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ORIGINAL RESEARCH

Lycium barbarum polysaccharide prevents cisplatin-induced MLTC-1 cell apoptosis and autophagy via regulating endoplasmic reticulum stress pathway

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Background: *Lycium barbarum* polysaccharide (LBP) has been reported to contribute to the recovery of male hypogonadism and infertility.

Aim: The aim of current study was to investigate the underlying mechanisms of LBP on male infertility recovery.

Methods: Recently, it is reported that cell apoptosis mediated by endoplasmic reticulum stress (ERS) was distinguished from that mediated by death reporters and mitochondria pathway, which could induce cell apoptosis independently. The possible signaling mechanisms were investigated using diversified molecular biology techniques, such as flow cytometry, western blotting, and immunofluorescence.

Results: In this study, we found that LBP protected Leydig MLTC-1 cells against cisplatin (DDP) by regulating ERS-mediated signal pathway, which was evidenced by downregulation of phosphorylation PERK, phosphorylation of eukaryotic translation-initiation factor 2α and activating transcription factor 4. Meanwhile, LBP decreased DDP-induced MLTC-1 cell apoptosis via reducing ERS apoptosis-relative proteins caspase 3, caspase 7, and caspase 12. In addition, the result of monodansylcadaverine staining indicated that LBP significantly inhibited DDP-induced autophagosome formation in MLTC-1 cells. Moreover, immunofluorescences and Western blot assays demonstrated that LBP reversed DDP-induced LC3II and Atg5 upregulation in MLTC-1 cells. Finally, the data of enzyme-linked immunosorbent assay showed that LBP markedly recovered MLTC-1 cells testosterone level even in the presence of DDP.

Conclusion: Thus, we suggest that LBP protected MLTC-1 cells against DDP via regulation of ERS-mediated apoptosis and autophagy.

Keywords: *L. barbarum* polysaccharide, endoplasmic reticulum stress, hypogonadism, DDP, MLTC-1 cell, autophagy, apoptosis

Introduction

In the recent years, approximately 6% of males are infected with male hypogonadism with its main features being testosterone deficiency and relevant clinical features. This trend is becoming more general and the incidence is increased.[1] Possibly, the main causes of male infertility are reproductive tract infection and inflammation.[2] In addition, hypogonadism can lead to a variety of diseases, such as sexual dysfunction, testicular failure, sleep disturbance, abdominal adiposity, and so on.[1,3] Along with increasing age, Leydig cell number and function will decline. Autocrine androgen action in Leydig cells is essential for all men's life, which plays a vital role in spermatogenesis and lifelong health of Leydig cells.[4] Testosterone is a main circulating androgen predominantly coming from Leydig cells in the gap space of the testes in mammals, which is vital

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for adjustment of different crucial processes such as sexual differentiation, sustained spermatogenesis, and reproductive function.[3,5] Therefore, enhancing the males' sperm energy and improving the hypogonadism are very important for male fertility, general health, and well-being.[1,6]

Lycium barbarum (*L. barbarum*) is a well-known, traditional Chinese medicinal herb, which has the homology of medicine and food.[7] The fruit of *L. barbarum* has been used for nourishing liver and kidney and improving eyesight.[8] The main active ingredient in *L. barbarum* is *L. barbarum* polysaccharide (LBP), which is composed of arabinose, glucose, galactose, mannose, xylose, and rhamnose.[8] LBP has the function of reproductive system protection, which can be achieved by increasing antioxidant enzyme activities and suppressing cell death to mediated male spermatogenic dysfunction induced by diabetes.[7] Moreover, it was found that LBP enormously increased the sperm count and vitality and enhanced the sexual function of male rats damaged by 60Co-γ irradiation.[9] LBP also have a protective role on spermatogenesis of rats with reproductive system damages induced by cyclophosphamide.[9]

Endoplasmic reticulum (ER) is an important organelle that is related to various cellular processes including protein synthesis, secreting, folding, and assemble. However, calcium ion homeostasis destruction, protein unfoldab misfolded, and accumulated in ER can induce endoplasmi reticulum stress (ERS).[10,11] Thereafter, serious ERS could induce apoptosis. In order to alleviate the ap ptosis f the cells, the subsequent response of this stimul on is ca d the unfold-protein reaction (UPR).[12] UPR ctivat ree transcription factors in mammalian cel RE1/ERN inositol requiring 1), PERK/PEK (PEK ke ER inase), and ATF4 (activating transcription fac r 4).[13] PERK an ER transmembrane protein kinas at inhibits protein translation via phosphorylating eukar ic tr slation-initiation factor 2α subunit. This is ey fac r for ac vating apoptosis in response to ER , acco panied ith nhancer-binding protein homologou protein HOP) upregulation.[10,14,15]

Autophagy y sosomal degradation pathway intracellular, and can eliminate or nelles and proteins, which was regulated by mTOR pathway and autophagy-related (ATG) family members.[16] Until now, several types of autophagy have been identified, such as mitophagy, microautophagy, macroautophagy, and chaperone-mediated autophagy.[17,18] They all have autophagy-related protein (ATG) components and these components have almost identical set.[19,20] All these different types of autophagy play vital roles in the different steps of autophagy.[19,20]

Although so many valuable studies about the biological activities and pharmacological functions of LBP have been published, there is scarcity of information concerning the protective effect of LBP on Leydig MLTC-1 cells. In this study, we aim to investigate the underlying mechanisms of how LBP prevents DDP-induced MLTC-1 cell apoptosis and autophagy.

