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Potential therapeutic effect of berberine and its metabolite nanoparticles on heavy metals induced testicular and prostatic lesions in rats.

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6. Summary and Conclusion

Male sexual hormones (androgens) and gametes are both produced primarily by the testes, which are the major reproductive organs of the male reproductive system; in addition, they synthesize male steroid hormones. One of the main auxiliary glands in the male reproductive system is the prostate gland, a walnut-sized organ located close to the underside of the urinary bladder. The prostate and seminal vesicles create the majority of the ejaculate. Metal exposure at work is a key contributor to severe morbidity and death among workers and can happen in a variety of sectors of industry and in a variety of ways. It was mentioned previously in various studies that exposure to toxic metals in the environment is frequently linked to impaired reproductive function. In terms of male infertility, the environmental pollutant cadmium (Cd) is regarded as a particularly hazardous element. It is known that contacting lead (Pb) or Cd at work or in the environment may have a negative effect on the hypothalamic-pituitary-testicular (HPT) axis, impairing spermatogenesis and suppressing the production of testosterone, which is most vulnerable and irreversible during pubertal development. Previous research has linked the oxidative harm and testicular toxicity brought on by Al to a decrease in the activity of the enzyme acetylcholinesterase (AChE) in the testis, which subsequently affects spermatogenesis.

Berberine (BBR) is isoquinoline alkaloid which treat different diseases It has been demonstrated that BBR comprises four principal metabolites, including berberrubine (BRB), which is thought to be the predominant one, following first-pass metabolism in the liver. However, the main barriers to their use related to the limited oral bioavailability, and the potentially deadly consequences of large alkaloids doses. To overcome these obstacles, nano carriers including bilosomes which are modified liposomes that comprise bile salts in their composition. They are able to more effectively cross biological membranes due to their high flexibility and stability than conventional liposomes. Additionally, bilosomes outperformed liposomes in terms of gastrointestinal tract (GIT) stability.

- **The aim of this study is to**

Formulate stable and flexible nano-structured biloalbuminosomes loaded with BBR, or its demethylated metabolite, BRB. To our knowledge, this is the first study to explore the efficacy of biloalbuminosomes as a nanocarrier for BRB delivery. Furthermore, the effectiveness of nano-structured BBR or BRB-loaded biloalbuminosomes in improving the therapeutic effect of free drugs was assessed by exploring the effect of free BBR, BRB, and their loaded biloalbuminosomes on alleviating the triggered oxidative stress, inflammation, apoptosis, and excessive autophagy in heavy metal- induced testicular and prostatic toxicity, along with observing any side effects related to these drugs by monitoring their effect on healthy rats.

- **Materials and methods**

First, BRB was chemically synthesized from BBR by chemical demethylation, followed by the preparation of BBR and BRB-loaded nano-structured biloalbuminosomes by SC and SG as bile salts with different amounts (10 mg, 20 mg, and 30 mg) using solvent evaporation techniques, followed by the characterization of the synthesized nano-vesicles through determining the VS, PDI, ZP, and EE%. Afterwards, the effects of free BBR, BRB, and the formulated biloalbuminosomes were studied on the heavy metals- induced testicular and prostatic deformities.

After one week of acclimation, the total number of experimental animals was 96 male rats, randomly divided into 12 groups as follows:

