COMT inhibitor tolcapone represses the migration and invasion of trophoblast and results in preeclampsia-like phenotypes in mice

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Abbreviations

YOPD, young onset Parkinson's disease; PD, Parkinson's disease; COMT, catechol O-methyltransferase; Tol, tolcapone; H-Tol, high-dose tolcapone; L-Tol, low-dose tolcapone; JZ, junction zone; LZ, labyrinth zone; SP, spongiotrophoblast; SBP, systolic blood pressure; DE, decidua; GC, trophoblast giant cell; CK8, cytokeratin 8; α-SMA, α-smooth muscle actin; EMT, epithelial–mesenchymal transition.

Abstract

Pregnancy in young-onset Parkinson's disease (YOPD) is rare; however, medication for this condition is critical for maternal and fetal health. Tolcapone is an effective antiparkinsonian drug. However, its safety studies on mothers and fetuses are limited. In this study, we aimed to investigate the effects of tolcapone on the mother and the developing fetus during different stages if gestation. Tolcapone was administered to pregnant mice at a dose of 60 (H-Tol) or 30 mg/kg/day (L-Tol) during early or mid-gestation. We observed that tolcapone administration during early gestation causes abortion and delays fetal development in a dose-dependent manner. During mid-gestation tolcapone barely caused embryo lethality; however, the mice developed preeclampsia-like phenotypes, including maternal hypertension, proteinuria, and fetal growth restriction. Histomorphological analysis of placentas from tolcapone treated mice revealed abnormalities in the trophoblast layer and the impaired trophoblast invasion in the decidua. Mechanistically, we revealed that tolcapone inhibits the invasion and migration of trophoblasts *in vitro*, with changes in the protein expression of Snail, Twist, and E-cadherin. In conclusion, tolcapone caused embryo lethality and growth restriction during early gestation, whereas it caused preeclampsia-like phenotypes in mice with defective trophoblast invasion in mid-gestation. Collectively, our study provides novel insights into the effects of tolcapone on pregnancy.

Introduction

Young onset Parkinson's disease (YOPD) is a type of Parkinson's disease (PD) diagnosed between 21 and 40 years[1]. The YOPD population comprises 5-7% of patients with PD in Western countries and approximately 10% in Japan[2]. Unlike late-onset PD, YOPD progresses slowly with regular and continuous care and treatments[3]. Pregnancy in YOPD is rare; however, clinicians should counsel women with YOPD of childbearing potential. Based on an observation of 74 pregnant women with PD, antiparkinsonian medications improved PD symptoms and reduced the incidence of deteriorated symptoms from 67% to 36%[4]. Antiparkinsonian medications are effective for controlling PD in pregnancy; however, knowledge of the safety of medications during pregnancy and the potential risk to the fetus remains limited[5].

Tolcapone, an inhibitor of catechol O-methyltransferase (COMT), is indicated in combination with levodopa/carbidopa therapy for PD[6-8]. The Food and Drug Administration (FDA) lists antiparkinsonian drugs in pregnancy category C[9]. Pregnancy category C implies that animal studies revealed a risk to the fetus, and studies on humans are unavailable. The potential benefits in maternal disease control may outweigh the potential risks to the fetus. Administration of tolcapone during animal gestation has been implicated in fetal growth restriction and malformation; however, the studies are limited, and the pathogenesis remains unknown[10]. Meanwhile, potential pregnancy complications in mothers have rarely been reported. Therefore, exploring the effects of tolcapone during gestation is necessary and may provide additional information for clinicians caring pregnant women with PD.

The placenta is vital for evaluating the tolcapone-mediated risks to the fetus. Notably, changes in the placental trophoblast layer, which is an important tool for understanding the mechanisms of reproductive and developmental toxicity, must be evaluated[11]. As the main cell type in the placenta, trophoblasts anchor the fetus to the uterine wall, synthesize steroids and hormones, and exchange nutrients and gases

121 between mother and fetus. The invasion of trophoblasts from the placenta to the
122 maternal decidua is critical for remodeling the uterine spiral artery into a dilated
123 vessel. Failure of invasive trophoblasts can lead to failed spiral artery transformation,
124 ischemia, and oxidative stress in placenta, with consequences for fetal growth
125 restriction and other pregnancy complications such as preeclampsia[12-14]. Therefore,
126 we aimed to elucidate the trophoblast- and placenta-derived
127 pathophysiological changes in the mother and fetus under the effect of tolcapone.
128

129 **Methods**

130 **Animals and experimental protocol**

131 C57BL/6 mice aged 8-10 weeks were housed overnight, and the presence of vaginal
132 spermatozoa confirmed pregnancy. Pregnant mice on gestational day 0.5 or day 9.5
133 were administered tolcapone (A4383, APExbio, USA) once daily via gastric gavage
134 until the end of gestation. A high dose (H-Tol, 60 mg/kg/day) of tolcapone for mice
135 was derived from the daily recommended human dose (100-200 mg, thrice a day)[8,15].
136 The low dose (L-Tol) of tolcapone administered to the mice was 30 mg/kg/day.
137

138 Blood pressure was measured with a Mouse Blood Pressure System (BP2010, Softron
139 Biotechnology, China) using the tail-cuff method. The mice were trained for 2-3 days
140 until they were accustomed to the cone animal holder and remained calm during the
141 measurements. Blood pressure was recorded from gestational day 0-17.5.
142

143 All animal experiments were approved by the Ethics Committee of Laboratory
144 Animal Sciences, Southern Medical University.
145

146 **Cell culture**

147 The human first-trimester placental trophoblast cell line HTR8/SVneo (ATCC
148 CRL-3271, USA) was cultured in RPMI 1640 medium containing 2 mM glutamine,
149 10% FBS, and 1% penicillin-streptomycin. The human choriocarcinoma trophoblast
150 cell line BeWo (ATCC CCL-98, USA) was cultured in F-12K medium containing 2