Materials and methods

Cell culture

The mouse Leydig MLTC-1 cell line was acquired from Stem Cell Bank of Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-164 edium (Sigma, St Louis, MO, USA) with 10% feta ovine se m (Thermo Fisher Scientific, Waltham, MA, U), penicillin 00 U/mL, Sigma) and streptomycin (1 µg/m Sigma) ells were grown at 37°C with 5% O_2 in a onsta e perature cell culture incubator (San Tok o, Japan). LBP was a gift from Professor L

CCK-8 assay of cell viability

The C assay (Doj do, Kumamoto, Japan) was used to d termine cell growth according to the protocol of ma facturer for dicated time points following treatment with BP with o without DDP in MLTC-1 cells. Briefly, MLTC-1 were seeded into 96-well plate at the density of 3 cells/well overnight. Two hundred microliters of LBP (0, 25, 50, 100 µg/mL) with or without DDP (10 µM) were added into each well in triplicate when cells were totally adhered.

EdU detection and autophagosome formation

MLTC-1 cells were exposed to LBP (50 µg/mL) and/or DDP (10 µM) for 48 hours, and EdU detection was tested according to the method described in the EdU kit (Thermo Fisher, C10638). Immunofluorescence assay was performed according to the previously described method.[22] Briefly, cells were incubated with primary antibodies for DAPI, EdU, or LC3 (Cell Signaling, Danvers, MA, USA; 1:200) at 4°C overnight and were incubated with Alexa 488-conjugated donkey anti-mouse secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) at 37°C for 1 hour. EdU-positive cells and autophagosome formation were observed by fluorescence microscopy (Olympus Corporation, Tokyo, Japan).

Western blot analysis

Following treatment with LBP (50 µg/mL) and/or DDP (10 µM) for 48 hours, MLTC-1 cells were collected and

lysed in RIPA buffer (Sigma). The extracted protein concentration was quantified using a BCA Protein Assay Kit (Beyotime, Shanghai, China) and separated on SDS-PAGE gels with 10%Tris-SDS gel. After the electrophoresis, the gel was transferred onto PVDF membranes (Thermo Fisher Scientific). The PVDF membrane was blocked in 5% defatted milk in TBST for 50 minutes and then probed with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-PERK (Cell Signaling; 1:1,000), anti-p-PERK (Cell Signaling; 1:1,000), anti-p-elF2α (Cell Signaling; 1:1,000), anti-elF2α (Cell Signaling; 1:1,000), anti-ATF4 (Cell Signaling; 1:1,000), and anti-β-actin (Santa Cruz Biotechnology; 1:1,000), active caspase-3 (Cell Signaling; 1:1,000), active caspase-7 (Cell Signaling; 1:1,000), caspase-12 (Cell Signaling; 1:1,000), LC3 (Cell Signaling; 1:1,000), and Atg 5 (Cell Signaling; 1:1,000). Immunodetection was carried out using anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibodies and enhanced chemiluminescence detection kit (Thermo Fisher Scientific).[23]

Flow cytometric analysis of cell apoptosis

Apoptotic cells were analyzed according to the formerly described method.[24] Briefly, MLTC-1 cells were exposed to LBP (50 μg/mL) and/or DDP (10 μM) for 48 hours. Subsequently, these three groups of MLTC-1 cells were collected and resuspended in PBS. Apoptotic cells were recognized with dual-staining of Annexin V-FITC and propidium iodide (PI; Thermo Fisher Scientific). For each group, the experiments were repeated in triplicate.

Monodansylcadaverine staining

MLTC-1 cells treated with LBP (50 μg/mL) and/or DDP (10 μM) at 37°C for 48 hours were stained with monodansylcadaverine (MDC, 50 μM) at 37°C for 30 minutes. After incubation, the cells were washed three times with PBS, and treated with 50 μM MDC in an incubator for 15 minutes. Autophagic vacuoles were immediately observed with a fluorescence microscope (Olympus Corporation) and quantified.[25]

Hormone assays

MLTC-1 cells were seeded in six-well plates at 2×10^5/well and treated with LBP (50 μg/mL) and/or DDP (10 μM) at 37°C for 12 hours. Subsequently, the culture medium was collected, and testosterone was analyzed by an enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN, USA).[23]

Statistical analysis

All experiments were performed at least three times and all data were expressed as the mean ± SD. One-way ANOVA followed by Dunnett's test or least significance difference was used to analyze the difference between groups with $P<0.05$ being regarded as a statistically significant difference ($^*P<0.05$, $^{**}P<0.01$).

