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase – helping in metal stress tolerance.

SOD is the first enzyme to get activated in response to oxidative stress. It plays a major role in catalysing the dismutation of superoxide anion (O_2^-) into O_2 and H_2O_2. SOD exists in several isoforms, including cytosolic copper–zinc SOD (SOD-1), mitochondrial manganese SOD (SOD-2) and extracellular iron SOD (SOD-3)[39].

Catalase is involved in detoxifying H_2O_2 (ref. 40). Catalase activity is majorly located in subcellular organelles like peroxisomes. Another major hydrogen peroxide (H_2O_2) detoxifying system in the cells is the ascorbate–glutathione (ASC–GSH) cycle, where ascorbate peroxidase (APx) plays a key role in catalysing H_2O_2 into H_2O using ascorbate as a specific electron donor. Several isoforms of APx are present in various subcellular compartments like mitochondria, peroxisome, cytosol and even in chloroplast[39]. Ascorbate, acting as electron donor, gets oxidized to monohydro/dihydro ascorbate (MDHA/DHA), which is catalysed by monohydro/dihydro ascorbate reductase (MDHAR/DHAR) to bring ascorbate back to the reduced form[38,39] using GSH (L-γ-glutamyl-L-cysteinylglycine) as reductant[41]. GSH is a tripeptide synthesized in two consecutive steps catalysed by γ-glutamyl-cysteine synthase and glutathione synthetase[42]. GSH is the most abundant cellular non-protein thiol in almost all eukaryotes[8]. This tripeptide provides the first line of defence against the metal ions[43]. GSH acts as substrate for glutathione S transferase (GST)[8,11]. The main function of GST enzymes is the conjugation of highly toxic compounds with GSH and converting them into relatively less toxic ones, thereby excreting them outside the cell by ATP-dependent efflux pumps[8]. GSH also acts as a substrate for glutathione peroxidase (GPx) to catalyse hydrogen peroxide[8].

In conjugation to chemical compounds such as Cu or hydrogen peroxide, GSH is oxidized and gets converted into GSSG (glutathione disulfide or di-L-γ-glutamyl-L-cysteinylglycine)[11,39]. GSSG formed in the presence of ROS is again reduced to regenerate GSH by glutathione reductase (GR) using NADPH as reducing agent[41]. This whole mechanism helps in maintaining the ratio of GSH : GSSG which is important to sustain the redox status in the cell[11]. However, when the level of ROS increases in the cell, the amount of GSSG increases significantly disrupting the ratio of GSH : GSSG. This ratio for a normal resting cell is 100 : 1 or more, while under oxidative stress the ratio decreases to 10 : 1 or 1 : 1 (ref. 44).

Changes in these antioxidant enzymes, including ASC–GSH cycle enzymes have been observed in various organisms exposed to different abiotic stresses, including heavy metals. It has been found that heavy metals like Cd interact with thiol group of glutathione (GSH), which results in the depletion of GSH and also inhibits the function of antioxidant enzyme, glutathione reductase[45]. Overexpression of any of these enzymes by a particular organism is an indicator of higher resistance to abiotic stresses compared to organisms under-expressing these enzymes[41]. In ciliates, studies on generation of ROS and other antioxidant enzymes in the presence of heavy metals have been carried out on *Euplotes* sp.[11,36], *Notohymena* sp.[36], *Paramecium* sp.[17], *Stylonychia mytilus*[43], *Tetrahymena thermophila*[21] and *Tetmemena* sp.[36].

After heavy metal exposure, increase in ROS has been detected in *Tetmemena* sp., *Notohymena* sp., *Euplotes* sp.[36], *Tetrahymena* sp. and *Colpoda steinii*[10] using various fluorophores like HE[10,31], DHE123 and DCFH[10].

When *Tetmemena* sp., *Notohymena* sp. and *Euplotes* sp. were exposed to heavy metals such as Cd and Cu, the levels of SOD and GPx increased significantly in a concentration-dependent manner[10]. In *Paramecium* sp., catalase and GST activity increased in response to heavy metal treatment[16]. Similarly, total GSH activity increased in heavy metal concentration-dependent manner in both *Stylonychia mytilus*[43] and *Euplotes crassus*[11]. Kim *et al.* noticed that expressions of GPx and GR increased in response to heavy metal treatment in *E. crassus* but at very high concentration, the expression of these antioxidant enzymes started decreasing due to cell death.