151 mM glutamine, 10% FBS, and 1% penicillin-streptomycin. The cells were maintained
152 at 37°C with 5% CO_2.
153
154 **Wound healing, Transwell, cell viability, and cell proliferation assays**
155 Wound healing and Transwell assays were performed using the invasive trophoblast
156 cell line HTR8/SVneo. The wound healing assay was conducted using an ibidi culture
157 silicone insert (81176, ibidi, Germany). HTR8/SVneo cells were cultured to 90%
158 confluence, and the insert was removed. The cells were maintained in a medium
159 containing tolcapone and 1% FBS to minimize cell proliferation. Cell migration was
160 examined after 24 h and 48 h. For Transwell migration assay, tolcapone-treated
161 HTR8/SVneo cells were suspended in a serum-free medium and seeded into a
162 Transwell chamber with 8μm pores (353097-8EA, Corning, USA). Medium
163 containing 10% FBS was supplied to the lower chamber to induce cells to pass
164 through the Transwell pores. The Transwell invasion assay was performed using a
165 Transwell chamber pre-coated with Matrigel (354234, Corning, USA). After 24 h of
166 incubation at 37°C with 5% CO_2, the cells on the lower surface of the transwell
167 membrane were fixed and labeled with crystal violet dye. Images were acquired using
168 a BX51 microscope (Olympus, Japan). Twenty stained images from each group were
169 quantified using Image-J software (NIH, USA).
170
171 A cell viability assay was performed using HTR8/SVneo cells. The cells were
172 exposed to 0-350 mM tolcapone for 24 h. Viable cells were assessed using the CCK-8
173 assay (C0039, Beyotime, China). For the cell proliferation assay, HTR8/SVneo cells
174 were cultured in a medium containing 0-45 mM tolcapone for 72 h. The numbers of
175 live cells were determined using the CCK-8 assays, and proliferation rates were
176 analyzed.
177
178 **Western immunoblotting**
179 Protein samples for western blotting were extracted as described previously[16]. Briefly,
180 proteins were extracted from homogenized mouse placental tissues or cell samples

using RIPA lysis buffer (89900, Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitors (A32961, Thermo Fisher Scientific, USA). Equal protein loading was confirmed using the intensity of the GAPDH blot. Western blotting experiments were performed in triplicate. Densitometry of the blots was performed using Image-J software (NIH, USA).

The primary antibodies used in this study were as follows: anti-E-cadherin (3195), anti-Twist1 (31174), and anti-Snail (3879) from Cell Signaling Technology, USA, and anti-GAPDH (sc-47724) from Santa Cruz, USA.

H&E staining, immunohistochemistry staining, and fluorescence staining

Paraffin-embedded sections of the whole mouse placentas were stained with hematoxylin and eosin staining (60524ES60, Yeasen, China). Consecutive mouse placental sections were used for immunohistochemical staining of cytokeratin 8 (CK8) and α-smooth muscle actin (α-SMA). The primary antibodies anti-CK8 (ab154301) and anti-α-SMA (ab5694) were from Abcam, USA. BrdU staining (6813S, Cell Signaling Technology, USA) of proliferating HTR8/SVneo and BeWo cells was performed. Nuclei were visualized by DAPI staining. Images were acquired using a fluorescence microscope (BX51 System Microscope, Olympus, Japan). Twenty stained images from each group were quantified using Image-J Software (NIH, USA).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Unpaired, two-tailed Student's *t*-test was used for two-group comparisons. One-way ANOVA, followed by the Student–Newman–Keuls method, was used for multiple-group comparisons. All analyses were conducted using GraphPad Prism 8.0 and SAS 9.4. Statistical significance was set at $P<0.05$.

Results

Tolcapone causes abortion, fetal growth restriction and placental abnormality in

early gestation

Administration of tolcapone in pregnant mice started at gestational day 0.5 and lasted until gestational day 17.5 (Fig. 1A). The dose of 60 mg/kg/day of tolcapone in mice was derived from the recommended daily human dose of 100-200 mg, thrice a day, which was set as the highest dose (H-Tol) in our animal study. To explore the minimum safe dose of tolcapone during pregnancy, we administered a low dose (L-Tol) of 30 mg/kg/day tolcapone. The body weights of L-Tol mice increased as gestation progressed; however, they were significantly lower from gestational day 7.5 to the end of gestation compared with those of the saline control group (Fig. 1B). The H-Tol mice displayed no change in body weight, indicating abortion or embryo loss (Fig. 1B). Therefore, we analyzed the placentas and fetuses of L-Tol mice in subsequent studies. The fetal size decreased at gestational day 17.5 in the L-Tol group compared with that in the saline control group (Fig. 1C). The number of live pups decreased, and the fetal resorption rate increased in L-Tol mice (Fig. 1D). Consistent with the smaller fetal size, the fetal weight of L-Tol mice decreased; however, placental weights increased (Fig. 1E). To explore the underlying mechanism, we examined the placental structure. Histomorphological analysis revealed a pathological change in the placentas of L-Tol mice, as collagen deposits were observed in the junction zone (JZ). Endothelial hyperplasia with thrombus formation was observed in large blood vessels (Fig. 1F). The ratio between the labyrinth zone (LZ) and the JZ, the two functional zones of the mouse placenta, decreased in the L-Tol group (Fig. 1G). A series of mouse blood pressure measurements were recorded during gestation and displayed no significant difference between the L-Tol and the control group (Fig. 1H). These data indicated that the administration of high-dose tolcapone in early gestation caused abortion in mice, whereas the low-dose caused fetal growth restriction in mice with placental damage.

Tolcapone causes preeclampsia-like phenotypes in mid-gestation

Considering the developmental toxicity of tolcapone during early gestation, we examined the effects of tolcapone administration from the middle to the end of

241 gestation. Pregnant mice received high- or low-dose tolcapone starting on gestational
242 day 9.5 and lasting until day 17.5 (Fig. 2A). Maternal body weight increased
243 consistently and showed no significant differences among the three groups (Fig. 2B).
244 Meanwhile, the number of live pups and the fetal resorption rate showed no
245 significant difference with or without tolcapone treatment (Fig. 2E, F), indicating that
246 administration of tolcapone at mid-gestation did not affect mouse abortion, even at a
247 high dose of 60 mg/kg/day. However, tolcapone treatment caused fetal growth
248 restriction even during mid-gestation, as evidenced by decreased fetal size and weight
249 (Fig. 2C, G). Fetal malformations were observed in H-Tol mice (Fig. 2D). Placental
250 weight did not differ with tolcapone treatment (Fig. 2G); however, the morphology of
251 the placentas changed significantly in response to H-Tol and L-Tol.
252 Spongiotrophoblasts, the main trophoblastic cell type comprising of a junction zone,
253 spread irregularly into the labyrinth zone in the placentas of tolcapone-treated mice
254 (Fig. 2H). In addition, pathological changes in the placenta caused the ratios of the
255 two functional zones to decrease (Fig. 2I).
256
257 Maternal blood pressure was examined to determine the function of tolcapone in
258 mothers. Notably, both the H-Tol and L-Tol groups exhibited significantly higher
259 systolic blood pressure (SBP) from gestational day 11.5 to the end of gestation (Fig.
260 2J). Moreover, H-Tol mice exhibited a significantly higher urine protein concentration
261 on gestational day 17.5, whereas L-Tol did not affect urinary protein excretion (Fig.
262 2K). These results indicated that tolcapone in mid-gestation led to fetal growth
263 restriction and maternal gestational hypertension, and might be associated with
264 proteinuria if high-dose tolcapone was administered. These symptoms are similar to
265 the typical phenotypes of preeclampsia.
266
267 **Tolcapone inhibits the invasion of placental trophoblast to maternal decidua in**
268 **mice**
269 The invasion of trophoblasts from the placenta to the maternal decidua is critical for
270 placentation and spiral artery transformation[12]. Therefore, we investigated trophoblast

