Placenta 75 (2019) 1–8

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Pravastatin improves fetal survival in mice with a partial deficiency of heme oxygenase-1

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ARTICLE INFO

Keywords:
Carbon monoxide
Placenta vasculature
Preeclampsia
Resorption
sFlt-1
Statins

ABSTRACT

Introduction: Statins induce heme oxygenase-1 (HO-1) expression *in vitro* and *in vivo*. Low HO-1 expression is associated with pregnancy complications, e.g. preeclampsia and recurrent miscarriages. Here, we investigated the effects of pravastatin on HO-1 expression, placental development, and fetal survival in mice with a partial HO-1 deficiency.

Methods: At E14.5, untreated pregnant wild-type (WT, n = 13–18), untreated HO-1$^{+/-}$ (Het, n = 6–9), and Het mice treated with pravastatin (Het + Pravastatin, n = 12–14) were sacrificed. Numbers of viable fetuses/resorbed concepti were recorded. Maternal livers and placentas were harvested for HO activity. Hematoxylin and eosin (H &E) and CD31 immunohistochemical staining were performed on whole placentas.

Results: Compared with WT, HO activity in Het livers (65 ± 18%, $P < 0.001$) and placentas (74 ± 7%, $P < 0.001$) were significantly decreased. Number of viable fetuses per dam was significantly lower in Untreated Het dams (6.0 ± 2.2) compared with WT (9.1 ± 1.4, $P < 0.01$), accompanied by a higher relative risk (RR) for concepti resorption (17.1, 95% CI 4.0–73.2). In Hets treated with pravastatin, maternal liver and placental HO activity increased, approaching levels of WT controls (to 83 ± 7% and 87 ± 14%, respectively). The number of viable fetuses per dam increased to 7.7 ± 2.5 with a decreased RR for concepti resorption (2.7, 95% CI 1.2–5.9). In some surviving Untreated Het placentas, there were focal losses of cellular architecture and changes suggestive of reduced blood flow in the labyrinth. These findings were absent in Het + Pravastatin placentas.

Discussion: Pravastatin induces maternal liver and placental HO activity, may affect placental function and improve fetal survival in the context of a partial deficiency of HO-1.

1. Introduction

Preeclampsia adversely affects 3%–5% of pregnancies and remains one of the leading causes of maternal and child mortality and morbidity [1]. Currently, the use of low-dose aspirin is the only therapy shown to be effective in reducing the risk of preeclampsia, with a 10%–20% decrease in women at moderate-to-high risk [2,3]. Pravastatin has been shown to reduce hypertension and proteinuria in mice overexpressing the anti-angiogenic protein soluble fms-like tyrosine kinase 1 (sFlt-1) [4,5], which has been implicated in the pathogenesis of preeclampsia [6–9]. Furthermore, a pilot randomized controlled clinical study assessing the safety of pravastatin for the prevention of preeclampsia in high-risk women has shown a favorable risk-benefit ratio [10].

Statins are known for their use in reducing cholesterol levels through the inhibition of the rate-limiting enzyme 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in patients at risk for cardiovascular diseases. However, their overall cardiovascular benefits appear to be greater than what might be expected from their lipid-

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; CO, carbon monoxide; E, embryonic day; FW, fresh weight; HDL, high density lipoprotein; H&E, hematoxylin and eosin; Het, HO-1 heterozygous; HMG-CoA, 5-hydroxy-3-methylglutaryl-coenzyme A; HO, heme oxygenase; IHC, immunohistochemistry; LDL, low density lipoprotein; PECAM1, platelet and endothelial cell adhesion molecule 1; RR, relative risk; sFlt-1, soluble fms-like tyrosine kinase 1; SP, spongiotrophoblast; WT, wild-type