Results

LBP reversed DDP-induced MLTC-1 cell growth inhibition

The viability of MLTC-1 cells were detected at indicated time points with LBP dose escalation (0, 25, 50, 100 μg/mL) with or without DDP (10 μM) using the CCK-8 assay. As shown in Figure 1A, 10 μM DDP inhibited MLTC-1 cell proliferation significantly, while 50 μg/mL LBP promoted MLTC-1 cell proliferation. When the MLTC-1 cells were treated with LBP (50 μg/mL) plus DDP, cell viability was significantly increased compared with DDP alone treatment (Figure 1A). Additionally, as indicated in Figure 1B, the quantity of MLTC-1 cells in LBP plus DDP group was much more than DDP alone group.

EdU is efficiently mixed with newly synthesized DNA and fluorescently labeled, which is an important index to evaluate cell activity. The EdU fluorescence assay revealed that the DDP could significantly decrease the EdU-positive cells, while LBP could dramatically increase the EdU-positive cells in DNA synthesis phase (Figure 1C and D). Meanwhile, LBP protected MLTC-1 cells against DDP, which was evidenced with the EdU-positive cells significantly increased in LBP plus DDP group compared with DDP alone group (Figure 1C and D). These results suggested that LBP could reverse DDP-induced cytotoxicity and increase MLTC-1 cell vitality.

LBP inhibited DDP-induced ERS-mediated proteins upregulation in MLTC-1 cells

To further demonstrate the effect of LBP on ERS-mediated cell apoptotic pathway, MLTC-1 cells were stimulated with LBP and/or DDP for 48 hours and harvested for western blotting analysis. The results showed that 50 μg/mL LBP alone could significantly decrease the proteins expressions of p-PERK, p-elF2α, and ATF4, while 10 μM DDP exerted the opposite effect on these proteins (Figure 2A–D). Meanwhile, DDP-induced p-PERK, p-elF2α, and ATF4 upregulation were dramatically reversed by LBP (Figure 2A–D). These results suggested that LBP inhibited DDP-induced ERS-mediated proteins upregulation in MLTC-1 cells.

