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Lithocholic acid activates mTOR signaling inducing endoplasmic reticulum stress in placenta during intrahepatic cholestasis of pregnancy

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ABSTRACT

Aims: Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disorder, which increases risks of adverse fetal outcomes. However, the pathophysiology is not fully understood. Here, we explored the roles of mTOR signaling and ER stress in placenta during ICP.

Materials and methods: Placental tissues were collected from normal and ICP pregnancies. mTOR signaling and endoplasmic reticulum stress were detected by immunohistochemistry in the placenta. The human placenta trophoblast cell line HTR-8/SVneo was used in vitro experiment.

Key findings: ICP placenta displayed histological abnormalities with fewer trophoblasts. Moreover, the expression of Bip and the phosphorylation of pS6(S235/236) or pAkt(S473) were higher comparing with normal placenta. In in vitro studies, the bile acids specifically to lithocholic acid rather than taurocholic acid or ursodeoxycholic acid, drastically increased the phosphorylation of pS6K1(T389), pS6(S235/236), or pAkt(S473), whereas the mTOR inhibitor can prohibit the upregulation. Similarly, the expressions of IRE1α and BiP increased sharply under lithocholic acid (20 μM) administration, while the same inhibitor can also decrease the expression. Additionally, transmission electron microscopy showed enlarged endoplasmic reticulum lumen under the lithocholic acid treatment. Furthermore, the cell viability reduced sharply under treatment with different dose of lithocholic acid. The mTOR inhibitor can reverse the decrease of cell viability to some extent.

Significance: Bile acid can activate mTOR signaling which resulted in endoplasmic reticulum stress, leading to trophocyte viability decrease. mTOR pathway activation may be associated with the pathophysiology of ICP.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-associated disorder that mainly develops during the second or third trimester of pregnancy. ICP affects 0.2–2% of all pregnant women worldwide, irrespective of ethnicity and geographical differences [1,2]. The major concern associated with this disease is the increased risk of adverse fetal outcomes, particularly in severe ICP cases with total bile acid level ≥40 μmol/L [3–5]. Elevated bile salts contribute to preterm delivery by increasing oxytocin bioactivity [6,7]. In addition, bile salts may play a role in unexplained intrauterine fetal death [8] or impairing fetal cardiomyocyte function [9]. However, the etiology and mechanisms underlying the fetal complications associated with ICP are not fully elucidated.

As the central regulator of maternal-fetal interaction, the placenta plays a crucial role in maintaining fetal health [10]. During the

Abbreviations: ICP, intrahepatic cholestasis of pregnancy; UDCA, ursodeoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid; mTOR, mechanistic target of rapamycin; mTORC1, mTOR Complex 1; mTORC2, mTOR Complex 2; ER stress, endoplasmic reticulum stress; UPR, unfolded protein response; HE, hematoxylin-eosin; SKs, syncytial knots; IHC, immunohistochemistry

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progression of ICP, placental function is severely disrupted by the accumulation of bile acids, especially toxic bile acids such as lithocholic acid (LCA), taurocholic acid (TCA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) [11–13]. High concentrations of bile acids can induce severe morphological abnormalities, including an increased number of syncytial knots, the expression of apoptotic markers and inflammation by activating the Gpbar1/NF-κB pathway in the placental tissue of ICP patients [14–16].

The mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase involved in two protein complexes known as mTOR Complex 1 (mTORC1) and 2 (mTORC2). mTORC1 functions as a nutrient/energy/redox sensor and promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). mTORC2 controls proliferation and survival primarily by phosphorylating several of the AGC family of protein kinases such as Akt [17]. Some previous reports showed that mTOR was an important transducer of endoplasmic reticulum (ER) stress [18]. ER stress has recently been identified as a major regulator of cell homeostasis through its involvement in post-translational protein modifications and folding, which involves inositol-requiring enzyme 1 (IRE1), RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and binding immunoglobulin protein (BiP) also known as 78 kDa glucose-regulated protein (GRP-78) [19–21]. Severe or prolonged ER stress leads to activation of the pro-apoptotic UPR and consequent cell death [22]. High concentrations of toxic hydrophobic bile salts within hepatocytes during cholestasis promote ER stress, contributing to hepatocellular injury [23]. In the present study, we aim to investigate the role of mTOR signaling and ER stress in placenta during ICP.

2. Materials and methods

2.1. Patients and sample collection

Prior informed consents for these investigations were obtained from all patients, and the study was approved by the Research Committee for Human Subjects, Southern Medical University. Forty clinical samples were obtained between December 2015 and December 2016 from Nanfang Hospital of Southern Medical University. ICP was diagnosed in women with the following symptoms: 1) presence of classical pruritus with no rash and quick disappearance after delivery. 2) Raised maternal serum bile-acid concentration ($> 10\,\mu M$). 3) Absence of itching skin disease. 4) Absence of any other causes of liver dysfunction, including preeclampsia, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, acute fatty liver of pregnancy, primary biliary cirrhosis, viral hepatitis and any ultrasound abnormality. Exclusion criteria for the recruitment of control subjects were similar to those for ICP cases.

To avoid artefacts from the effects of labor, only placental samples obtained from women who had not undergone labor were selected. All samples were collected immediately after cesarean sections and randomly cut near the maternal surface villous lobule to yield 5–6 sections. The size of sections was approximately 1 cm × 1 cm × 1 cm.

2.2. Cell culture and treatment

The human placenta trophoblast cell line HTR-8/SVneo was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma Chemical Co. St. Louis, MO, USA) at 37 °C with 5% CO_2. The cells were seeded into 24-well plates overnight, and were serum starved for 24 h, followed by treatment with different doses of LCA, TCA, and UDCA (Sigma-Aldrich, St. Louis, MO, USA) for different times or rapamycin (RAP) and wortmannin (WORT) (Selleck, Shanghai, China) as indicated in the results section. Then, the cells were harvested, and protein was extracted for western blotting.

