Original Article

Human Sperm Quality and Metal Toxicants: Protective Effects of some Flavonoids on Male Reproductive Function

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Abstract

Background: Metals can cause male infertility through affection of spermatogenesis and sperm quality. Strong evidences confirm that male infertility in metal-exposed humans is mediated via various mechanisms such as production of reactive oxygen species (ROS). Flavonoids have antioxidant and metal chelating properties which make them suitable candidates for neutralizing adverse effects of metals on semen quality. In the current study, we have evaluated the effects of five types of flavonoids (rutin, naringin, kaempferol, quercetin, and catechin) on recovery of sperm motility and prevention of membrane oxidative damage from aluminum chloride ($AlCl_3$), cadmium chloride ($CdCl_2$), and lead chloride ($PbCl_4$).

Materials and Methods: In this experimental study, motility and lipid peroxidation of metal-exposed sperm was investigated in the presence of different concentrations of five kinds of flavonoids. Malondialdehyde (MDA) production was assessed as a lipid peroxidation marker.

Results: Aluminum chloride ($AlCl_3$), cadmium chloride ($CdCl_2$), and lead chloride ($PbCl_4$) diminished sperm motility. Treatment of metal-exposed sperm with rutin, naringin, and kaempferol attenuated the negative effects of the metals on sperm motility. Quercetin and catechin decreased the motility of metal-exposed sperm.

Conclusion: Based on the MDA production results, only $AlCl_3$ significantly induced lipid peroxidation. Treatment with rutin, naringin, and kaempferol significantly decreased MDA production.

Keywords: Metal Toxicity, Sperm Motility, Lipid Peroxidation, Flavonoids, Semen Quality

Introduction

Metals are one of the main constituents of an industrialized lifestyle that have a wide range of applications. Metals such as lead (Pb), aluminum (Al) and cadmium (Cd) induce toxicity in humans and other living organisms by impacting enzyme activity and generation of free radical production. However, in terms of their unique characteristics, their applications are expansive, even in medical and drug industries (1, 2).

Metals can affect male and female fertility by induction of reactive oxygen species (ROS) production. Therefore, antioxidant therapy that inhibits metal-induced toxicity is under active investigation (3). Flavonoids are a broad group of natural antioxidant compounds with flavan nucleus and a benzo-ý-pyrone structure. These compounds are low molecular weight polyphenols ubiquitously synthesized by green plants that may show various pharmacological attributes according to their chemical structures (4). Direct antioxidant effects and the ability of flavonoids to chelate metal ions have been previously researched (5-7). Researchers report the existence of a cardioprotective role (8, 9) and free radical scavenging potential of flavonoids (10). Until now, over 4000 natural flavonoids have been identified in leaves, seeds, barks, and flowers

of different plants (11). Protection against ultraviolet (UV) light, pathogens, herbivores, and the attraction of pollinating insects are major proposed roles for flavonoids in various plants (12-14). Flavonoids can occur both in the free form and as glycosides. Their structure is composed of a basic C_6-C_3-C_6 phenyl-benzopyran backbone (Fig.1). The position of the phenyl ring relative to the benzopyran moiety, oxidation of central ring, hydroxylation profile, and degree of polymerization determine chemical properties of a flavonoid (15).
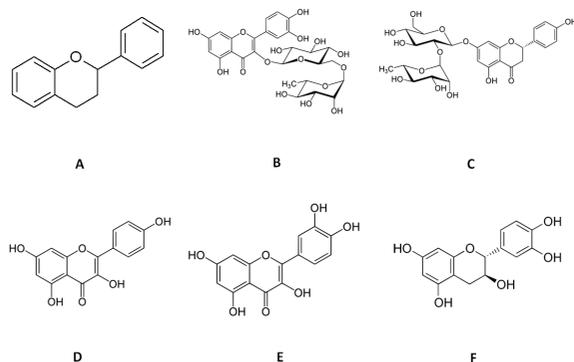

Fig.1: Chemical structure of flavonoids. **A.** Basic structure of a flavonoid with two benzene rings and a heterocyclic pyran ring as the linker. Chemical structures of: **B.** Rutin, **C.** Naringin, **D.** Kaempferol, **E.** Quercetin, and **F.** Catechin.

