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Original article

The protective role of apigenin against cadmium toxicity: study of metallothionein gene expression and antioxidant enzyme activity in mouse testicular tissue

Zahra Zarei[1], Mohammad Karimian[2,*], Parisa Davoodi[1]

[1]*Department of Biology, Faculty of Science, University of Mazandaran, Babolsar, Mazandaran, Iran*
[2]*Department of Cellular and Molecular Biology, Faculty of Science, University of Mazandaran, Babolsar, Mazandaran, Iran*

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**** Corresponding Author:***
Tel: +98 (11) 35302452
Fax: +98 (11) 35302452
mdkarimian@umz.ac.ir

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Abstract

Objective: The present research explored how apigenin may exert a protective role against cadmium toxicity by evaluating the expression of metallothioneins (*Mts*) and metal regulatory transcription factor 1 (*Mtf1*) genes, along with assessing the activity of antioxidant enzymes Superoxide dismutase (Sod), Glutathione peroxidase (Gpx), and Catalase (Cat) in testicular tissue of male mice, followed by bioinformatics analysis.

Materials and methods: Within this experimental setup, male Balb/C mice received administration of cadmium and apigenin for 14 consecutive days. *Mt1*, *Mt2*, and *Mtf1* gene expression, as well as Sod, Gpx, and Cat antioxidant enzyme activities, were measured in the testis. Molecular docking and gene network analysis were employed to investigate apigenin-metallothionein interactions and their associated gene connections.

Results: The expression of *Mt1* and *Mt2* genes was markedly elevated in the cadmium-treated group; however, apigenin administration significantly attenuated this up-regulation. In the case of *Mtf1*, cadmium exposure enhanced its expression, while apigenin exerted no noticeable effect. Moreover, cadmium treatment led to a reduction in the activity of Sod, Gpx, and Cat enzymes, whereas their activities were improved when apigenin was co-administered. Molecular docking revealed that apigenin interacted with both *Mt1* and *Mt2* proteins in the primary binding sites of the proteins. GeneMANIA revealed links between the three target genes and 20 others, mainly through physical interactions and co-expression, suggesting direct interaction and co-regulation.

Conclusion: Apigenin can regulate the expression of metallothionein genes and preserve the activity of antioxidant enzymes Sod, Gpx, and Cat in mice subjected to cadmium exposure.

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Introduction

Heavy metals persist in natural ecosystems for long periods and are among the most difficult contaminants to manage. Their presence compromises the safety of air, water, and food supplies, ultimately endangering the health and viability of living organisms. Since metals are not subject to metabolic breakdown, they accumulate within the tissues of living organisms (Briffa et al. 2020). Metals such as cobalt, manganese, nickel, mercury, lead, chromium, and cadmium which have a relatively large atomic mass, are categorized as heavy metals (Tchounwou et al. 2012). Cadmium (Cd) is considered among the leading harmful and hazardous heavy metals for living organisms. Over the past few years, a noticeable surge of interest has emerged regarding cadmium because of its significant impact as a pollutant originating from industrial activities and environmental sources (Bhardwaj et al. 2024). Due to its solubility in water, cadmium can easily spread in the environment and infiltrate biological systems in the form of Cd^{2+} via plants and aquatic organisms, displaying bioaccumulation potential (Kubier et al. 2019). In humans and animals, cadmium uptake occurs mainly through dietary intake, while tobacco use also serves as an important source of long-term exposure (Vizuete et al. 2019).

Cadmium can accumulate and localize in several tissues after entering the human body. One of the organs that cadmium can damage is the male reproductive system (Genchi et al. 2020). This hazardous element has been reported to function as an endocrine disruptor, influencing hormones that regulate sperm production (Benoff et al. 2000). Cadmium can also cause disturbances in sperm parameters and ultimately infertility through triggering oxidative stress within male reproductive tissues such as the testes (Ali et al. 2022). Although oxygen is essential for the aerobic metabolism of spermatogenic cells, it can also harm them by generating reactive oxygen species (ROS). A substantial positive association has been observed between elevated ROS levels and abnormal sperm morphology, such as defects in the head, acrosome, midpiece, and tail regions (Kobayashi and Suda 2012).

