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Possible Effects of Metallosis on Spermatozoal Apoptotic Genes Expression in Individuals with Intramedullary Nailing Prosthesis

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Abstract Seminal quality could be affected by metallosis caused by intramedullary nailing (IMN). Our objectives were to estimate metal ion levels in the seminal plasma of subjects with IMN, to determine their effects on semen parameters and on spermatozoal apoptotic gene expression, and to determine whether these expressed genes could be used as candidate biomarkers of seminal deterioration in individuals with IMN or not. Semen samples were collected from 60 subjects with IMN and 30 age-matched healthy controls. Seminal plasma contents of cobalt (Co), chromium (Cr), and molybdenum (Mo) were assayed. Spermatozoal Bcl-2 and Bax gene expressions were determined. Studied semen parameters were significantly lower in subjects with IMN for ≥5 years in relation to controls while the concentrations of Co, Cr, and Mo in the seminal plasma samples were significantly higher. There were significantly lower spermatozoal Bcl-2 expression, higher Bax expression, and lower Bcl-2/Bax ratio in subjects with IMN for ≥5 years than in controls. In subjects with IMN for ≥5 years, receiver operating characteristic (ROC) curve analysis of studied gene expressions and Bcl-2/Bax ratio were done showing priority of the ratio with 86.7 % sensitivity, 100 % specificity, 100 % positive predictive value, and 93.8 % negative predictive value at cutoff values ≤0.777. Co, Cr, and Mo metals are found at high concentrations in the seminal plasma of individuals with IMN leading to increased spermatozoal apoptotic activity. Spermatozoal Bcl-2/Bax ratio could be used as a candidate biomarker of reproductive disorders in individuals with intramedullary nailing.

Keywords Semen · Intramedullary nailing · Apoptosis · Bcl-2 · Bax

Abbreviations

Co	Cobalt
Cr	Chromium
FSH	Follicular stimulating hormone
IMN	Intramedullary nailing
Mo	Molybdenum
ROC	Receiver operating characteristic

Introduction

Heavy metals are possible pollutants that may be harmful to semen quality because of their direct effect on testicular function or hormonal alterations [1]. The materials used in modern fixation of fractures are well tolerated by the human body; however, it is not totally biologically inert. All metal implants may undergo corrosion and wear in vivo, which may result in the production of metal ions and surface degradation particles [2]. In fracture fixation devices such as intramedullary nails (IMN), movement and micromotion of the implant may facilitate the shedding of metal ions and degradation particles and greatly increase the total surface area of contact between the implanted material and the biological environment. Sequentially, small quantity of metallic ions from the implants

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will be released into the local soft tissues and general circulation [3].

Infertility affects approximately 10–15 % of reproductive age couples. Poor semen quality contributes to about 25 % of infertile cases. The causes of poor semen quality are complex, and an increasing number of reports suggest that the environmental, industrial, and dietary agents may affect male fertility in human [4]. One of these factors is heavy metals, which can affect the testis size, semen quality, the secretory function of the prostate and seminal vesicles, and the reproductive endocrine function [5].

Apoptosis is an important mechanism that prevents damaged cells from proliferating and also controls cell populations. The Bcl-2 family includes both proapoptotic (such as Bax) and antiapoptotic (such as Bcl-2) effector proteins [6]. Bcl-2 protein is a potent repressor of apoptosis, contributing to the selection and maintenance of long living cells. The proapoptotic Bax protein resides in the cytosol or is loosely attached to cell membranes. In response to cytotoxic signals, it translocates into the mitochondria where it triggers cytochrome c release which in turn activates the caspase-dependent DNA fragmentation [7]. Bcl-2 and Bax share homology and heterodimerize to antagonize the effects of each other. The Bcl-2/Bax ratio has been considered to be an apoptotic index, and it seems to be a main determinant of relative resistance to cell death-inducing stimuli [8].

The aim of the current study is to estimate metal ion levels in the seminal plasma of subjects who had undergone intramedullary nailing, to determine the effect of metal ions on semen quality and parameters, to investigate the possible effects of seminal metallosis on spermatozoal apoptotic gene expression, and to determine whether these expressed genes could be used as candidate molecular biomarkers of reproductive disorders in individuals with intramedullary nails or not.

