

## Effect of the combination between empagliflozin and calcipotriol on cadmium-induced testicular toxicity in rats

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### Keywords

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### Abstract

The aim of this study was to assess the effect of empagliflozin and/or calcipotriol on cadmium-induced testicular toxicity in rats. Sixty male Wistar rats were divided into 6 equal groups: Control; cadmium; cadmium + empagliflozin; cadmium + calcipotriol; cadmium + Carboxymethyl cellulose and cadmium + empagliflozin + calcipotriol. Testicular enzymes, serum testosterone, luteinizing hormone and follicle stimulating hormone were measured. Also, testicular tissue antioxidant enzymes, interleukin-6 (IL-6), transforming growth factor beta 1 (TGF- $\beta$ 1) and sperm characteristics were determined. Parts of the testes were subjected to histopathological and immunohistochemical examination. Empagliflozin/calcipotriol combination restored the testicular enzymes, sperm characteristics, hormonal profile and the antioxidant defenses compared to the use of each of these drugs alone. Also, this combination significantly ameliorated the inflammatory processes and induced significant improvement of the histopathological, and immunohistochemical picture compared to the use of each of these drugs alone. So, empagliflozin/calcipotriol combination might represent a promising therapeutic modality for amelioration of cadmium-induced testicular toxicity.

## INTRODUCTION

Cadmium is one of the toxic heavy metals that was used for a long time as a corrosion-resistant plating on steel and as red, orange and yellow pigments to color glass and to stabilize plastic [1]. Cadmium use is generally decreasing due to its toxic effects on various body systems even if it was absorbed at relatively low doses [2]. Although its main repository organ is the kidney, its toxic effects are demonstrated in the testes even before pathological changes occur in other organs [3]. The mechanisms of cadmium-induced testicular toxicity include induction of oxidative stress, increased production of the proinflammatory cytokines and induction of apoptosis in the spermatogenic cells. These effects may lead to damage of the gonadal functions which may significantly affect male fertility [4].

Empagliflozin is one of the anti-diabetic drugs that selectively inhibits sodium glucose cotransporter-2 [5]. It has a minimal possibility to produce hypoglycaemia than other antidiabetic drugs due to its insulin-independent mechanism of action [6]. Recent studies reported that empagliflozin may have potent antioxidant, anti-inflammatory and antiapoptotic effects [7,8]. Moreover, empagliflozin may regulate different enzymes involved in DNA replication, transcription and protein synthesis which are vitally needed for

spermatogenesis [9]. These properties may give a role to empagliflozin in amelioration of cadmium-induced testicular toxicity.

Calcipotriol is one of the synthetic derivatives of calcitriol (Vitamin D) which is widely used for treatment of certain disorders of the skin [10]. Vitamin D and its derivatives were reported to have potent antioxidant, anti-inflammatory and anti-apoptotic properties that may ameliorate oxidative stress and inflammation in various body tissues [11]. Moreover, calcipotriol was proven to antagonize TGF- $\beta$ 1 signaling via vitamin D receptor/Smad3 genomic crosstalk which blocks Smad residency on chromatin and inhibits acetylation of histone H<sub>3</sub> leading to suppression of the pro-inflammatory cytokines at the gene expression level [12]. The aim of this study was to assess the effect of empagliflozin and/or calcipotriol on cadmium-induced testicular toxicity in rats.

## 2. Materials and Methods

### 2.1. Chemicals and drugs

Cadmium sulfate salt was purchased from Guangzhou Fischer Chemical Co., Ltd, Guangdong, China. Empagliflozin was purchased from Boehringer Ingelheim, Germany. Calcipotriol was obtained from Hölzel Diagnostika (Hohenzollernring, Germany). Carboxymethyl cellulose (CMC) was purchased from ADWIC Co., Cairo, Egypt. All other chemicals were purchased

from Sigma Aldrich Co., Saint Louis, Missouri, USA. All reagents and chemicals used were of analytical grade. Cadmium sulfate was dissolved in distilled water. Empagliflozin and calcipotriol were suspended in 0.5% CMC solution.

### **2.2. Experimental animals**

This study was carried out on 60 male adult sexually mature fully grown Wistar rats weighing about 120-200 grams. They were allowed to acclimatize for two weeks before starting the experiment. Rats were kept in a special room at a constant temperature of  $25 \pm 3^{\circ}\text{C}$  with relative humidity of  $55 \pm 5\%$  and were exposed to 12 h light/dark cycle. They were fed with standard diet and distilled water provided ad libitum. All the experiments were conducted according to the National Research Council's guidelines. This study was approved by the Research Ethics Committee of faculty of medicine, Tanta University, Egypt. Animal handling was followed according to Helsinki declaration of animal ethics.