Major endogenous antioxidant systems, including superoxide dismutase (Sod), catalase (Cat), and glutathione peroxidase (Gpx), are critical in neutralizing metal-induced oxidative damage (Karimian et al. 2024). In the testes, the activity of these enzymes has been documented to rise in Cd-exposed rats (Cupertino et al. 2017). However, other studies indicate a decline in the functional performance of antioxidant enzymes in the testis of Cd-exposed rats (Faraji et al. 2019; Jahan et al. 2014), highlighting the complexity of Cd-triggered oxidative stress, cellular defense mechanisms, and the possible regulatory functions of these enzymes (Cuypers et al. 2010). Several molecular responses are triggered by heavy metal-induced free radicals. Metallothioneins (MTs) constitute a group of small, cysteine-rich proteins that serve essential functions in maintaining intracellular metal homeostasis and have protective functions against heavy metal-induced damage, oxidative stress, and DNA damage (Si and Lang 2018). Metallothionein was originally characterized in horse kidney cortex as a protein capable of selectively associating with cadmium ions. In mammals, this protein consists of 61-68 amino acid including 18-23 cysteine residues. In mammals, there are four distinct types of metallothioneins known as *MT1*, *MT2*, *MT3*, and *MT4*, among which *MT1* and *MT2* isoforms are commonly transcribed in most soft tissues (Moffatt and Séguin 1998; Ruttkay-Nedecky et al. 2013). The expression of metallothionein genes is controlled via the metal regulatory factor *Mtf1*. Elevated heavy metals activate *Mtf1*, which attaches to metal-responsive elements located within the promoter regions of *MT* genes and activates them. Consequently, Mtf1 plays a role in protecting cells against various types of cellular stress signals, including cadmium toxicity (Talukder et al. 2021). *Mtf1*, *MT1*, and *MT2* proteins are crucial for sperm health and fertility. These factors protect sperm and contribute to the production of healthy sperm by regulating zinc, reducing heavy metal toxicity, and reducing oxidative stress. Deficiency in these factors correlates with reduced sperm count and quality, reduced motility, and increased sperm DNA damage. Therefore, increasing levels of these factors can help maintain male reproductive health (Andrews 2000; Ruttkay-Nedecky et al. 2013). In a study, cells with high metallothionein expression were shown to be resistant to cadmium toxicity, while cells unable to synthesize MTs were sensitive to cadmium (Enger et al. 1986).

Many natural compounds are capable of alleviating oxidative stress induced by heavy metals, including apigenin (AP) as an antioxidant. Apigenin belongs to the flavonoid family and is present in many vegetables and fruits, including aromatic herbs, celery, apples, fennel, and wheat germ (Shankar et al. 2017). It is also categorized as a phytoestrogen and, due to its chemical structure, has many pharmacological impacts, including anti-inflammatory, antioxidant, anticancer, and antidepressant features. Studies have reported that AP affects some toxins that cause tissue damage (Dang et al. 2017). The biological function of AP in various tissues is dose-dependent and operates through different mechanisms. Evidence suggests that AP at certain concentrations can enhance the oxidative capacity in rat testicular tissue or have antioxidant effects in various organs (Azmoonfar et al. 2024; Kashyap et al. 2022).

This research sought to study the influence of apigenin on the expression of *Mt1*, *Mt2*, and *Mtf1* genes and the activity of Cat, Gpx, and Sod enzymes in the testicular tissue of mice subjected to cadmium.

Materials and Methods

Animals and experimental design

In this experiment, 49 male Balb/C mice having a body weight spanning 20 to 25 g were employed. After procurement, the animals were transferred to the animal facility and housed under controlled conditions for one week to adapt to the new environment. Throughout this period, they were maintained at 24±2˚C under a 12-hr light-dark schedule, with unrestricted access to water and food. To perform the experiment, AP was first dissolved in propylene glycol, and cadmium in distilled water. The mice were subsequently assigned at random to the following seven groups, with seven animals assigned to each category including: 1- control group, 2- sham group treated with propylene glycol and distilled water, 3- the group receiving a 20 mg/kg dosage of apigenin (AP20), 4- group receiving a 1 mg/kg dosage of cadmium (Cd), 5- group treated with 1 mg/kg cadmium together with 5 mg/kg apigenin (Cd+AP5), 6- group receiving a 1 mg/kg dosage of cadmium together with 20 mg/kg apigenin (Cd+AP20), and 7- group receiving a 1 mg/kg dosage of cadmium together with 50 mg/kg apigenin (Cd+AP50). The treatment regimen was continued for 14 successive days. In the groups that were administered with both cadmium and AP concurrently, the mice were first administered cadmium intraperitoneally, followed by intraperitoneal administration of AP two hours later. The entire experimental process adhered to the ethical regulations set forth by the Bioethics Committee of the University of Mazandaran.

Tissue preparation and quantitative real-time PCR

After 14 days of treatment, the animals were rendered unconscious through an intraperitoneal injection containing ketamine (100 mg/kg) together with xylazine (10 mg/kg). Following dissection, one testis was preserved at -80˚C for enzymatic assay studies, while the contralateral testis was kept at -80˚C to preserve RNA integrity for subsequent expression analyses. For homogenizing testicular tissue, the sample was pulverized within a chilled vessel, employing liquid nitrogen for rapid freezing and grinding.

Real-time PCR was employed to examine the expression of *Mt1*, *Mt2*, and *Mtf1* genes. Total RNA was isolated from the testis by utilizing a commercial extraction kit (Pars-Tous, Iran) and reverse-transcribed into cDNA with another kit from the same manufacturer, following the provided instructions. All procedures were done strictly following the guidelines supplied by the producer. Then, primers specific to the mouse *Mt1*, *Mt2*, and *Mtf1* genes, along with *Gapdh* as the internal control, were designed by utilizing Primer3 software. For this purpose, the complete sequences of each of the mRNAs of the above genes were first deduced from the NCBI database. After designing the primers, their specificity was checked using BLAST. The primer sequences and their characteristics are presented in Table 1.

