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Curcumin down regulates INHBB and enhances the anti-oxidant defense system in Testes of Lead-treated Mice

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ABSTRACT

Infertility is a widespread health problem worldwide and the level of serum inhibin B is one of the main factors affects infertility in males. Alternatively, exposure to lead element has shown the strong induction of infertility. In the present study, for the first time we evaluated the effects of curcumin, a herbal compound which recently attracted much attention in herbal medicine, on the level of inhibin B expression in lead pre-exposed mice. Furthermore, we were seeking to determine if there is any relationship between the level of inhibin B and the presence of lead and how curcumin may exert synergistic effect. According to our data, curcumin remarkably decreased the negative effects of lead in lead-treated mice and reduced the expression of inhibin B. Here, we showed the effect of curcumin on testes of lead-treated mice and evaluated how it neutralizes oxidative stress in mice caused by exposure to lead. Curcumin is a strong candidate and is able to be used as a therapeutic compound in treatment of infertility.

Key words: curcumin, anti-oxidant, inhibin *B*

INTRODUCTION

Inhibin is a heterodimer glycoprotein hormone and a member of the TGF-β family, composed of two different subunits (α and either β_A or β_B) linked together by disulphide bonds [1, 2]. Although many molecular species are found in the circulation, only dimeric forms, inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$), are biologically active [3]. Inhibin B is the most accessible and an appropriate endocrine marker of spermatogenesis in subfertile men and is the principal circulating form generated in the fetal and adult males, whereas inhibin A is undetectable. Inhibin B secreted by Sertoli cells, regulates the level of FSH in serum through a negative feedback loop on the anterior pituitary. Serum level of inhibin B hereupon could conceivably be applied to directly study testicular function, and in combination with FSH measurement, it could be used as an indirect index for evaluation of reproductive hormones regulation [4].

In recent decades human occupational and environmental exposures to heavy metals such as lead have had adverse effects on their reproduction [5]. Several studies have reported the abnormalities of spermatogenesis as a result of lead exposure [6]. Furthermore, it has been shown that amounts of the blood lead ≥40 µg/dL are associated with reduction in semen volume, sperm count, motility and morphological alterations [7]. There are little data to suggest any relationship between lead toxicity and the production of inhibin B [8]. In 1992, Nathan et al. found that lead acetate weakly inhibits the total production of inhibin in Sprague Dawley rats [9] and the next year, in 1993, Foster et al. observed a decrease in the total inhibin/FSH ratio in a lead-treated monkey [10]. Later in 2005, Mahmoud et al.

suggested that exposure of Sertoli cells of 68 workers to excessive amounts of lead in the workplace resulted in overproduction of inhibin B, which might disrupt spermatogenesis [7]. Recently in 2009, Hsieh et al. found that spermatogenesis in male workers with a long-term exposure to lead was indirectly affected [11]. According to the harmful effects of heavy metals such as lead, finding a solution to retreat the patients and also to prevent the progression of the adverse effects is the must.

Over the past few years researchers have received lots of attention to herbal medicine. The reason may lie in the fact that active ingredients in herbal remedies usually have a biological balance, and hence, do not accumulate in the body and have fewer side effects. Furthermore, there are significant advantages for herbal medicine against chemical drugs such as effectiveness with chronic conditions, lower costs, and widespread availability [12]. Curcumin (diferuloylmethane), an active component and yellow pigment extracted from the rhizoma of turmeric (Curcuma longa) has been shown to remarkably prevent the generation of reactive oxygen species (ROS) and neutralize free radicals [13]. It has been demonstrates that curcumin has antioxidant, anti-proliferative, anti-carcinogenic and anti-tumor properties in a variety of cell lines and animals [14, 15]. These characteristics may make curcumin a suitable and potential candidate to treat infertility. Therefore, in the present study we seek to evaluate the ameliorating effects of curcumin on expression of inhibin B gene in lead-contaminated male NMRI mice.

EXPERIMENTAL SECTION

Chemicals and Reagents

Lead acetate, curcumin and corn oil were purchased from Sigma-Aldrich, Germany. RNA extraction kit (Qiagen, USA), cDNA synthesis kit (ThermoScientific, USA) and Real-Time PCR MasterMix (Bioneer, Korea) were purchased.