ROS induce cellular membrane instability (16), destruction of DNA structures, and promotion of transformation, (17) ultimately resulting in cellular aging (18), mutagenesis (17), carcinogenesis (19), induction of coronary heart disease (CHD) (4), and infertility (20). In addition to ROS, nitrogen reactive species (NOS) can cause cardiovascular diseases (CVD) through oxidation of LDL particles (21, 22) and increased release of matrix metalloproteinase-2 (MMP-2) in the coronary effluent (23). Based on the scientific findings, a flavonoid-rich diet is highly recommended to decrease CVD and other ROS-/NOS-induced myocardial injuries (4).

Recent interest in flavonoids arises from the potential health benefits attributed to the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (4, 11). The chelating of metals can be crucial in prevention of radical generation which damage target biomolecules (11). In the current study, we have evaluated the effects of five types of flavonoids (rutin, naringin, kaempfer-

ol, quercetin, and catechin) on recovery of sperm motility and prevention of membrane oxidative damage from aluminum chloride ($AlCl_3$), cadmium chloride ($CdCl_2$), and lead chloride ($PbCl_4$).

Materials and Methods

Materials

For this experimental study, $AlCl_3$, $CdCl_2$, $PbCl_4$, naringin, kaempferol, and quercetin were obtained from Merck (Darmstadt, Germany). Rutin, catechin and the remainder of chemicals and reagents used in this research were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection and preparation of sperm suspension

Sperm samples considered compatible to the world health organization (WHO) reference value for human semen (volume ≥ 3.0, sperm concentration/ml $\geq 50 \times 10^6$, forward motility $\geq 60\%$, and atypical forms $\leq 40\%$) (24) were collected and pooled from 40 healthy, non-smoking volunteers, that resided in Ahvaz, Khuzestan Province, Iran. We compared the effects of flavonoides on motility and lipid peroxidation of metal-exposed sperms using laboratory studies. The Institutional Ethics Committee of Ahvaz University of Medical Sciences reviewed and approved the protocol. All participants in the current study signed informed consents. Collected sperm samples were separated from semen plasma for assessment of clinical attributes by washing three times with an equal volume of M_6 solution and subsequent centrifugation for 10 minutes at 1600 g (25). M_6 solution contained (per liter, pH=7.4): 0.55% NaCl, 0.03% KCl, 0.019% $CaCl_2$, 0.016% K_3PO_4, 0.029% $MgSO_4$, 0.031% $NaHCO_3$, 0.496% HEPES, 0.26% sodium lactate, $36 \times 10^{-4}\%$ sodium pyruvate, 0.11% glucose, 0.4% bovine serum albumin, $60 \times 10^{-4}\%$ penicillin, and $50 \times 10^{-4}\%$ streptomycin. Separated pellets were suspended in M_6 solution at a density of 100 million sperm/ml and freshly were used. Sperm counts were performed by a MMC-SK Sperm Counting Chamber (Saint Petersburg, Russia).

Incubation of sperm samples with aluminum chloride, cadmium chloride, and lead chloride

We evaluated the effects of $AlCl_3$, $CdCl_2$, and $PbCl_4$ on sperm motility and lipid peroxidation of sperm cells at different concentrations (125 µM,

250 µM, 500 µM, 1 mM, and 5 mM) of the metal salts. The metal salt solutions were prepared in M_6 solution. Sperm samples were incubated in the presence of defined concentrations of these metals for 2 hours at 37˚C. From the examined concentrations of metals, we selected those that significantly impacted sperm motility for additional experiments with the flavonoids (P≤0.05).