Quantitative real-time PCR analyses were done in 20 µl reaction mixtures comprising 10 µl of 2X SYBR Green master mix, 0.5 µl of sense and antisense primers, and 20 ng of cDNA, using the Roche LightCycler 96 platform (Roche, Germany). Specificity of the amplified products was confirmed through melting-curve profiling. The thermal protocol included an initial denaturation at 94˚C for 5 min, followed by 35 amplification cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec, and concluded with a final extension step at 72˚C for 5 min. Relative transcript abundance was determined by calculating ΔCt values (target gene Ct minus reference gene Ct), and differences between experimental groups were subsequently derived using the ΔΔCt method.

Table 1. Sequence and characteristics of the specific primers

Gene name	Accession no.	Primer sequence (5’ to 3’)	Annealing temperature (˚C)	PCR product size (bp)
Mt1	NM_013602.3	F: CAGATCTCGGAATGGACCCC R: CTTTGCAGACACAGCCCTG	59	165
Mt2	NM_008630.2	F: TGCTGGCCATATCCCTTGAG R: AGGAGCGTGATGGAGAGAAG	60	152
Mtf1	NM_008636.4	F: TCCCACCATAACCATCACCC R: CACCTGCTCCACTTTCGATG	59	154
Gapdh	NM_001411844.1	F: CGACTTCAACAGCAACTCCC R: GAGTTGGGATAGGGCCTCTC	59	217

Mt1: Metallothionein 1, Mt2: Metallothionein 2, Mtf1: Metal regulatory transcription factor 1, Gapdh: Glyceraldehyde-3-phosphate dehydrogenase, bp: Base pair

Assessment of enzymatic activity

Activity of Cat was quantified via monitoring the decomposition rate of H_2O_2 based on Aebi’s analytical principle. For this assay, the enzyme preparation (10 µl) was combined with sodium phosphate buffer at pH 7 (50 mM) and an H_2O_2 working solution (30 mM). The absorbance at 240 nm was measured at 30-sec intervals over a total period of 3 min (Aebi 1984). Superoxide dismutase activity was determined by the Marklund and Marklund pyrogallol auto-oxidation assay using Tris-HCl buffer (50 mM, pH 8.2), 1 mM EDTA, and 0.2 mM pyrogallol, monitoring absorbance at 420 nm at 30-second intervals for 5 min (Marklund and Marklund 1974). Glutathione peroxidase activity was measured using a commercial kit (Hamedan, Iran) after homogenizing 10-20 mg of testicular tissue in phosphate-buffered saline and centrifuging at 12,000 rpm for 15 min at 4˚C. Absorbance was recorded at 340 nm at 1-min intervals for 5 min, as per the kit protocol.

Bioinformatics analysis

Molecular docking of AP with the metallothionein proteins Mt1 and Mt2 was performed. The three-dimensional structure of mouse Mt1 was generated using the SWISS-MODEL server according to the amino acid sequences obtained from UniProt (accession number P02802). The template 4Mt2.1.A, sharing 78.69% sequence identity, was applied for homology modeling. Model quality was assessed using the GMQE score (0.73), confirming its reliability. The resulting Mt1 structure was analyzed in Molegro (v6.0) to identify key regions, including the binding site. For Mt2, the experimental crystal structure was retrieved from the PDB database (ID: 4Mt2). Prior to docking, crystal water molecules, non-relevant ligands, and excess ions were removed using UCSF Chimera (v1.15rc) and PyMOL (v3.1.1), followed by the addition of polar hydrogens and assignment of partial charges using the AMBER ff14SB force field. The structure was stabilized through energy minimization with 1,000 steps of steepest descent.

The three-dimensional conformation of AP (PubChem CID: 5280443) was obtained in SDF format and subsequently subjected to geometric optimization in Avogadro using the UFF universal force field. After completing the energy-minimization procedure, the optimized structure was exported in PDB file type through Open Babel.

The docking process was executed utilizing AutoDock Vina integrated into Chimera. The grid box dimensions and center coordinates were specified in the vicinity of the predicted active site (Table 2). Docking settings, including the run number and exhaustiveness, were improved to ensure thorough sampling. Each ligand was docked individually with Mt1 and Mt2. The optimal docking configuration was identified by selecting the pose with the minimum binding energy (kcal/mol), followed by examining how the ligand was oriented within the active pocket. Intermolecular contacts, including hydrogen bonding, hydrophobic interactions, and van der Waals forces, were subsequently characterized using the PDBsum platform. Protein-Ligand Interaction Profiler (PLIP), Discovery Studio Visualizer (v21.1.0.20298), and PyMOL. Both two- and three-dimensional representations of the complexes were generated, highlighting key residues involved in hydrogen and hydrophobic interactions. Furthermore, to gain deeper insight into the potential functional relationships among the studied genes, we employed GeneMANIA to analyze three target genes: *Mt1*, *Mt2*, and *Mtf1*. This integrative bioinformatics platform utilizes a wide range of biological datasets, including gene co-expression profiles, participation in shared signaling or metabolic pathways, physical protein-protein interactions, and conserved protein domains, to construct a functional gene interaction network.

Statistical analysis

Following data acquisition, statistical analyses were carried out via SPSS (version 22). Group differences were assessed through one-way ANOVA, and when significant, Tukey’s post hoc test was applied for pairwise comparisons. Data are presented as the mean ± standard deviation, with significance defined at $p<0.05$. All charts and graphical outputs were generated in Microsoft Excel.