271 invasion in the placental and decidual tissues of mice treated with tolcapone.
272 Immunostainings of cytokeratin 8 (CK8) and α-smooth muscle actin (α-SMA) were
273 conducted in consecutive sections of placental tissue to label placental trophoblasts
274 and maternal decidua, respectively (Fig. 3A). Compared with the saline control, L-Tol
275 mouse placentas showed a significant decrease in trophoblast invasion. The invasion
276 area was further reduced in H-Tol mouse placentas (Fig. 3A, B). In addition to the
277 histomorphological evidence, the EMT marker E-cadherin protein expression was
278 increased in H-Tol mouse placentas (Fig. 3C). These results suggested that tolcapone
279 inhibited trophoblast invasion *in vivo*, potentially through E-cadherin-mediated EMT.
280 To test this hypothesis, we explored the effects of tolcapone on the trophoblast
281 HTR8/SVneo cell line.
282
283 **Tolcapone represses cell invasion in HTR8/SVneo trophoblast cell line**
284 HTR8/SVneo trophoblasts were exposed to different tolcapone concentrations, and
285 cell viability was examined (Fig. 4A). Based on the cell viability curve, we
286 investigated the cell invasion and migration at tolcapone concentrations of 25mM and
287 35mM. In our study, tolcapone suppressed the migration of HTR8/SVneo cells in a
288 dose- and time-dependent manner (Fig. 4B-D). We further investigated the invasive
289 ability of HTR8/SVneo cells. Tolcapone inhibited cell invasion in the Matrigel-coated
290 Transwells (Fig. 4E). The EMT signaling pathway is a classic regulator of trophoblast
291 invasion and migration. Thus, we explored the potential underlying mechanism by
292 examining Snail, Twist, and E-cadherin protein expressions. The tolcapone-induced
293 inhibition of cell invasion was associated with increased E-cadherin expression and
294 decreased Snail and Twist expressions (Fig. 4F). These data were consistent with
295 observations in mouse placentas and revealed that EMT regulation might be involved
296 in tolcapone-repressed trophoblastic invasion and migration.
297
298 **Low concentration of tolcapone induces proliferation in HTR8/SVneo and BeWo**
299 **trophoblast cell lines**
300 We next determined whether the tolcapone-induced inhibition of cell invasion was

301 followed by the suppression of cell proliferation. HTR8/SVneo cells were treated with
302 0 mM to 45 mM tolcapone for 72 h and analyzed using cell viability assays. Notably,
303 low concentrations of 5 mM, 15 mM, and 25 mM tolcapone showed proliferation
304 activities, whereas 35 mM tolcapone had no effect on cell viability and 45 mM
305 tolcapone showed evident growth-suppressive activity (Fig. 5A). HTR8/SVneo cells
306 displayed active proliferation when exposed to 25 mM tolcapone (Fig. 5A). To
307 confirm these findings, we performed BrdU staining of HTR8/SVneo cells and
308 observed increased BrdU incorporation into the proliferating nucleus after treatment
309 with 25 mM tolcapone (Fig. 5B). We also tested the effects of tolcapone in a different
310 trophoblast cell line, BeWo. Low concentrations of tolcapone also induced the
311 proliferation of BeWo cells (Fig. 5C).
312

313 **Discussion**

314 Pregnancy in YOPD is rare, and the clinical experience of caring for pregnant women
315 with PD is limited[17]. However, as the reproductive age increases, it may become a
316 common occurrence[4]. Tolcapone is an effective antiparkinsonian drug used in
317 combination with levodopa/carbidopa. Safety studies of tolcapone in humans and
318 animals have focused on its potential hepatotoxicity; however, the risks to the mother
319 and fetus have not been fully elucidated[10,18]. Our study demonstrated that tolcapone
320 restricts fetal development and embryo death during early gestation in a
321 dose-dependent manner. The embryo survived if the mother took tolcapone during
322 mid-gestation; however, preeclampsia-like phenotypes associated with fetal growth
323 restriction occurred. One possible mechanism involves EMT signaling-modulated
324 trophoblast invasive dysfunction. Our study provides additional information on the
325 use of tolcapone in pregnancy, including dosage, gestational stage, and adverse effects
326 on the mother and fetus.
327

328 Patients with YOPD are more predisposed to levodopa-induced dyskinesias (motor
329 fluctuations)[19]. To overcome the loss of levodopa, two COMT inhibitors, entacapone
330 and tolcapone, are widely used as adjunctive therapies for PD[6]. Based on therapeutic

evidence in PD patients with motor fluctuations, tolcapone displays greater efficacy than entacapone[6]. However, evidence regarding the use of tolcapone during pregnancy in women with PD is limited; nonetheless, entacapone has been used in some patients with PD during pregnancy[9,17,20]. Animal studies have suggested that entacapone has teratogenic effects on fetal development[21]. Based on these results, women with PD were administered entacapone combined with levodopa/carbidopa starting at week 21 of gestation after the fetal organogenesis period and consequently delivered healthy babies[9,20]. Our study suggested that tolcapone caused fetal malformation and delayed development in mice, depending on the dosage and gestational stage. Our data showed that low-dose tolcapone in mid-gestation might be safer for fetal formation, although smaller fetuses were observed. In addition, tolcapone induces hypertension in pregnancy, which has not been reported in studies on antiparkinsonian drugs. Maternal preeclampsia-like complications should receive special attention in future studies.

Preeclampsia (PE) is a pregnancy-associated disorder that occures after 20 weeks of gestation with new-onset hypertension, proteinuria, and fetal intrauterine growth restriction[22]. It is widely accepted that preeclampsia is caused by shallow trophoblast invasion. Failure of trophoblast invasion into decidual arteries causes placental hypoxia and releases anti-angiogenic cytokines into the maternal blood, and consequently resulting in maternal hypertension[23,24]. We observed preeclampsia-like phenotypes in tolcapone-treated mice. Tolcapone administration began at mid-gestation, which is the period of trophoblast invasion, and hampered trophoblast invasion into the placenta and decidua. The *in vitro* experiments verified that tolcapone inhibits the migration and invasion of trophoblasts through changes in EMT signaling. Consistently, excessive E-cadherin hampered trophoblast invasion and has been implicated in patients with preeclampsia[25,26]. These findings suggest that tolcapone-induced productive and developmental toxicity share a similar mechanism of preeclampsia etiology.