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https://doi.org/10.1016/j.placenta.2018.11.001
Received 13 July 2018; Received in revised form 21 September 2018; Accepted 1 November 2018

lowering effects alone. Statins have been shown to improve endothelial function, decrease oxidative stress and inflammation, enhance stability of atherosclerotic plaques, and inhibit the thrombogenic response [11]. These "pleiotropic" properties of statins are believed to be mediated by the inducible heme oxygenase (HO) isozyme, HO-1. HO is a ubiquitously-expressed stress-response protein that degrades heme into carbon monoxide (CO), iron, and biliverdin, which is ultimately reduced to bilirubin [12]. HO not only prevents the accumulation of excess free heme [13]; but also, its bioactive byproducts can mediate antioxidant [14], anti-inflammatory [15], vasodilatory [16], and pro-angiogenic functions [17]. We and others have shown that various statins can increase the expression of HO-1 *in vitro* [18,19] and *in vivo* [20,21] in a statin- and tissue-specific manner, and thereby increase CO and bilirubin levels [21]. We even observed that statin treatment can reverse certain complications related to HO-1 deficiency, such as progression of abdominal aortic aneurysms (AAAs) [22].

Polymorphisms of the human HO-1 gene promoter region, such as the length of a (GT)n dinucleotide repeat sequence, have been shown to affect HO-1 expression – with longer (GT)n repeat lengths associated with decreased expression and a number of pathologic conditions [23]. For example, women possessing long (GT)n repeat lengths have an increased risk for recurrent pregnancy loss [24] as well as for non-severe and late-onset preeclampsia [25]. Moreover, the presence of long (GT)n repeats in the fetus (cord blood) has also been found to be associated with severe and early-onset preeclampsia [26]. Furthermore, HO-1 is upstream of sFlt-1 and has been shown to negatively regulate the expression of sFlt-1 [17]. Therefore, targeting HO-1 expression may be a viable option in the prevention of recurrent pregnancy loss and preeclampsia.

We have been studying the role of HO-1 in pregnancy and placental development using a HO-1-deficient mouse model [27–30]. We have demonstrated that the breeding of HO-1 heterozygous (Het, HO-$1^{+/-}$) pairs results in abnormal placental vascular development [28,30], smaller litter sizes, high maternal diastolic pressures, and elevated plasma sFlt-1 levels [30]. Therefore, in this study, we evaluated the potential of pravastatin to increase HO-1 expression and lead to improved placental development and fetal survival in pregnant mice with a partial deficiency of HO-1.

2. Materials and methods

2.1. Study design

2.1.1. Optimal treatment duration and dosing of pravastatin (Fig. 1A)

Non-pregnant wild-type (WT) female mice were randomly assigned to receive drinking water without (Con) or with pravastatin (5 or 10 mg/kg/day) supplementation. Subgroups of mice were sacrificed after 2 and 3 weeks of treatment. Blood immediately collected for measurements of plasma liver enzymes and lipid levels. Livers, spleens, and uteri were then harvested for measurements of HO activity.

2.1.2. Effect of pravastatin on pregnant het dams (Fig. 1B)

Three groups of pregnant mice were compared: (i) WT dams receiving plain drinking water (WT); (ii) Het dams receiving plain drinking water (Untreated Het); and (iii) Het dams receiving drinking water supplemented with pravastatin (10 mg/kg/day, one week prior to breeding and 2 weeks thereafter for a total of 3 weeks, Het + Pravastatin). All dams were sacrificed at E14.5. Placentas from each dam were then harvested from each uterine horn and randomly assigned for measurements of HO activity or for hematoxylin and eosin (H &E) staining and CD31 immunohistochemistry (IHC).

2.2. Animals

FVB/n WT mice (6–8 weeks-old) were obtained from Charles River Laboratories (Wilmington, MA). FVB/n Het mice that carry a targeted deletion of the Hmox1 gene were obtained from our mouse colony. Mice were allowed food (Teklad Rodent Diet #2018, Envigo, Hayward, CA) and pre-filled acidified drinking water (Aquavive®, Innovive, San Diego, CA) *ad libitum*. For pregnancy studies, gestational ages were determined by vaginal plug day representing embryonic day (E) 0.5. All studies were approved by Stanford University's Institutional Animal Care and Use Committee (Protocol #32266) and conducted in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals.

2.3. Genotyping

Genomic DNA was isolated from adult mouse tails clippings for genotyping adult mice [30] and from a subset of fetal placentas (viable fetuses only [13,30] with corresponding HO activity and histology) for genotyping using the Tissue DNeasy kit (Qiagen, Germany). Two sets of primers specifically designed for the WT and mutants were used for PCR. Presence of WT (510 bp) or mutant (390 bp) bands were confirmed by gel electrophoresis.