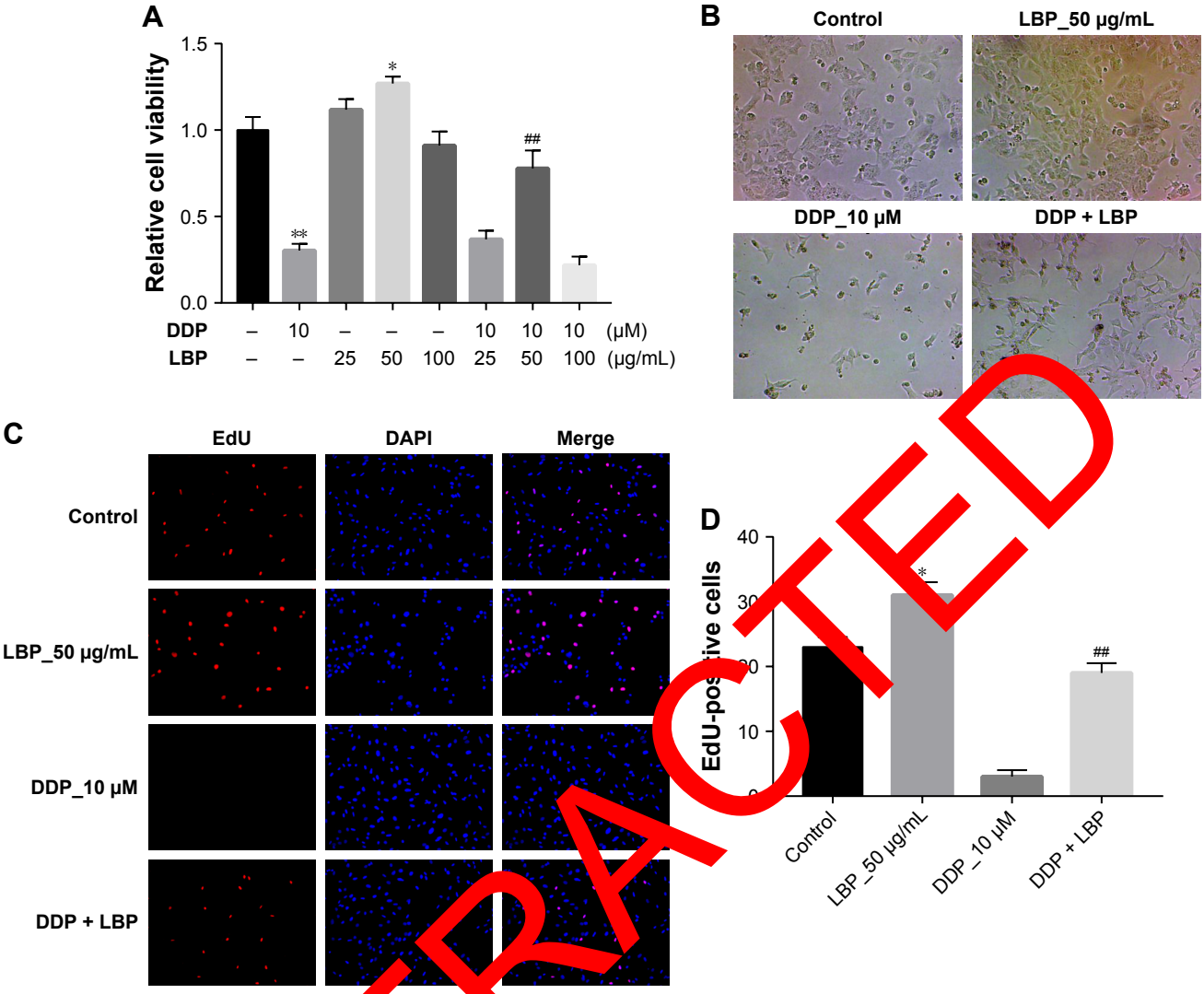

Figure 1 LBP increased MLTC-1 cell viability as[illegible]platin (DDP).
Notes: (**A**) Cell viability was determined usi[illegible]CK-8 [illegible]ay in MLTC-1 c[illegible]s treated with LBP (0, 25, 50, 100 μg/mL) combined with or without DDP (10 μM) for 48 hours. (*P<0.05, **P<0.01 compared with the 0 μg/mL LBP grou[illegible] ##P<0.01 compared with the 50 μg/mL LBP groups.). (**B**) Images of MLTC-1 cells treated with LBP (50 μg/mL) with or without DDP for 48 hours. Ma[illegible]ification ×200. (**C**) I[illegible]es of the MLTC-1 cells stained with EdU and DAPI in an EdU detection and immunofluorescence assay; cells were treated with LBP (50 μg/mL) [illegible]th or without DDP for 48 [illegible]ours. Magnification ×200. (**D**) Quantification of the EdU-positive MLTC1 cells. (*P<0.05 compared with control groups; ##P<0.01 compa[illegible]d with the [illegible]μg/mL LBP groups).
Abbreviation: LBP, *Lycium barb*[illegible]n poly[illegible]charide.

LBP inhibit[illegible]d D[illegible]DP-i[illegible]u[illegible]ced ERS-mediate[illegible] apop[illegible]sis in MLTC-1 cells

We next asse[illegible] if LBP could decrease ERS-mediated apoptosis induced [illegible]y DDP in MLTC-1 cells. As shown in Figure 3A and B, 50 μg/mL LBP alone did not trigger any apoptosis in cells. In contrast, 10 μM DDP significantly induced cell apoptosis (31.89%) compared with control group. However, the cell apoptosis rate in LBP plus DDP group was reduced from 31.89% to 12.05% compared with DDP alone group (P<0.01). In addition, the results of Western blot revealed that DDP alone activated caspase 3, caspase 7, and caspase 12 proteins in cells. Similarly, the levels of caspase 3, caspase 7, and caspase 12 in cells were downregulated in the presence of LBP (Figure 3C–F). These data indicated that LBP could reverse DDP-induced ERS-mediated apoptosis in MLTC-1 cells.

LBP reversed DDP-induced autophagy in MLTC-1 cells

ERS also activated autophagy-associated proteins LC3II and Atg5 expressions. Thereby, we performed MDC assay in order to further confirm the effect of LBP on autophagic vacuoles formation in cells. As shown in Figure 4A and B, 50 μg/mL LBP had no effect on MLTC-1 cells compared with control group, while 10 μM DDP significantly increased the autophagic vacuoles formation in cells. As expected,