Animals

Totally 40 male NMRI mice, at 6-8 weeks age (Laboratory Animals Production Complex, Pasteur Institute of Iran, Tehran, Iran) were used in this study in full accordance to ethical guidelines for using laboratory animals. The mice were kept in cages with the ambient temperature of (24±2) °C, relative humidity of (60±10) %, and in 12-hour light-dark cycle. Free access to food in the form of dry pellets and water was allowed. Before the beginning of the experiment, all mice received basal diet for 10 days for adaptation and to ensure normal growth and behavior.

Animal protocol

Mice were divided into four groups, each contained 10 mice. The first group (group I) served as control and received vehicle, corn oil only (5 ml/kg body weight). Mice of the second group (group II) received lead acetate dissolved in drinking water at the dose of 25mg/L with 3 ml of 5% acetic acid per litre to keep the lead salt in solution. Mice of the third group (group III) received curcumin dissolved in corn oil at a dose of 15 mg/5 ml/kg body weight for two months by gastric gavages. Mice of the fourth group (group IV) received a combination of lead acetate and curcumin at the same dose and route used for groups II and III. At the end of the experimental period animals were left night fasted and at the next day they were euthanized following protocols and ethical procedures then testes were removed from the body.

RNA extraction

Testes tissue was frozen at -80^{oC}, and RNA was extracted using RNeasy Mini Kit. RNA quality was verified by ThermoScientific^{TM}NanoDrop 2000 UV-Vis Spectrophotometer (260/280 nm ratio) and ThermoScientific^{TM}Owl^{TM}EasyCast^{TM} B2 Mini Gel Electrophoresis Systems. These also showed the quality of the RNA extracted from the tissues. Only RNA samples with no degradation were processed further.

cDNA synthesis

Reverse transcription was carried out on 3–5 μγ of RNA using the Revert Aid TM First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. The cDNA was amplified by PCR using primers for the INHBB gene and for the β-actin gene (TAG Copenhagen A/S).

Quantitative Real-Time PCR

PCR was performed on BIONEER Exicycler^{TM} 96 Quantitative Real-Time PCR system. Commercial reagents (BIONEER AccuPower$^{®}$GreenStar^{TM}qPCRPreMix) and conditions were employed according to the manufacturer's protocol. PCR was performed under the following conditions: step 1, 95 ^{o}C (20 s) for 1 cycle; step 2, 95 ^{o}C (3 s); 60 ^{o}C (30 s) for 40 cycles. All samples were run in duplicate and were all within the standard curve. A serial dilution of known copy numbers of a PCR product served as reference (standard curve) providing a relative quantification of the unknown samples. Target gene expression was related to the housekeeping genes β-actin (forward: 5´-AAACTGGAACGGTGAAGGTG-3´ reverse: 5´-TATAGAGAAGTGGGGTGGCT-3´) and INHBB (forward: 5´-

TCTGCATCATTGCAGAGACAG-3´ reverse: 5´-TTCTTCTCCACCACATTCCAC-3´), following the manufacturer's instructions. Values were standardized to β-actin and expressed as a percentage of the control. Similar data were obtained when the values were standardized to INHBB. Suitable controls were carried out to eliminate any potential for spurious amplification from contaminated genomic DNA.

Immunoblotting for INHBB

Testis tissue homogenates were prepared in protease inhibitor cocktail (P8340, Sigma-Aldrich, Germany) in 0.9% NaCl. The tissue was homogenized in suspension buffer. Then, the lysate was centrifuged at 300 g for 10 minutes. The salts were removed, and again the supernatant was undergone a centrifugation at 10000 g for 30 minutes. The supernatant was collected and the eluate was then freeze-dried at -70 ^{o}C. The protein was diluted to 1:2 in a solution of 10% sodium dodecyl sulfate (SDS), glycerol, 10% bromophenol blue, and 5% b-mercaptoethanol in 0.5 M TRIS buffer, pH 6.8. The samples were boiled for 5 minutes. Denaturated 12% SDS polyacrylamide gels were prepared, and 50 mL samples were loaded into the wells. Gels were run for 90 minutes at 100 volt and then proteins were transferred to a nitrocellulose membrane for 60 minutes by using a 250mA current. The membrane was blocked using blocking buffer for 1 hour at room temperature. Then the membrane was incubated overnight at 4oC with anti INHBB antibody (Abcam, Ireland), diluted 1:1000. After incubation, the membrane was washed with PBS 0.05% Tween solution three times and then was incubated for 1 hour in 1:1000 dilution of horseradish peroxidase–conjugated goat anti-rabbit (Abcam, Ireland). Strips were washed three times with PBS 0.05%Tween solution and the proteins of interest was detected using li-cor imager.