2.3. Cell viability assay

Cell viability was assessed using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, 3000 cells were seeded in 96-well plates, incubated in culture medium overnight, starved for 24 h with serum-free medium, and treated with different doses of LCA or RAP and WORT as shown in the results section. Light absorbance was measured at 450 nm using BioTek™ ELx800™ Absorbance Microplate Readers. All experiments were carried out in triplicate.

2.4. Western blotting

Whole cell lysate preparation and western blot analysis were completed as described previously [24,25]. The primary antibodies against p-AKT (Ser 473) (1:2000), p-p70S6K (T389) (1:1500), p-PS6(S235/236) (1:3000), BiP (1:2000), and IRE1α (1:2000) used in experiments were obtained from Cell Signaling Technology (Danvers, MA, USA) and antibodies against AKT (1:1500), p70S6K (1:1500), S6 (1:3000), and ACTIN (1:5000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Hematoxylin-eosin staining and immunohistochemistry

The placentas were collected immediately after cesarean delivery. Tissue samples were briefly rinsed in ice-cold phosphate-buffered saline, fixed in 4% paraformaldehyde, and embedded in paraffin. Blocks were sectioned at 4 μm and stained with hematoxylin-eosin (HE). For immunohistochemistry, placenta sections were deparaffinized, rehydrated, and incubated with primary antibody at 4 °C. The same concentration of normal mouse IgG served as a negative control. After washing in PBS, the sections were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and immunoreactivity was visualized with 3,3′-Diaminobenzidine (DAB) staining. Finally, slides were counterstained with HE. For quantitation of immunoreactivity, 15 consecutive nonoverlapping fields at 200× magnification were scored in a blinded fashion.

2.6. Analysis of placental syncytial knots

Syncytial knots were regarded as multilayered aggregates of > 10 syncytiotrophoblast nuclei which project from surface villi and not in direct contact with nearby villi. For each morphometric measurement, ten fields for each tissue section (5 per placenta) were examined and average data were calculated.

2.7. Transmission electron microscope

After treatment, the cells were harvested and fixed with 2.5% glutaraldehyde at 4 °C for 2 h, and then were suspended in PBS containing 1% osmic acid at 4 °C for 2 h. Following dehydration and embedding, ultrathin sections (65–70 nm) were prepared on uncoated copper grids using an Ultrotome and stained with uranyl acetate and lead citrate. Images were photographed using a TEM (H7650, Hitachi Limited, Japan).

2.8. Statistical analyses

Data were expressed as the mean ± SEM. The Student's *t*-test or two-way analysis of variance was used to analyze differences between groups. A value of $p < 0.05$ was considered statistically significant.

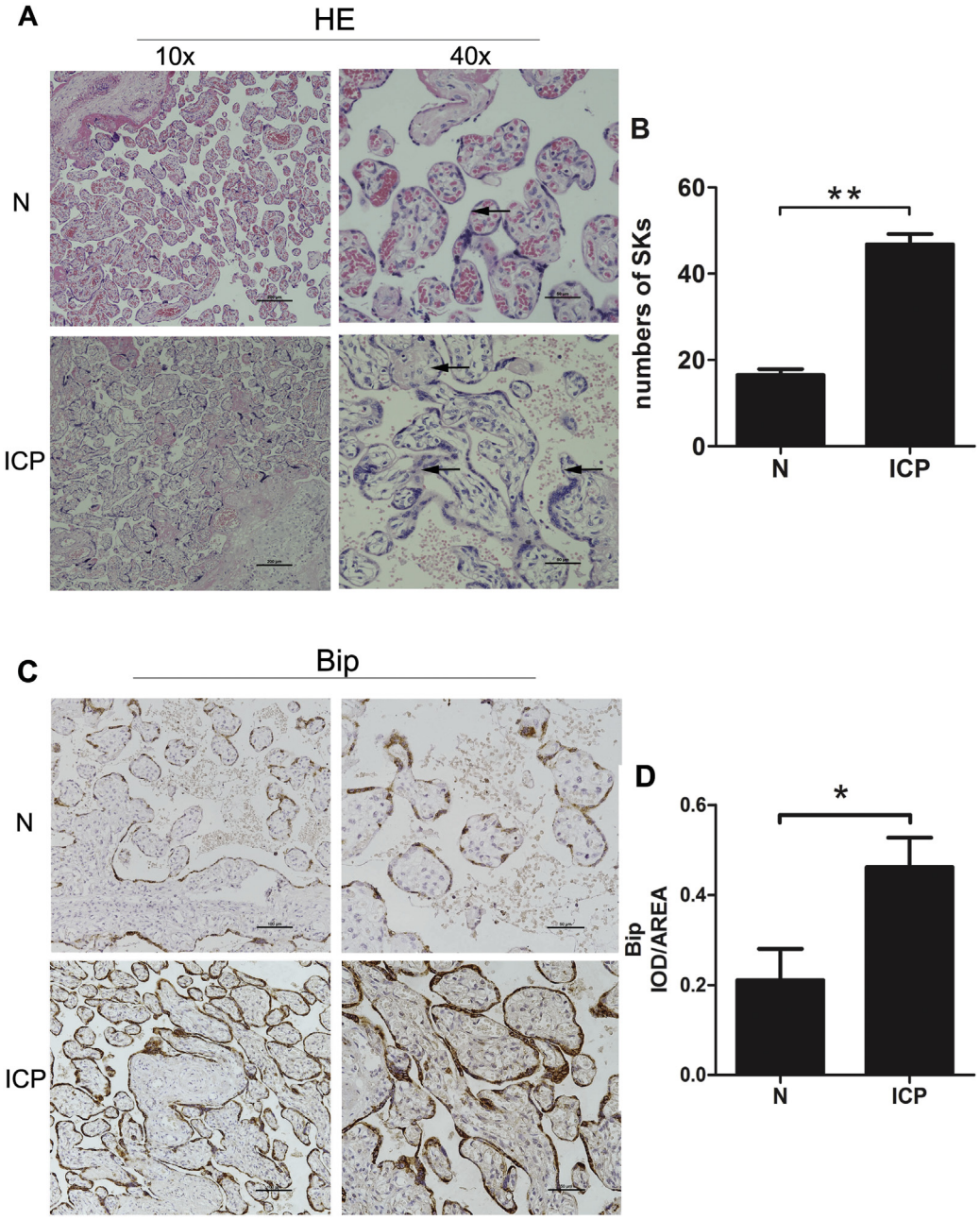

Fig. 1. ICP placenta displayed histological abnormalities and severe ER stress. (A) The histological properties of the trophoblasts in normal and ICP placentas were assessed. The arrow indicated the syncytial knots (SKs). (B) The number of SKs in placenta was counted in at least 4 fields per section (n = 4–9). (C) The expression of Bip was detected using immunohistochemistry (IHC) in normal and ICP placentas. (D) Digital images were acquired and the integrated optical density (IOD) of semi-quantitative analysis for Bip was performed. *: $P < 0.05$, **: $P < 0.01$.