### **2.3. Experimental design**

Animals were randomly divided into six equal groups of 10 rats each as follows:

Group 1: Control group; received daily intraperitoneal injection of distilled water at 0.2 mL/100 g for 3 consecutive days.

Group 2: Received intraperitoneal injection of cadmium sulfate in a dose of 0.3 mg/kg body weight for 3 days [13].

Group 3: Cadmium sulfate + Empagliflozin group; received empagliflozin in a dose of 10 mg/kg/day by oral gavage for six weeks before starting cadmium injection and continued for 3 days concurrently with cadmium sulfate [14].

Group 4: Cadmium sulfate + Calcipotriol group; received calcipotriol in a dose of 20  $\mu\text{g}/\text{kg}/\text{day}$  by oral gavage for six weeks before starting cadmium injection and continued for 3 days concurrently with cadmium sulfate [15].

Group 5: Cadmium sulfate + CMC group; received 0.5 % CMC solution daily by oral gavage for six weeks before starting cadmium injection and continued for 3 days concurrently with cadmium sulfate.

Group 6: Cadmium sulfate + Empagliflozin + Calcipotriol group; received empagliflozin concomitantly with calcipotriol in the above mentioned doses for six weeks before starting cadmium injection and continued for 3 days concurrently with cadmium sulfate.

### **2.4. Assessment of serum biochemical parameters**

At the end of the study, rats were anaesthetized with thiopental sodium (30 mg/kg body weight, intraperitoneal). Blood was collected from the retro-orbital plexus, centrifuged and sera were separated and used for assessment of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) using ELISA kits obtained

from DRG Diagnostics, Marburg, Germany according to the manufacturer's protocol. Also, serum testosterone was assessed using ELISA kits obtained from Microlisa AMGENIX Int, Inc. USA according to the instructions of the manufacturer.

### ***2.5. Preparation of the testicular tissue samples***

The testis and the epididymis were removed, cleared and weighed. The left testis was rinsed with ice-cold saline, weighed and homogenized. Then, the homogenate was centrifuged at 3000 rpm for 20 min and the supernatant was used for assessment of the tissue biochemical parameters. The right testis was used for histopathological and immunohistochemical examination

### ***2.6. Assessment of testicular tissue oxidative stress parameters, interleukin 6 (IL-6) and transforming growth factor beta 1 (TGF- $\beta$ 1)***

Tissue catalase (CAT) was measured according to Sinha [16]. Tissue glutathione reductase (GR) was assessed using kits purchased from Sigma Aldrich Co., USA, according to the instructions of the manufacturer. Tissue thiobarbituric acid derivatives (TBARS) were determined using kits purchased from Cell Biolabs, Inc., San Diego, USA according to the instructions of the manufacturer. Tissue IL-6 and TGF- $\beta$ 1 were measured using ELISA kits supplied by

Sigma Aldrich Co. according to the manufacturer's instructions.

### ***2.7. Assessment of the activity of the testicular enzymes***

Acid phosphatase (ACP), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and glucose-6-phosphate dehydrogenase (G6PD) activities were measured using kits supplied by Sigma Aldrich Co. according to the instructions of the manufacturer. Sorbitol dehydrogenase (SDH) was assessed using ELISA kits supplied by Wuhan USCN Business Co., Ltd., according to the manufacturer's protocol. Protein content was determined according to Lowry et al. [17].

### ***2.8. Assessment of the sperm characteristics***

Epididymal sperms were collected by slicing the epididymis in 5 mL of Ham's F10 then incubated for 5 min at 37°C in 5% CO<sub>2</sub> to allow sperms to swim out of the epididymal tubules. Then, one drop of the sperm suspension was put on a glass slide and a cover slip was placed. Ten microscopic fields at 400 $\times$  magnification were observed using light microscope and the percentage of the motile sperms was evaluated microscopically within 2-4 min of their isolation from the epididymis [18].