Since the gene expression of these antioxidant enzymes increases significantly with response to heavy metals, these can be used as cellular tools to evaluate metal toxicity.

Bioaccumulation of heavy metals and the role of metallothionein gene

MT was first discovered in the cortex of horse kidney as a cadmium-binding protein[46]. *MTs* are ubiquitous, hydrophilic, cytosolic, cysteine-rich, low molecular weight stable proteins[47]. They act as antioxidants by protecting against stress, DNA damage and apoptosis[47,48]. *MTs* help in scavenging free hydroxyl and superoxide radicals, and this reaction rate is 340-fold higher than GSH[48]. Important biological role of *MTs* is in detoxification of heavy metals by protecting against excess reactive heavy metal ions, free radical scavenging and in the maintenance of metal ion homeostasis[47,49].

MTs are also known to be involved in heavy metal bioaccumulation in ciliates[8]. This mechanism has been studied in different ciliates like *Colpoda steinii*, *C. elongata*, *C. inflata* in the presence of Cd, Zn and Cu. The species showing maximum accumulation of heavy metals are more resistant to metal toxicity[13]. *MTs* bind to heavy metal ions (mainly Cd, Zn and Cu) via metalthiolate clusters[49] and are involved in intracellular sequestration by forming metal–protein complexes which are accumulated in the vacuoles[8]. Finally, they are released from the cell in the form of non-toxic metallic complexes[15,48,50].

MT gene of *T. thermophila*[48,51,69], *T. pyriformis*[51], *T. pigmentosa*[52], *T. trophicalis lahorensis*[53], *T. rostrata*[54] and *Paramecium* sp. has been sequenced[55]. Typically, classic *MTs* (present in majority of organisms) have 7–21 highly conserved cysteine residues, and lack aromatic amino acids and histidine[8]. However, ciliate *MTs* present exclusive features with regard to classic *MTs*: they are longer proteins (60–193 aa) having higher cysteine content (22–54 residues) and aromatic amino acids are present in several of them[48]. So far, study of *MTs* in ciliates has been restricted to genera *Tetrahymena*[47,52] and *Paramecium*[56]. In *Paramecium* sp., *MT* gene is not well characterized.

Ciliate *MTs* are known to belong to family 7 of the *MT* superfamily[57]. *MTs* are reportedly divided majorly into two subfamilies on the basis of their Cys residue pattern and on the positioning of amino acids: subfamily 7a (cadmium metallothionein – Cd*MTs*) and subfamily 7b (copper metallothionein – Cu*MTs*)[8,48,57]. They are further divided into different isoforms on the basis of their structure[8]. Subfamily 7a contains seven isoforms of Cd*MTs*: Cd*MT*-1 to Cd*MT*-7 (ref. 57) and subfamily 7b contains four isoforms of Cu*MTs*[8], two major (Cu*MT*-1 and -2) and two minor (Cu*MT*-3 and -4) isoforms[47]. One of the main structural changes between the two subfamilies is cysteine clusters. In Cd*MTs*, CCC and XCCX clusters are maximally found, whereas in Cu*MTs*, CXC clusters are present in abundance. Another important structural feature difference is the presence and location of lysine. Lys residues are normally seen adjacent to Cys residues in subfamily 7b, whereas in subfamily 7a this association is rarely noticed[57].

MT genes are regulated by one or more copies of MREs (metal response elements) sequences present at the 5′ flanking sites of these genes. The multiple MREs are located within the first 150 bp of the promoter region