Oxidative status of the cell

The testes of animals were removed, after they were put to sleep with ketamine injection of 100 mg/kg m.c. and dislocation of cervical vertebrae, and used for further investigation. They were weighed and frozen at −80 °C. For performing biochemical tests, the samples were thawed, washed with 0.9% NaCl plus EDTA, homogenized in ice-cold 20 mM Tris-HCl Buffet (pH = 7.4), and centrifuged for 10 min at 4 °C and 14600 g. To measure the level of reduced glutathione (GSH) Bioxytech GSH-400 kit (OXIS) was used. For evaluation of the SOD enzymatic activity in testes tissue, aliquots of 500 mg of the frozen samples were homogenized in phosphate buffered saline (PBS) and centrifuged at 4000 g at 4 ^{o}C. Afterwards, SOD concentrations were assayed using specific enzyme-linked immunosorbent (ELISA) assay.

Statistical analysis

Comparisons between groups were made using unpaired Tukey's HSD post-hoc test. Results were considered statistically significant if the $P<0.05$. Where appropriate the data were also analyzed by one-way ANOVA.

RESULTS

Quantitative analysis of Inhibin B gene expression

To investigate the expression of Inhibin B Real time PCR performed. First, to ensure the quality of the extracted RNA, gel electrophoresis was used for both the Lead+Curcumin treated and control mice. As illustrated in the fig. 1 the presence of 18S and 28S is an indicative of the high quality of the performance. To quantitatively confirm the extracted mRNA, the absorbance was read at 280 and 260 nm using NanoDrop. The ratio was calculated in the range of 1.9-2 which confirm the absence of contamination.

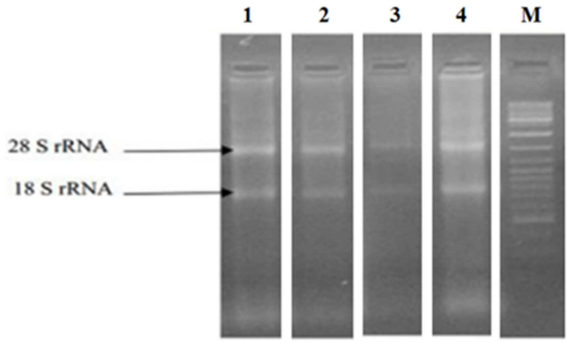


Figure 1. Qualitative analysis of the extracted RNA. Lanes 1 to 4; samples which introduced respectively in Material and Method and M is Marker

Effects of Lead and Curcumin on expression of Inhibin B using Real-Time PCR

The curves of Real-Time PCR confirm the formation of the products. Moreover, the products were analyzed through melt curves and gel electrophoresis. The pattern of melt curve for Inhibin B is at 90/65 ^{o}C and for β-actin is at 86/46 ^{o}C. Furthermore, the specific amplification of the desired fragments were evaluated by gel electrophoresis (Fig. 2).

The change in gene expression was evaluated in Lead+Curcumin treated mice and control group. The ratios of Inhibin B expression to reference gene (β-actin) were 7.06±0.57 ($p<0.001$) for Lead-treated mice, 0.2±0.23 ($p<0.001$) for Curcumin-treated mice and 0.516±0.13 ($p<0.001$) for Lead+Curcumin treated mice. The level of Inhibin B and β-actin expression were 1.09±0.76 and 1.08±0.94 respectively in control group which shows that Curcumin exclusively has much more effect on reduction of Inhibin B expression rather than in combination with lead (Fig. 3).