2.4. Pravastatin treatment

Based on a mean water consumption of 4 ml/day calculated for pregnant WT and Het mice, pravastatin (Sigma-Aldrich, St. Louis, MO) was added to water bottles to yield concentrations of 0.044 and 0.087 mg/ml so that each mouse would receive approximately 5 or 10 mg pravastatin/kg/day, respectively.

2.5. Assessment of viable fetuses and resorbed concepti

At E14.5, the number of viable fetuses and resorbed concepti was recorded for each dam. Resorbed concepti were identified by their hemorrhagic/necrotic appearances and absence of fetuses as described by Zenclussen et al. [13].

2.6. HO activity measurements

Total HO activity was determined as previously described [31]. In brief, 80–100 mg of tissue was weighed, diluted 10-fold with 0.1 M potassium phosphate buffer (pH 7.4), and sonicated using a Microson Ultrasonic Cell Disruptor (Farmingdale, NY). For uteri, tissues taken from both horns were used, sonicated, centrifuged at 10,000 × *g* for 1 min, and total HO activity in the supernatant was measured. For placentas, fetal sides were identified, isolated, and pooled (n = 1 or 2 combined for each measurement) to yield adequate tissue for HO activity assays. For a subset of Het placentas, corresponding embryos were also genotyped.

20-μl sonicates (livers, spleens, and placentas) or supernatant (uteri) were incubated for 15 min at 37 °C in vials containing equal (20 μl) volumes of NADPH (4.5 mM) and methemalbumin (150-μM heme/15-μM BSA). Reactions were stopped with the addition of 5-μl 15% sulfosalicylic acid (w/v), and vials placed in ice. CO released into the vial headspace was determined by gas chromatography [31]. Liver, spleen, and placental HO activity was calculated as pmol CO/h/mg fresh weight (FW). Protein concentrations in uterine supernatants were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Uterine HO activity was then calculated as nmol CO/h/mg protein [31].

2.7. Plasma liver enzymes and lipid profiles

After sacrifice, blood was immediately collected by intracardiac puncture, transferred to Terumo™ Capiject™ tubes (Fischer Scientific, Pittsburgh, PA), and then centrifuged to obtain plasma. Liver enzymes [alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)] and lipid profiles [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and