Table 2. Coordinates of the Grid Box around the protein active site

Protein/Ligand	Coordinates/Size	X	Y	Z
Mt1/Ap	Center	17.827	11.3541	35.2941
	Size	10.7849	9.59256	14.2894
Mt2/Ap	Center	19.219	11.1274	34.4031
	Size	10.1549	8.48391	15.8373

Mt1: Metallothionein 1, Mt2: Metallothionein 2, AP: Apigenin

Results

Gene expression changes of *Mt1*, *Mt2*, and *Mtf1* in testicular tissue

The expression levels of the target genes in testis tissue were quantified by the qPCR method. The *Mt1* expression data demonstrated that animals receiving AP alone at a dose of 20 mg/kg did not show a substantial change compared to the control group. In the cadmium-exposed group, this gene revealed a marked up-regulation relative to the control group ($p<0.0001$). Co-treatment with cadmium and AP led to a significant down-regulation of *Mt1* compared to cadmium alone, at doses of 20 mg/kg ($p<0.05$) and 50 mg/kg ($p<0.0001$). Also, the expression of this gene in the group receiving cadmium and AP 5 mg/kg ($p<0.01$) was still higher than the control group. Moreover, a significant change in *Mt1* expression was detected between the Cd+AP5 and Cd+AP50 groups ($p<0.01$) (Figure 1A).

Mt2 expression levels showed a similar response. The outcomes of *Mt2* gene expression examination revealed that in the group administered AP alone at 20 mg/kg, gene expression showed no statistically detectable deviation relative to the control group. Cadmium exposure strongly upregulated *Mt2* expression in testicular tissue ($p<0.0001$). However, in the groups that simultaneously received cadmium and AP at doses of 5 ($p<0.05$), 20 ($p<0.001$), and 50 mg/kg ($p<0.0001$), the expression level of *Mt2* exhibited a markedly decreased level in comparison with the cadmium group. Also, the expression of this gene in the groups receiving cadmium and AP 5, 20, and 50 mg/kg was still higher than the control group ($p<0.0001$). No meaningful statistical variation was detected among the Cd+AP5, Cd+AP20, and Cd+AP50 groups (Figure 1B).

Regarding *Mtf1* gene, the cadmium-treated group revealed significantly elevated level of this gene’s expression in testicular tissue relative to the control group ($p<0.05$). However, no other substantial changes were detected between the studied groups in the mouse testicular tissue (Figure 1C). The group receiving AP at a dose of 20 mg/kg did not show substantial changes relative to the control group. Furthermore, the simultaneous intake of cadmium and AP did not affect the expression of this gene. These findings indicate that AP supplementation does not modulate *Mtf1* regulation in testicular tissue under the studied conditions.

Antioxidant enzyme activity in testicular tissue

The enzymatic activity levels of Cat, Sod, and Gpx was assessed in the testis of mice treated with cadmium and AP, the results of which are shown in Figure 2. Our data showed that cadmium exposure markedly reduced the Cat enzyme activity in the cadmium group relative to the control group ($p<0.0001$). However, co-treatment with cadmium and AP 5, 20, and 50 mg/kg significantly increased the catalase activity relative to the cadmium group ($p<0.0001$). However, there was still a noteworthy difference in the Cat activity between the Cd+AP5 ($p<0.01$) and Cd+AP20 ($p<0.05$) groups relative to the control group. Furthermore, a significant change was detected in Cat activity between the Cd+AP5 and Cd+AP50 groups ($p<0.01$) (Figure 2A).

In the case of Sod, cadmium was able to significantly reduce the enzyme activity relative to the control group ($p<0.0001$). Though co-treatment of cadmium and AP at doses of 20 ($p<0.01$) and 50 mg/kg ($p<0.05$) markedly elevated this enzyme’s activity relative to the cadmium group. Importantly, Sod activity in the Cd+AP5, Cd+AP20, and Cd+AP50 groups revealed no meaningful change relative to the control group (Figure 2B).

For Gpx, cadmium administration was also able to significantly reduce the enzyme activity relative to the control group ($p<0.0001$). Co-administration of cadmium and AP at doses of 5 ($p<0.01$), 20 ($p<0.01$), and 50 ($p<0.001$) was able to increase the activity of this enzyme relative to the cadmium group. However, Gpx enzyme activity in the Cd+AP5, Cd+AP20, and Cd+AP50 groups displayed no statistically meaningful variation relative to the control group (Figure 2C).