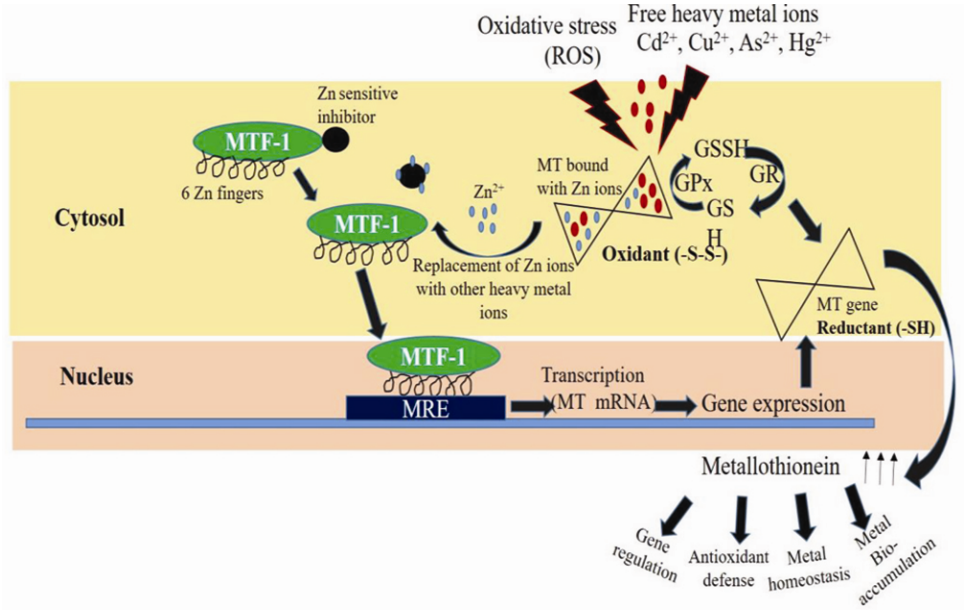

Figure 3. Mechanism of metallothionein gene activation in the presence of heavy metals.

upstream of the TATA box. All these copies of MREs are shown to act cooperatively. MTF-1 (metal transcription factor/MRE-binding transcription factor-1), in the absence of metal ions, remains in inactive bounded state with Zn sensitive inhibitor[58]. When the heavy metal enters the cell, it quickly replaces zinc ions associated with *MTs*. These free Zn ions bind to the MTF-1 factor, thereby removing the Zn-sensitive inhibitor. This activates the MTF-1 factor, which simultaneously binds to MRE and regulates the transcription for more *MT* proteins[59] (Figure 3). When *MTs* are produced in sufficient amounts, the zinc ions are removed from the inhibitor which allows it to bind to MTF-1, once again inhibiting the transcription process[60].

It has been reported that ciliate *MTs* are intronless[48,57] and due to the absence of introns in *MTs*, their expressions rapidly change with response to environmental stress[49]. Expression analysis of *MT* genes with respect to different heavy metals suggests a time-dependent as well as dose-dependent variation in the production of *MT* mRNA. Relative *MT* induction has been studied by various researchers by Northern blotting and real-time qPCR techniques. Till now experimental data related to gene expression of *MTs* are only available for *Tetrahymena* sp.[52]. In *T. pigmentosa*, gene expression of Cd*MT*-1 is fast and within 30 min of Cd exposure, the expression increases up to tenfold[49,52]. Around 210-fold increase in the expression of *MT* has been shown after 30 min of Cd expression in *T. thermophila*. Similarly, 21-fold and 14-fold increased gene expression has been found in Hg and Cu respectively[61]. In *T. thermophila*, by fusing GFP with the *MT* promoter, production of green fluorescence (509 nm) increases significantly in the presence of Cd, whereas very low emission has been detected in the absence of Cd[62] indicating increased expression of *MT* gene in the presence of Cd. In all the cases, Cd is shown to be a potent and quick inducer of Cd*MT* genes.

Since the promoter of this *MT* gene is induced strongly in the presence of heavy metals, these genes could be used as molecular tools to design whole-cell biosensors to evaluate metal toxicity[49].

Heat shock proteins

HSPs were first discovered in *Drosophila melanogaster* when exposed to heat shock. HSPs or stress proteins have received much attention due to their vital functions in the eukaryotic and prokaryotic cells[63]. Although some HSPs are expressed under normal cellular conditions, these proteins are known to show significant response when cells are exposed to different environmental stresses like temperature variation, heavy metals, salinity, etc.[64,65]. Genes like *hsp* are generally classified into different families on the basis of their molecular weights: *hsp*100, *hsp*90, *hsp*70, *hsp*60, *hsp*40 and small *hsp* families[66]. These ATP-dependent molecular chaperones help in normal protein folding and assembly, and also in the protection and repair of proteins during stress conditions[64,67]. Among the *hsp* gene families, *hsp*70 and *hsp*90 play a major role in protein folding, assembly and distribution[67]. Due to their vital role, these *hsp* genes are highly conserved in all prokaryotes and eukaryotes[63], and their use as biomarkers of environmental pollutants has been suggested[68].