Subjects and Methods

Selection of Subjects

This case control study was performed according to the principles of Declaration of Helsinki, and the procedures were approved by the local ethics committee. All subjects had provided written informed consent. The study was carried out on 60 subjects with intramedullary nailing ranging in age from 29 to 58 years and 30 age-matched healthy controls (group I) with no metal implant in their bodies. Subjects with previous history of occupational exposure, administration of drug containing metal formula, varicocele, and smoking were excluded from the study. Subjects with intramedullary nailing were classified into two subgroups: individuals with IMN of less than 5 years (group IIa) and those with IMN of more than 5 years (group IIb), according to Nikolaou et al. [9].

Samples Collection

Semen samples were taken from all participants who were enrolled from Andrology and Infertility as well as Orthopedic Clinics, Mansoura University Hospital and referred to Medical Biochemistry Department, Mansoura Faculty of Medicine. All semen samples were collected by masturbation after an abstinence period of 48–72 h and allowed to liquefy completely for 15 min at 37 °C.

Semen Analysis

After complete liquefaction, computer-assisted semen analysis (Autosperm, Fertipro, Belgium) [10] was used to assess sperm quality parameters, including semen quantity, sperm count, sperm motility, and sperm morphology according to the World Health Organization (WHO) method [11]. The criteria for normozoospermia were a concentration of $\geq 15 \times 10^6$/ml, with sperms of progressive motility more than 32 % of spermatozoa, and normal morphology with oval-shaped head and no irregularities of tail in at least 4 % of the spermatozoa. Sperm morphology was evaluated by phase contrast microscope and sperm Mac stain (Fertipro, Belgium). Peroxidase-positive white blood cells (WBCs) were detected by peroxidase stain [12].

Assay of Metal Ions in Seminal Plasma

According to Sarada and Ramasastri [13], 2–5 μl of each seminal plasma sample were diluted with 1 N HCl to about 20 % by volume for digestion. Samples were kept in polyethylene containers that were cleaned using multistep acid leaching and frozen immediately at −20 °C until analysis. The concentrations of chromium (Cr(VI)), cobalt (Co), and molybdenum (Mo) samples were quantified using inductively coupled plasma-mass spectroscopy (ICP-MS) analysis (LC-MS-MS system, Agilent 7700S) according to the manufacturer's instructions. The standards for calibration of the heavy metals (Cr(VI), Co, and Mo) were purchased from Sichuan Xinju Mineral Resource Development Stock Co., Ltd. The calibration range was 1 to 1,000 μg/l. Standard solutions were prepared from single-element stock for each metal (1,000 μg/l) and diluted in the same HCl as the samples. Standard calibration curves were produced by the equipment.

Bcl-2 and Bax Gene Expressions

Total RNA Extraction

After liquefaction, 1 ml of each collected sample was taken into tubes containing 2 ml of RNA later solution (Sigma). Cells were pelleted by centrifugation (500*g*, 10 min, 4 °C).

Total RNA was isolated by the RNeasy Plus Micro Kit (catalogue number 74134, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The concentration of isolated RNA was assessed spectrophotometrically by measuring the optical density (OD) at 260 nm (Jenway, Genova, UK). The RNA yield (μg/μl)=A260×40×dilution factor [14]. The quality of extracted RNA was assessed by using formaldehyde agarose gel electrophoresis.

Semiquantitative RT-PCR

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the QIAGEN OneStep RT-PCR Kit (QIAGEN Inc., 28159 Avenue Stanford, Valencia, USA) according to the method of McPherson and Moller [15]. The reaction passed as follows:

Synthesis of cDNA A reaction mix (40 μl/reaction) was prepared as follows: 2 μl of first strand primer, provided by the kit; 3 μl containing 30 pmol of PCR gene-specific primer (sense); 3 μl containing 30 pmol of PCR gene-specific primer (antisense); 2 μl OneStep RT-PCR enzyme mix; 10 μl 5× OneStep RT-PCR buffer; and 20 μl of DEPC-treated water to obtain a total volume of 40 μl. Then, 10 μl of each template RNA were added in each tube and mixed well. One tube was prepared as a negative control reaction to test for DNA contamination. The reactions were transferred to TECHEN TC-312 thermal cycler and incubated at 50 °C for 30 min for synthesis of complementary DNA (cDNA) followed by incubation at 95 °C for 15 min to inactivate the reverse transcriptase and completely denature the template.