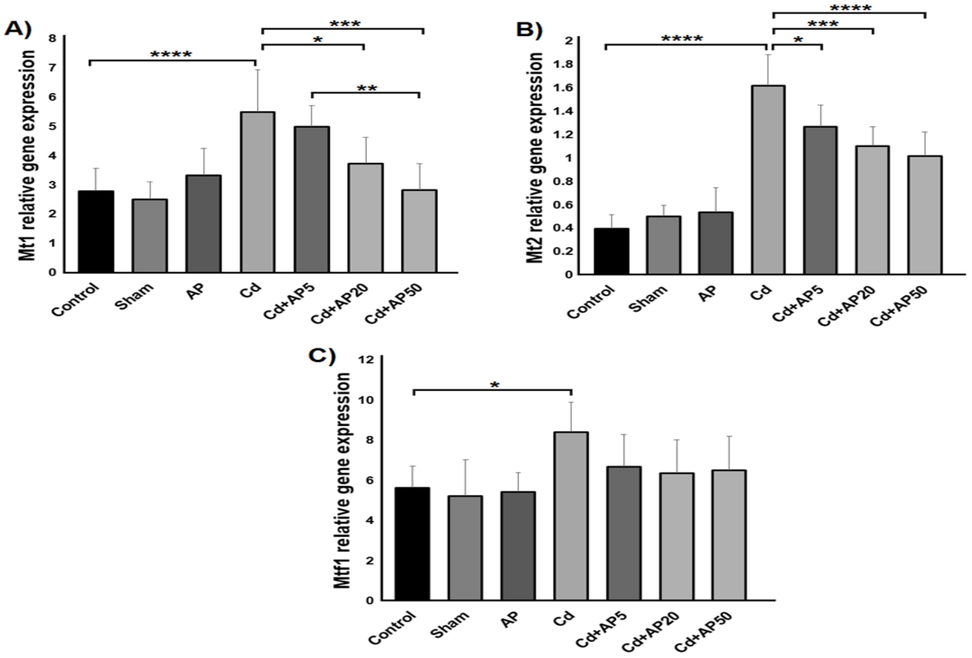

Figure 1. . Results of *Mt1*, *Mt2*, and *Mtf1* gene expression in testicular tissue after treatment of mice with cadmium and apigenin. Changes in Mt1 (A), Mt2 (B), and Mtf1 (C) gene expression after treatments. The symbols *, **, ***, and **** indicate significant differences less than 0.05, 0.01, 0.001, and 0.0001, respectively (n=7). Cd: Cadmium, AP: Apigenin. Mt1: Metallothionein 1, Mt2: Metallothionein 2, Mtf1: Metal regulatory transcription factor 1.

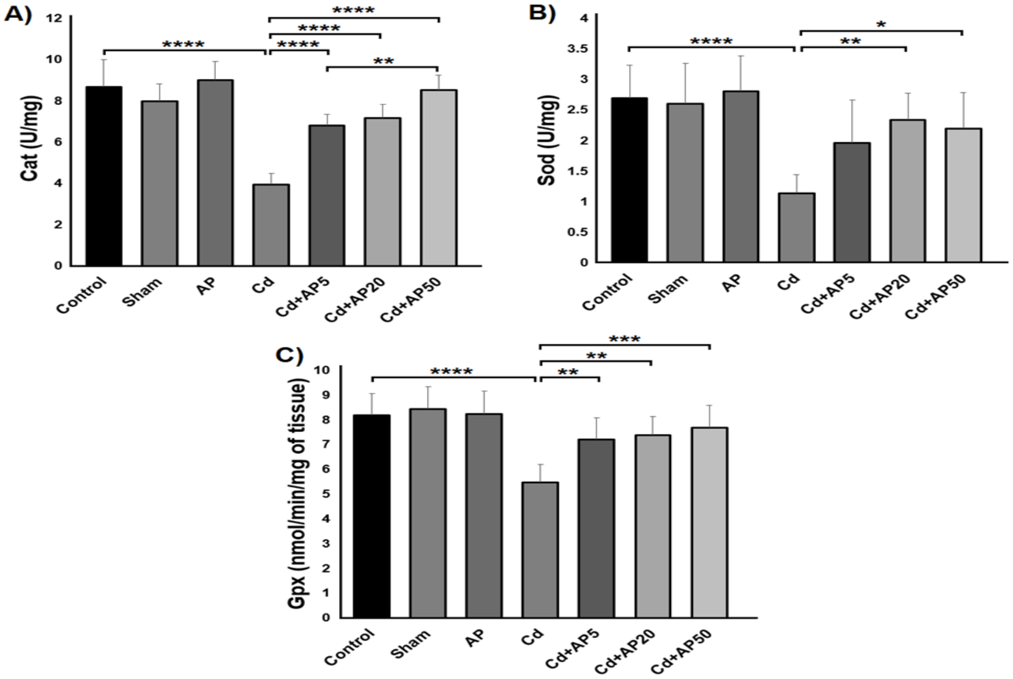

Figure 2. Results of the activities of catalase, superoxide dismutase, and glutathione peroxidase enzymes in testicular tissue after treatment of mice with cadmium and apigenin. Representation of changes in the activities of catalase (A), superoxide dismutase (B), and glutathione peroxidase (C) enzymes after treatments. The symbols *, **, ***, and **** indicate significant differences less than 0.05, 0.01, 0.001, and 0.0001, respectively (n=7). Cd: cadmium, AP: Apigenin. Cat: Catalase, Sod: Superoxide dismutase, Gpx: Glutathione peroxidase.

Interaction of apigenin with Mt1 and Mt2 proteins and networking approach

Molecular docking served to assess the strength of binding interactions of AP with Mt1 and Mt2 proteins. Apigenin exhibited binding affinities of -6.1 and -6.2 kcal/mol for Mt1 and Mt2, respectively, with docking precision confirmed by RMSD values of 0.0 for both proteins. Two-dimensional interaction maps indicated that AP engages in hydrogen bonding and van der Waals contacts with both proteins. In the 3D structural analyses, AP established one hydrogen bond with Mt1 and two hydrogen bonds with Mt2, alongside hydrophobic interactions with distinct residues in their active sites. These interactions are depicted in two-dimensional and three-dimensional presentations (Figures 3). Specific binding energies, hydrogen bonds, hydrophobic interactions, and involved amino acids are summarized in Table 3.