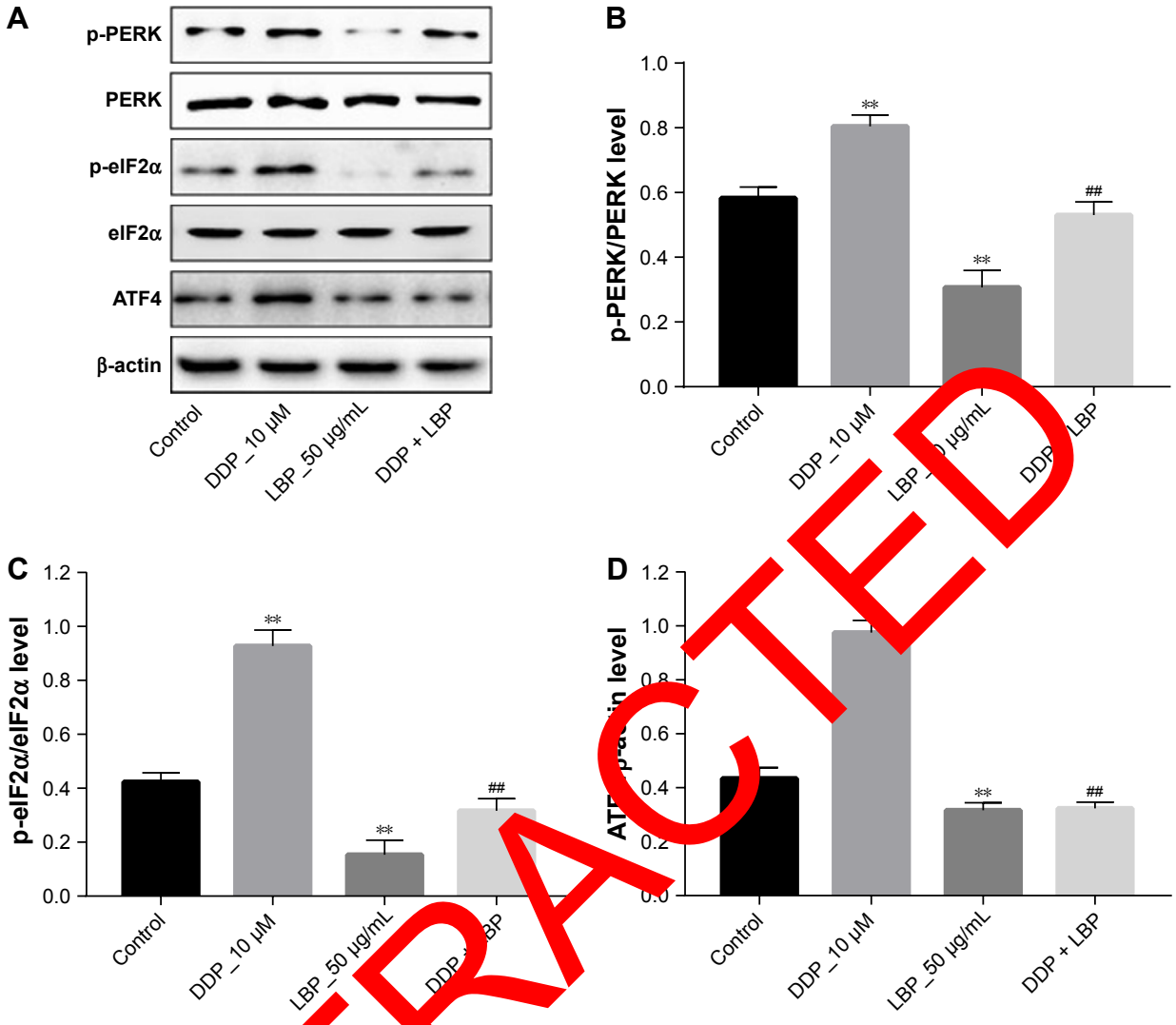

Figure 2 LBP inhibited cisplatin (DDP)-ind ERS activation MLTC-1 cell line.
Notes: (**A**) Expressions of p-PERK, PER eIF2 -eIF2α, and AT analyzed by western blotting after 48 hours LBP or/and DDP treatment in MLTC-1 cells. (**B**) Relative proteins expressions were quantified by p-PERK PERK (n=3; $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group). (**C**) Relative proteins expressions were quantified by p 2α to eIF2α (n= $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group). (**D**) Relative proteins expressions were quantified by ATF4 to β n (n=3; $^{**}P<0.01$ comp d with control group; $^{\#\#}P<0.01$ compared with DDP group).
Abbreviations: ATF4, act ng transc ion factor 4; ERS, endoplasmic reticulum stress; LBP, *Lycium barbarum* polysaccharide.

MDC-po ive lls w significantly decreased by LBP t tment e en in the presence of DDP. The immunofluores and Western blot data also confirmed that DDP-induce LC3II and Atg5 increases were inhibited by LBP (Figure C–F). All these results suggest that DDP induced ERS-mediated autophagy, which was partly reversed by LBP in MLTC-1 cells.

LBP increased the level of testosterone in MLTC-1 cells

To assess the sperm motility effect of LBP on MLTC-1 cells in vitro, we detected the testosterone level using ELISA. As shown in Figure 5, 10 μM DDP significantly decreased the level of testosterone in culture medium, while that was dramatically reversed by LBP. These data demonstrated that LBP could protect the level of testosterone in MLTC-1 cells against DDP.

Discussion

LBP has various biological characteristics including improving the male hypogonadism.[7,9,26] However, the underlying mechanisms of how LBP regulated ERS-mediated apoptosis and autophagy were not fully illuminated. Our results suggested that 50 μg/mL LBP treatment stimulated cell growth, while 100 μg/mL LBP inhibited cell growth. This condition might be because of the high glucose in cell culture media.