Effects of flavonoids on the motility of metal-exposed sperm

Sperm samples were treated for 2 hours at 37˚C with $AlCl_3$ (1.0 mM), $CdCl_2$ (500 µM) or $PbCl_4$ (250 µM) in the presence of various concentrations (25, 50, 100, 200, 500, and 1000 µM) of rutin, naringin, kaempferol, quercetin, and catechin. Subsequently, we assessed sperm mobility by MMC Sperm. In order to increase solubility, all flavonoids were solvated in a 1:1 (v/v) of Dimethyl sulfoxide (DMSO): M_6 solution prior to their treatment of the sperm cells.

Effects of flavonoids on lipid peroxidation of metal-exposed sperm

Induction of lipid peroxidation was evaluated in sperm samples in the presence of various concentrations of $AlCl_3$, $CdCl_2$, and $PbCl_4$. Between treated groups, sperm samples treated with 20 mM of $AlCl_3$ were simultaneously incubated with 25 µM, 50 µM, 100 µM, 200 µM, 500 µM, and 1 mM each of rutin, naringin, kaempferol, quercetin, and catechin for 2 hours at 37˚C. After incubation, we assessed for lipid peroxidation of the sperm cells according to the indicated approach.

Analytical methods

Assessment of sperm motility

Evaluation of sperm motility was performed by MMC Sperm (MultiMedia Catalog Sperm). MMC Sperm is an automated image analysis software package for sperm quality analysis according to parameters recommended by the WHO laboratory manual (26).

Measurement of lipid peroxidation

Lipid peroxidation was measured using malondialdehyde (MDA) and thiobarbituric acid-reactivity (27, 28). Briefly, 50 µl of 0.2% butylated hydroxytoluene (dissolved in ethanol) and 1.0 ml of 15% aqueous trichloroacetic acid were successively added to $2.0×10^7$ sperm. The mixture was then centrifuged at 4000 g for 15 minutes at 4˚C. An aliquot of 500 µl of the deproteinized supernatant was added to 1.0 ml thiobarbituric acid (0.375% in 0.25 M HCl) and the mixture was heated at 100˚C for 20 minutes. After cooling, the solution was analyzed by a spectrophotometer at 532 nm.

Statistical analysis

All treatments were performed in triplicate. Each experiment was run at least three times. Results were expressed as mean ± SE. Significance of difference between treatment groups was determined by the student's t test. P<0.05 was considered statistically significant.

Results

Effects of aluminum chloride, cadmium chloride, and lead chloride on sperm motility

$AlCl_3$ is an abundant metal in the earth which has toxic effects. High concentrations of $AlCl_3$ induce free radical-mediated cytotoxicity and can be toxic for the male reproductive system (29, 30). In previous studies, it has been shown that treatment with $AlCl_3$ could decrease ejaculate volume, sperm concentration, and sperm motility (31). $CdCl_2$ is a well-known nephrotoxin and carcinogen (32, 33) that can induce ROS production. Exposure to $CdCl_2$ may result in decreased sperm concentration, diminished sperm motility, creation of abnormal forms of sperm following long-term exposure to $CdCl_2$ (3, 34), and infertility in treated male mice (35). $PbCl_4$ poisoning can result in decreased sperm motility. A number of reports discuss DNA fragmentation in sperm cells exposed to this metal *in vitro* (36). Our *in vitro* studies have confirmed the above mentioned findings where different concentrations of $AlCl_3$, $CdCl_2$ and $PbCl_4$ significantly decreased sperm motility (P≤0.05, Fig.2). Mean sperm motility after a 2-hour incubation period in the presence of 5.0 mM $AlCl_3$, $CdCl_2$, and $PbCl_4$ were 93% ($AlCl_3$), 75% ($CdCl_2$), and 41% ($PbCl_4$) less than the control groups. As seen in Figure 2, the effect of Pb on sperm motility was higher at the same concentrations of the three tested metals $AlCl_3$, at the 1.0 mM concentration, significantly affected sperm motility (P≤0.0013). The 500 µM concentration of $CdCl_2$ significantly affected sperm motility (P≤0.032), whereas $PbCl_4$ significantly affected motility at the 250 µM (P≤0.0005) concentration (Fig.2). The adverse effects of all three metals on sperm motility were completely dose-dependent.