Amplification of cDNA by PCR Gene-specific primers were purchased from Biolegio BV, Nijmegen, the Netherlands. The oligonucleotide primers for Bcl-2, forward (21 mer) 5′-TTGT GGCCTTCTTTGAGTTCG-3′ and reverse (21 mer) 5′-TACT GCTTTAGTGAACCTTTT-3′, were designed on the basis of the human Bcl-2 (332 bp). Also, the oligonucleotide primers for Bax, forward (21 mer) 5′-AGACAGGGGCCCTTTTGC TTC-3′ and reverse (21 mer) 5′-GAGCACTCCCGCCACA AAGAT-3′, were designed on the basis of the human Bax (482 bp) [16]. Two oligonucleotide primers, forward (20 mer) 5′-AAGAGAGGCATCCTCACCCT-3′ and reverse (20 mer) 5′-TACATGGCTGGGGTGTTGAA-3′, were also used to amplify β-actin as an internal control (218 bp) [17]. Thermal cycling reaction was performed with the following program: 32 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C (Bcl-2), 58 °C (Bax), and 60 °C (β-actin) for 1 min, and an extension at 72 °C for 1 min then a final extension at 72 °C for 10 min.

Detection of Amplified RT-PCR Products

Specific PCR products were subjected to agarose gel electrophoresis using 2 % agarose stained with ethidium bromide and visualized via light UV Transilluminator (Model TUV-20) and photographed under fixed conditions (the distance, the light, and the zoom) (Fig. 1). Minus RT controls were permitted to rule out genomic contamination. Similarly, no products were detected when the RT-PCR step was carried out with no added RNA, indicating that all reagents were free target sequence contamination. The intensities of Bcl-2, Bax, and β-actin bands were determined using ImageJ software which performs bands detection and conversion to peaks. Areas under each peak were calculated in square pixels and used in quantification. Calculating the relative ratio of expression of Bcl-2 and Bax was done in relation to the endogenous reference gene β-actin (by calculating the ratio between the square pixel values of Bcl-2/β-actin and Bax/β-actin).

Statistical Analysis

The data were expressed as mean ± standard error of mean (mean ± SEM). Data were processed and analyzed using MedCalc version 9.3. Results were compared by using one-way analysis of variance (ANOVA) test and the two-tailed Student's *t* test. Pearson correlation coefficient was used to study the correlation between different variables in all groups. A minimum level of significance is considered if $P \leq 0.05$. Receiver operating characteristic (ROC) curve analysis was used to assess the value of Bcl-2 and Bax expressions as well as Bcl-2/Bax ratio in the screening of apoptosis as a possible adverse effect of seminal metallosis.

Results

In the current study, the included subjects aged from 29 to 58 years with mean age of 36.13±1.26, 41.87±2.23, and 39.80±1.49 for group I, group IIa, and group IIb, respectively. The mean durations of IMN were 2.63±0.27 years for group IIa and 6.73±0.37 years for group IIb. As shown in Table 1, comparing subjects in group IIb with controls shows that all studied semen parameters were significantly lower while the concentrations of cobalt, molybdenum, and chromium in the seminal plasma samples were significantly higher.

Spermatozoal Apoptotic Gene Expression

Regarding Bcl-2, Bax gene expression, and Bcl-2/Bax ratio, samples were significantly different in between all groups ($P<0.001$). There were significantly lower Bcl-2 expression ($P<0.0001$), higher Bax expression, and lower Bcl-2/Bax ratio in semen samples of subjects having IMN for ≥5 years