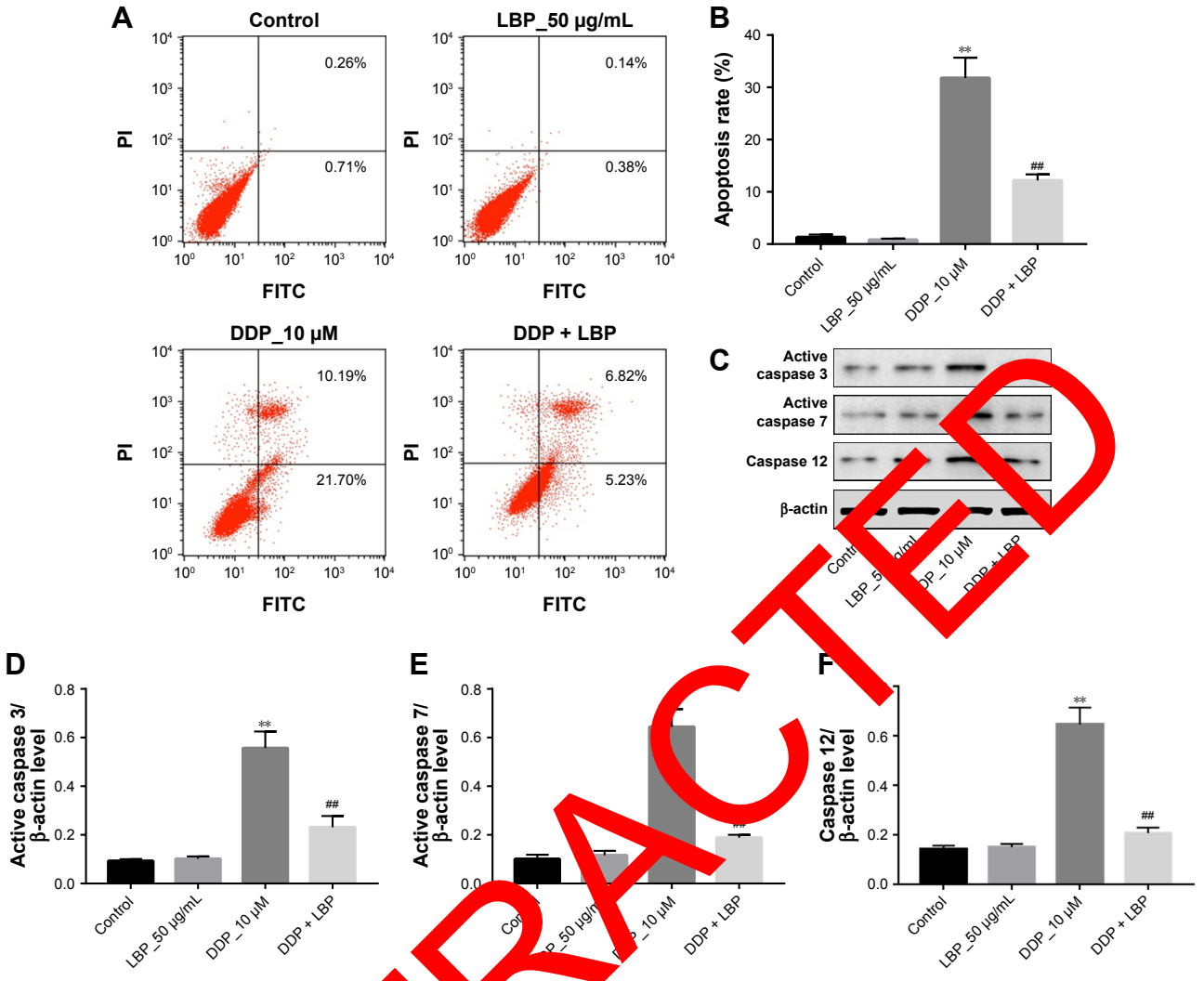

Figure 3 LBP decreased cell apoptosis induced by cisplatin (DDP) in MLTC-1 cells.
Notes: (**A**) MLTC1 cells were exposed to LBP (50 μg/mL) with or without DDP (10 μM) for 48 hours. Apoptotic cells were indicated with Annexin V and PI double staining. (**B**) The apoptosis cell rates were calculated. $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group). (**C**) Expressions of active caspase 7 and caspase 12 in MLTC-1 cells were analyzed by western blotting after 48 hours culture. (**D**) Relative proteins expressions were quantified by active caspase 3 to β-actin (n=3; $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group). (**E**) Relative proteins expressions were quantified by active caspase 7 to β-actin (n=3; $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group). (**F**) Relative proteins expressions were quantified by caspase 12 to β-actin (n=3; $^{**}P<0.01$ compared with control group; $^{\#\#}P<0.01$ compared with DDP group).
Abbreviations: LBP, *Lycium barbarum* polysaccharide; PI, propidium iodide.