Epididymal sperm counts were assessed according to Badkoobeh et al. [19]. 20  $\mu$ l of the sperm suspension was mixed with 20  $\mu$ l of 0.05% eosin-Y and incubated

for 2 min at room temperature for calculation of the percentage of dead sperms. Then, the mixture was placed on a glass slide and examined by bright-field microscope at 400 $\times$ . Two hundred sperms were counted in each sample and viability percentages were calculated [20].

For assessment of the morphological abnormalities of the sperms, smears were prepared on clean and grease-free slides and allowed to air dry overnight. Then, the slides were stained with 1% eosin-Y/5% nigrosin and examined at 400 $\times$  for morphological abnormalities such as bicephalic, coiled, amorphous, hook less or abnormal tails [21].

### **2.9. Histopathological examination**

The right testes were kept in 10% formalin solution for 24 h, dehydrated in ethanol and embedded in paraffin blocks. Then, sections were cut at 5 micron thickness, placed on glass slides, stained with hematoxylin and eosin and examined using Leica DM750 Camera Microscope, Leica Microsystems GmbH, Wetzlar, Germany.

#### **2.10. Assessment of nuclear factor kappa-B (NF- $\kappa$ B) (p65) immunostaining**

Sections from the right testes were fixed in 10% neutral buffered formalin. Then, paraffin sections were prepared and stained with rat Anti-NF- $\kappa$ B (p65) antibody purchased from RayBiotech, USA and the slides were examined under light microscope. The activated subunit p65 of

NF- $\kappa$ B was determined in the examined tissues and the immunostaining was detected according to the intensity of staining as (+1) weak when nuclear staining was visible at (x200) magnification, (+2) when visible at (x100) magnification and (+3) strong when visible at (x40) magnification [22].

#### **2.11. Statistical analysis**

The statistical package for the social sciences (SPSS) version 21.0 was used for statistical analysis of the obtained results. Values of the measured parameters were expressed as mean  $\pm$  standard error of mean (SEM). For comparison between the different groups, one way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test was used. Pearson's correlation coefficient ( $r$ ) was used to correlate between tissue TBARS and the sperm characteristics. P-values less than 0.05 were considered statistically significant.

### **3. Results**

#### **3.1. Effect of different treatments on testicular tissue IL-6 and TGF- $\beta$ 1 in the studied groups**

Cadmium induced significant increase in tissue IL-6 and TGF- $\beta$ 1 compared to the control group. Addition of CMC induced non-significant effect on these parameters compared to rats treated with cadmium alone. Administration of empagliflozin and/or calcipotriol induced significant decrease in these parameters

compared to rats treated with cadmium alone. This decrease was significant with empagliflozin/calcipotriol combination compared to the use of each of these drugs alone (Table 1).

### *3.2. Effect of different treatments on testicular tissue oxidative stress parameters in the studied groups*

Administration of cadmium induced significant decrease in tissue CAT and GR associated with significant increase in tissue TBARS compared to the control group. Empagliflozin and/or calcipotriol induced significant increase in tissue CAT and GR associated with significant decrease in tissue TBARS compared to rats treated with cadmium alone. Administration of CMC induced non-significant effect on the above mentioned parameters compared to rats treated with cadmium alone. Empagliflozin/calcipotriol combination induced significant increase in tissue CAT and GR associated with significant decrease in tissue TBARS compared to the use of each of these drugs alone (Table 1).

### *3.3. Effect of different treatments on the activity of the testicular enzymes in the studied groups*

Administration of cadmium induced significant decrease in tissue ALP, ACP, G6PD, LDH, and SDH compared to the

control group. Empagliflozin and/or calcipotriol induced significant increase in these markers compared to rats treated with cadmium alone. Administration of CMC induced non-significant effect on these markers compared to rats treated with cadmium alone. Concomitant administration of empagliflozin and calcipotriol induced significant increase in the above-mentioned markers compared to the use of each of these drugs alone (Table 2).

### *3.4. Effect of different treatments on serum testosterone, LH and FSH in the studied groups*

Cadmium induced significant decrease in serum testosterone, FSH and LH compared to the control group. Addition of CMC induced non-significant effect on these parameters compared to rats treated with cadmium alone. Administration of empagliflozin and/or calcipotriol induced significant increase in the above-mentioned parameters compared to rats treated with cadmium alone. This increase was significant with empagliflozin/calcipotriol combination compared to the use of each of these drugs alone (Table 3).