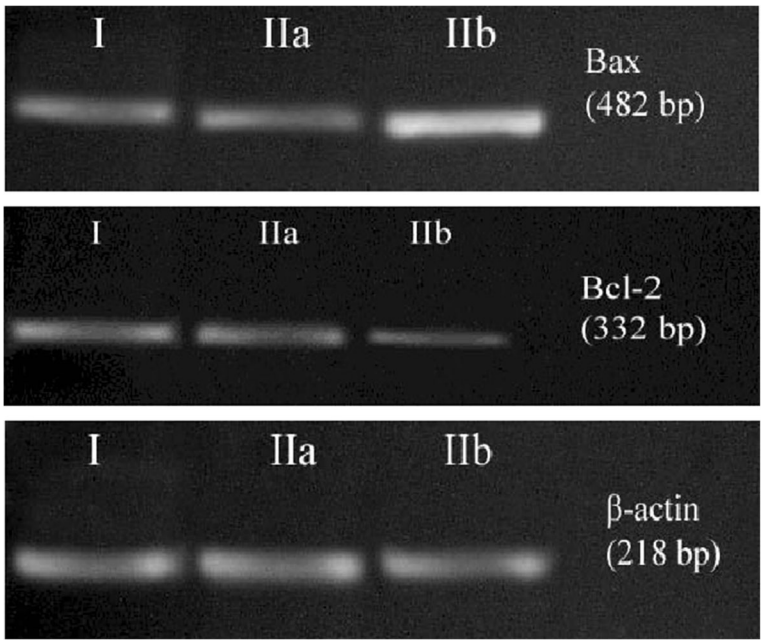

Fig. 1 Bcl-2 and Bax gene expressions in seminal samples of all groups: *I* control group, *IIa* subjects with IMN for <5 years, *IIb* subjects with IMN for ≥5 years

(group IIb) than those in controls (group I). However, nonsignificant differences were found, in exception of molybdenum ($P=0.0454$), between subjects having IMN for <5 years (group IIa) and controls. On the other hand, our results showed significantly lower levels of semen parameters, Bcl-2 expression, and Bcl-2/bax ratios in semen samples of subjects of group IIb than in subjects of group IIa while all studied metals and Bax expression were significantly higher in group IIb (Table 1).

With regards to the Pearson correlation coefficient, both Bcl-2 expression and Bcl-2/Bax ratio have significant negative correlation with the duration of nailing in years as well as

Table 1 Seminal analysis data and gene expression study of both Bcl-2 and Bax in seminal samples of all studied groups

Variable	Group I ($n=30$)	Group IIa ($n=30$)	Group IIb ($n=30$)	F P (ANOVA test)
Sperm concentration (million/ml)	136.17±13.68	90.84±13.32[a]	62.43±13.21[b,c]	7.696 0.001
Total sperm count (millions)	268.02±49.10	243.55±27.16	147.29±20.6[b,c]	3.421 0.042
Progressive motility (%)	58.46±2.56	56.73±2.18	44.51±3.82[b,c]	6.690 0.003
Normal morphology (%)	64.58±2.03	58.52±2.57	55.15±2.45[b]	4.094 0.024
Cobalt (μg/l)	1.96±0.13	2.06±0.0094	3.15±0.031[b,c]	77.161 <0.001
Molybdenum (μg/l)	1.87±0.10	2.12±0.06[a]	5.69±0.28[b,c]	148.406 <0.001
Chromium (μg/l)	42.55±2.73	47.46±0.35	85.42±6.13[b,c]	36.596 <0.001
Bcl-2 expression	0.55±0.017	0.51±0.018	0.34±0.035[b,c]	20.256 <0.001
Bax expression	0.23±0.017	0.298±0.033	0.54±0.028[b,c]	36.907 <0.001
Bcl-2/Bax ratio	2.62±0.26	1.98±0.22	0.71±0.13[b,c]	21.947 <0.001

Group I control group, *Group IIa* group with IMN of <5 years, *Group IIb* group with IMN of ≥5 years

[a] P significance of IMN of <5 years compared to control (*t* test)

[b] P significance of IMN of ≥5 years compared to control (*t* test)

[c] P significance of IMN of <5 years relative to IMN of ≥5 years (*t* test)

the studied metal levels. Moreover, significant positive correlations were found between Bax expression and the duration together with the studied metal levels (Table 2).