In the current study, we demonstrated that 50 μg/mL LBP treatment significantly inhibited DDP-induced MLTC-1 cell apoptosis by flow cytometry analysis with Annexin V-FITC/PI staining. Shi et al also revealed that LBP inhibited streptozotocin-induced apoptosis in diabetic male mice, which is consistent with our results.[7] EdU is a kind of thymidine nucleoside, which can insert into DNA molecules during cell reproduction. In the current study, LBP increased MLTC-1 cell vitality via increasing DNA synthesize in S phase.

At the cellular level, we illustrated that pathological ERS activation could be the key signaling mechanism responsible for DDP-induced MLTC-1 cells apoptosis. The hallmarks of ERS, p-PERK, p-elF2α, and ATF4 were all downregulated in LBP-treated MLTC-1 cells even in the presence of DDP. Type I ERS induced cell death and apoptosis accompanied with a series steps of elF2α phosphorylation mediated by PERK and ATF4. In the current study, LBP inhibited DDP-induced MLTC-1 cell apoptosis via downregulation of p-PERK/p-elF2α/ATF4 pathway, which was further validated by cytometry analysis. Shi et al reported that LBP protected male sexual dysfunction and fertility impairments by activating hypothalamic pituitary gonadal axis in streptozotocin-induced type 1 diabetic male mice.[23] Consistent with this work, LBP also protected MLTC-1 cells against DDP.

Caspase 7 was major mainly in spermatogenic cells and have apoptotic function in spermatogenic dysfunction.[27]