**Table 1: Effect of different treatments on the testicular tissue IL-6, TGF- $\beta$ 1 and markers of oxidative stress**

	Control	Cadmium	Cadmium + Empagliflozin	Cadmium + Calcipotriol	Cadmium + CMC	Cadmium + Empagliflozin + Calcipotriol
Tissue IL-6 (pg/ mg protein)	122.1 $\pm$ 5.5	431.2 $\pm$ 21.3*	310.6 $\pm$ 15.62 <sup>#</sup>	341.2 $\pm$ 17.2 <sup>#</sup>	438.7 $\pm$ 22.1	231.5 $\pm$ 13.5 <sup>##</sup> •
Tissue TGF- $\beta$ 1 (pg/ mg protein)	21.15 $\pm$ 1.32	95.2 $\pm$ 5.14*	68.41 $\pm$ 3.52 <sup>#</sup>	71.82 $\pm$ 3.67 <sup>#</sup>	92.64 $\pm$ 4.65	45.24 $\pm$ 2.39 <sup>##</sup> •
Tissue CAT (U/mg tissue)	28.3 $\pm$ 1.41	13.25 $\pm$ 0.6*	17.35 $\pm$ 0.77 <sup>#</sup>	18.79 $\pm$ 0.89 <sup>#</sup>	12.43 $\pm$ 0.54	23.26 $\pm$ 1.13 <sup>##</sup> •
Tissue GR (U/g/min)	215.4 $\pm$ 10.2	124.67 $\pm$ 6.5*	145.8 $\pm$ 7.35 <sup>#</sup>	155.74 $\pm$ 7.6 <sup>#</sup>	127.35 $\pm$ 6.8	184.83 $\pm$ 8.7 <sup>##</sup> •
Tissue TBARS ( $\mu$ M/g tissue)	8.26 $\pm$ 0.33	62.31 $\pm$ 3.4*	49.5 $\pm$ 2.4 <sup>#</sup>	43.56 $\pm$ 2.24 <sup>#</sup>	64.61 $\pm$ 3.7	32.76 $\pm$ 1.7 <sup>##</sup> •

Values were represented as mean  $\pm$  SEM

\* Significant compared to the control group (p-value less than 0.05); <sup>#</sup> Significant compared to untreated cadmium group (p-value less than 0.05);

<sup>§</sup> Significant compared to cadmium + empagliflozin group (p-value less than 0.05); • Significant compared to cadmium + calcipotriol group (p-value less than 0.05)

**Table 2: Effect of different treatments on the activity of the testicular enzymes**

	Control	Cadmium	Cadmium + Empagliflozin	Cadmium + Calcipotriol	Cadmium + CMC	Cadmium + Empagliflozin + Calcipotriol
Tissue ACP (U/mg protein)	131.42 $\pm$ 6.25	75.4 $\pm$ 3.4*	101.2 $\pm$ 5.3 <sup>#</sup>	91.4 $\pm$ 4.92 <sup>#</sup>	72.5 $\pm$ 3.37	115.4 $\pm$ 5.35 <sup>##</sup> •
Tissue ALP (U/mg protein)	144.75 $\pm$ 7.41	88.5 $\pm$ 4.21*	113.2 $\pm$ 5.86 <sup>#</sup>	102.9 $\pm$ 5.4 <sup>#</sup>	86.3 $\pm$ 4.1	128.8 $\pm$ 6.42 <sup>##</sup> •
Tissue G6PD (U/mg protein)	7.17 $\pm$ 0.33	4.21 $\pm$ 0.26*	5.46 $\pm$ 0.25 <sup>#</sup>	5.21 $\pm$ 0.22 <sup>#</sup>	4.26 $\pm$ 0.24	6.34 $\pm$ 0.31 <sup>##</sup> •
Tissue LDH (U/mg protein)	5.32 $\pm$ 0.23	3.14 $\pm$ 0.11*	3.86 $\pm$ 0.15 <sup>#</sup>	3.78 $\pm$ 0.16 <sup>#</sup>	2.99 $\pm$ 0.12	4.67 $\pm$ 0.22 <sup>##</sup> •
Tissue SDH (U/mg protein)	4.98 $\pm$ 0.26	3.42 $\pm$ 0.13*	3.92 $\pm$ 0.19 <sup>#</sup>	3.86 $\pm$ 0.18 <sup>#</sup>	3.35 $\pm$ 0.12	4.57 $\pm$ 0.24 <sup>##</sup> •