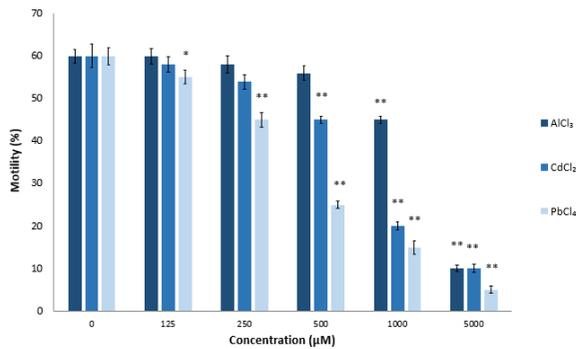**Fig.2:** Effects of aluminum chloride (AlCl$_3$), cadmium chloride (CdCl$_2$), and lead chloride (PbCl$_4$) on sperm motility. We evaluated the effects of these compounds on sperm motility at different concentrations (125 μM, 250 μM, 500 μM, 1 mM, and 5 mM) of metal salts. Sperm samples were incubated in the presence of the defined concentrations of metals for 2 hours at 37˚C. *; P<0.05 and **; P<0.01 compared to the untreated control.

Effects of flavonoids on motility of aluminum chloride-exposed sperm

Previous studies reported an *in vitro* protective effect of ascorbic acid (vitamin C) and tocopherol (vitamin E) on AlCl$_3$-treated sperm (31, 37). As seen in Figure 2, 1000 μM of AlCl$_3$ significantly decreased sperm motility by 15% (P≤0.0013). Therefore, we used this concentration for additional studies with flavonoids. We used different concentrations of rutin, naringin, kaempferol, quercetin, and catechin for motility recovery of AlCl$_3$-exposed sperm. Compared to the untreated control group, rutin increased sperm motility by 9% at the 50 μM concentration and 18% at the 200 μM concentration. Naringin, at a final concentration of 100 μM, significantly increased sperm motility by 9% (P≤0.038). There was a gradual increase in recovery of sperm motility when the concentration of naringin increased to 500 μM (Fig.3). Kaempferol showed the most protective effect of all the tested flavonoids. There was 10% recovery of sperm motility at the kaempferol concentration of 25 μM. On the other hand, effects of quercetin and catechin on the sperm mobility completely differed from the other tested flavonoids - rutin, naringin and kaempferol. The antioxidants, quercetin and catechin did not protect sperm cells from heavy metal-mediated damages; rather, they showed inhibitory effects on sperm motility. When we increased the concentrations of quercetin and catechin from 0 to 1000 μM, there was a gradual decrease in sperm motility compared to the untreated control group. Mean motility of AlCl$_3$-exposed

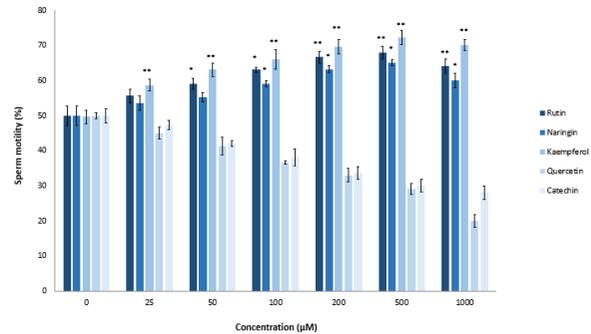sperm after a 2 hours incubation period in the presence of 1000 μM quercetin was 22% and for catechin, it was 28%.

Fig.3: Effects of rutin, naringin, kaempferol, quercetin, and catechin on aluminum chloride (AlCl$_3$)-exposed sperm. Sperm samples were treated for 2 hours at 37˚C with AlCl$_3$ (1.0 mM) in the presence of various concentrations (25, 50, 100, 200, 500, and 1000 μM) of rutin, naringin, kaempferol, quercetin, and catechin. Sperm mobility was assessed by MMC Sperm. *; P<0.05 and **; P<0.01 compared to the flavonoid untreated control.