ROC Curve Analysis of Apoptotic Gene Expression

In subjects with IMN for ≥5 years, ROC curve analysis of Bcl-2 gene expression in seminal samples showed 86.67 % sensitivity, 93.33 % specificity, 86.7 % positive predictive value, and 93.3 % negative predictive value at cutoff values ≤0.427 ($P<0.0001$). Also, ROC curve analysis of Bax gene expression showed 93.33 % sensitivity, 90 % specificity, 82.4 % positive predictive value, and 96.4 % negative predictive value at cutoff values >0.317 ($P<0.0001$). Moreover, ROC curve analysis revealed that Bcl-2/Bax ratio had 86.7 % sensitivity, 100 % specificity, 100 % positive predictive value, and 93.8 % negative predictive value at cutoff values ≤0.777 (Table 3 and Fig. 2b).

Discussion

The human male has a relatively low fertility potential compared with other mammals and is much more susceptible to metal toxicity [18]. Metals may affect the male reproductive system directly, when they target specific reproductive organs, or indirectly, when they act on the neuroendocrine system [19]. However, data on reproductive toxicity in men are scanty for most metals [20, 21].

Metal Ions in Seminal Plasma

The current study aimed to assay metal ion levels in seminal plasma of subjects who had undergone IMN and to determine the effect of metal ions on semen quality and parameters, as well as to investigate the possible effects of seminal metallosis on apoptotic gene expression of spermatozoa and to determine whether it could be used as a candidate molecular biomarker of male reproductive disorder in individuals with intramedullary nailing.

The results of the current study showed that the concentrations of cobalt, molybdenum, and chromium VI in the seminal plasma samples were higher in subjects having intramedullary nails being compared to control subjects. The presence of the studied metals in the control subjects can be explained by the presence of these trace elements in food, drinking water, and in multivitamin/multimineral supplements [22]. On the other hand, it is reported by Patton et al. [3] that all metal implants in vivo may undergo corrosion and wear, which will result in the production of metal ions and surface degradation particles. In fracture fixation devices such as intramedullary nails, this phenomenon may be facilitated further by the movement and micromotion of the implant, galvanic corrosion between materials of different composition in close proximity, and fretting corrosion between the nail-locking screw interface [3]. Also, the significantly high level of those metals in the studied groups with IMN goes in accordance with the results of Nikolaou et al. [9], who stated that the increase of Co and Cr levels is a well-proven fact in patients with metal-on-metal total hip arthroplasty, as a result of wear of the implants and their release into the blood, urine, and other body fluids. They found that Co and Cr ion levels were elevated by five and three times, respectively, compared with their reference groups. Jacobs et al. [23] reported that there is a ninefold elevation in serum Cr and threefold elevation in serum Co concentrations in patients with long-term use (more than 20 years) of hip arthroplasties, as compared to controls. These results are in agreement with the results of the current study as the increase in seminal plasma level of these elements is higher in those with IMN of a period more than 5 years than those with less than 5 years (Table 1).

Metallosis Effects on Seminal Parameters

In the current study, data of seminal parameters revealed decrease in sperm concentration and progressive motility as well as deterioration of sperm morphology in the studied group (less and more than 5 years IMN) when compared to the control group and in those with IMN of more than 5 years being compared to those with less than 5 years. However, all studied semen parameters were within normal ranges provided by WHO 2010 criteria (Table 1). These results coincide with the results of Meeker et al. [22], Li et al. [24], Kumar et al. [25], and Geoffroy-Siraudin et al. [26]. The influence of metals especially chromium VI on semen parameter could be explained by the fact that they increase follicular stimulating hormone (FSH) level in the serum which in turn leads to reduction of sperm count and sperm density. Also, disruption in germ cell arrangement within the seminiferous tubules and the decrease in the diameter of the seminiferous tubules are

Table 2 Correlations between different studied variables in all studied groups

		Bcl-2 expression	Bax expression	Bcl-2/Bax ratio
Duration of nailing	*r*	−0.330	0.571	−0.469
	P	0.0750	0.0010[a]	0.0090[a]
Cobalt (μg/l)	*r*	−0.676	0.690	−0.540
	P	<0.0001[a]	<0.0001[a]	0.0001[a]
Molybdenum (μg/l)	*r*	−0.769	0.843	−0.726
	P	<0.0001[a]	<0.0001[a]	<0.0001[a]
Chromium (μg/l)	*r*	−0.638	0.726	−0.643
	P	<0.0001[a]	<0.0001[a]	<0.0001[a]