Values were represented as mean  $\pm$  SEM

\* Significant compared to the control group (p-value less than 0.05); <sup>#</sup> Significant compared to untreated cadmium group (p-value less than 0.05);

<sup>§</sup> Significant compared to cadmium + empagliflozin group (p-value less than 0.05); • Significant compared to cadmium + calcipotriol group (p-value less than 0.05)

### 3.5. Effect of different treatments on the sperm characteristics in the studied groups

Following administration of cadmium, significant decrease in sperm count and motility with significant increase

in the percentage of dead sperms and abnormal sperm forms were observed compared to the control group. Addition of CMC induced non-significant effect on the sperm characteristics compared to rats treated with cadmium alone. Administration

of empagliflozin and/or calcipotriol induced significant increase in sperm count and motility associated with significant decrease in the percentage of dead sperms and abnormal sperm forms compared to rats treated with cadmium alone. Empagliflozin/calcipotriol combination induced significant improvement in the above-mentioned sperm characteristics compared to the use of each of these drugs alone (Table 4).

### 3.6. Correlation between tissue TBARS and the sperm characteristics

There was significant positive correlation ( $P < 0.05$ ) between the percentage of dead sperms and abnormal sperm forms, and tissue TBARS while there was significant negative correlation ( $P < 0.05$ ) between the sperm count and motility, and tissue TBARS (Table 5).

### 3.7. Histopathological and immunohistochemical results

Administration of cadmium induced significant disorganization in the

seminiferous tubules associated with significant decrease in the germinal cells, increased vacuolization and immature germinal epithelial cells in the lumen (Fig. 1b). This was associated with significant increase in NF- $\kappa$ B (p65) immunostaining (Fig. 2b) compared to the control group. Addition of CMC induced non-significant effect on the histopathological and immunohistochemical picture compared to the untreated cadmium group (Fig. 1c, 2c). These changes were significantly improved in the groups treated with empagliflozin or calcipotriol (Fig. 1d, e) with significant decrease in NF- $\kappa$ B (p65) immunostaining (Fig. 2d,e) compared to rats treated with cadmium alone. Empagliflozin/calcipotriol combination induced significant improvement in the histopathological and immunohistochemical picture compared to the use of each of these drugs alone (Fig. 1f, 2f).

**Table 3: Effect of different treatments on serum testosterone, LH and FSH**

	Control	Cadmium	Cadmium + Empagliflozin	Cadmium + Calcipotriol	Cadmium + CMC	Cadmium + Empagliflozin + Calcipotriol
Serum testosterone (ng/dl)	226.3±11.7	75.3± 3.74*	121.4±5.77 #	102.45±5.12 #	69.93±3.64	172.4±9.12 #*•
Serum LH (mIU/ml)	0.57± 0.04	0.32± 0.02 *	0.43±0.03 #	0.40±0.02 #	34.52±0.02	0.52±0.03 #*•
Serum FSH (mIU/ml)	0.24± 0.02	0.11± 0.01 *	0.17±0.01 #	0.16±0.01 #	0.12±0.01	0.21±0.02 #*•

Values were represented as mean  $\pm$  SEM

\* Significant compared to the control group (p-value less than 0.05); # Significant compared to untreated cadmium group (p-value less than 0.05);

\$ Significant compared to cadmium + empagliflozin group (p-value less than 0.05); • Significant compared to cadmium + calcipotriol group (p-value less than 0.05)



**Table 4: Effect of different treatments on the epididymal sperm count, motility, % of dead sperms and % of abnormal sperms**

	Control	Cadmium	Cadmium + Empagliflozin	Cadmium + Calcipotriol	Cadmium + CMC	Cadmium + Empagliflozin + Calcipotriol
Sperm count (x10 <sup>6</sup> /mL)	168.72± 9.12	118.42± 6.15*	142.28±7.2#	136.4±7.32#	113.6±5.8	156.8±8.43#*•
Motility (%)	77.6 ± 4.61	45.2± 2.87*	62.3±3.4#	58.3±3.2#	47.4±2.9	68.81±3.6#*•
Dead sperms (%)	8.12 ± 0.44	24.2± 1.52*	16.4±0.83#	17.43±0.88#	23.9±1.58	11.23±0.62#*•
Abnormal sperms (%)	6.81 ± 0.35	16.95±1.13*	11.56±0.63#	12.71±0.73#	17.43±0.98	8.14±0.38#*•