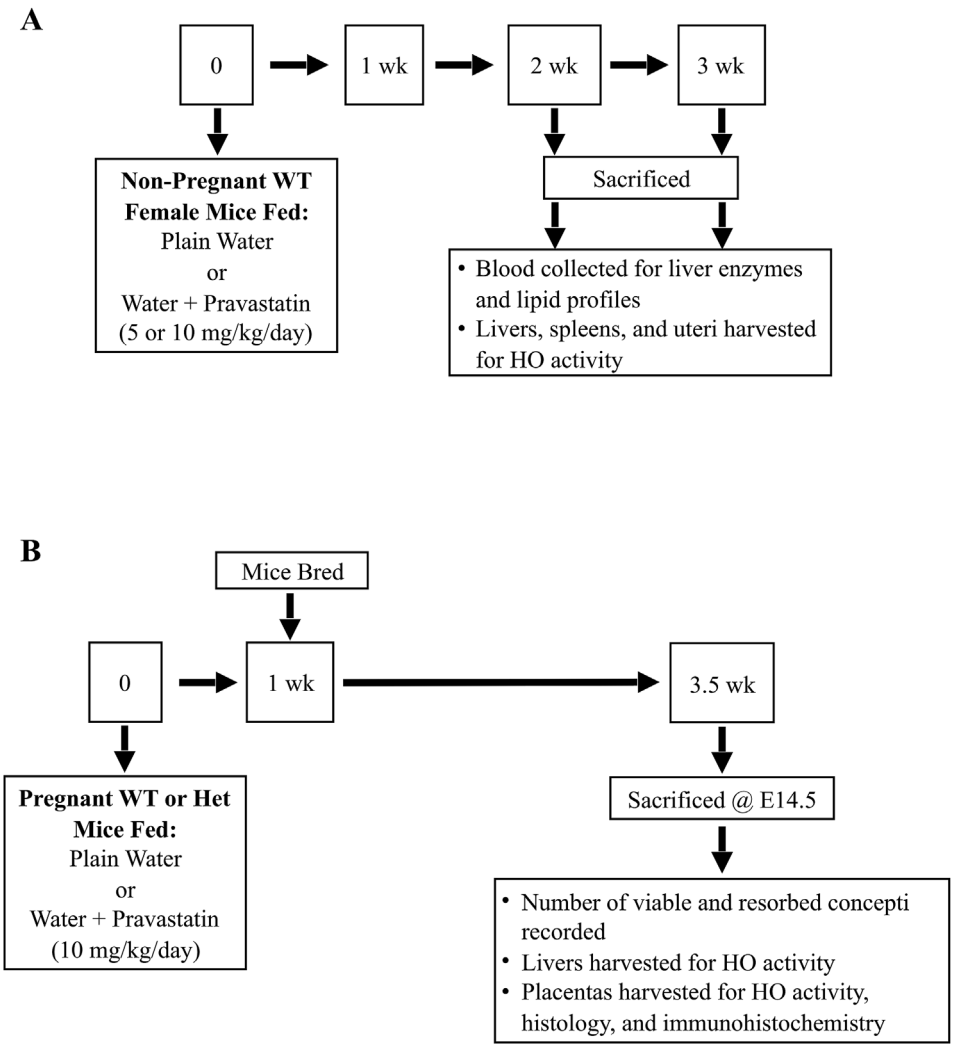

Fig. 1. (A) Schematic showing the design of the study performed to establish the optimal treatment duration and dose of pravastatin for non-pregnant WT female mice. (B) Schematic showing the design of the study performed to evaluate the effect of pravastatin on pregnant WT, Het, and Het + Pravastatin dams.

triglycerides] were measured by the Animal Diagnostic Laboratory (Dept. of Comparative Medicine, Stanford University, Stanford, CA) and expressed as IU/ml and mg/dl, respectively.

2.8. Histology and IHC

Histology and IHC were performed on a subset of randomly-selected whole placentas from WT (n = 9), surviving Untreated Het (n = 7), and Het + Pravastatin (n = 10) pregnancies as well as resorbed Het concepti (n = 2). Only Het placentas with a fetal Het placenta were used. To eliminate litter biases, no more than 2 placentas per pregnancy were obtained. After harvest, placentas were placed in 10% (v/v) neutral buffered formalin (Fisher Scientific). After 24 h, tissues were transferred to 70% ethanol and then stored at room temperature until shipment to HistoWiz, Inc. (Brooklyn, NY) as per their tissue preparation protocol for sectioning, H&E staining, and CD31 IHC. Placentas were sectioned along midline (perpendicular to the fetal and maternal placental layers), paraffin-embedded, and sectioned at 4-μm.

Two blinded veterinary pathologists performed all histologic assessments. The criteria used for histologic evaluation (Supplementary Table S1) of H&E stained placentas were based on evaluation of the following: necrosis/apoptosis, arterial sinuses, glycogen trophoblast cells in the spongiotrophoblast (SP) layer. SP layer thickness for each placenta was calculated as the mean of 3 measurements taken at evenly-spaced locations.

Because CD31, also known as platelet and endothelial cell adhesion molecule 1 (PECAM1), is a transmembrane receptor highly expressed in endothelial cell junctions [32], CD31 IHC was used to evaluate vascular and endothelial differences between the groups. Primary rabbit polyclonal CD31 antibody (Abcam 28364) and secondary anti-rabbit Poly-HRP-IgG antibody (Leica Biosystems) were used [33]. CD31 immunoreactivity was deemed positive by dark-brown staining of vascular endothelium. The intensity and distribution of vascular immunoreactivity (*i.e.* vascular density) [34,35] within and between all placental layers were compared.

2.9. Statistical analyses

Unpaired Student's t-tests or ANOVAs were used for all comparisons. Total numbers of resorbed concepti and implantations (viable fetuses *plus* resorbed concepti) were used to calculate the relative risk (RR) for concepti resorption of Het and Het + Pravastatin groups compared with the WT group (referent). A P-value < 0.05 was deemed statistically significant.