[a] Significant *P* in Pearson correlation coefficient

Table 3 ROC curve analysis of Bcl-2 and Bax gene expressions as well as Bcl-2/Bax ratio in seminal samples of subjects with IMN for more than 5 years

	AUC (95 % CI)	Cutoff value	Sensitivity (%)	Specificity (%)	+PV (%)	−PV (%)	*P*
Bcl-2 expression	0.873 (0.740–0.954)	≤0.427	86.67	93.33	86.7	93.3	<0.0001
Bax expression	0.949 (0.839–0.992)	>0.317	93.33	90	82.4	96.4	<0.0001
Bcl-2/Bax ratio	0.949 (0.839–0.992)	≤0.777	86.67	100	100	93.8	<0.0001

AUC area under curve, *+PV* positive predictive value, *−PV* negative predictive value

possible suggested effects of Cr(VI) which lead to the decrease in sperm counts [24]. Cr(VI) caused a decrease in zinc concentration in seminal plasma which plays an important role in the oxidant defense system, an effect that may impair the maturation of spermatid cells. In addition, in vitro studies have demonstrated that Cr(VI) can cause a variety of cellular injuries, including DNA strand breaks, lipid peroxidation, and protein modification through Cr(VI)-mediated free radical reactions [27]. In addition, Kumar et al. [25] reported accumulation of chromium in the interstitial tissue of the gonads of male mice, degeneration of the outer layer of semineferous tubules, and reduction of seminal plasma zinc level. All of these findings had adverse effects on sperm motility, morphology, and physiologic functions. The effects of chromium were confirmed by Geoffroy-Siraudin et al. [26], who showed that Cr(VI) is toxic for meiotic cells even at low concentrations, and its toxicity increases in a dose-dependent manner.

With regard to molybdenum, Meeker et al. [22] reported that it is an important cofactor for a limited number of human enzymes and has demonstrated reproductive toxicity in animal studies. Pandey and Singh [28] reported degeneration of testicular morphology, function, and dose-dependent declines in sperm concentration, motility, and normal morphology in rats after oral administration of sodium molybdate. Previous animal studies suggested a chelating effect of Mo on Cu resulting in lower semen volume and sperm concentration, motility, and normal morphology in a Cu-deficient group [22, 29]. As far as we know, this was the first study to illustrate the possible toxic effects of cobalt on sperm parameters in human.

Metallosis Effects on Spermatozoal Apoptotic Gene Expression

The results of current study demonstrated that there is a significant decrease in Bcl-2 (antiapoptotic gene) and increase in Bax gene expression (proapoptotic gene) with significant decrease in Bcl-2/Bax ratio indicating diminished antiapoptotic activity and increased apoptotic activity of the spermatozoa in individuals with IMN of more than 5 years when compared with those with IMN of less than 5 years and with the control group. Also, there are significant negative correlations between Bcl-2 gene expressions as well as Bcl-2/Bax ratio and the seminal plasma levels of cobalt, chromium, and molybdenum while a significant positive correlation was found between Bax expression and those metals. These results are in agreement with the study of Kanaji et al. [30] who supported the hypothesis that metal implant debris can induce apoptosis through the production of proinflammatory cytokines, IL-6, and TNFα in osteocytes. Also, they reported that Co-Cr-Mo alloy particles have a direct effect by increasing TNFα gene expression and protein secretion in vitro. TNFα has been reported to induce apoptosis which involves activation of caspase 3 and 7 [31]. In 2008, Ogata et al. [32] studied the effect of poly-oxo-molybdates on pancreatic cancer cells and reported that poly-oxo-molybdates can induce apoptosis and autophagy through induction of apoptotic gene and production of apoptotic proteins (Bax and Bak) as well

