Metallothioneins in Earthworms: The Journey So Far

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Abstract

Earthworms play important roles in terrestrial ecosystems including evaluating the health status of the soil in environmental studies. Its regulation and detoxification of metallic metals and the non-essential metal ion are associated with the possession of Metallothioneins (MTs). Three isoforms of MTs are induced in some species of earthworms under stress in the soil; wMT1, wMT2, and wMT3 (found in cocoons). Though reports on the nucleotide sequences, mechanisms of action and entire functions of two earthworm MTs isoforms exist, the precise mechanism of action and entire functions of wMT3 are still obscure. Metals and stress are known inducers of MTs in earthworms. In recent times, Contaminants of Emerging Concerns (CECs) and the advent of nanotechnology has occasioned a handful of studies evaluating their effect in the environment using biomarkers like metallothioneins. More research focusing on CECs’ and nanoparticles’ ecotoxicological impact in the environment by monitoring biomarkers like earthworm metallothioneins is encouraged. The detection and quantification of MTs involve a wide array of techniques including analytical, instrumentation and molecular analyses which remains the most commonly used. This review evaluates the various methods and highlights their pros and cons.

Introduction

The soil is a major repository for contamination where terrestrial organisms are exposed to pollution. Earthworms are important organism in the terrestrial ecosystem and their ecological functions are indispensable as they participate in various processes in soil. They form significant biomass in the terrestrial ecosystem and they occupy a sensitive position in the food chain. Lumbricus terrestris, L. rubellus, Eisenia fetida and E. andrei are relevant earthworm species for monitoring environmental pollution [1] in terrestrial ecotoxicology studies. This is attributed to their capability to accumulate and tolerate elevated amounts of toxic metals within a certain threshold without experiencing significant damages [2]. Their survival and tolerance are dependent on the regulation/excretion of metallic trace elements and detoxification of non-essential toxic metal ions.

Earthworms are affected by soil contaminants at the various levels of biological organization from sub–organismal, individual to population levels. The passageways of contact with contaminants are majorly through the skin from the interstitial pore or from the ingestion of soil particles into their guts [3]. In their adaptive responses to such environmental stress, they exhibit non–transferable physiological adaptations which could induce metabolic modifications making them more tolerable to such environmental changes [4] like metal contamination [5] and [6]. On the other hand, the coping mechanisms could involve changes that would be transferable to offspring hence forming ecotypes of earthworm species based on location found [7].

There are standardized protocols for earthworm acute and sublethal testings of chemicals in contaminated soils [1] based on their responses and behavioural patterns [9]. Advances in molecular biology make use of biomarkers as rapid diagnostic and predictive tools in environmental assessments [10]. The use of genetic biomarkers gives better insight into ecotoxicological assessments as gene expression underscores changes in functionality at all levels of organizations and the predictive effect on the ecosystem. A protocol developed from a target gene can be extrapolated and used for similar genes in other related organisms [11]; hence this approach is more reliable than conventional earthworm testings [12]. Molecular markers are generally used because they typically indicate the susceptibility of organisms to contaminants or stressors. The molecular biomarkers monitored in earthworm ecotoxicological studies include Carboxylesterase (CES), Acetylcholinesterase (AChE), Catalase (CAT) and Glutathione S Transferase (GST) activity, the concentration of glutathione (GSH), [13]. Other genetic markers used in such studies are metallothioneins, annetocin [14]. Their presence and levels in organisms are

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indicative of tolerance to metal, stress and other physiological forms of pollution hence their suitability as biomarkers and indicators of environmental status and pollution.

Metallothioneins (MTs) are genetic biomarkers commonly monitored in invertebrates and vertebrates as well as plants and fungi. They are capable of binding metals tightly, thus reducing their toxic effects. MTs are low molecular weight proteins, encoded by a single gene, and expressed by organisms under stress condition especially to heavy metal ions. MTs are involved in metal detoxification, metal ion transport, maintaining redox pool, scavenging of radicals and regulation of expression. They have structures containing metal–cysteine clusters at the N- and C terminals. The functional domains are linked with varying lengths of amino acid sequences; these linkers determine the structural stability of the MT.