Changes in placental morphology are common in pregnant disorders. Severe placental defects were found in 68% of the embryonic lethal genetic mutant mice[27]. The mutant gene in trophoblast cells caused a higher incidence of embryonic lethality[27]. We noted the abnormal spreading of spongiotrophoblasts in the placentas of tolcapone-treated mice. Consistent with our observations, the infiltration of spongiotrophoblast cells in the labyrinth zone was also detected in *Chtop*-/- and *Pth1r*-/- placentas with obvious fetal developmental delays[27,28], and to date, its pathogenesis is unknown. The biofunction of the spongiotrophoblast layer is poorly understood; however, it could be a supportive structure of the labyrinth that changes the vessels in the placenta and modulates exchange between the mother and fetus[29].

In our study, the data indicated that tolcapone promotes the proliferation of HTR8/SVneo and BeWo cells. One possible explanation is that the hyperactivated proliferation of trophoblasts results in an increased number of spongiotrophoblasts. However, detecting proliferating trophoblasts in mouse placentas is necessary to validate this hypothesis.

Conclusion

In the present study, we confirmed that tolcapone caused abortion and fetal growth restriction, and uncovered that tolcapone also resulted in maternal hypertension and proteinuria in mice, depending on the dosage and gestational stage at which they received tolcapone. Tolcapone inhibited the invasion of placental trophoblasts *in vivo* and *in vitro*, which was attributed to the regulation of the Snail/Twist/E-cadherin signaling pathway. Our study provides information on using tolcapone in pregnancy with PD, and provides novel insights into the pathogenesis of tolcapone-mediated developmental toxicity.

Declaration of interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author Contributions

L.C., M.P., Q. J. and F. S. performed experiments and analyzed data. X.Y., Y. M., L. H. designed research and wrote the manuscript. All the authors have read and approved the final version of the manuscript.

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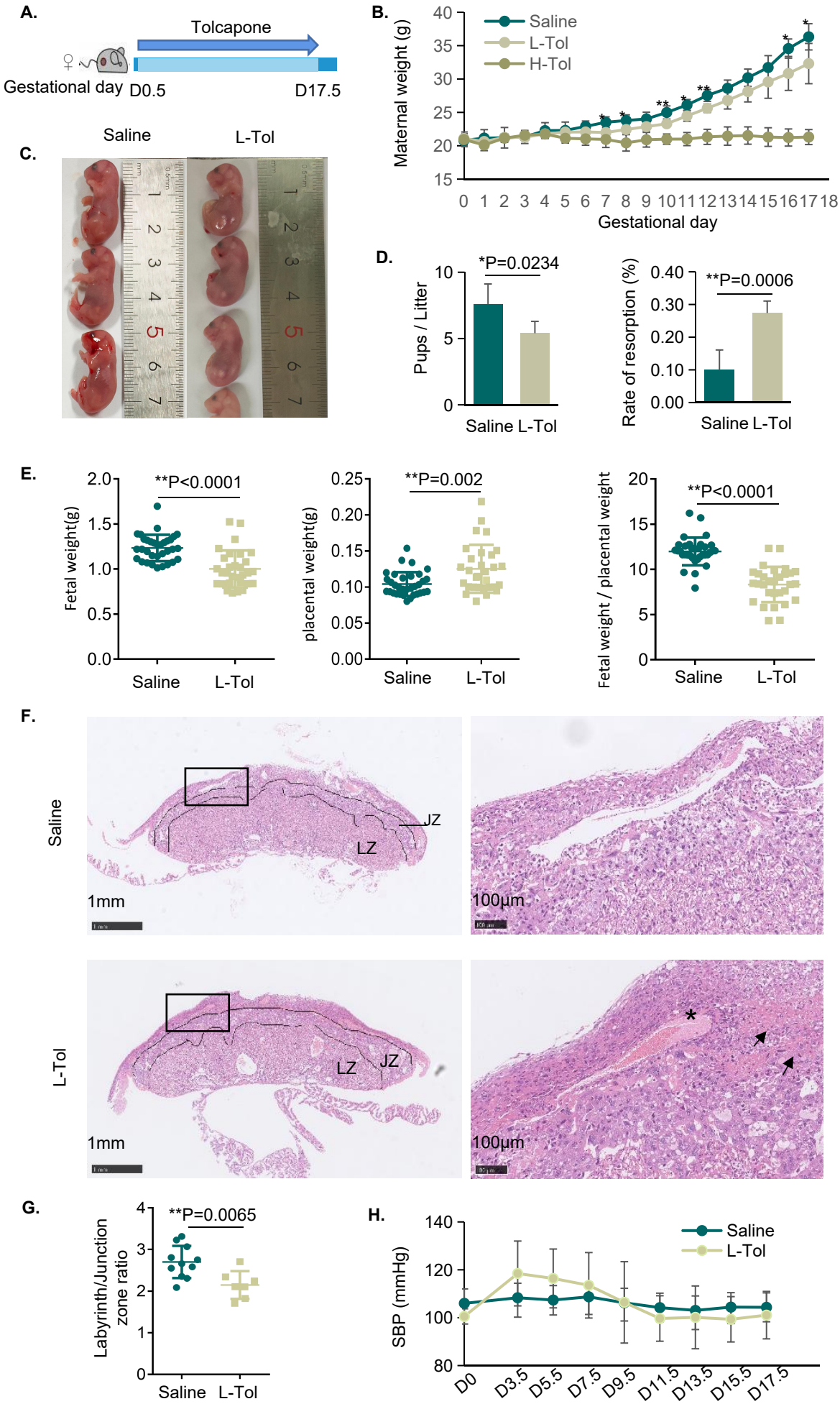
514 **Figure 1. Administration of tolcapone at early gestation resulted in abortion,**
515 **fetal growth restriction, and placental damage. A.** Schematic representation of
516 administration of tolcapone. Tolcapone was applied by i.h. injection for 17 days
517 starting at gestational Day 0.5. **B.** Maternal weight during pregnancy with or without
518 tolcapone. H-Tol, high-dose tolcapone; L-Tol, low-dose tolcapone; * indicates $p<0.05$
519 in a comparison of saline control group with L-Tol group; ** indicates $p<0.01$ in a
520 comparison of saline control group with L-Tol group. **C.** Smaller fetal size was
521 observed in the mice with L-Tol comparing with that in the saline control group. **D.**
522 Number of live pups and fetal resorption rate per litter. **E.** Fetal weight and placental
523 weight in mice with or without tolcapone. **F.** Representative H&E staining images of
524 placental tissue sections. Labyrinth zone (LZ) and junction zone (JZ) were indicated
525 by dotted line. The right panel images were zoomed from the left panel showing
526 increased fibrin deposit (marked with arrows) and vascular thrombus formation
527 (marked with a star) in the placentas of L-Tol group. **G.** Ratio of the areas of labyrinth
528 zone over junction zone in placentas. **H.** Maternal systolic blood pressure (SBP) of
529 mice with or without tolcapone treatment was measured during the whole gestation.