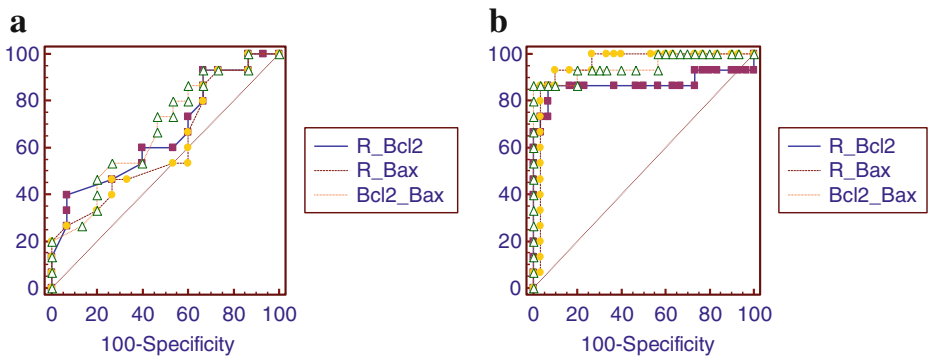

Fig. 2 ROC curve analysis of Bcl-2 and Bax gene expressions as well as Bcl-2/Bax ratio in seminal samples of subjects with IMN for <5 years (**a**) and for ≥5 years (**b**)

as inhibition of antiapoptotic gene (Bcl-2) that leads to shift from apoptosis to autophagic cell death [33]. These data coincide with our results and could explain the possible apoptotic role of molybdenum in the present study.

With regard to the apoptotic activity of chromium, it was studied and confirmed by García-Rodríguez et al. [34]. They observed that Cr(VI) given orally to mice could induce dose- and time-dependent hepatic oxidative stress and hepatocyte apoptosis through induction of genomic DNA damage and increase production of 8-hydroxydeoxyguanosine, which is a form of oxidative DNA damage.

Cobalt proved to have an apoptotic activity through its genotoxic effects including DNA strand breaks, sister chromatid exchanges, and aneuploidy [35]. Besides, elevated cellular inflammatory response has been recorded following exposure to cobalt nanoparticles [36], such as the proinflammatory cytokine. These inflammatory response factors mediate inflammation under normal physiological conditions and are important protective defenses against tissue injury or infection. However, they are also capable of promoting DNA damage and may lead to altered gene expression profile [35]. These data were confirmed by Alarifi et al. [37] who stated that a synergistic effect of cobalt and tungsten carbide enhanced production of reactive oxygen species (ROS) and induced DNA fragmentation. They explained this finding by the fact that ROS is an important factor in the apoptosis process as well as in DNA damage and oxidative stress-induced cellular damage.

ROC Curve Analysis

In the current study, ROC curve analysis was used to assess the value of Bcl-2 and Bax expressions as well as Bcl-2/Bax ratio in the screening of apoptosis as a possible adverse effect of seminal metallosis. In subjects with intramedullary nails for ≥5 years, ROC curve analysis revealed that Bcl-2 gene expression has higher specificity while Bax gene expression has higher specificity, but it is better to use Bcl-2/Bax ratio which has 86.7 % sensitivity, 100 % specificity, 100 % positive predictive value, and 93.8 % negative predictive value at cutoff values ≤0.777. This result is in need for further investigation to confirm it in a larger number of cases.

Conclusions

It could be concluded that Co, Cr, and Mo metals that are used for manufacturing of stainless steel intramedullary nails are present in high concentration in the seminal plasma of individual utilizing these fracture fixation devices. The spermatozoal apoptotic activity measured by gene expression is increased and positively correlated with the levels of these metals and with the duration of exposure. Lastly, spermatozoal Bcl-2/Bax ratio could be used as a candidate molecular biomarker of reproductive disorders in males with intramedullary nails.

Limitations and Recommendations

Few research articles were concerned with the effects of the studied metals on the semen quality, and most of them were animal studies. To our knowledge, this is the first study that tried to find a possible molecular biomarker that can diagnose the toxic effect of metallosis caused by prolonged intramedullary nailing on semen quality. Further investigations are needed for comparison and interpretation. Another limitation of the present study is the small number of the included subjects because smokers and occupationally exposed subjects were excluded. So, we recommend repetition of that work on larger number and longer durations for either confirmation or not.

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Conflicts of interest We declare that no benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. We also declare that we have no conflicts of interest in connection with this paper.

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