**Metallothioneins**

The first metallothionein was identified by Margoshes and Vallee [18] and a myriad of research followed with a focus on vertebrate and mammalian isoforms [19] and [20]. Their roles in the medical field are well reported [21] and [22]. Since its first report, more than 11500 articles on metallothioneins are cited in PubMed, and about one–tenth of these are related to environmental studies. MTs are low molecular weight cysteine–rich (up to 33% by composition) ubiquitous proteins expressed by organisms under stress condition especially when induced by metals at certain levels, making them very well–studied targets. They are heat–stable [23] and have approximately 70 amino acids [24,25]. MTs are encoded by a multigene family which vary in their responses to different inducers including heavy metals, glucocorticoids, hormones, oxidants, strenuous exercise, superoxide and hydroxyl radicals generated by gamma radiation and cold exposure [26]. The major roles of MTs include the homeostasis of trace metals (Zn, Cu, Mn, Fe etc), protection against oxidative stress and detoxification of xenobiatic metals (Pd, Cd etc) [27] and [28], metal ion transport, maintaining redox pool, scavenging of radicals and regulation of expression as explained and depicted in Figure 1 [29]. They are found in a range of organisms from microbes to mammals and reports on invertebrate MTs include nematodes [30], annelids [31]; insects [32]; the oysters [33] and various species of gastropods [34,35].

MTs have shown functional variability among organisms and significant sequence heterogeneity [36] between taxa but notable conserved regions within phylogenetically related taxa [30]. Extensive reports on their detection, roles, mechanisms of action and stoichiometry in a wide variety of organisms [37] are available.

**The basic functions of metallothionein**

The structures of proteins depict their functionalities. Metallothionein has a chemical configuration often occurring as a straight polypeptide chain of cysteine (cys–cys) or cystine having other amino acids within the chain (cys – x – cys) [38]. This makes it form better binding cluster since cysteine possesses the thiolate – SH end for metal attachment [39]. Individual cysteine residue required for metal ion binding is typically insufficient, hence the cluster forming tetrahedral binding arrangement using bridging sulphur binding ligands. The sulphur groups of cysteines are usually positioned adjacent to themselves hence encouraging the clustering.

Their chemical configurations of various families of MTs are reported but the 3D depictions are scarce [40]. Although there are structural diversity among MTs in organisms, the functional domains (C- and N-) for metal binding is usually common, appearing as “dumbbell”. The functional domains only form 3D structures upon metal coordination, and when there are no metal ions, (apo–thionein or apo–T), the domains usually appear unstructured; their structure depicts their functionality [41]. One elucidated Mt 3D structure is the mammalian MTs; they have two metal–binding domains that form metal–cysteine clusters at the N– and C terminals [42]. They have structures configured to form folded metal–binding domains with the α–domain closer to C–terminal and more stable while the other is a more reactive β–domain, which is closer to N–terminal. The metal clusters formed are named “M4Cyst1 (α–domain) and M3Cys9 (β–domain)” where M represents a divalent metal ion like Zn2+ or Cd2+ [43]. The functional domains are linked with varying lengths of amino acid sequences; these linkers determine the structural stability of the MT.

**Earthworm metallothioneins structure**

The mechanisms of tolerance of earthworms to metal by accumulation are attributed to expression of MTs and their formation of metal–rich granules (MRGs) [44]. Metal toxicity will only occur when the capacity of these mechanisms to bind metals is exceeded [45]. Unlike vertebrate MTs where similarities occur structurally, invertebrates MTs show inter / intra – structural diversities hence they have distant phylogenetic relationships. This diversity could be due to their evolutionary
MTs do exist in homologues and are referred to as isoforms in the literature [46]; invertebrates like snails and earthworms have eight and three MT isoforms respectively. The most-reported earthworm MT isoform are wMT1 and wMT2. They are often described to have a reverse mammalian MT arrangement “C- M4Cys11, α-domain and N- M3Cys9, β-domain” just as most vertebrates. Instead, there is an “N-terminal α-domain (M4Cys11-cluster); C-terminal β-domain (M3Cys9-cluster)” arrangement depicted in Figure 2. These two isoforms (wMT1 and wMT2) have greater than 75% similarities in their sequences but differ considerably in the length and composition of their linker sequences. wMT1 have longer linker regions (6 residues), and it is less stable than wMT2 with shorter linker sequences (4 residues) wMT2 has shown more stability in its metal retention with a wider range of pH and its effectiveness in cadmium toxicity protection than wMT1 [47].