3. Results

3.1. ICP placenta displayed histological abnormalities and severe ER stress

Firstly, the histological properties of the placenta from normal and ICP pregnancies were assessed. HE staining showed there were no differences in the sizes or numbers of terminal villi and the placental trophoblasts aggregated around the blood vessels in both groups. However, there were fewer trophoblasts and more SKs in ICP placental tissue than in normal controls (Fig. 1A and B). The clinical characteristics of the individuals involved in our study were shown in Table 1.

Numerous studies have confirmed that endoplasmic reticulum stress contributes to the etiology of many human diseases [26]. The expression of Bip which played a key role in ER stress was detected using

Table 1
Clinical characteristics of pregnancies and neonates.

Characteristic	Normal (n = 20)	ICP (n = 20)	p value
Maternal age (years)	29.64 ± 4.31	27.67 ± 2.34	0.252
BMI of mother	27.03 ± 4.75	25.17 ± 2.54	0.047
Gravidity	2.36 (1–4)	1.87 (1–3)	0.181
Parity	1.57 (1–2)	1.4 (1–3)	0.432
Gestational age (weeks)	39.24 ± 0.24	37.51 ± 1.69	0.001
TBA at delivery (μmol/L)	15.28 ± 2.85	45.034 ± 19.45	< 0.001
Birth weight (g)	3411.43 ± 459.75	2815.33 ± 457.08	0.002
Placenta weight (g)	528.46 ± 22.82	522.67 ± 38.45	0.622
Apgar at 5 min	9.86 (9–10)	9.47 (7–10)	0.176
Male (%)	8/14 (57.1%)	7/15 (46.7%)	0.715

Data are expressed as medians (range), mean ± SD or numbers (%).

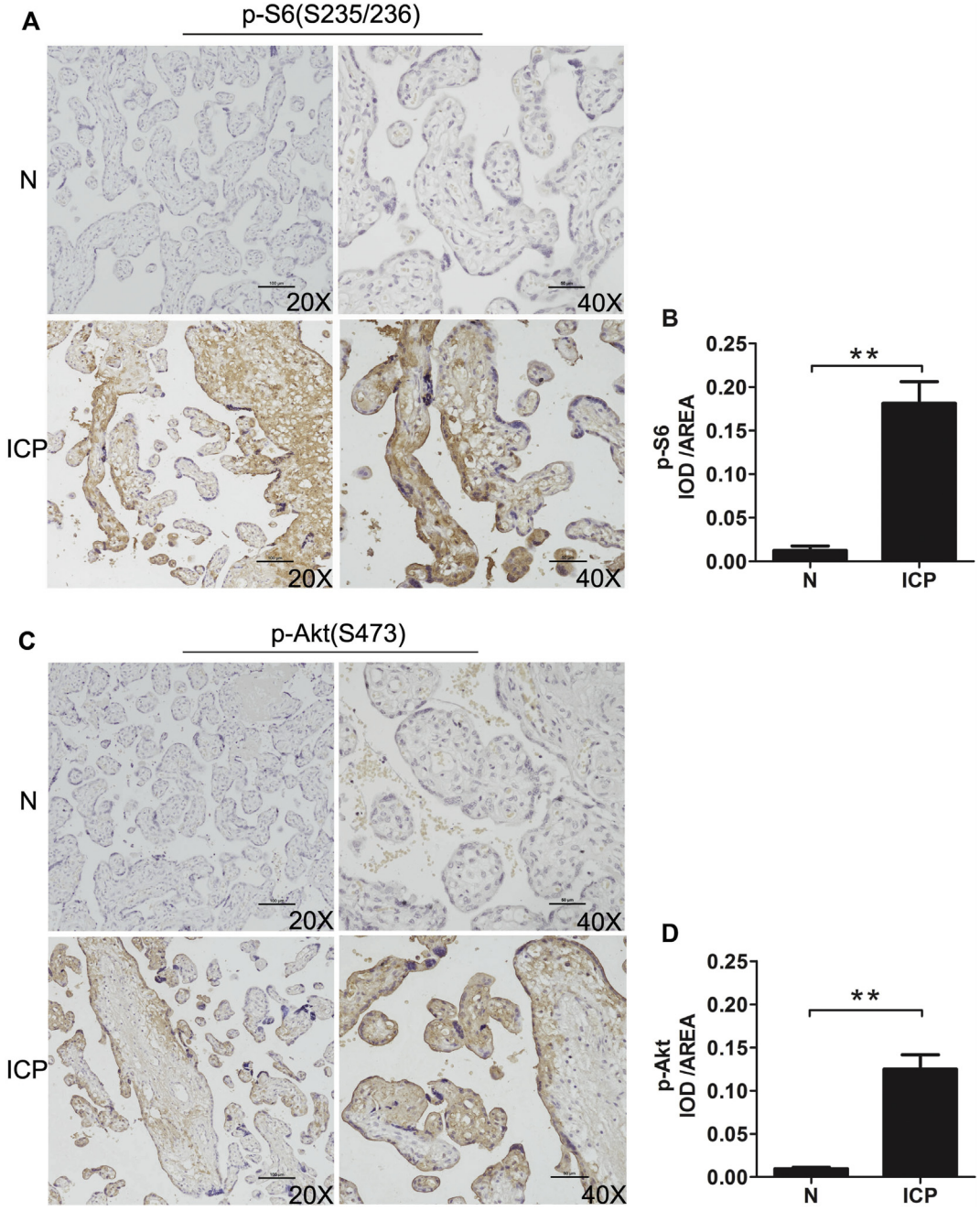

Fig. 2. mTOR signaling activity was upregulated in the ICP placenta. The p-S6 (S235/236) (A) and p-Akt (S473) (C) were detected in normal and ICP placentas by immunohistochemistry. Digital images were acquired and the integrated optical density (IOD) of semi-quantitative analyses for p-S6(S235/236) (B) and p-Akt (S473) (D) was performed. **: $P < 0.01$.