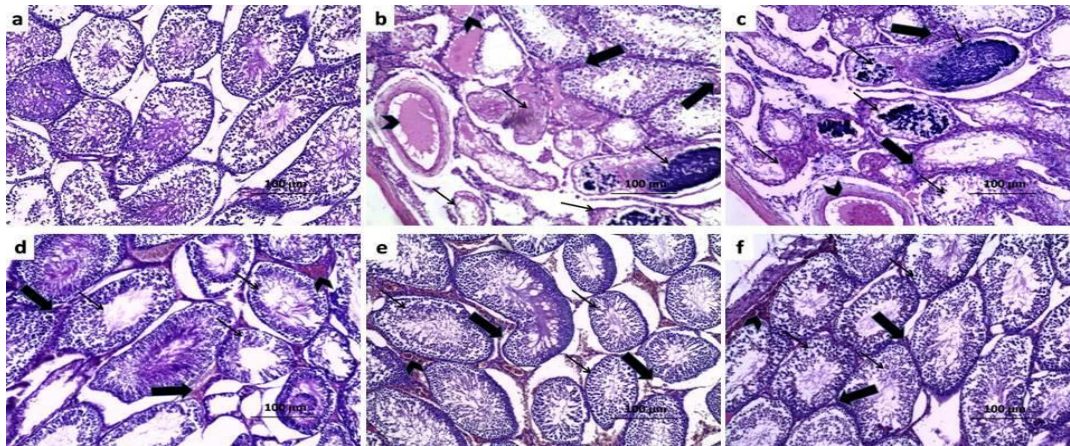
Values were represented as mean ± SEM

\* Significant compared to the control group (p-value less than 0.05); # Significant compared to untreated cadmium group (p-value less than 0.05);

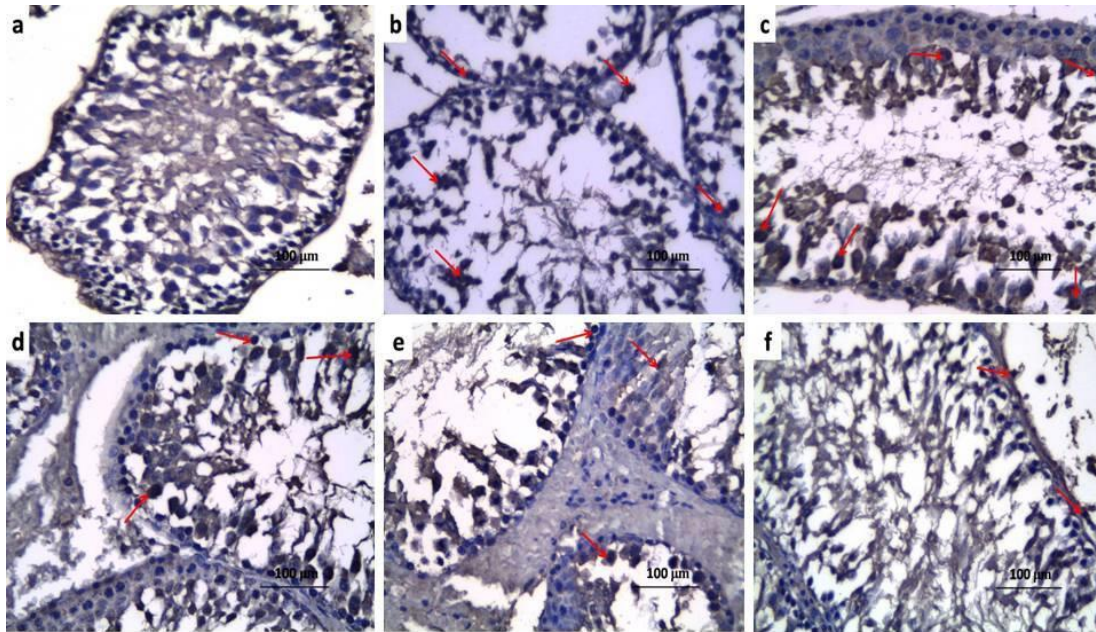
§ Significant compared to cadmium + empagliflozin group (p-value less than 0.05); • Significant compared to cadmium + calcipotriol group (p-value less than 0.05)

**Table 5: Correlation between tissue TBARS and the sperm characteristics**

Variables	Tissue TBARS	Pearson's (r)	P-value
Sperm count		- 0.624	0.025
Motility		- 0.436	0.036
Dead sperms		0.547	0.032
Abnormal sperms		0.611	0.022