Induction of metallothioneins in earthworms

Ecotoxicology studies involving earthworms earlier attributed major forms of cellular management of excess heavy metal to the possession of chloragosomes [48,49]. Figure 3 depicts a conceptual model of impacts on soil metal chemistry due to exposure of earthworms to metal contaminated soils.

One of the tolerance mechanisms of genetic origin is the induction of metallothionein and it is reported in several earthworm species. They include E. fetida [50,51], E. Andrei, [52,53] and Libyodrilus violaceus [54]. The genetic origin of resistance is attributed to evolutionary changes in MT gene and researches suggest that MTs are the basis of metal resistance and tolerance in these organisms [55]. Earthworm MTs mainly function in metal detoxification and evidence indicate that. Studies had shown metallothionein induction and their regulation in insects and vertebrates were conserved [57,58], it involved the binding of metal transcription factor 1 (MTF-1) to metal responsive elements (MREs) usually found in the MT genes promoter. It was however established that the transcriptional activation of MTs in invertebrate is not consistent with that of the insects and vertebrates [59] but the exact mechanism is unclear. Instead, MREs were found in the invertebrate MT gene promoters in Lumbricus rubellus [60] and cAMP responsive element (CRE) was found to be involved in Cd-induced Wmt2 transcription and acted as a transcriptional activator of invertebrate MTs. Metallothionein as biomarker

are monitored in earthworms for Cd contamination [61,62] and other metals like mercury and CuSO4 [63,64] and metallothionein monitoring in earthworm ecotoxicological studies is common.

Earthworm metallothioneins induction by metals

In earthworms, metallothionein induction of two metal responsive proteins is known. They have nucleotide and amino acid sequences similarities of 80.9% and 74.7%, respectively but a distinctive deletion/insertion of two amino acids [65]. Their coding regions show a conserved arrangement of the cysteine residues which lack aromatic amino acids. The sequences of the two isoforms (wMT1 and wMT2) are structurally similar to other invertebrate MTs. The Metallothionein gene, Wmt2, is known to express the most responsive protein among wMTs. Wmt3 is a third isoform of earthworm metallothioneins derived from an EST library generated from developing cocoon and highly expressed in embryonic development. It is 67% similar and 56% identical with wMT1 and wMT2 however, their role remains unclear. The three wMTs isoforms differ in their expression patterns and levels when exposed to metal ions.

After the first report on earthworm wMTs, their modes of action needed further elucidation; presently, with the advent of ecotoxicogenomic approaches, a handful of such reports are available. Studies reveal that wMT1 and wMT2 bind approximately six [6] Cd2+ in two domains and the report also indicates that recombinant WMTs coordinates seven [7] Cd2+ (Cd3Cys9 and Cd4Cys11); the MT contain 20 cysteines. These MTs are like the 20-cysteine in mammals, but the overall protein structures are different being that their 11-cys and 9-cys segments are at alternate positions (i.e. the N – and C – terminus).

A study of their biological function including biophysical properties, affinities to particular metals and protein folding of WMT2 revealed there are significant differences in the
Earthworm metallothionein induction by Contaminants of Emerging Concerns (CECs)

Substances other than metals are known contaminants found in the environment where they cause detrimental effects on the biota, among such are organic secretions like toxins and drugs, especially antibiotics; they are grouped as CECs. Investigating metallothionein induction due to these contaminants is of interest in recent times and a few reports are available. van OmmenKloeke [67] reported expression of MTs in E. Andrei induced by low concentrations of 2-phenylethyl-isothiocyanates (ITCs), a known natural toxin. MT was recommended as an early biomarker of ITCs contamination even at low concentrations. Colistin is a feed additive used by animal farmers as antibiotics and nutrient enhancer [68]. Its suppression of MTs is shown by Guo, et al. [69] and they indicated that colistin in soils interfered with other molecular markers in metal ecotoxicity study, but MT served as early biomarker for colistin contamination. Enrofloxacain is another antibiotic used in veterinary but did not induce MT in E. fetida [70]. Not all CECs are inducers of MTs. The CECs in the environment have gained attention in environmental studies because of their health implication. Their induction of molecular markers like MT indicated by few reports therefore implies that environmental monitoring with biomarkers like MT in earthworm for CECs is plausible. Elaborate investigation on other CECs as inducers of MT is therefore encouraged as only a few literature exist presently.