immunohistochemistry (IHC). As expected, the expression of Bip elevated sharply in ICP placenta compared with normal placenta (Fig. 1C). The IHC scores were shown in Fig. 1D. The current results indicated that ER stress was aggravated in placenta with ICP.

3.2. mTOR signaling was upregulated in the placenta with ICP

Secondly, the activity of mTOR signaling was examined by immunohistochemistry (IHC). As shown in Fig. 2A and C, the phosphorylation of p-S6 (S235/236) and p-Akt (S473) were dramatically intensified in the ICP placenta comparing with normal placenta. Likewise, there was significant difference in IHC scores between two groups (Fig. 2B and D). These results revealed that the mTOR pathway was activated in the ICP placenta. To explore the roles of mTOR pathway and the ER stress during ICP, the in vitro cell experiments were adopted further.

3.3. Taurocholic acid (TCA) and ursodeoxycholic acid (UDCA) had no effect on mTOR signaling

In the subsequent cell experiments, three types of bile acids including TCA, UDCA and LAC were adopted. First of all, TCA and UDCA were used to treat the human placenta trophoblast cell line HTR-8/SVneo. Unfortunately, the TCA had no effect on phosphorylation of p-S6 (S235/236) and p-Akt (S473) at different doses (25 μM, 50 μM, 100 μM, 150 μM and 200 μM) for 1.5 h or at 100 μM for different time (0.5 h, 1 h, 1.5 h, 2 h, 4 h), as it was shown in the Fig. 3A and B. The UDCA also did not affect the phosphorylation of p-S6 (S235/236) and p-

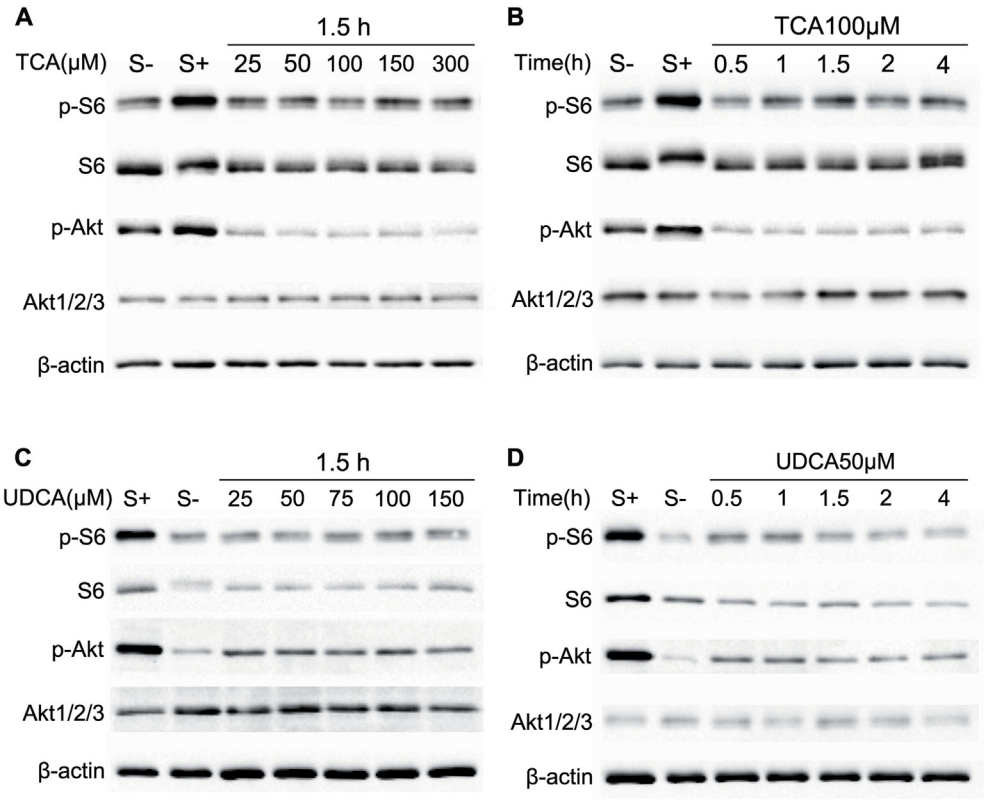

Fig. 3. TCA or UDCA had no effect on mTOR signaling in vitro studies. (A) Under treatment with 25 μM, 50 μM, 100 μM, 150 μM, and 300 μM TCA in 1.5 h (A) or 100 μM TCA in 0.5 h, 1 h, 1.5 h, 2 h and 4 h (B), the p-S6 (S235/236), p-Akt (S473), S6, Akt, or Actin were tested by western blot. Under treatment with 25 μM, 50 μM, 75 μM, 100 μM, and 150 μM UDCA in 1.5 h (D) or 50 μM UDCA in 0.5 h, 1 h, 1.5 h, 2 h and 4 h (E), the p-S6 (S235/236), p-Akt (S473), S6, Akt, or Actin were tested by western blot.

Akt (S473) at different doses (25 μM, 50 μM, 75 μM, 100 μM and 150 μM) for 1.5 h or at 50 μM for different time (0.5 h, 1 h, 1.5 h, 2 h, 4 h), as it was shown in the Fig. 3C and D. The two types of bile acids had no profound impact on the expression of S6, Akt, or Actin.