Effects of flavonoids on motility of cadmium chloride-exposed sperm

Previous studies by El-Demerdash et al. (3) in male rats showed beneficial effects of vitamin E and β-carotene in reducing the toxic effects of CdCl$_2$ on the male reproductive system. In the current study, we observed that treatment with rutin, naringin and kaempferol resulted in recovery of motility in CdCl$_2$-exposed sperm cells. Our results showed that rutin, naringin, and kaempferol at 25-500 μM significantly increased (P≤0.05) motility of CdCl$_2$-exposed sperm cells in a dose-dependent manner (Fig.4). In contrast, quercetin and catechin did not induce any protective effect against CdCl$_2$ toxicity; they reduced the motility of CdCl$_2$-exposed sperm compared to the untreated control samples (Fig.4). These results disagreed with an *in vivo* study by Farombi et al. (38) about the antioxidative nature of quercetin. They showed that administration of the biflavonoid, kolaviron, or quercetin prevented Cd-mediated decreased sperm motility in adult male rats. Other researchers reported the positive effects of quercetin on sperm capacity under both *in vitro* and *in vivo* conditions (39). Supplementation of quercetin restored the decrease in glutathione (GSH) level, and superoxide dismutase (SOD) and GSH peroxidase activities in Cd-exposed mice. This discrepancy between *in vitro* and *in vivo* results might be attributed to the difference in quercetin exposure time or to *in situ* metabolic alteration of quercetin (40).
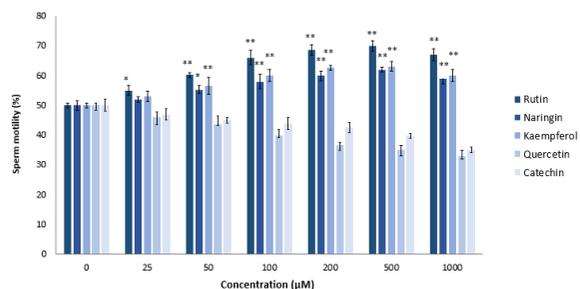
Fig.4: Effects of rutin, naringin, kaempferol, quercetin, and catechin on cadmium chloride (CdCl$_2$)-exposed sperm. Sperm samples were treated for 2 hours at 37˚C with CdCl$_2$ (500 μM) in the presence of various concentrations (25, 50, 100, 200, 500, and 1000 μM) of rutin, naringin, kaempferol, quercetin, and catechin. Sperm mobility was assessed by MMC Sperm. *; P<0.05 and **; P<0.01 compared to the flavonoid untreated control.

Effects of flavonoids on motility of lead chloride-exposed sperm

Toxic effects of PbCl$_4$ on sperm quality, motility, DNA fragmentation, and acrosome reaction have been investigated extensively in mice and humans (36, 41-44). According to our results (Fig.2), PbCl$_4$ compared to AlCl$_3$ and CdCl$_2$ had more adverse effects on sperm motility at the 0.125 to 5.0 mM concentrations. We used the 250 μM concentration of PbCl$_4$ for additional experiments with flavonoids. Quercetin and catechin decreased motility of PbCl$_4$-exposed sperm cells in a dose-dependent manner. However, as seen in Figure 5, the 500 μM concentration of rutin, naringin, and kaempferol significantly increased sperm motility to 65% (rutin), 60% (naringin) and 63% (kaempferol). Rutin was more efficient in fortifying sperm cells against PbCl$_4$-induced harmful attacks.
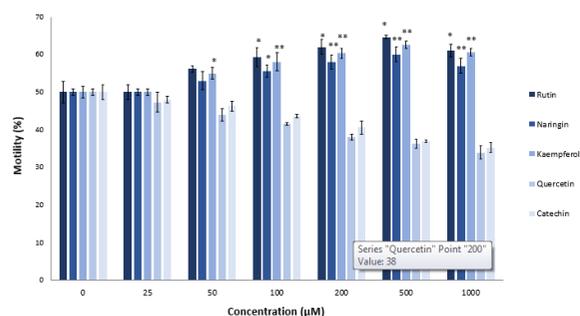