**Figure 1:** Sections of the testis (H&E x200) from **a**) Control group with normal morphology of the seminiferous tubules, interstitial tissue, spermatogenic cells and spermatozoa; **b**) Cadmium group showing degenerated interstitial tissue (Thick arrow) with marked congestion (Arrow head) and disorganization of the epithelium of the seminiferous tubules with decrease in the germinal cells and immature germinal epithelial cells in the lumen (Thin arrow); **c**) Cadmium + CMC group showing significant degeneration of the seminiferous tubules (Thin arrow) with degeneration of the interstitial tissue (Thick arrow) with marked congestion (Arrow head); **d**) Cadmium + Empagliflozin group showing significant decrease in the degeneration of the interstitial tissue (Thick arrow) with most of the seminiferous tubules showing organized epithelium (Thin arrow) with mild congestion (Arrow head); **e**) Cadmium + Calcipotriol group showing significant improvement in the morphology of the seminiferous tubules (Thin arrow) with apparently normal interstitial tissue (Thick arrow); **f**) Cadmium + Empagliflozin + Calcipotriol group showing apparently normal seminiferous tubules (Thin arrow) and interstitial tissue (Thick arrow) with minimal congestion (Arrow head).



**Figure 2:** Testicular sections of immunohistochemical staining of NF- $\kappa$ B (p65) (x400) of **a)** Control group revealing negative immunostaining for NF- $\kappa$ B (p65); **b)** Cadmium group revealing strong positive immunostaining for NF- $\kappa$ B (p65) (Arrow); **c)** Cadmium + CMC group revealing strong positive immunostaining for NF- $\kappa$ B (p65) (Arrow); **d)** Cadmium + Empagliflozin group revealing moderate positive immunostaining for NF- $\kappa$ B (p65) (Arrow); **e)** Cadmium + Calcipotriol group revealing moderate positive immunostaining for NF- $\kappa$ B (p65) (Arrow); **f)** Cadmium + Empagliflozin + Calcipotriol group revealing weak positive immunostaining for NF- $\kappa$ B (p65) (Arrow).

#### 4. Discussion

In the present study, cadmium induced significant deterioration of the testicular functions compared to the control group. This was in agreement with Babaknejad et al. [23] and Wu et al. [24] who found that cadmium induces significant atrophy in the seminiferous tubules and markedly affects energy production in the spermatogenic cells. Zhang et al. [25] reported that cadmium interferes with the division of spermatogenic cells and induces denaturation of the epithelium of the seminiferous tubules and phagocytosis of Sertoli cells together with significant regression in testicular development and

weight. These changes were ameliorated in our study with administration of empagliflozin and/or calcipotriol. Liu et al. [26] reported that supplementation with vitamin D and its derivatives may significantly improve the testicular functions. Also, Purcell and Moley [27] suggested that targeting sodium glucose cotransporter-2 in the testis may have a positive impact on the testicular functions.

In the present study, cadmium administration induced significant decrease in serum testosterone, FSH and LH compared to the control group. This was in agreement with Siu et al. [4] who reported that cadmium induced significant decrease in serum testosterone, LH and FSH which in

turn will affect the process of spermatogenesis as well as on the morphology of the seminiferous tubules. This decrease was ameliorated in the present study with administration of calcipotriol which was in accordance with Nimptsch et al. [28] who stated that vitamin D and its derivatives have the ability to improve the serum levels of sex hormones due to their potent antioxidant effects. Also, empagliflozin in our study induced significant increase in serum testosterone, FSH and LH compared to the group treated with cadmium alone. This may be attributed to the antioxidant and anti-inflammatory properties of empagliflozin which may ameliorate the toxic effects of cadmium on the synthesis of androgens [29].

In the present study, cadmium administration induced significant decrease in the sperm motility and count together with significant increase in the percentage of dead and abnormal forms of sperms compared to the control group. This was in agreement with Mahmoudi et al. [30] who reported that cadmium administration disrupts the maturation of the spermatogenic cells and alters spermatogonia DNA and sperm morphology. Also, it was suggested that cadmium decreases the number of Sertoli cells and causes widespread death of the germ cells which is usually followed by significant deterioration in testicular morphology and the sperm characteristics

[31]. This was attenuated in the present study with administration of calcipotriol which was in the same line with BaSalamah et al. [32] who reported that vitamin D and its derivatives can restore the normal functions of the spermatogenic cells and antagonize the deleterious effects of cadmium and lead on Sertoli cells and germ cells. Also, empagliflozin in our study had the ability to improve the sperm characteristics compared to cadmium-treated group. This may be due to the ability of empagliflozin to improve energy production by the mitochondria and inhibit DNA damage induced by cadmium administration to rats [33].