3.4. Lithocholic acid (LCA) dramatically activated mTOR signaling

Moreover, LCA was used in the same condition. As shown in Fig. 4A, the phosphorylation of p-S6K1 (T389) and p-S6 (S235/236) increased sharply in response to different doses (10, 15, 20, 30, and 40 μM) of LCA at 1.5 h compared with control, and similar results were observed

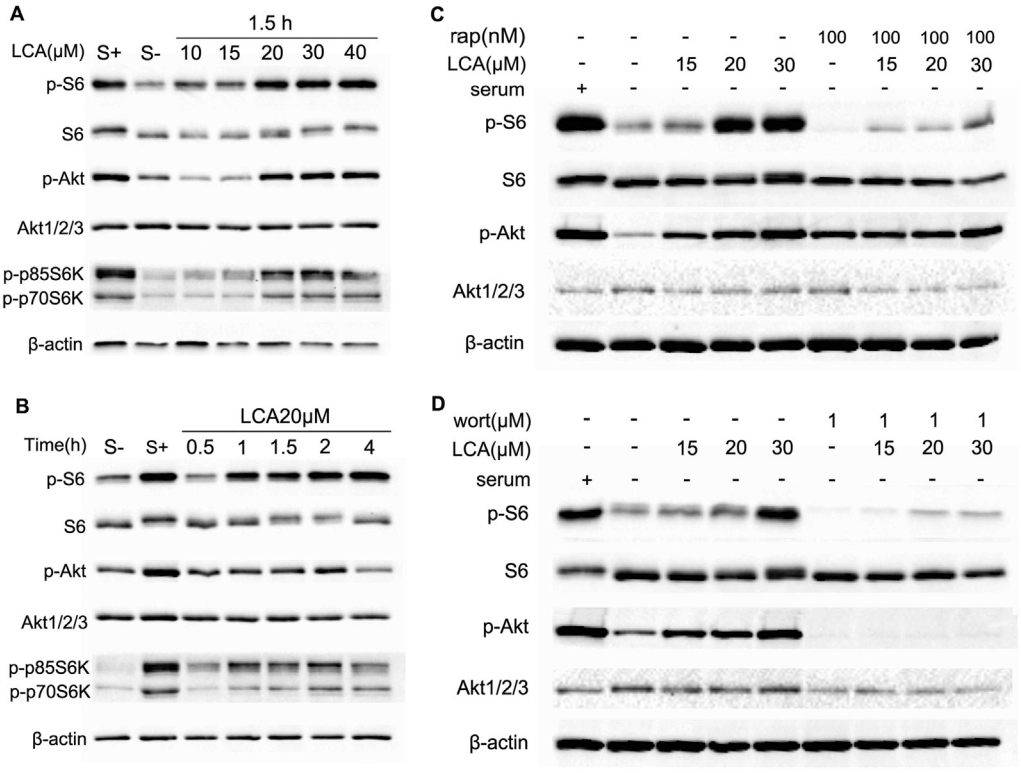

Fig. 4. LCA activated mTOR signaling in vitro experiments. Under treatment with 10 μM, 15 μM, 20 μM, 30 μM, and 40 μM LCA in 1.5 h (A) or 20 μM LCA in 0.5 h, 1 h, 1.5 h, 2 h and 4 h (B), the p-S6K1 (T389), p-S6 (S235/236), p-Akt (S473), S6, Akt, or Actin were analyzed by western blot. Rapamycin (C) or wortmannin (D) treatment followed by 15 μM, 20 μM, or 30 μM LCA administration, the p-S6 (S235/236), p-Akt (S473), S6, Akt, or Actin were analyzed by western blot. rap: rapamycin, wort: wortmannin.