- **Group I**: **Sham control group** (n=6), healthy rats; they were kept on free access to a normal, regular diet and tap water.
- **Group II: Vehicle Group** (n=6), vehicle biloalbuminosomes were injected intraperitoneally, at a dose of 365 mg biloalbuminosomes/kg body weight daily for 45 days.
- **Group III: Berberine (BBR) control group** (n=6); free BBR was injected intraperitoneally, at a dose of 25 mg/kg body weight daily for 45 days, where LD_{50}= 205 mg/kg according to Singh & Sharma, 2018 (Singh & Sharma, 2018).
- **Group IV**: **Berberrubine (BRB) control group** (n=6); free BRB was injected intraperitoneally, at a dose of 25 mg/kg body weight daily for 45 days (Yu *et al.*, 2018).
- **Group V**: **BBR-Bilosomes Control group** (n=6), BBR-loaded biloalbuminosomes were injected intraperitoneally, at dose of 365 mg biloalbuminosomes/kg containing 25 mg BBR/kg body weight daily for 45 days.
- **Group VI: BRB-Bilosomes Control group** (n=6), BRB-loaded biloalbuminosomes were injected intraperitoneally, at dose of 365 mg biloalbuminosomes/kg containing 25 mg BRB/kg body weight daily for 45 days.
- **Group VII: Heavy Metals Induced group** (n = 60), a mixture of heavy metals containing cadmium chloride ($CdCl_2$), 5 mg/kg, lead acetate ($Pb(CH_3COO)_2$), 20 mg/kg, and aluminum chloride ($AlCl_3$), 10 mg/kg, were dissolved in distilled water and orally administered to rats for 90 consecutive days (Anyanwu *et al.*, 2020; Cheraghi *et al.*, 2017; Hussien *et al.*, 2018).
- **Group VIII**: **BBR-treated group** (n = 10); free BBR was intraperitoneally injected to heavy metal-induced rats following the end of the induction period, at a dosage of 25 mg/kg body weight daily for 45 days.
- **Group IX**: **BRB-treated group** (n = 10); free BRB was intraperitoneally injected to heavy metal-induced rats following the end of the induction period, at a dosage of 25 mg /kg body weight daily for 45 days.
- **Group X**: **BBR-Bilosomes-Treated group** (n=10), BBR-loaded biloalbuminosomes were intraperitoneally injected to heavy metal-induced rats following the end of the induction period, at dose of 365 mg biloalbuminosomes/kg containing 25 mg BBR/kg body weight daily for 45 days.

- **Group XI: BRB-Bilosomes-Treated group** (n=10), BRB-loaded biloalbuminosomes were intraperitoneally injected into heavy metal-induced rats following the end of the induction period, at dose of 365 mg biloalbuminosomes/kg containing 25 mg BRB/kg body weight daily for 45 days.
- **Group XII: Vehicle-treated** (n=10), vehicle biloalbuminosomes were intraperitoneally injected to heavy metal-induced rats following the end of the induction period, at dose of 365 mg biloalbuminosomes/kg body weight daily for 45 days.

After 24 hours from the end of the animal treatment or induction period, rats were scarified under ether anesthesia and blood and tissue samples were collected. Blood samples were collected from each rat by cardiac puncture at the aorta section in serum tubes and centrifuged at 3000 r.p.m. for 10 minutes. The obtained serum samples were stored at -80°C for analysis of testosterone, TAP and PAP enzymes, total proteins, liver function tests ALT, AST, ALP, T.B, D.B, and albumin, and kidney function tests (urea, creatinine, and uric acid). Prostate and testicular tissues are decapsulated and homogenized. The homogenates were centrifuged at 13,000g for 15 min at 4°C, then the supernatant was collected and stored at -80°C until used in the biochemical investigations of oxidative stress status, apoptotic markers, autophagy markers and pro-inflammatory cytokines. Epididymis was isolated for computer assisted semen analysis (CASA) to investigate the semen quality. Parts of the testicular and prostatic tissues are stored in modified Davidson's fluid for histopathological analysis.

- **The results of the present study revealed that:**

The best prepared loaded nano-structured biloalbuminosomes were the 30 mg SC formulations for both BBR and BRB; BLP-3 and MLP-3 formulations, respectively, which possess the best EE%, VS, PDI, and ZP. The biochemical parameters were estimated, and the results showed that heavy metals negatively affect the liver and kidney functions and the treatments successfully improved these functions, since the heavy metals-induced group's serum total protein and albumin revealed a substantial decline in their levels, accompanied by an elevation in liver injury markers such as ALT AST and ALP activities, along with T.B and D.B levels. In contrast, in the BBR, BRB, or their loaded biloalbuminosome-treated groups, the levels of these parameters decreased considerably in comparison to heavy metals -induced group.

The levels of serum urea, creatinine, and uric acid were noticeably higher in the heavy metals -induced rats compared to their corresponding sham control values, whereas the levels of these parameters were significantly lower in the free BBR, BRB, or their loaded biloalbuminosomes-treated groups in comparison to heavy metals -induced rats. Additionally, treatment of rats with BRB-loaded biloalbuminosomes normalized urea levels. While BBR or BRB-loaded biloalbuminosomes injection into normal rats resulted in a considerable reduction in urea level when compared to sham control. The testicular and prostatic thiobarbituric acid reactive substances (TBARS), and nitric oxide (NO) contents were significantly higher in the heavy metals-induced group than the sham control group, while antioxidants such as glutathione-S-transferase (GST),

glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities were significantly decreased. All treatments successfully improved the antioxidant capacity, where they were substantially elevated, and TBARS and NO contents displayed a significant decrease. Interestingly, BBR and BRB-loaded biloalbuminosomes-treated groups showed improvement in oxidative status in a better manner than treatment with free drugs, specifically NO content, which was normalized in both tested tissues.