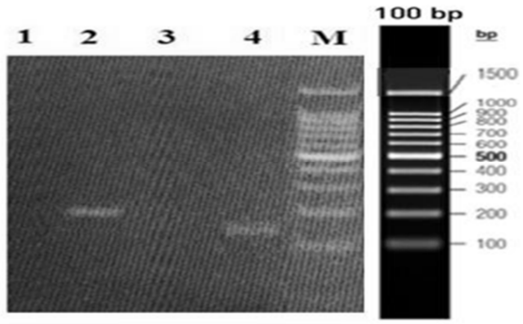


Figure 2. Gel electrophoresis of amplified β-actin and INHBB genes. Lane 1: lack of template reaction for β-actin; Lane 2: product of amplified INHBB gene (145 bp fragment); Lane 3: lack of template reaction for INHBB, Lane 4: product of amplified β-actin gene (195 bp fragment)

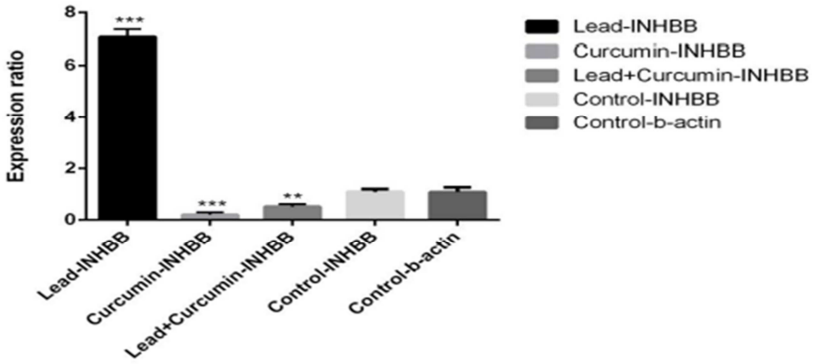
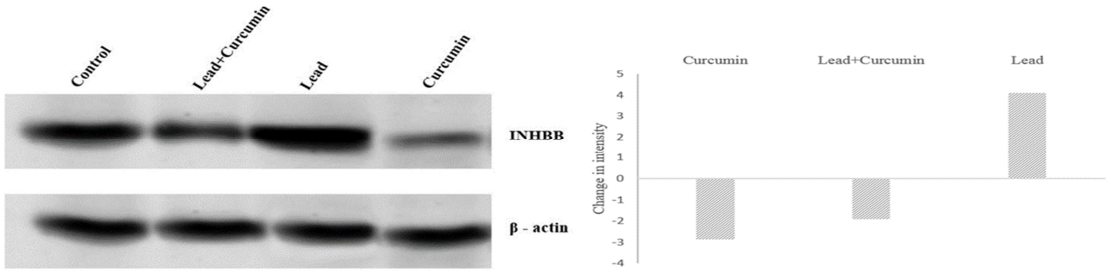


Figure 3. The level of INHBB expression in four groups of experiment include lead treated, curcumin treated, lead+curcumin treated and non-treated mice as a control group

The rate of expression compared with housekeeping gene β-actin (P<0.05, P<0.01**, P<0.001***: n=10).*

Immunoblotting for INHBB

As shown in figure. 2 the presence of INHBB was detected in different groups of mice. In order to further analysis of the level of proteins in each group, using ImageJ software the intensity of bands were quantified. Accordingly, the level of INHBB was increased 4.1 fold in lead treated mice, whereas in curcumin treated mice and those of co-treated with curcumin and lead, the level of INHBB decreased 2.86 and 1.91 fold respectively. All the fold changes were calculated compare to control group (fig. 4).



A **B**

Figure 4. Immunoblotting for INHBB. (A) Immunoblotting analysis of INHBB (top) in different groups of mice, each treated with different substance and β-actin as a control (bottom). (B) Quantification of immunoblotting data using ImageJ to further demonstrate the changes in the protein level

Level of GSH and superoxide dismutase concentration

Figure 6 shows that GSH concentration decreased 55.44 percent compared to control mice. In addition, the level of GSH was not significantly affected by curcumin treatment. In curcumin and lead co-treated mice GSH lowered 11.6 percent in comparison with control group. In parallel to these data, the concentration of SOD was increased significantly in those mice treated with lead (1.92 fold) as compared to control mice, whereas, a significantly decreased level of SOD concentration was observed in the group which had been co-treated with curcumin and lead (1.35 fold) with respect to non-treated group. Here, curcumin decreased the effect of lead in increasing the SOD concentration 1.42 fold. The SOD concentration was not increased significantly in those mice treated with curcumin and the level of SOD concentration seems to be at similar level as control group (Fig. 5, 6).