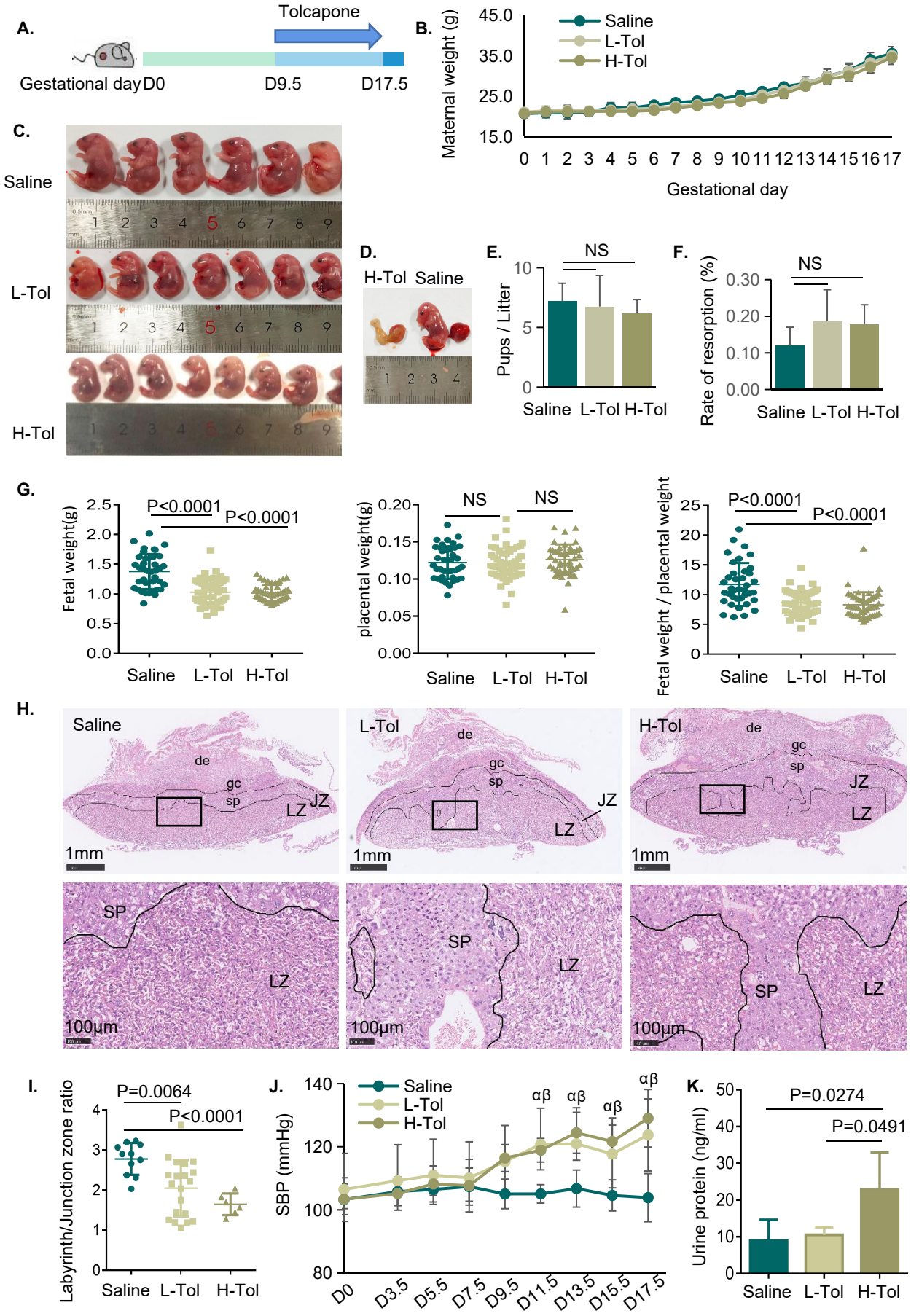
530

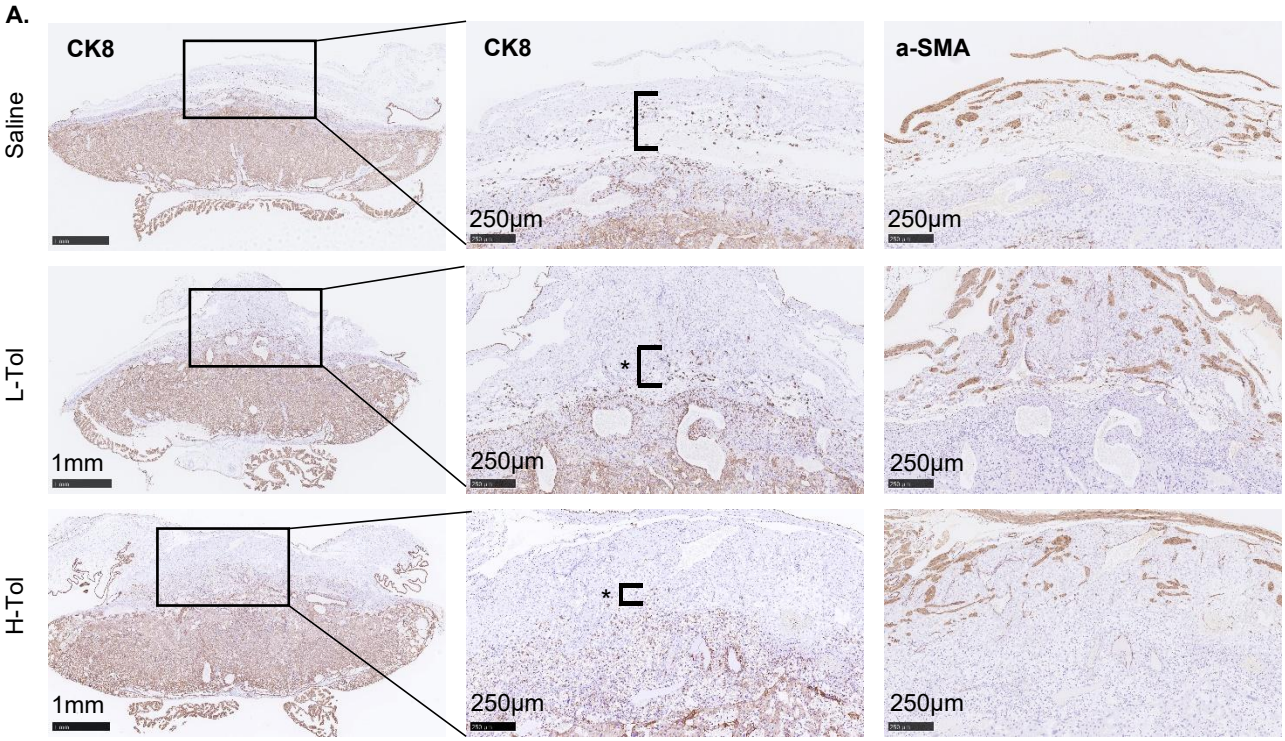
531 **Figure 2. Administration of tolcapone at mid-gestation resulted in fetal growth**
532 **restriction, maternal gestational hypertension, proteinuria, and placental**
533 **abnormalities. A.** Schematic representation of administration of tolcapone. Tolcapone
534 was applied by i.h. injection starting at gestational Day 9.5 until Day 17.5. **B.**
535 Maternal weight during pregnancy with or without tolcapone. **C.** Both L-Tol mice and
536 H-Tol mice showed smaller fetal size when compared with that in the saline control
537 group. **D.** Malformation of embryo from H-Tol mice. **E.** Number of live pups per litter.
538 **F.** Quantification of fetal resorption rate per litter. **G.** Fetal weight and placental

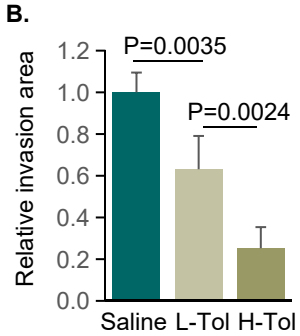
539 weight in the mice of the three groups. **H.** Representative H&E staining images of
540 placental tissue sections. Labyrinth zone (LZ) and junction zone (JZ) were indicated
541 by dotted line. The penetration of spongiotrophoblast cells (SP) in LZ were shown in
542 the placentas of L-Tol and H-Tol mice at the lower panel. de, decidua; gc, trophoblast
543 giant cell. **I.** Ratio of areas of labyrinth zone over junction zone of placentas. **J.**
544 Maternal systolic blood pressure (SBP) of mice was evaluated among the three groups
545 during the whole gestation. α indicates $p<0.05$ in a comparison of saline group with
546 L-Tol group; β indicates $p<0.05$ in a comparison of saline group with H-Tol group. **K.**
547 Urinary protein concentration of maternal mice was examined among the three groups
548 at gestational day 17.5.
549
550 **Figure 3. Tolcapone repressed placental trophoblast invasion in mice. A.**