Furthermore, the results of the present investigation showed that all treatments reciprocated the toxic effect of exposure to heavy metals, which exacerbates inflammation, as evidenced by a substantial increase in the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the testicular and prostatic tissues of the heavy metals -induced group. Treatment with BBR or BRB-loaded biloalbuminosomes restored testicular IL-6 and IL-1β, as well as prostatic IL-1β levels, to their respective normal sham control levels. However, testicular and prostatic TNF-α levels, along with prostatic IL-6 were significantly decreased in BBR and BRB-loaded biloalbuminosome-treated groups when compared to the heavy metals-induced group.

Heavy metal exposure led to an increase in apoptosis in testicular and prostatic tissues, as indicated by the up-regulation of the BAX gene, while BCL-2 was down-regulated. On the contrary, all treatments reversed the apoptotic effect of heavy metals, where testicular BCL-2 expression was normalized in all treatment groups, while prostatic BCL-2 expression was sharply augmented in BBR, BRB, and their loaded biloalbuminosome-treated groups. Free BBR, BRB, and their loaded biloalbuminosome treatments showed downregulation of testicular BAX expression and successfully approached the expression of the sham control group. However, prostatic BAX expression showed remarkable downregulation in the free BBR and free BRB-treated groups, while it was normalized in BBR- and BRB-loaded biloalbuminosome-treated groups.

Autophagy was highly activated in the heavy metals-induced group; however, the treatment, especially with BBR- or BRB-loaded nano-structured biloalbuminosomes, markedly inhibited autophagy by elevating the protein levels of P62, mTOR, and PI3K and diminishing AMPK. Intraperitoneal injection of biloalbuminosomes loaded with either BBR or BRB into heavy metals-induced rats showed the maximal results among all the treatment groups in both tissues, where testicular and prostatic mTOR, P62, and pI3K protein levels approached their respective control levels. Likewise, the protein level of the autophagosome formation marker (LC3-II) was also determined, and the results revealed that it was significantly decreased in all treatment groups than in the heavy metals-induced group and approached the normal levels in the BBR or BRB-loaded biloalbuminosomes groups.

Results of male infertility parameters matched with autophagy and oxidative stress markers, where testosterone level was significantly reduced in the heavy metals-induced group, while it returned to increase after treatment with BBR, BRB, and their loaded biloalbuminosomes. Prostatic injury markers, including TAP and PAP activities, were significantly elevated in the heavy metals-induced group; however, their activities showed a remarkable decrease in the treated groups when

compared to the heavy metals-induced group as well. The results of histopathology and semen analyses supported these findings, where semen analysis showed improvement in all tested parameters, including sperm concentration, morphology, motility, and vitality, in all treatment groups when compared to the heavy metals-induced group, which showed a huge decline in the aforementioned parameters in comparison to the sham control results.

Histopathology results displayed loss in testicular architecture accompanied by degeneration of seminiferous tubules, and only a few spermatogonia with many intercellular vacuolations were obviously seen in the testicular section of the heavy metals-induced group; however, treatments significantly restored the testicular tissue damage. Prostatic tissue exhibited an atypical hyperplastic structure associated with an increase in epithelial cell thickness and luminal diameters in the lateral and dorsal prostate lobes of heavy metals-induced group sections. Interestingly, all the treatments showed improvement in the prostatic hyperplasia, especially the BRB-loaded biloalbuminosomes group, which displayed the best improvement.

▪ Conclusion:

The accumulation of Cd, Pb, and Al in the hypothalamus, as well as their accumulation in the testicular and prostatic tissues, affected male reproductive functions and eventually caused oxidative stress and inflammation, which in turn led to excessive autophagy and eventually increased apoptosis in the testicular and prostatic tissues. According to the measured parameters, the findings of the current study demonstrated that BBR and its active metabolite (BRB) possessed anti-apoptotic, anti-autophagic, anti-oxidant, and anti-inflammatory activities. Interestingly, the treatment of rats with BBR or BRB-loaded biloalbuminosomes successfully displayed higher potencies than the free drugs as a result of the higher ability of biloalbuminosomes to entrap these drugs and their higher accumulation in tissues, which in turn increased their treatment efficacy (Figure **62**).