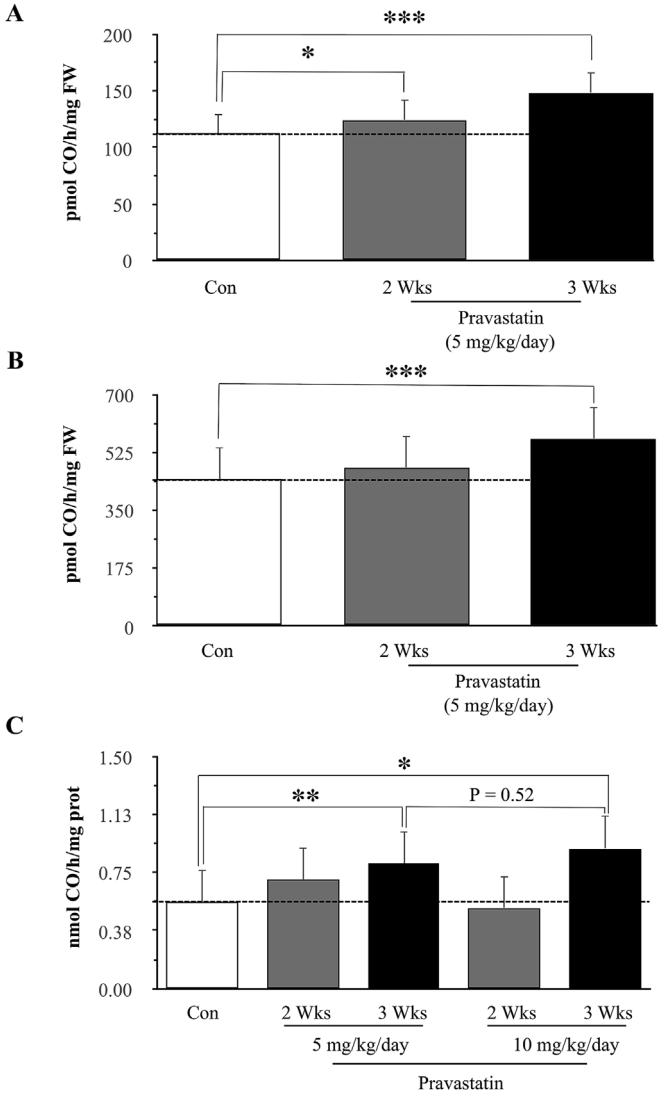

Fig. 2. HO activity in livers, spleens, and uteri of non-pregnant WT female mice increased after 2 and 3 weeks of treatment with pravastatin (5 mg/kg/day) compared with age-matched untreated WT controls. (A) Liver, (B) spleen, and (C) uterine HO activity were measured in non-pregnant adult female WT FVB mice fed regular water (Con, n = 22, 18, and 22, respectively) or with water supplemented with 5 mg/kg/day of pravastatin for 2 (n = 12, 12, and 12, respectively) or 3 weeks (n = 12 for liver and spleen, and 10 for uterus). (C) Treatment with 10 mg/kg/day of pravastatin for 3 weeks did not decrease the time required to reach significant induction of uterine HO activity. The higher 10 mg/kg/day dose resulted in a higher induction (1.61 ± 0.46-fold, n = 5) than Con (P = 0.055) and the 5 mg/kg/day dose (1.43-fold) that was not statistically significant (P = 0.52).
P < 0.05; **P < 0.01; ***P < 0.001 vs age-matched controls.

3. Results

3.1. Optimal treatment duration and dosing of pravastatin for non-pregnant mice

3.1.1. Treatment duration

2 weeks of treatment with 5 mg/kg/day of pravastatin increased HO activity in the liver (1.11 ± 0.08-fold, n = 12, P < 0.05), spleen (1.08 ± 0.14-fold, n = 12), and uterus (1.24 ± 0.43-fold, n = 12) compared with Con (1.00 ± 0.16-fold, n = 22, Fig. 2A, B, and C, respectively). After 3 weeks of pravastatin treatment, HO activity increased significantly in the liver (1.32 ± 0.19-fold, n = 12, P < 0.001), spleen (1.28 ± 0.12-fold, n = 12, P < 0.001), and uterus (1.43 ± 0.35-fold, n = 10, P < 0.01) compared with Con (n = 22, 18, and 22, respectively).