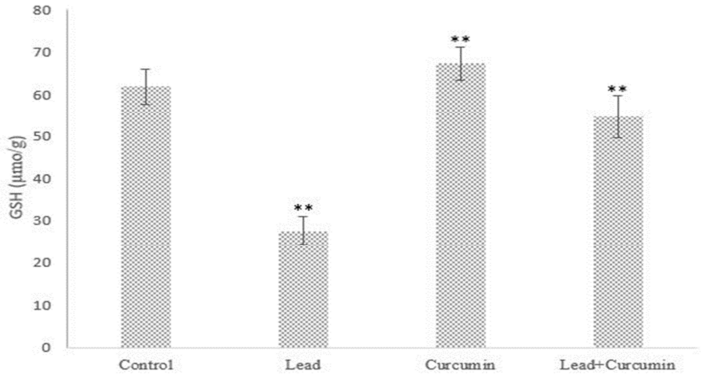


Figure 5. Concentration of reduced glutathione in testicular homogenates of four groups of mice (P<0.01)**

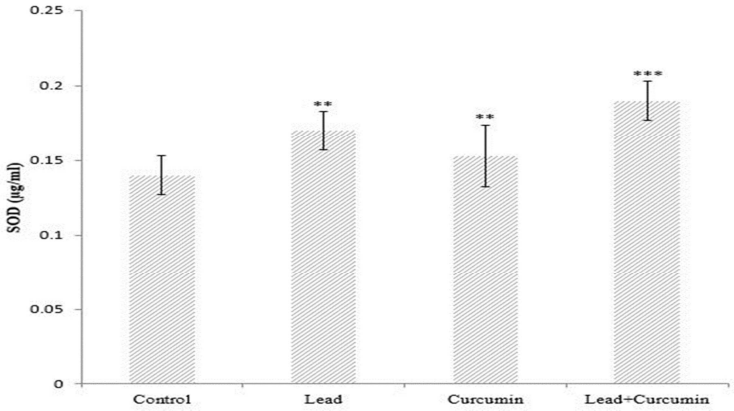


Figure 6. Concentrations of superoxide dismutase in four groups of mice, each treated with different substances (P<0.01, P<0.001***)**

DISCUSSION

Infertility is one of the most serious social problems in both developed and developing countries, affecting almost 15 percent of couples, in half of whom a male factor is involved. Social factors such as marriage at older ages, environmental factors such as pollution, and genetic factors such as chromosomal disorders and mutations all contribute to increasing infertility. According to a WHO report, the rate of infertility ranges between 12 to 15 percent worldwide. This is an important issue in developing countries such as Iran, which is confronting a high rate of contamination.

Inhibin B is one of the endocrine hormones synthesized and secreted from Sertoli cells in the male testis and is a member of TGF-β family. It is a heterodimer glycoprotein with one α and two distinct β subunits, attached by a disulfide bridge (Barakat et al., 2008). The subunits of inhibin appear to be regulated by FSH both *in vivo* and *in vitro*, as FSH receptors stimulate cAMP production, which suggests that inhibin B subunits may be regulated through signals dependent on protein kinase A [16]. It has been reported that the α subunit is stimulated in a protein kinase A-mediated mechanism and simultaneous actions of protein kinase A and C, which increases the βA subunit via AP-1 and attachment to CRE [17]. The role of cAMP in Sertoli cells is not yet clear. Inhibin B regulates the secretion of FSH from pituitary through negative feedback, while FSH inversely stimulates the secretion of inhibin B [18]. As FSH is one of the main hormones in the stimulation of spermatogenesis, any increase or decrease in the synthesis and secretion of FSH may disrupt that process. Therefore, in any number of conditions—whether

environmental, physiological, pharmaceutical, or pathological—the expression of inhibin B and its secretion may increase and disrupt the spermatogenesis. Many researchers believe that inhibin B is a more sensitive indicator for revealing sperm in azoospermic patients than the prevalent methods of testis size and serum FSH [19].

Lead is a dangerous and toxic element used in many industries. In human bodies, it prevents heme synthesis, disrupts calcium homeostasis, increases reactive oxygen species (ROS), and concentrates inhibin B in serum [20]. Lead can cause infertility in males through interaction with Na+/K+ channels in the testes or by inhibition of alkaline phosphatase activity [21]. It has been proven that exposure to industrial chemicals and metals such as cadmium and lead cause oxidative stress in the testes. Mammalian testes and spermatozoa contain polyunsaturated fatty acids that are the reason for sperm fluidity but also make them vulnerable to oxidative stress [22]. As a result, ROS may induce disturbance in the structure and function of spermatozoa. ROS production acts as a stimulator for FSH secretion, which in turn suppresses or inhibits completely the generation of ROS [23]. Subsequently, the increased amounts of FSH stimulate the secretion of inhibin B by binding to receptors on Sertoli cells. Therefore, as indicated in Figure 3, an increase in the level of inhibin B expression is observed when treated by lead.