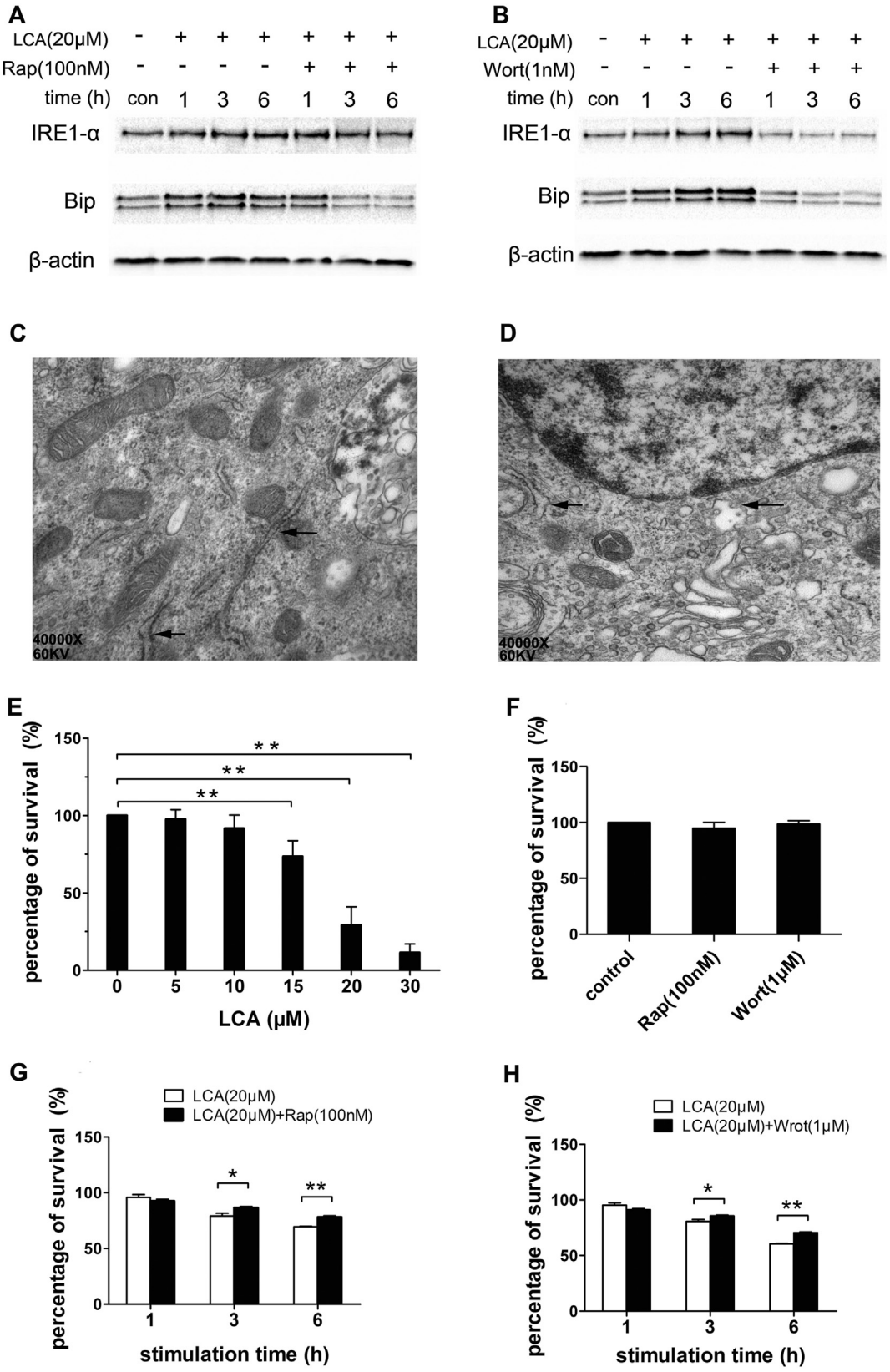

Fig. 5. ER stress induced by LCA promoted cell viability decrease. Treatment with 20 μM LCA in 1 h, 3 h, and 6 h with or without rapamycin (rap) (A) or wortmannin (wort) (B), the IRE1α and BiP were analyzed by western blot. The ultrastructure of endoplasmic reticulum was observed by transmission electron microscope with 20 μM LCA treatment (D) or without (C). ← : endoplasmic reticulum. (E) HTR-8/SVneo cells were treated with 5 μM, 10 μM, 15 μM, 20 μM or 30 μM LC for 12 h, and the cell viability was assessed using a Cell Counting Kit-8. (F) HTR-8/SVneo cells were treated with rap or wort for 6 h, the cell viability was tested. Under treatment with 20 μM LCA in 1 h, 3 h, and 6 h followed by rap (G) or wort (H), the cell viability was also tested. All experiments were carried out in triplicate. rap: rapamycin, wort: wortmannin. *$P < 0.05$; **$P < 0.01$.

for p-Akt (S473). Treatment with LCA at 20 μM for different times (0.5, 1, 1.5, 2, and 4 h) also upregulated the phosphorylation of p-S6K1 (T389), p-S6 (S235/236) and p-Akt (S473), as shown in Fig. 4B. The expression of S6, Akt, and Actin was not affected by LCA.

In the following experiments, the two specific mTOR inhibitors were used in the presence of LCA. As shown in Fig. 4C, treatment with rapamycin followed by 15, 20, or 30 μM LCA administration markedly decreased the phosphorylation of p-S6 (S235/236) to a level similar to the control group, whereas the levels of p-Akt (S473) increased compared with the control group under the same treatment. However, treatment with wortmannin followed by different doses of LCA prevented the increase of p-S6 (S235/236) and p-Akt (S473) (Fig. 4D). The expression of S6, Akt, and Actin was not affected by LCA, rapamycin, or wortmannin. These results suggested that LCA can dramatically activate the mTOR pathway, not only mTORC1 but also mTORC2.

3.5. ER stress induced by LCA activation promoted cell viability decrease

As mentioned above, mTOR is an important transducer of endoplasmic reticulum (ER) stress. Firstly, the ER stress response under treatment with LCA was tested. As shown in Fig. 5A, the expression of ER stress markers such as IRE1α and BiP increased sharply in response to LCA at 20 μM for different times (1, 3, and 6 h) comparing with control in HTR-8/SVneo cells. In addition, followed by rapamycin treatment, the levels of BiP decreased sharply especially at 3 h or 6 h, however, rapamycin had no profound impact on the levels of IRE1α (Fig. 5A). In the same situation, wortmannin treatment decreased the expression of IRE1α and BiP significantly (Fig. 5B). Additionally, Transmission electron microscope showed the rough endoplasmic reticulum was prominent in control cells, of which the membranes were continuous with the outer nuclear membrane. After treatment with 20 μM LCA, the endoplasmic reticulum vesicles got severely enlarged (Fig. 5C and D). Simultaneously, the cell viability decreased sharply with 15, 20, or 30 μM LCA treatment for 12 h compared with the control group, whereas 5 and 10 μM LCA had no significant effect on cell viability (Fig. 5E). Under treatment with LCA (15, 20 or 30 μM) followed by rapamycin or wortmannin, the decrease in cell viability can be reversed especially at 3 h or 6 h as it was shown in the Fig. 5G and H. The rapamycin or wortmannin treatment alone had no adverse effect on cell viability in a short time (Fig. 4F).

4. Discussion

ICP is a disease of the late second or third trimester of pregnancy, which is characterized by pruritis, elevated serum bile acids, and abnormal liver function tests [4]. High concentrations of bile acids can induce several morphological abnormalities, including an increased number of syncytial knots and the expression of apoptotic markers in the placental tissue [5,6]. However, the underlying molecular mechanisms are still unclear.

Extensive research over the past two decades led to the elucidation of the central role of mTOR in regulating many fundamental cell processes, and dysregulation of mTOR signaling is implicated in the pathogenesis of many diseases [27–29]. In this study, we showed that the mTOR signaling was hyperactivated in the ICP placenta, and our in vitro experiments also revealed that the LCA but not TCA or UDCA can activate the mTOR pathway greatly in HTR-8/SVneo cells. Previous studies have discovered that bile acids can affect the mTOR pathway in cancer cells [25,30].