GeneMANIA-based gene network analysis demonstrated that the *Mt1*, *Mt2*, and *Mtf1* were functionally linked to 20 other related genes (Figure 4). Notably, a substantial proportion of these associations (45%) were classified as physical interactions, suggesting the potential for direct protein-protein interactions between the products of the studied genes and those of related genes. Moreover, co-expression patterns represented a considerable portion of the network (17.96%), implying that these genes may be co-regulated or simultaneously expressed under specific cellular conditions or in response to common biological stimuli.

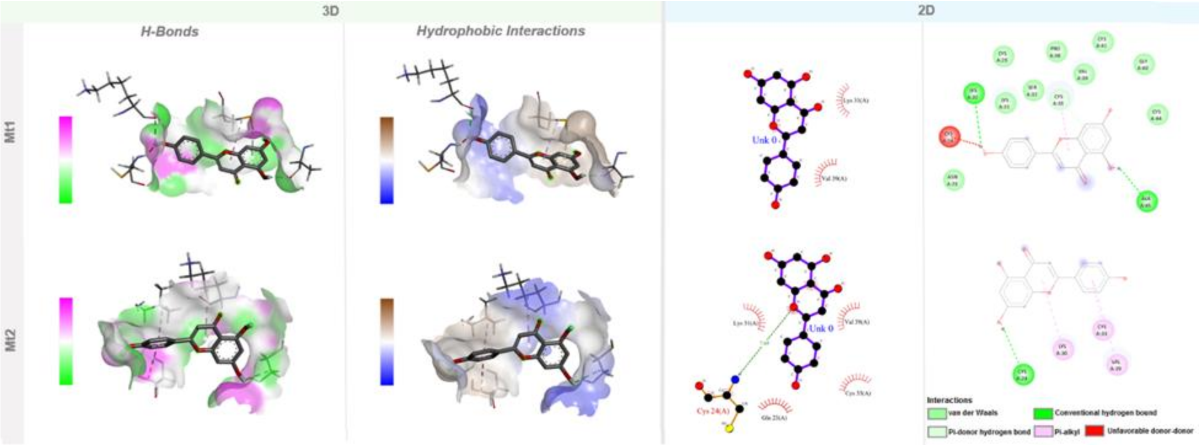

Figure 3. The molecular interactions of ligands M1 and M2 with the target protein. Hydrogen bonding (H-Bonds) is depicted with pink (donor) and green (acceptor) dashed lines. A 3D presentation highlights hydrophobic interactions, with color intensity ranging from blue (low) to brown (high). A 2D schematic delineates hydrophobic interactions (green circles) and π-π interactions (red circles), with dashed lines indicating hydrogen bonds. A comprehensive 2D interaction map identifies key amino acid residues (e.g. Cys and Val) involved in the binding interface. Scale and interaction types are annotated accordingly. Mt1: Metallothionein 1, Mt2: Metallothionein 2.

Table 3. Binding energy and key interactions of apigenin with Mt1 and Mt2 proteins

Receptor	Binding Affinity (kcal/mol)	H-Bond Count	RMSD l.b.	RMSD u.b.	Hydrophobic Interactions			Hydrogen Bonds			
					Residue	AA	Distance	Residue	AA	Distance H-A	Distance D-A
Mt1	-6.1	1	0.0	0.0	39A	VAL	3.89	22A	LYS	2.02	3.05
								24A	CYS	2.14	3.12
								41A	CYS	3.61	4.06
								41A	CYS	3.04	3.89
								45A	ALA	2.23	2.89
Mt2	-6.2	2	0.0	0.0	30A	LYS	3.98	23A	GLN	2.29	3.10
								24A	CYS	2.68	3.06
					39A	VAL	3.47	41A	CYS	3.35	3.71

RMSD l.b. and RMSD u.b.= 0.0 indicate high docking precision; AA: Amino acid residue number; H-A: Hydrogen-acceptor distance; D-A: Donor-acceptor distance. Mt1: Metallothionein 1, Mt2: Metallothionein 2.