Herbal compounds are widely used for treatment of large number of disorders [24-26]. Curcumin is among the strongest antioxidants. The most important feature of curcumin is that it is highly effective pharmaceutically, especially with regard to ROS, with no known side effects. Research has demonstrated that curcumin decreases the expression of NF-κB, an antiapoptotic factor, and increases the production of ROS, which in turn increases NF-κB. These effects have been observed in mice treated with curcumin alone. Under curcumin treatment, ROS generation increases and influences cells towards apoptosis [27]. Here, curcumin exerts its effect through the activation of p53, a tumor-suppressing protein. p53 induces the oligomerization of BAX and Bak, which are responsible for the release of cytochrome c and the initiation of intrinsic apoptosis pathways [28]. On the other hand, p53 has an antagonistic effect on Bcl-2 and Bcl-XL, which prevent the leakage of mitochondrial contents. Furthermore, curcumin directs cells towards apoptosis through the activation of caspases-3, -7, and -9 [27, 29]. As a result of these processes, cells undergo apoptosis and, as demonstrated in Figure 3, inhibin B expression will be less than in the control group of non-treated mice. When mice were treated with lead and curcumin simultaneously, the level of inhibin B expression decreased remarkably compared to mice treated with lead alone. This might be due to the antioxidant properties of curcumin. It has been reported that curcumin is able to inhibit the transcription factor AP-1(Activator Protein 1), which plays a role in inhibin B expression, so the rate of inhibin B expression decreases as a result.

Heavy metals such as lead cause oxidative stress due to the generation of the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•) [30]. To protect organisms against these harmful compounds, there is a complex defensive system of enzymatic antioxidants including superoxide dismutase (SOD), which is responsible for the dismutation of O_2•− anions into H_2O_2 and molecular oxygen, glutathioneperoxidase (GPx), which can reduce hyperoxides, glutathione reductase, and catalase, which detoxifies H2O2 into water; non-enzymatic antioxidants including glutathione (GSH) and vitamins C and D have been developed [31]. It appears that curcumin is able to modify these defensive enzymes in order to act with greater efficiency. Some possible explanations can be offered. First, curcumin may attach itself to the enzyme and change the conformational structure in such a way that the catalytic site is exposed to substrates much more easily. Second, the presence of curcumin may act as an additive in cell contents, which can lead to increasing enzymatic activity. This phenomenon appears to be a result of improvement in the kinetic properties of the enzymes. It is possible that curcumin increases the stability of the enzymes noted above, which may lead to a longer-term presence of the specific number of enzyme molecules in the cell. Moreover, treatment with curcumin may increase the affinity of enzymes with substrates and catalytic activity. In other words, at specific time intervals, ROS compounds are neutralized in greater numbers. The last possible reason may be the effect of curcumin on reducing the inhibitory effects of various agents in the cell. Inhibitory effects of intracellular compounds on the activity of enzymes have been reported previously for different types of enzymes. To investigate further the effects of curcumin on the enzymatic defensive system, in vitro studies are required; our most recent results will be published in the near future.

As noted above, this study aims to determine the role of curcumin in the expression of the inhibin B gene and its involvement in spermatogenesis and male fertility in the context of lead contamination. In the present study, lead-treated mice showed a sevenfold increase in the expression of inhibin B compared to the control group, which is statistically significant. This result shows that lead can increase the level of inhibin B expression. In curcumin-treated mice the rate of expression decreased almost five and a half times when compared to the control group. In lead+curcumin-treated mice, two-fold decrease was observed for the expression of inhibin B. Based on the results obtained in mice treated with curcumin and lead simultaneously, curcumin decreased the effects of lead and therefore reduced the expression of inhibin B, compared to control group.

CONCLUSION

Curcumin significantly decreased the negative effects of lead in lead-treated mice and reduced the expression of inhibin B. Since decreases in the expression of this gene increase the synthesis and secretion of the inhibin B hormone and trigger FSH secretion, this might play a role in improving spermatogenesis and fertility. Curcumin, with its powerful ability to inhibit ROS, is effective in the promotion of spermatogenesis.

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