551 Representative images for immunostainings of cytokeratin 8 (CK8) and α-smooth
552 muscle actin (α-SMA) in consecutive sections of placental tissues. Stars indicated the
553 invasion distance of trophoblasts (CK8 positive cells) in maternal decidua (α-SMA
554 labeled zone). **B.** Relative area of trophoblast invasion into decidua was quantified. **C.**
555 Protein expression level of E-cadherin was detected in placental tissues.
556
557 **Figure 4. Tolcapone inhibited cell invasion in HTR8/SVneo cells. A.** Cell viability
558 assay. HTR8/SVneo cells were treated with various doses of tolcapone for 48 h. Cell
559 viability was measured by CCK8 assay. **B.** Wound healing assay of HTR8/SVneo
560 cells treated with 25mM or 35mM tolcapone for 48 h. **C.** Quantification of relative
561 healing wound width is shown. **D.** Measurement of cell migration by Transwell assay.
562 HTR8/SVneo cells were treated with 25mM or 35mM tolcapone for 24 h. **E.**
563 Measurement of cell invasion by Transwell assay. HTR8/SVneo cells treated with
564 25mM or 35mM tolcapone for 24 h. **F.** Protein expressions of E-cadherin, Snail, and
565 Twist were detected in cells treated with 35mM tolcapone.
566
567 **Figure 5. Tolcapone induced proliferation in trophoblasts. A.** Cell viability assay
568 for the HTR8/SVneo cells treated with various doses of tolcapone for 72 h. α indicates

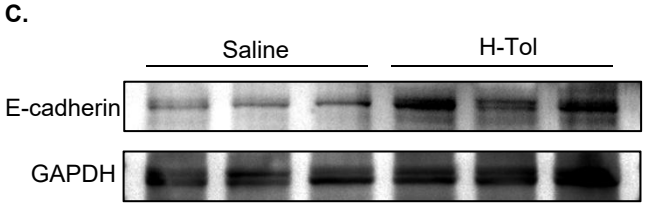
569 $p<0.05$ in a comparison of 25 mM group with 0 mM group; β indicates $p<0.05$ in a
570 comparison of 5 mM group with 0 mM group; γ indicates $p<0.05$ in a comparison of
571 15 mM group with 0 mM group. **B.** Representative images of BrdU stained
572 HTR8/SVneo cells treated with tolcapone. **C.** Representative images of BrdU stained
573 BeWo cells treated with tolcapone.