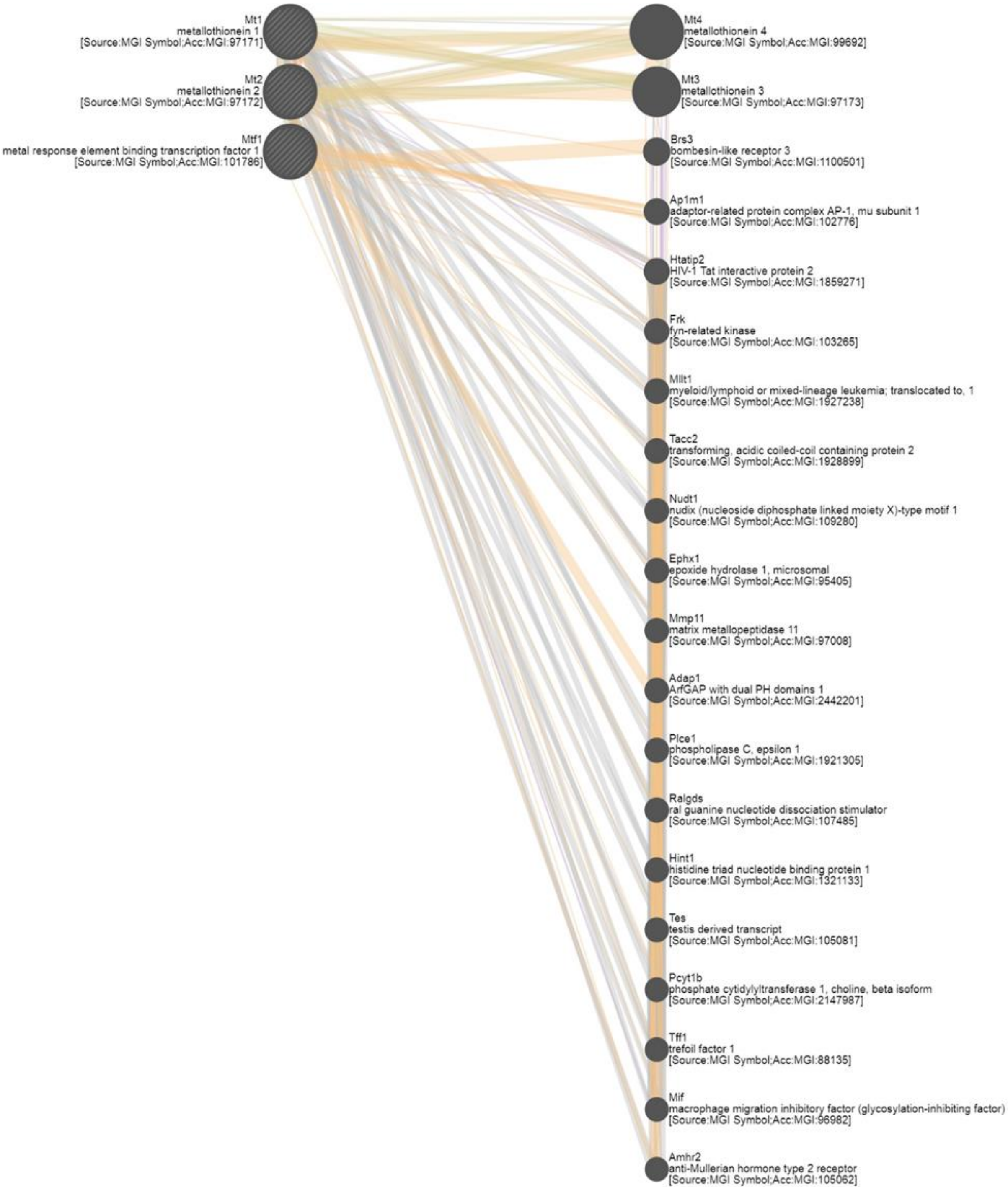

Figure 4. Gene network obtained from GeneMANIA. General diagram of the network obtained from the GeneMANIA database for *Mt1*, *Mt2*, and *Mtf1* genes. These three genes are connected to 20 other genes, with the majority of these interactions being of the physical type. Mt1: Metallothionein 1, Mt2: Metallothionein 2, Mtf1: Metal regulatory transcription factor 1.

Discussion

Cadmium is a widespread environmental pollutant, and animals and humans could be exposed to its toxicity via contaminated air, water, or food (Mitra et al. 2022). Current evidence suggests that the testes show pronounced sensitivity to cadmium, which can disrupt the vascular system and epigenetic regulation disrupts the functions of somatic and germ cells, ultimately leading to infertility or reduced male fertility (Ali et al. 2022; Bhardwaj et al. 2021; Wanjari and Gopalakrishnan 2024). This study aimed to examine the impact of AP on the expression of *Mt1*, *Mt2*, and *Mtf1* genes as well as the activity of Cat, Sod, and Gpx enzymes in the testicular tissue of mice subjected to cadmium. Overall, we found that AP has the potential to regulate metallothionein gene expression and preserve the activities of the antioxidant enzymes in cadmium-exposed mice.

The antioxidant properties of MTs are attributed to their high cysteine content, which allows them to react with ROS and other free radicals and prevent cellular damage (Ruttkay-Nedecky et al. 2013). A study has demonstrated that MTs can interact with other antioxidant enzymes, including Sod, Cat, and Gpx, which, through these interactions, could improve the total antioxidant capacity of the cell and contribute to the defense against oxidative stress (Ruttkay-Nedecky et al. 2013). Our findings demonstrated that exposure to cadmium may elevate the expression of *Mt1*, *Mt2*, and *Mtf1* genes. A study demonstrated that low doses of cadmium stimulated the Mt expression in the testes, whereas a significant reduction in Mt was observed in mice treated with higher doses of cadmium (Xu et al. 2005). Another study reported that cadmium exposure stimulated the expression of the major isoforms Mt1 and Mt2 in mouse testes. This expression was time-dependent in Sertoli and spermatogenic cells, with a stronger response observed in Sertoli cells than in spermatogenic cells (Ren et al. 2003). These findings indicate that cadmium-induced Mt expression is dependent on cadmium concentration, cell type, and time.