More interestingly, we discovered that the expression of BiP was upregulated in ICP placenta. Additionally, our cell experiments also confirmed that the expressions of BiP and IRE1α increased sharply with LCA treatment, and rapamycin or wortmannin administration can reverse their increase. Recent studies have highlighted pathological situations where cell toxicity by ER stress is coupled with the activation of mTORC1 [18,31,32]. During intrahepatic cholestasis, accumulation of toxic hydrophobic bile acids activates ER stress, leading to induction of the pro-apoptotic UPR and cell death [23]. In rat model of ICP, the damage of mitochondria and ER was observed, and the inflammatory cytokines including IL-1β, TNF-α and IFN-γ were increased in placenta [33]. In our study, subsequent experiments proved that LCA markedly decreased HTR-8/SVneo cell viability and use of mTOR or PI3K inhibitor can attenuate cell viability decrease at some degree. One preliminary comparative proteomics analysis showed that proteins associated with apoptosis and oxidative stress were upregulated in placentas with ICP [34].

The reason why LCA but not TCA or UDCA activated the mTOR pathway was not illustrated in this study. One study reported that LCA significantly induced placental tumor necrosis factor-α upregulation and syncytiotrophoblast cell apoptosis in intrahepatic cholestasis of pregnancy [35]. The other study showed that high concentration of taurocholic acid induced apoptosis in HTR-8/SVneo cells via overexpression of ERp29 and activation of p38 [36]. Unfortunately, the underlying molecular mechanism was not elucidated in these studies. We suspected that it may be associated with the solubility of bile acids or the subcellular localization of the bile acid receptor. Taken together, our data discovered a new regulatory mechanism involved in mTOR pathway and ER stress during pathogenesis of ICP. The etiology of ICP is multifactorial and may be linked to increasing estrogen levels in pregnancy as well as altered expression of hepatobiliary transport proteins [37]. According to our experiments, the higher level of bile acids especially to fat-soluble bile acid may be one of the etiological factors of ICP. In addition, the pathogenesis of ICP probably involved complex cell signal transduction and kinase inhibitors may have potential therapeutic value.

5. Conclusion

In conclusion, the results from this study clearly showed that mTOR pathway and ER stress were upregulated in placenta with ICP. Bile acid can activate mTOR signaling which resulted in ER stress, leading to trophocyte viability decrease. mTOR pathway activation may be associated with the pathophysiology of ICP.

Conflict of interest declaration

The authors declare no conflict of interest.

Acknowledgments

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References

[1] A.E. Kremer, R. Bolier, P.H. Dixon, V. Geenes, J. Chambers, D. Tolenaars, C. Ris-Stalpers, B.M. Kaess, C. Rust, J.A. van der Post, C. Williamson, U. Beuers, R.P. Oude Elferink, Autotaxin activity has a high accuracy to diagnose intrahepatic cholestasis of pregnancy, J. Hepatol. 62 (2015) 897–904.

[2] L. Brouwers, M.P. Koster, G.C. Page-Christiaens, H. Kemperman, J. Boon, I.M. Evers, A. Bogte, M.A. Oudijk, Intrahepatic cholestasis of pregnancy: maternal and fetal outcomes associated with elevated bile acid levels, Am. J. Obstet. Gynecol. 212 (2015) 100.e101–100.e107.

[3] V. Geenes, L.C. Chappell, P.T. Seed, P.J. Steer, M. Knight, C. Williamson, Association of severe intrahepatic cholestasis of pregnancy with adverse pregnancy outcomes: a prospective population-based case-control study, Hepatology 59 (2014) 1482–1491.

[4] V. Geenes, C. Williamson, Intrahepatic cholestasis of pregnancy, World J. Gastroenterol. 15 (2009) 2049–2066.

[5] A. Glantz, H.U. Marschall, L.A. Mattsson, Intrahepatic cholestasis of pregnancy: relationships between bile acid levels and fetal complication rates, Hepatology 40