Earthworm metallothionein induction by nanoparticle

Nano-particles are described as substances in nanometer scales which are 1000 times smaller than normal bacteria. These nanoparticles are used in designing and manufacturing of various consumer products [71,72]. Natural nanoparticles of clay minerals, metal (hydr) oxides, humic substances are well–known examples of natural nanoparticles in soils. These nanoparticles because of their small size and large surface area have unique and novel properties. They are used in a wide variety of products from agrochemicals, food, textiles to solar panels and waste water treatment plants. The properties of nanoparticles can be further enhanced by surface coating of biocompatible molecules and stabilizing the surface, hence their surface charges, solubility and/or hydrophobicity changes depending upon the kind of biomolecule or the process of stabilization [73,74]. Global production of nanoparticles is projected to increase hence the usage and disposal of these materials will be enormous. Commonly used nanoparticles include AgNPs, CoNPsCuNPs, ZnNPs and AuNPs. Study on their environmental impact is of necessity, especially in the soil ecosystem where they are subject to transformations, aggregation/agglomeration and reaction with other biomolecules, exchange of surface elements and other redox reactions [74]. These properties make them behave differently with living organisms with respect to their parent metal.

A few nano-related ecotoxicology studies monitoring molecular markers in earthworms are available. This include assessing levels of biomarkers like Lipid Peroxidation (LPO), total, reduced and oxidized glutathione content (TG, GSH and GSSG), the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx),glutathione S–transferases (GSTs) and cholinesterases (ChEs) in Enchytraeus albidus exposed to ionic copper and copper nanoparticles (75). Such studies also include genotoxic (comet assay) and oxidative effects (SOD activity, TBARS) of functionalized–QDs and cadmium chloride on Hedistedi versicolor and Eisenia fetida coelomocytes [76].

Just a handful of investigations involve MTs’ use as biomarkers in nano – related ecotoxicological studies with few focusing on the detection and quantification of metallothioneins in earthworms. Inductions of MT in earthworms are recorded in recent studies of Urine, et al. [77,78]. Other such investigations include Enchytraeus crypticus exposed to AgNP [79], Lumbriicus rubellus and their coelomocytes impacted by AgNPs (NM–300 K) [80] and AgNPs exposure to E. fetida causing transcriptional expression of MT [81]. The presence of nanoparticles, drugs and toxins in the environment and their impact are areas of interest in recent time, such studies involving earthworm MTs are under reported hence more investigations in this area are encouraged.

Methods of Metallothionein (Mts) Detection and Quantification

The earliest detection of organic substances like cystine was by Heyrousky polarography [82] while the first detection of metallothioneins was by Differential pulse polarography - DPP method [83]. In the earlier approach, cystine was the only amino acid that showed a polarographic reaction in a solution of ammonium chloride, ammonia and cobaltous chloride (Brdicka electrolyte). Conversely, cystine and other thiocids act catalytically in the Brdcka solution which they owe to their sulphydryl groups, the technique involves the catalysis of hydrogen in the presence of a protein containing SH– groups. Using this technique, the quantification of cystine and others were reported by Brdicka [84,85] hence the subsequent use of the term “Brdcka reaction” by Thompson and Cosson [83]. With this method, Cystine and cysteine were quantified in pure solutions and hydrolysates of organic substances in work by Stern, et al. [82]. Several efforts have been made in the modification of DPP technique which had yielded better results like better detection limits, rapid assays, increased sensitivity etc [86].

Series of techniques including colorimetric, fractionation, paper electrophoresis etc were involved in the detection

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of the first MTs [18]. Brdicka reaction (with several modifications – AdTS, AdTS CV, AdTS DPV) was commonly used in metallothionein detection and quantification in various organisms [87,88]. Other MT detection involved using metal saturation assays in monitoring Mt in fish [89] and terrestrial organisms [90]. The method involves equating the quantity of MTs as a total saturation of their sulphhydryl groups by metal ions. This estimation was misleading as other metal–binding ligands also exist in these biological systems could interfere with the estimation [91,92].