Cadmium can increase antioxidant activity in several ways, one of which is its destructive effect on antioxidant enzymes. For example, an animal model study showed that cadmium in the gonad reduced the activities of Cat, Gpx, and Sod (Amara et al. 2008). Other animal studies have reported results indicating a decrease in antioxidant enzymes upon exposure to cadmium (Almeer et al. 2018; Bashir et al. 2019; Shi and Fu 2019). However, some animal studies have reported that cadmium reduces the activity of antioxidant enzymes in testicular tissue (Almeer et al. 2018; El-Refaei and Abdallah 2021; Momeni and Eskandari 2020). This variability in results is likely due to differences in cadmium dose, exposure duration, animal species, or methodological factors. Cadmium may damage enzymes through various mechanisms. For example, cadmium mainly affects proteins undergoing synthesis or folding, likely through its interaction with sulfhydryl groups present in proteins that are not yet properly folded (Jacobson et al. 2017). Conversely, cadmium may substitute essential metals within intracellular enzymes, leading to impaired activity of antioxidant enzymes. As a result, the body's ability to combat free radicals is reduced, and oxidative damage occurs (Yang et al. 2025). Beside, in some cases, the cadmium ion can become tightly bound to the active site, preventing any substrate from reaching the enzyme (Attaallah and Amine 2022; Ragunathan et al. 2010).

Medicinal plants have long been used in traditional healing practices for centuries to alleviate numerous illnesses, including disorders of the male reproductive system. In recent decades, more scientific research has focused on the potential of medicinal plants to combat conditions compromising male reproductive performance, including erectile dysfunction, low sperm count, endocrine disturbances, and infertility (Boroujeni et al. 2022). Flavonoids are natural plant metabolites that have specific colors and are produced secondarily from phenylalanine (Havsteen 2002). Research has demonstrated that AP acts as a potent trigger of apoptosis and suppressor of cell proliferation in numerous cancer cell types, and it has also been shown to reduce oxidative injury, gamma-radiation effects, and related hematological disturbances (Begum et al. 2012; Song et al. 2011). Additional reports show that it reduces ROS and malondialdehyde levels, thereby boosting the activity of antioxidant enzymes, including Sod, Cat, and Gpx, and elevating the levels of antioxidant response proteins such as Nrf2 and activated kinase (Thiruvengadam et al. 2021; Yi 2018). Another study also indicated that AP boosts testosterone production and protects sperm from harmful environmental insults, such as pollution and toxins (Kaltsas 2023). Our study also confirmed that AP supports antioxidant enzyme activity in cadmium-exposed tissues. It functions as a scavenger of ROS such as the superoxide anion, hydroxyl radical, and hydrogen peroxide (Kalivarathan et al. 2017). By lowering oxidative burden, this flavonoid provides cellular defense against damage while augmenting the performance of enzymatic antioxidants (Ayaz et al. 2024; Güçlü and Ayan 2023; Kashyap et al. 2022). Moreover, earlier studies have noted that other flavonoids, such as genistein and biochanin A, could increase metallothionein levels (Kuo et al. 1998). Hence, the flavonoid AP may also have this effect on metallothionein expression, as was observed in our study.

Assessing molecular interactions exclusively through laboratory-based techniques is often challenging and labor-intensive. However, the use of computational methods can be a promising approach to overcome this issue (Vahidinia et al. 2022). Therefore, in a separate phase of our research, we performed a molecular docking approach to evaluate the binding affinity and key interactions between AP and Mt1 and Mt2 proteins. The compound exhibited both hydrogen bonding and van der Waals forces with the proteins. In 3D conformations, one hydrogen bond with Mt1 and two hydrogen bonds with Mt2 were observed, along with hydrophobic interactions in the active sites. Generally, flavonoid-protein associations are established through non-covalent mechanisms such as hydrogen bonds, van der Waals interactions, hydrophobic interactions, and ionic interactions. These interactions can affect protein structure and enzymatic activity (Tang et al. 2017). Consequently, beyond its gene-regulatory impact, AP could modulate protein through interaction with metallothionein.

Apigenin, at varying concentrations, was able to effectively reduce the detrimental effects of cadmium on mice testicular tissue through modulating the expression of metallothionein genes and maintaining the activity of Sod, Gpx, and Cat antioxidant enzymes. Therefore, AP may be proposed as a promising therapeutic compound for eliminating oxidative damage caused by cadmium. However, further cellular and molecular research is still essential to fully clarify its underlying mechanisms of action.

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Conflicts of interest

The authors had no competing interests.

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Ethical Considerations

All stages of the experiment were carried out according to the guidelines of the Bioethics Committee of the University of Mazandaran.

Code of Ethics

IR.UMZ.REC.1403.102

Authors' Contributions

Zahra Zarei performed the laboratory experiments and participated in the initial drafting. Mohammad Karimian supervised the project, conceptualized the study, conducted data analysis, and contributed to drafting the manuscript. Parisa Davoodi contributed to data analysis and manuscript preparation. All authors read and approved the final version of the manuscript.

Abbreviations

AP: Apigenin, Cd: Cadmium, Mt: Metallothionein, ROS: Reactive oxygen species, Cat: Catalase, Gpx: Glutathione peroxidase, Mtf1: Metal regulatory transcription factor 1, Sod: Superoxide dismutase

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