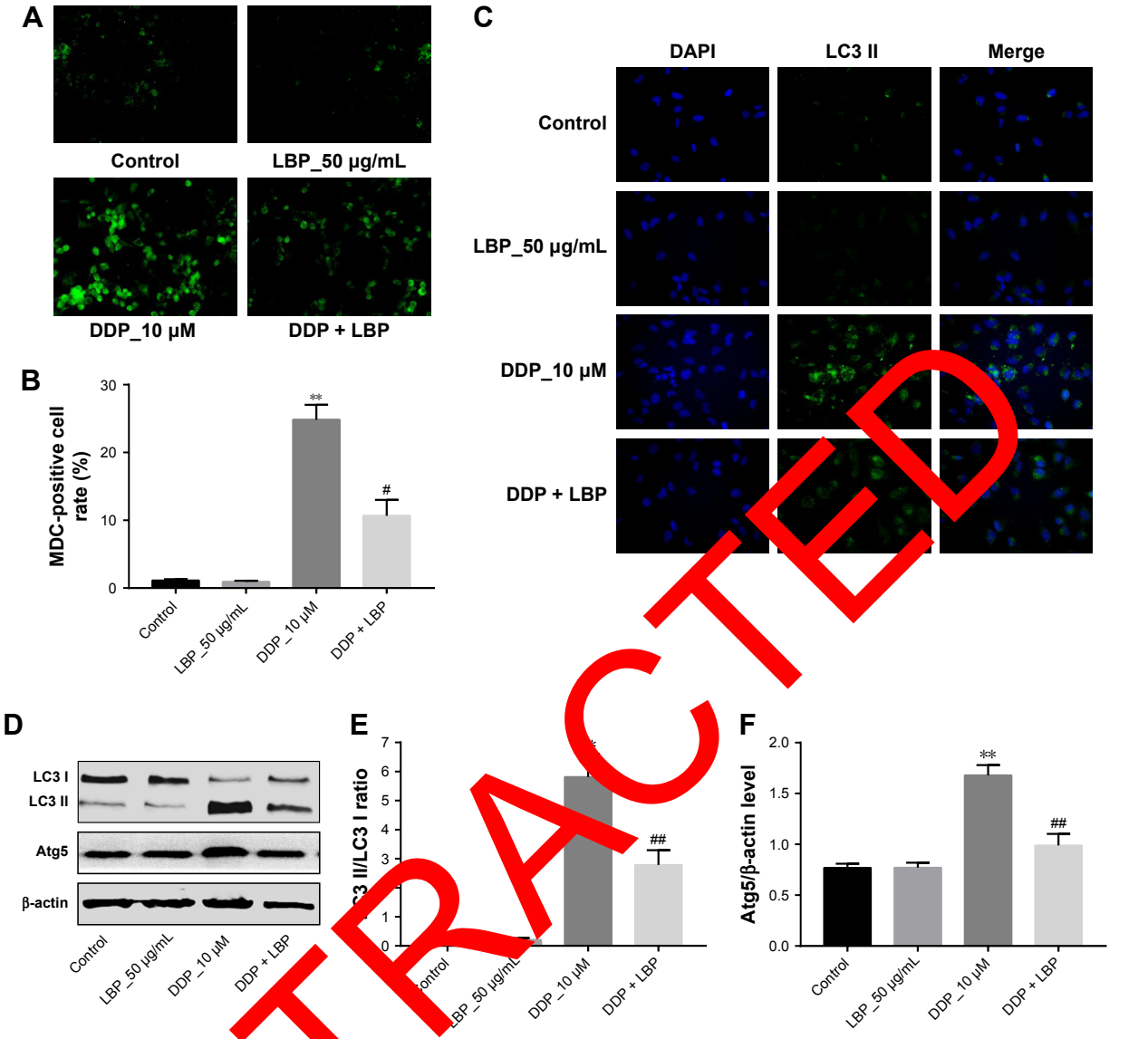

Figure 4 LBP decreased cell autophagy induced by cisplatin (DDP) in MLTC-1 cells.

Notes: (**A**) MLTC1 cells were exposed to LBP (50 μg/mL) with or without DDP (10 μM) for 48 hours and subjected to MDC staining. (**B**) Quantification of the autophagosomes after MDC staining. (**$P<0.01$ compared with control group; #$P<0.01$ compared with DDP group.) (**C**) Images of the MLTC1 cells stained with LC3 and DAPI in immunofluorescence assay; MLTC1 cells were exposed to LBP (50 μg/mL) with or without DDP for 48 hours. Magnification ×200. (**D**) Expressions of LC3 I/II and Atg5 in MLTC1 cells were analyzed by Western blotting after 48 hours of treatment. (**E**) Relative proteins expressions were quantified by LC3II/I (n=3; **$P<0.01$ compared with control group; ##$P<0.01$ compared with DDP group). (**F**) Relative proteins expressions were quantified by Atg5 to β-actin (n=3; **$P<0.01$ compared with control group; ##$P<0.01$ compared with DDP group.

Abbreviations: LBP, *Lycium barbarum* polysaccharide; MDC, monodansylcadaverine.

In the current study, we found that LBP could reverse DDP-induced caspase 3, caspase 7, and caspase 12 upregulation. In addition, the formation of autophagic vacuoles was detected using immunohistochemistry and MDC staining. We illustrated that the numbers of MDC and autolysosome were increased by DDP alone treated compared with control cells, while LBP could reverse this kind of increases in the cells. Liu et al reported that LBP could protect mTOR-mediated autophagy in diabetic rats; LC3 and p62 were involved; the expression of LC3II was decreased and p62 was increased, indicating a decreased activation of autophagy.[28] Similarly, the expressions of LC3II and Atg5 were both downregulated by LBP in MLTC-1 cells even in the presence of DDP in the present study.

It is reported that LBP exerted protective effects on the male spermatogenic dysfunction, and reproductive system of male rats might have a relationship with testosterone level.[7,9,26] Here, our data confirmed that LBP also preserve testosterone level against DDP in MLTC-1 cell medium. This finding was consistent with Shi et al.[7]

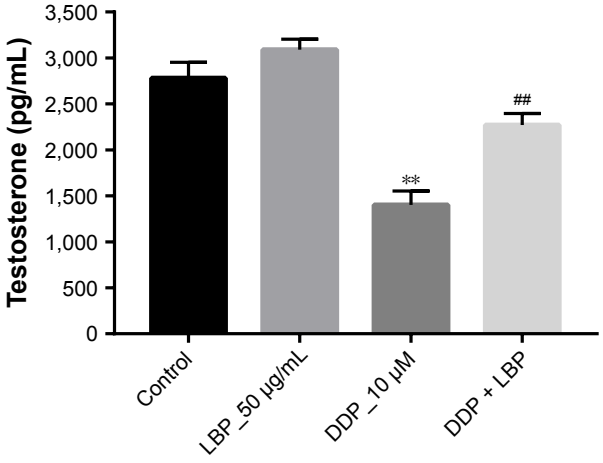

Figure 5 LBP increased the level of testosterone in MLTC-1 cells mediated by cisplatin (DDP).
Notes: MLTC-1 cells were treated LBP (50 μg/mL) with or without DDP (10 μM) for 12 hours. The levels of testosterone in culture mediums were detected by ELISA. The results are given as the means ± SD from three independent experiments. (**$P<0.01$ compared with control group; ##$P<0.01$ compared with DDP).
Abbreviations: ELISA, enzyme-linked immunosorbent assay; LBP, *Lycium barbarum* polysaccharide.

In conclusion, we found that the LBP could reverse DDP-induced ERS activation-mediated autophagy and apoptosis in MLTC-1 cells. In addition, LBP effectively protected testosterone production against DDP in MLTC-1 cell medium. Our findings provide a new insight into the protective eff mechanisms of LBP on spermatogenic dysfunction an propose that LBP may be an alternative medicine for the treatment of male infertility and hypogonadism diseases.

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Disclosure

The authors report no conflicts of interest in this work.

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