The present–day technique used in the detection and quantification of MTs range from electroanalytical to bioanalytical and molecular methods. These methods involve procedures like ELISA, enzyme–linked assays, chromatography, electrophoresis, mass spectrometry, inductive coupled plasma mass spectrometry, electrochemistry, etc. Most of these techniques, however, do have their pros and cons. The immunoochemical technique was the most commonly reported in publications in metallothionein detection between 2001 and 2010 [29], it is specific and sensitive however limited by the difficulty to obtain MTs antibodies among other disadvantages [93]. The electrochemical techniques like AdTS, AdTS CV, AdTS DPV [94,95] were sensitive and could detect MT peaks but require the use of analyser such as AUTOLAB Analyzer [24].

The improvement of fluorescent technique for MT detection resulted in detecting trace amount of MTs where fluorescent agents like ammonium–7fluorobenzo–2–oxa– 1, 3–diazole– 4–sulfonate (SDB–F) [96] and monobromobimane (mBBr) [97] are derived. Geng, et al. [98] further improved on the fluorimetric method for MT quantification; it was sensitive to a wide range of MT concentrations and gave a relatively accurate estimation of MT. It however required tandem column system to separate the derived compound to eliminate interference or require a prior MT purification before derivation. Also, improved colorimetric method for detecting metallothioneins (MTs) was developed by Qian, et al. [99]. It involves using a thymine (T)–rich oligonucleotide (TRO)–Hg–AuNP system. The thiol groups of MTs could interact with mercury from the T–Hg2+–T complex to release TRO, resulting in a colour change of the system. MTs concentration of the range 2.56 x 10^8 to 3.08 x 10^7 mol/L and the detection limit of 7.67 x 10^9 mol/L were possible. This method allows direct analysis of the samples by the naked eye without costly instruments, and it is reliable, inexpensive, and sensitive.

The advent of high–performance liquid–phase chromatography–electrospray tandem mass spectrometry (HPLC ESI MS) and high–performance liquid chromatography–inductively coupled plasma–mass spectrometry (HPLC–ICP–MS) promised more accurate quantification of metallothioneins. The high costs and technicalities of this equipment remain an imperative factor to consider in their use for the advancement of biological research. Biomolecular method, e.g. ELISA, MT – mRNA (PCR and QT–PCR) are standard method used in detecting and quantifying of metallothioneins; they are simple, less technical and accessible. They can be used to distinguish Mt–isoforms but the mRNA concentration does not give an accurate estimate of the protein concentration [93].

The first detection of earthworm MTs reported in 1998 [16] required the combination of gel chromatographic techniques and “novel” molecular methodologies (Directed Differential Display and quantitative PCR. Recent reports on earthworm MTs detection and quantification indicate molecular based kit as the most commonly used method. They are reliable but require devices like PCR and QPCR. These equipments and their consumables are relatively expensive.

**Conclusion**

Metallothioneins among other biomarkers impact on pollution tolerance and management in the ecosystem are well documented. Techniques involving High performance liquid–phase chromatography–electrospray tandem mass spectrometry (HPLCESI–MS), high performance liquid chromatography–inductively coupled plasma–mass spectrometry (HPLC–ICP–MS) are used for the detection and quantification of MT; they are expensive, requires technical – know – how and are not readily available. Other methods include fluorimetric method and biomolecular methods but the biomolecular method is the most accessible and commonly in used. Earthworms play vital role in metal detoxification and maintenance and this functionality is associated with MTs. Studies have indicated that three MTs isoforms of earthworms (Wmt1, Wmt2 and Wmt3). They differ in their affinity, expression patterns and levels when exposed to metal ions and Wmt2 is the most responsive protein among Wmts especially to Cd. Though earthworm metallothioneins are well studied and documented, the mechanism of gene induction and mechanism of action need more scientific investigation, Wmt3 remains the least understood and it is under reported. Also, with the advent of nanotechnology, a handful of studies have evaluated the effect of nanoparticles in the environment using metallothioneins and a few focused–on earthworms an important entity of the soil ecosystem. Nanoparticles ecotoxicological impact are not well elucidated and remains an area that require more research attention. Other specific areas are vMTs induction, mechanism of action and their entire functions in nanoparticle impacted environment. Research is an ongoing process and the grey areas in earthworm metallothioneins research highlighted in this review are area that can be elucidated.

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