Oxidative stress is thought to be the main mechanism incriminated in the testicular damage induced by cadmium [2]. Patra et al. [34] reported that cadmium induces significant DNA damage with subsequent production of reactive oxygen species (ROS) which in turn suppress the activities of the antioxidant enzymes leading to significant deterioration of the sperm characteristics and testicular functions. These effects were attenuated in the present study with administration of calcipotriol which was in the same line with Saedisomeolia et al. [35] who stated that calcipotriol may exert significant antioxidant effects by enhancing the activity of the antioxidant enzymes and decreasing ROS production. Also, empagliflozin in our study

had the ability to increase the activity of the antioxidant enzymes and decrease TBARS production which was in agreement with Zhou et al. [9] who attributed the antioxidant effects of empagliflozin to activation of AMP-activated protein kinase which subsequently increases the activity of the antioxidant enzymes leading to significant amelioration of oxidative stress.

In the present study, tissue TBARS levels showed significant positive correlation with the percentage of dead sperms and abnormal sperm forms and showed significant negative correlation with the sperm count and motility. This was in the same line with Alkan et al. [36] and Cruz et al. [37] who reported that the increase verified in oxidative stress parameters such as tissue TBARS concentration is correlated with the disturbed sperm characteristics observed in semen analyses because lipid peroxidation interferes with the integrity of the spermatozoa membrane which in turn will be reflected on various sperm characteristics.

Coinciding with the results of the present study, Du et al. [38] found that cadmium increased mRNA expression of TGF- $\beta$ 1, TNF- $\alpha$  and IL-6 which in turn induces severe inflammatory reactions leading to significant damage of the testicular tissues. Moreover, Freudlsperger et al. [39] reported that there is a strong relationship between TGF- $\beta$ 1 and NF- $\kappa$ B

signaling pathways which may significantly modulate the inflammatory cascade. They found that the significant increase in TGF- $\beta$ 1 levels induces NF- $\kappa$ B gene activity which was in the same line with the results of the present study where cadmium administration induced significant increase in TGF- $\beta$ 1, IL-6 and NF- $\kappa$ B levels compared to the control group.

The present study proved the anti-inflammatory properties of empagliflozin and/or calcipotriol where administration of each of these agents resulted in significant decrease in TGF- $\beta$ 1, IL-6 and NF- $\kappa$ B levels compared to rats treated with cadmium alone. Segart et al. [40] attributed the anti-inflammatory properties of calcipotriol to its inhibitory actions on the effects of Th1 and Th17 cytokines, which subsequently inhibits the secretion of TNF- $\alpha$  and IL-17. Moreover, calcipotriol was proven to attenuate TGF- $\beta$ 1/NF- $\kappa$ B signaling pathways [41]. Also, Jojima et al. [42] reported that empagliflozin can inhibit the expression of the proinflammatory cytokines, possibly through affection of mRNA expression of NF- $\kappa$ B and TGF- $\beta$ 1.

In the present study, empagliflozin/calcipotriol combination induced significant improvement of the testicular functions, sperm characteristics, hormonal profile and the antioxidant defenses associated with amelioration of the inflammatory processes and improvement of

the histopathological and immunohistochemical picture compared to the use of each of these drugs alone. This may be due to the combined antioxidant and anti-inflammatory properties of both drugs together with their ability to affect TGF- $\beta$ 1/NF- $\kappa$ B signaling which may have a positive impact on the testicular functions and the sperm characteristics. This may be due to the hypothesis that sodium glucose cotransporter-2 (SGLT2) may regulate the absorption and metabolism of vitamin D and its derivatives such as calcipotriol which in turn may affect the testicular functions [43].

## 5. Conclusion

Empagliflozin/calcipotriol combination might represent a promising therapeutic modality for amelioration of cadmium-induced testicular toxicity, possibly due to their antioxidant and anti-inflammatory properties together with their ability to improve the hormonal profile, sperm characteristics and testicular functions.

## Conflict of interest

The authors had no conflict of interest to declare.

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