Fig.5: Effects of rutin, naringin, kaempferol, quercetin, and catechin on lead chloride (PbCl$_4$)-exposed sperm. Sperm samples were treated for 2 hours at 37˚C with PbCl$_4$ (250 μM) in the presence of various concentrations (25, 50, 100, 200, 500, and 1000 μM) of rutin, naringin, kaempferol, quercetin, and catechin. Sperm mobility was assessed by MMC Sperm. *; P<0.05 and **; P<0.01 compared to flavonoid untreated control.

Sperm lipid peroxidation in the presence of aluminum chloride, cadmium chloride and lead chloride

Sperm membranes are rich in polyunsaturated fatty acids (PUFAs) (45). Previous *in vivo* studies have demonstrated that Al could increase peroxidation of PUFAs in sperm samples (31, 46). The presence of a high level of PUFA in the sperm plasma membrane is required for membrane fusion events associated with fertilization. Loss of fluidity as a result of lipid peroxidation can diminish the rates of sperm-oocyte fusion (47). Our *in vitro* studies have shown that AlCl$_3$ at concentrations higher than 0.5 mM significantly induced MDA production after 1 hour of incubation (P≤0.0008, Fig.6). MDA is an end-product of enzymatic and oxygen radical-initiated oxidative decomposition of PUFAs and most frequently used as an indicator of lipid peroxidation. We have shown that the effect of AlCl$_3$ on sperm lipid peroxidation was dose- and time-dependent (Fig.6). There were no significant changes in sperm MDA formation observed following incubation with 0.5-30 mM of CdCl$_2$ or PbCl$_4$ (data not shown). Therefore, we only investigated the effects of flavonoids on MDA formation in AlCl$_3$-exposed sperm cells.
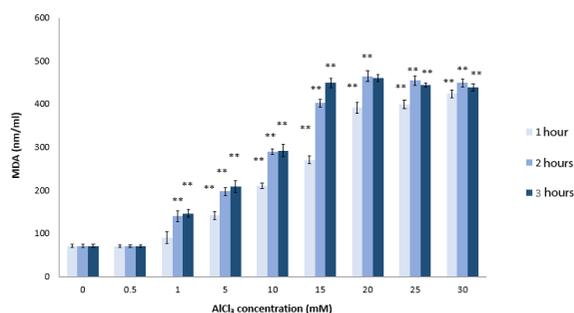

Fig.6: Sperm lipid peroxidation in the presence of aluminum chloride (AlCl$_3$). Sperm samples were treated with AlCl$_3$ (20 mM) for 2 hours at 37˚C. After incubation, we assessed the amount of lipid peroxidation of the sperm cells with MDA. **; P<0.01 compared to the untreated control group and MDA; Malondialdehyde.

Effects of flavonoids on lipid peroxidation of aluminum chloride-exposed sperm

Researchers previously reported the protective effect of ascorbic acid as an antioxidant against induction of lipid peroxidation by AlCl$_3$ in sperm cells (46). However, to the best of our knowledge there was no report about the protective effect