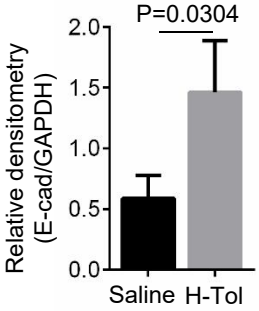

A.
Tolcapone
Gestational day D0.5
D17.5
B.
Saline
L-Tol
H-Tol
Maternal weight (g)
Gestational day
C.
Saline
L-Tol
D.
*P=0.0234
Pups / Litter
Saline L-Tol
**P=0.0006
Rate of resorption (%)
Saline L-Tol
E.
**P<0.0001
Fetal weight(g)
Saline
L-Tol
**P=0.002
placental weight(g)
Saline
L-Tol
**P<0.0001
Fetal weight / placental weight
Saline
L-Tol
F.
Saline
JZ
LZ
1mm
100μm
L-Tol
LZ
JZ
1mm
100μm
G.
**P=0.0065
Labyrinth/Junction zone ratio
Saline
L-Tol
H.
Saline
L-Tol
SBP (mmHg)
D0
D3.5
D5.5
D7.5
D9.5
D11.5
D13.5
D15.5
D17.5

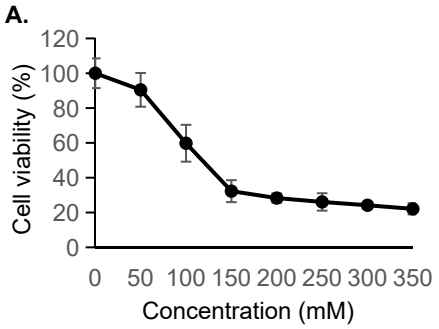

A.
Tolcapone
Gestational day D0
D9.5
D17.5
B.
Maternal weight (g)
Saline
L-Tol
H-Tol
Gestational day
C.
Saline
L-Tol
H-Tol
D.
H-Tol Saline
E.
NS
Pups / Litter
Saline L-Tol H-Tol
F.
NS
Rate of resorption (%)
Saline L-Tol H-Tol
G.
P<0.0001
P<0.0001
Fetal weight(g)
Saline L-Tol H-Tol
NS
NS
placental weight(g)
Saline L-Tol H-Tol
P<0.0001
P<0.0001
Fetal weight / placental weight
Saline L-Tol H-Tol
H.
Saline
de
gc
sp
LZ
JZ
1mm
L-Tol
H-Tol
SP
100μm
I.
P=0.0064
P<0.0001
Labyrinth/Junction zone ratio
Saline L-Tol H-Tol
J.
Saline
L-Tol
H-Tol
αβ
SBP (mmHg)
D0 D3.5 D5.5 D7.5 D9.5 D11.5 D13.5 D15.5 D17.5
K.
P=0.0274
P=0.0491
Urine protein (ng/ml)
Saline L-Tol H-Tol