In ciliates, the structural features of only *hsp*70 and *hsp*90 proteins have been reported so far[69]. The conserved structural features of these proteins consist of three domains: N-terminal domain to bind and hydrolyse ATP,

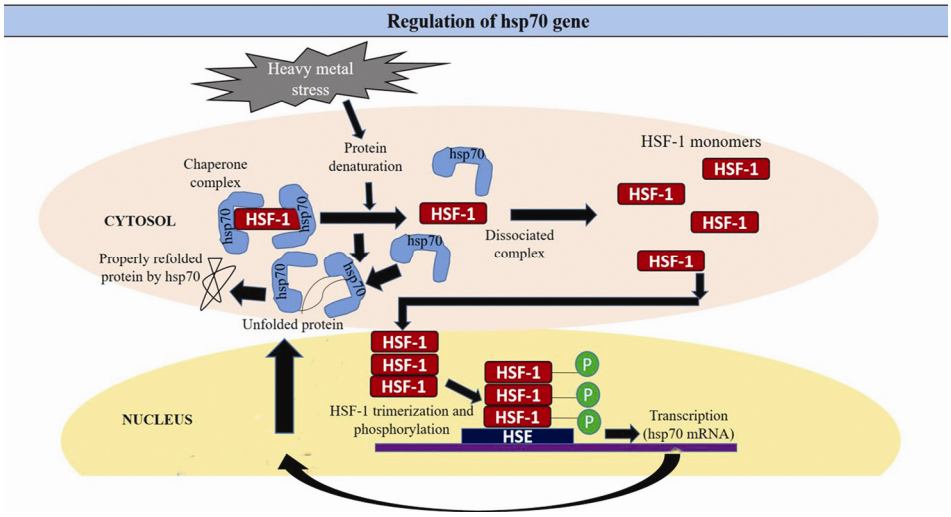

Figure 4. Diagrammatic representation of regulation and activation of *hsp70* gene under heavy metal stress.

substrate-binding domain specific for substrate interactions, and a C-terminal domain (CTD) which acts as a lid for substrate-binding domain[69].

When the cells are exposed to heat shock or heavy metal stress, they experience changes in protein conformation. Heavy metals like Cd are known to get accumulated in the cells and interact with thiol group of the target protein which generates abnormal or denatured protein[68]. This abnormal protein sends a signal for the induction of *hsp* genes[65]. The induction of *hsp* genes is regulated at the transcriptional level primarily by heat shock transcription factors (HSFs) binding to heat shock promoter elements (HSEs) present at the 5′ flanking regions of *hsp* genes[70,71]. In normal condition, monomeric HSFs are bound to HSPs in cytoplasm. Exposure to stress leads to elevated levels of denaturing proteins which compete for cytoplasmic HSPs, and this frees HSFs. The HSFs enter the nucleus and trimerize, then bind to HSEs thereby activating the transcription of *hsp* genes[71] (Figure 4). In *T. thermophila*, additional cluster of GATA motifs along with HSEs in the promoter region of the gene has been found, and their association is necessary for induction of *hsp*70 gene transcription[70].

The expression level of *hsp* genes might be maintained at an increased extent until the cells are completely protected and adapted to heavy metal toxicity by correcting the protein conformation[68]. However, when the cell experiences a strong stress from heavy metal accumulation beyond a certain level, mRNA expression level of *hsp* gene families starts decreasing and thus the organisms cannot achieve the appropriate levels of metabolic function[72].

In ciliates, the expression of HSPs has been recorded by exposing the cells to thermal stress, salinity stress and cold stress. So far, however, very little information is known about the regulation of *hsp* gene expression in ciliates with response to heavy metal.

Characterization and effect of *hsp*70 gene with respect to heat and Cd shock have been studied in *Oxytricha nova*. Significant increase in *hsp*70 transcripts is noticed by Northern blot hybridization in the presence of Cd and heat shock stress indicating that the expression of *hsp*70 gene is regulated at transcriptional level[73].

Being a stress gene, expression of *hsp*70 is known to increase with heavy metal exposure in a concentration dependent manner, and thus can act as a better molecular tool to evaluate heavy metal toxicity.

Conclusion

The article clearly suggests that ciliates can be used to understand the effects of heavy metal toxicity in both freshwater and terrestrial ecosystems. Absence of cell wall in ciliates makes them more sensitive towards heavy metal stress. As the level of sensitivity is considered to be the most important factor for designing a classic or whole-cell biosensor, these microorganisms have a greater advantage over other prokaryotes to be used as biosensors/cellular tools.

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