(2004) 467–474.
[6] G. Mazzella, N. Rizzo, F. Azzaroli, P. Simoni, L. Bovicelli, A. Miracolo, G. Simonazzi, A. Colecchia, G. Nigro, C. Mwangemi, D. Festi, E. Roda, Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers, Hepatology 33 (2001) 504–508.
[7] A.M. Germain, S. Kato, J.A. Carvajal, G.J. Valenzuela, G.L. Valdes, J.C. Glasinovic, Bile acids increase response and expression of human myometrial oxytocin receptor, Am. J. Obstet. Gynecol. 189 (2003) 577–582.
[8] W.H. Sepulveda, C. Gonzalez, M.A. Cruz, M.I. Rudolph, Vasoconstrictive effect of bile acids on isolated human placental chorionic veins, Eur. J. Obstet. Gynecol. Reprod. Biol. 42 (1991) 211–215.
[9] C. Williamson, J. Gorelik, B.M. Eaton, M. Lab, M. de Swiet, Y. Korchev, The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intrauterine fetal death in obstetric cholestasis, Clin. Sci. (Lond.) 100 (2001) 363–369.
[10] J.J. Marin, R.I. Macias, O. Briz, M.J. Perez, A.G. Blazquez, M. Arrese, M.A. Serrano, Molecular bases of the fetal liver-placenta-maternal liver excretory pathway for cholephilic compounds, Liver Int. 28 (2008) 435–454.
[11] M.A. Serrano, R.I. Macias, O. Briz, M.J. Monte, A.G. Blazquez, C. Williamson, R. Kubitz, J.J. Marin, Expression in human trophoblast and choriocarcinoma cell lines, BeWo, Jeg-3 and JAr of genes involved in the hepatobiliary-like excretory function of the placenta, Placenta 28 (2007) 107–117.
[12] T. Zhang, C. Zhao, L. Luo, J. Xiang, J. Cheng, T. Wang, D. Chen, High concentraction of taurocholic acid induced apoptosis in HTR-8/SVneo cells via overexpression of ERp29 and activation of p38, Placenta 35 (2014) 496–500.
[13] J.J. Marin, R.I. Macias, M.A. Serrano, The hepatobiliary-like excretory function of the placenta. A review, Placenta 24 (2003) 431–438.
[14] M.J. Perez, R.I. Macias, J.J. Marin, Maternal cholestasis induces placental oxidative stress and apoptosis. Protective effect of ursodeoxycholic acid, Placenta 27 (2006) 34–41.
[15] E. Wikstrom Shemer, M. Thorsell, E. Ostlund, B. Blomgren, H.U. Marschall, Stereological assessment of placental morphology in intrahepatic cholestasis of pregnancy, Placenta 33 (2012) 914–918.
[16] Y. Zhang, Y. Pan, C. Lin, Y. Zheng, H. Sun, H. Zhang, J. Wang, M. Yuan, T. Duan, Q. Du, J. Chen, Bile acids evoke placental inflammation by activating Gpbar1/NF-kappaB pathway in intrahepatic cholestasis of pregnancy, J. Mol. Cell Biol. 8 (2016) 530–541.
[17] R.A. Saxton, D.M. Sabatini, mTOR signaling in growth, metabolism, and disease, Cell 169 (2017) 361–371.
[18] E. Bachar, Y. Ariav, M. Ketzinel-Gilad, E. Cerasi, N. Kaiser, G. Leibowitz, Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1, PLoS One 4 (2009) e4954.
[19] C. Xu, B. Bailly-Maitre, J.C. Reed, Endoplasmic reticulum stress: cell life and death decisions, J. Clin. Invest. 115 (2005) 2656–2664.
[20] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell Biol. 8 (2007) 519–529.
[21] D.T. Rutkowski, R.J. Kaufman, A trip to the ER: coping with stress, Trends Cell Biol. 14 (2004) 20–28.
[22] R. Kim, M. Emi, K. Tanabe, S. Murakami, Role of the unfolded protein response in cell death, Apoptosis 11 (2006) 5–13.
[23] H. Malhi, R.J. Kaufman, Endoplasmic reticulum stress in liver disease, J. Hepatol. 54 (2011) 795–809.
[24] C.H. Jia, M. Li, J. Liu, L. Zhao, J. Lin, P.L. Lai, X. Zhou, Y. Zhang, Z.G. Chen, H.Y. Li, A.L. Liu, C.L. Yang, T.M. Gao, Y. Jiang, X.C. Bai, IKK-beta mediates hydrogen peroxide induced cell death through p85 S6K1, Cell Death Differ. 20 (2013) 248–258.
[25] C.J. Yen, J.G. Izzo, D.F. Lee, S. Guha, Y. Wei, T.T. Wu, C.T. Chen, H.P. Kuo, J.M. Hsu, H.L. Sun, C.K. Chou, N.S. Buttar, K.K. Wang, P. Huang, J. Ajani, M.C. Hung, Bile acid exposure up-regulates tuberous sclerosis complex 1/mammalian target of rapamycin pathway in Barrett's-associated esophageal adenocarcinoma, Cancer Res. 68 (2008) 2632–2640.
[26] M. Wang, R.J. Kaufman, Protein misfolding in the endoplasmic reticulum as a conduit to human disease, Nature 529 (2016) 326–335.
[27] Y. Guri, M.N. Hall, mTOR signaling confers resistance to targeted cancer drugs, Trends Cancer 2 (2016) 688–697.
[28] F. Di Domenico, A. Tramutola, C. Foppoli, E. Head, M. Perluigi, D.A. Butterfield, mTOR in Down syndrome: role in ass and tau neuropathology and transition to Alzheimer disease-like dementia, Free Radic. Biol. Med. 114 (2018) 94–101.
[29] A. Kaur, S. Sharma, Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases, Inflammopharmacology 25 (2017) 293–312.
[30] J.J. Marin, A. Hernandez, I.E. Revuelta, E. Gonzalez-Sanchez, J.M. Gonzalez-Buitrago, M.J. Perez, Mitochondrial genome depletion in human liver cells abolishes bile acid-induced apoptosis: role of the Akt/mTOR survival pathway and Bcl-2 family proteins, Free Radic. Biol. Med. 61 (2013) 218–228.
[31] U. Ozcan, L. Ozcan, E. Yilmaz, K. Duvel, M. Sahin, B.D. Manning, G.S. Hotamisligil, Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis, Mol. Cell 29 (2008) 541–551.
[32] N. Ito, Y. Nishibori, Y. Ito, H. Takagi, Y. Akimoto, A. Kudo, K. Asanuma, Y. Sai, K. Miyamoto, H. Takenaka, K. Yan, mTORC1 activation triggers the unfolded protein response in podocytes and leads to nephrotic syndrome, Lab. Investig. 91 (2011) 1584–1595.
[33] F. Han, L. Xu, Y. Huang, T. Chen, T. Zhou, L. Yang, Magnesium sulphate can alleviate oxidative stress and reduce inflammatory cytokines in rat placenta of intrahepatic cholestasis of pregnancy model, Arch. Gynecol. Obstet. 298 (2018) 631–638.
[34] T. Zhang, Y. Guo, X. Guo, T. Zhou, D. Chen, J. Xiang, Z. Zhou, Comparative proteomics analysis of placenta from pregnant women with intrahepatic cholestasis of pregnancy, PLoS One 8 (2013) e83281.
[35] Q. Du, Y. Zhang, Y. Pan, T. Duan, Lithocholic acid-induced placental tumor necrosis factor-α upregulation and syncytiotrophoblast cell apoptosis in intrahepatic cholestasis of pregnancy, Hepatol. Res. 44 (2014) 532–541.
[36] T. Zhang, C. Zhao, L. Luo, J. Xiang, J. Cheng, T. Wang, D. Chen, High concentraction of taurocholic acid induced apoptosis in HTR-8/SVneo cells via overexpression of ERp29 and activation of p38, Placenta 35 (2014) 496–500.
[37] M.J. Bicocca, J.D. Sperling, S.P. Chauhan, Intrahepatic cholestasis of pregnancy: review of six national and regional guidelines, Eur. J. Obstet. Gynecol. Reprod. Biol. 231 (2018) 180–187.