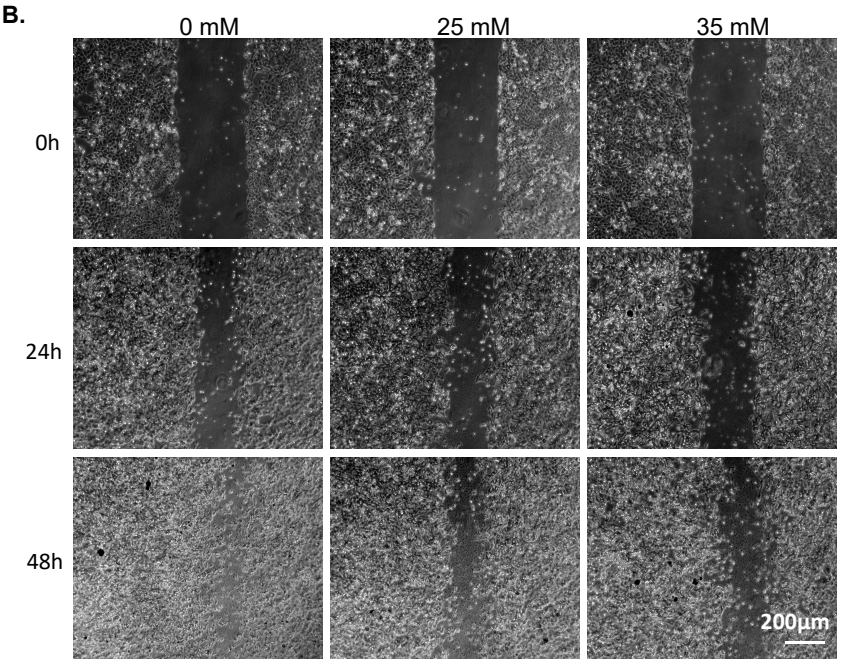

A.
CK8
CK8
a-SMA
Saline
L-Tol
H-Tol
250μm
250μm
250μm
1mm
250μm
250μm
1mm
250μm
250μm

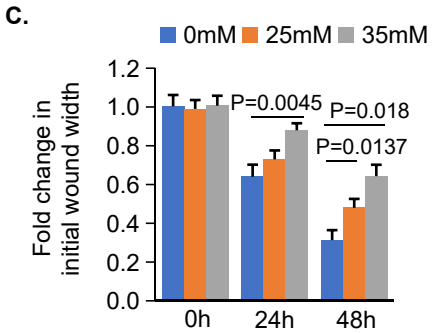

B.
Relative invasion area
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.0035
P=0.0024
Saline
L-Tol
H-Tol

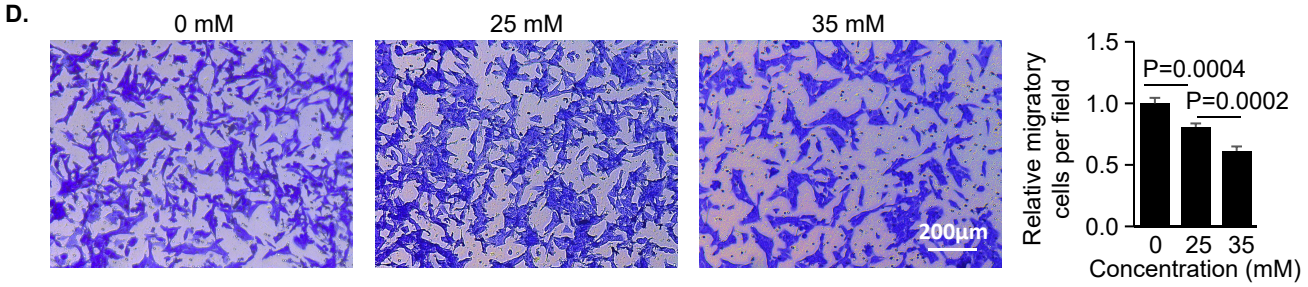

C.
Saline
H-Tol
E-cadherin
GAPDH

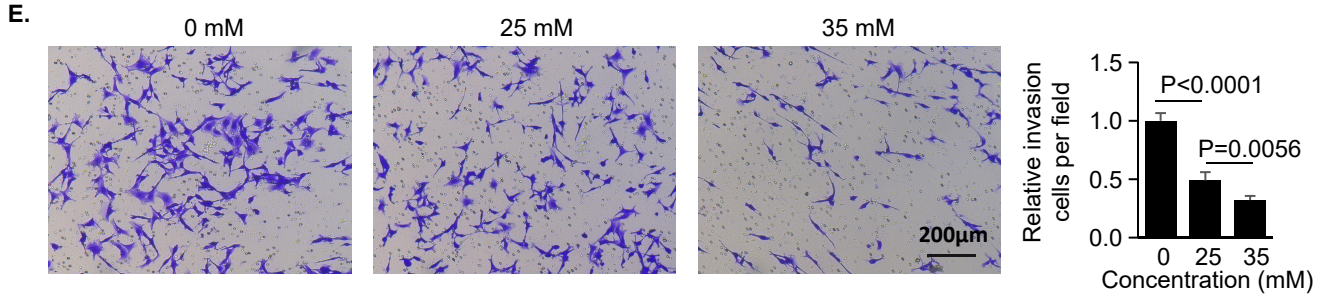

Relative densitometry
(E-cad/GAPDH)
2.0
1.5
1.0
0.5
0.0
P=0.0304
Saline
H-Tol

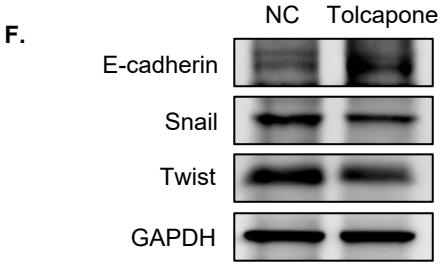

A.
Cell viability (%)
120
100
80
60
40
20
0
0 50 100 150 200 250 300 350
Concentration (mM)


B.
0 mM
25 mM
35 mM
0h
24h
48h
200μm

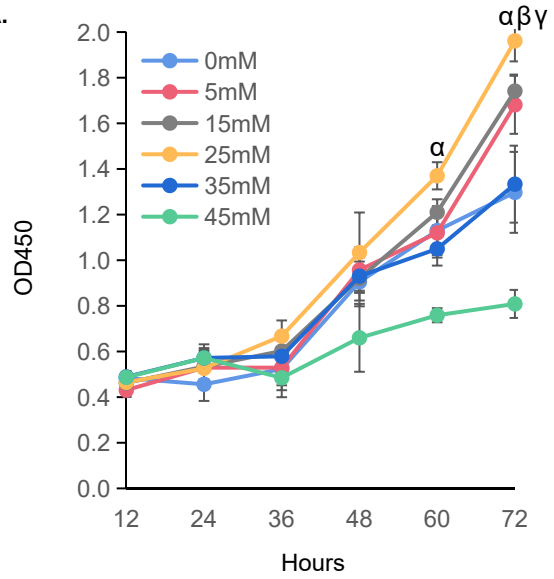

C.
0mM 25mM 35mM
Fold change in initial wound width
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.0045
P=0.018
P=0.0137
0h
24h
48h


D.
0 mM
25 mM
35 mM
200μm
Relative migratory cells per field
1.5
1.0
0.5
0.0
P=0.0004
P=0.0002
0 25 35
Concentration (mM)

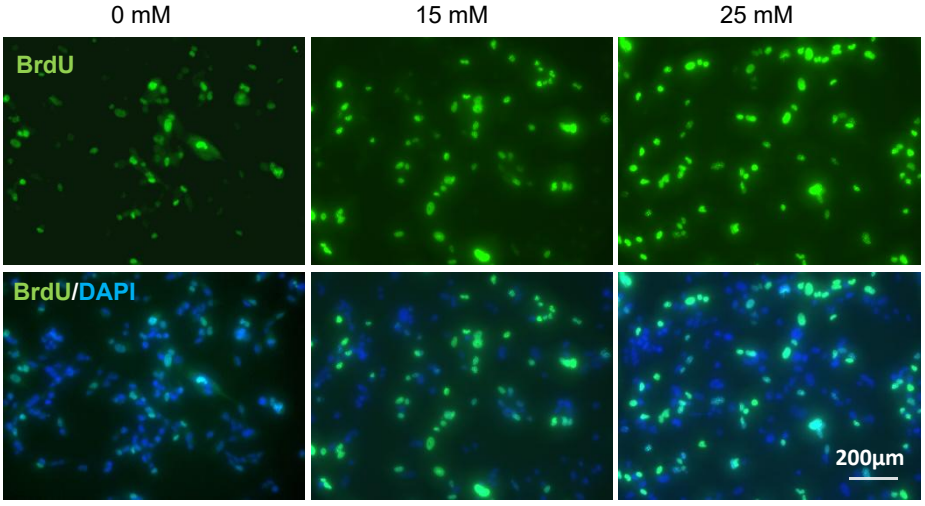

E.
0 mM
25 mM
35 mM
200μm
Relative invasion cells per field
1.5
1.0
0.5
0.0
P<0.0001
P=0.0056
0 25 35
Concentration (mM)


F.
NC
Tolcapone
E-cadherin
Snail
Twist
GAPDH

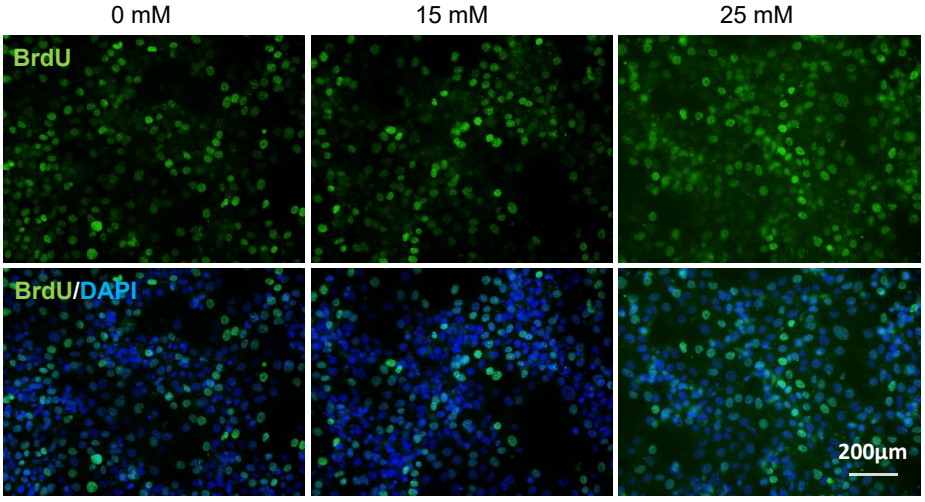
A.


αβγ
α
0mM
5mM
15mM
25mM
35mM
45mM
OD450
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
12
24
36
48
60
72
Hours

B.


0 mM
15 mM
25 mM
BrdU
BrdU/DAPI
200μm

C.


0 mM
15 mM
25 mM
BrdU
BrdU/DAPI
200μm