of flavonoids against lipid peroxidation in Al-exposed sperm cells. Moretti et al. showed that quercetin, rutin and, to a lesser extent, naringenin, significantly decreased tert-butyl hydroperoxide induced lipid peroxidation in human sperm (48). Their studies indicated that epicatechin was not efficacious as an antioxidant to protect sperm cells against oxidants. Our investigations showed that kaempferol was the most effective amongst the tested products in protection of sperm cells against $AlCl_3$-induced lipid peroxidation (Fig.7). Kaempferol, at a concentration of 100 μM, reduced MDA production from 250 nmol/ml (in untreated cells) to approximately 80 nmol/ml. Naringin and rutin were less effective in protection of $AlCl_3$-exposed sperm cells against lipid peroxidation compared to kaempferol. We observed that quercetin and catechin did not protect sperm. Quercetin, as an antioxidant, did not protect sperm cells against lipid peroxidation; rather, it had inhibitory effects on sperm motility. Khanduja et al. (49) have reported a significant decrease in sperm Ca^{2+}-ATPase activity following quercetin treatment. Ca^{2+}-ATPase is the responsible enzyme that provides energy for progressive movement of sperm cells. Inhibition of Ca^{2+}-ATPase activity has been shown to result in Ca^{2+} accumulation in the cells and blockage of the sperm motility apparatus (50).
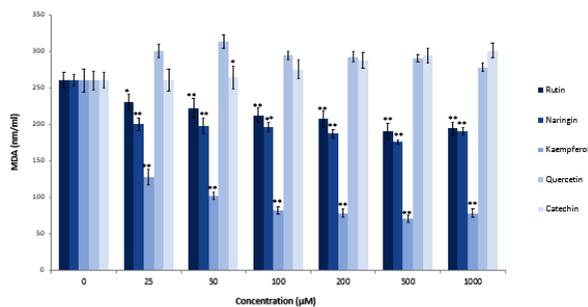

Fig.7: Effects of rutin, naringin, kaempferol, quercetin, and catechin on lipid peroxidation of aluminum chloride ($AlCl_3$)-exposed sperm. Sperm samples were treated with $AlCl_3$ (20 mM) and simultaneously incubated with different concentrations of rutin, naringin, kaempferol, quercetin, and catechin for 2 hours at 37˚C. After incubation, we assessed the lipid peroxidation of sperm cells with MDA. *; P<0.05, **; P<0.01 compared to the flavonoid untreated control group and MDA; Malondialdehyde.

Discussion

The impact of heavy metal toxicity, even at low concentrations, on the male reproductive system has been extensively investigated and confirmed

(51-54). Sperm motility depends on the synchronized actions of proteins, sugars, ions, and small organic molecules. It is one of the main factors that facilitates the journey of sperm toward the egg and the subsequent fertilization process (55). Defects in sperm motility are a common reason for infertility in humans (56). In the current study we have shown that $AlCl_3$, $CdCl_2$ and $PbCl_4$ significantly affected sperm motility. $PbCl_4$ had the most toxic effect.

Infertility due to metal toxicity usually occurs as a result of ROS induction (57). Therefore, antioxidant therapy is a promising strategy for treatment of individuals with heavy metal poisoning (58). Among natural antioxidants, flavonoids are more likely to exert protective activities against metal toxicity compared to carotenoids and vitamin E (37, 59). Based on our results, three flavonoids, rutin, naringin, and kaempferol have been shown to restore motility of $AlCl_3$-, $CdCl_2$-, and $PbCl_4$-exposed sperm cells. The other two flavonoids, catechin and quercetin, had no positive effects on motility of metal-exposed sperm; rather, they decreased sperm motility compared to untreated control samples.

We conducted additional research on the protective effects of flavonoids as antioxidant agents against heavy metal-induced lipid peroxidation. MDA formation was assessed in $AlCl_3$-exposed sperm cells treated with the five above mentioned flavonoids. Among flavonoids, quercetin due to its free radical scavenging and metal chelating abilities has been extensively investigated (60). However, according to the obtained results, quercetin and catechin did not protect sperm cells from ROS-mediated damages. They adversely affected sperm motility. Inhibition of sperm motility without considerable effects on peroxidation of PUFAs would indicate involvement of other inhibitory mechanisms. In contrast, increased motility of Al-exposed sperm cells treated with rutin, naringin and kaempferol was accompanied by decreased levels of MDA formation. We have concluded that antioxidant or chelating properties were not sufficient to protect sperm cells against the harmful damages of heavy metals. Flavonoids, as naturally occurring compounds may have some inhibitory effects on enzyme activities (49) or exert their growth inhibitory activities through binding to human receptors (61). Therefore, it is essential to

know the exact mechanisms of metal-induced toxicity and the properties of flavonoids before prescribing medications to combat the adverse effects of heavy metals on infertility.

Acknowledgments

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