3.1.2. Dosing

For the uterus, we evaluated if a higher dose of pravastatin (10 mg/kg/day) would decrease the treatment duration and/or result in a higher induction of HO activity. After 3 weeks of treatment, we found that the higher dose did not shorten treatment duration but did result in a higher (albeit not significant, P = 0.52) increase of uterine HO activity to 1.61 ± 0.46-fold (n = 5) compared with 1.43 ± 0.35-fold at the 5 mg/kg/day dose (Fig. 2C).

3.1.3. Liver enzymes and lipid profiles

Following 3 weeks of pravastatin treatment at both doses, plasma liver enzymes were similar to Con (Supplementary Table S2). For lipid profiles (Supplementary Table S3), 5 mg/kg/day of pravastatin did not affect total cholesterol, LDL, HDL, or triglycerides levels; however, at the 10 mg/kg/day dose, there was a statistically significant decrease in LDL levels (P = 0.034).

3.2. Effect of pravastatin in pregnant Het dams

3.2.1. Liver and placental HO activity

In pregnant Untreated Het dams, liver HO activity was significantly lower (104 ± 29 pmol CO/h/mg FW, n = 8) than pregnant WT dams as expected, being only 65 ± 18% of WT levels (160 ± 24, pmol CO/h/mg FW, n = 13, P < 0.001). Based on our duration and dosing studies, Het dams were given the 10 mg/kg/day dose of pravastatin one week prior to breeding and 2 weeks thereafter for a total of 3 weeks. After this treatment regimen, liver HO activity significantly increased to 132 ± 12 pmol CO/h/mg FW (n = 12) approaching levels of WT (83 ± 7%, P < 0.001) and was also significantly higher than that of Untreated Het dams (P < 0.05, Fig. 3A). Similarly, placental HO activity in Untreated Het dams was significantly lower (194 ± 17 pmol CO/h/mg FW, n = 6), being 74 ± 7% of WT levels (P < 0.001). Pravastatin treatment significantly increased placental HO activity to 228 ± 38 pmol CO/h/mg FW (n = 13), also approaching levels of WT (87 ± 14%, P < 0.05) and was significantly higher than that of Untreated Het dams (P < 0.05, Fig. 3B).

3.2.2. Number of viable fetuses and resorbed concepti

At E14.5, numbers of viable fetuses per dam were significantly reduced in Untreated Het (6.0 ± 2.2, n = 9) compared with WT (9.1 ± 1.4, n = 18, P < 0.01, Fig. 4) pregnancies. This difference was accompanied by an increase in resorbed concepti of Untreated Het (1.6 ± 1.7, n = 9) compared with WT (0.1 ± 0.3, n = 18, P < 0.05) pregnancies. In Het + Pravastatin dams, numbers of viable fetuses increased to 7.7 ± 2.5 compared with Untreated Het dams (P = 0.12) and accompanied by a concomitant decrease in resorbed concepti (0.6 ± 0.8, n = 14, P = 0.19). The number of implantations (viable fetuses *plus* resorbed concepti) per pregnancy was significantly lower in Untreated Het (7.6 ± 1.3) compared with WT (9.2 ± 1.3, P = 0.010) pregnancies. In Het + Pravastatin pregnancies, total implantations increased to 8.4 ± 2.2.

In WT dams, only 2 concepti were resorbed from 166 implantations (1.2%). In contrast, 14 concepti in the Untreated Het group were resorbed from 68 implantations (20.6%). When RR was calculated for concepti resorption using WT as the referent, we found a RR of 17.1 (95% CI 4.0–73.2) for Untreated Het dams. In Het + Pravastatin dams, only 9 resorbed concepti were found from 117 implantations (7.7%), which translated to a 6-fold reduction in RR (2.7, (95% CI 1.2–5.9).

Of 41 from a total of 68 viable fetuses from untreated Het pregnancies, 13 (31.7%) embryos were WT, 28 (68.3%) were Het, and none (0%) were knockouts (KO, HO-1$^{-/-}$). The genotype frequencies of Het pregnancies are similar to our previous findings of 25.5% WT, 72.1% Het, and 2.4% KO [30]. In that study, the number of resorbed concepti were not recorded. Of 55 from a total of 117 viable fetuses from Het

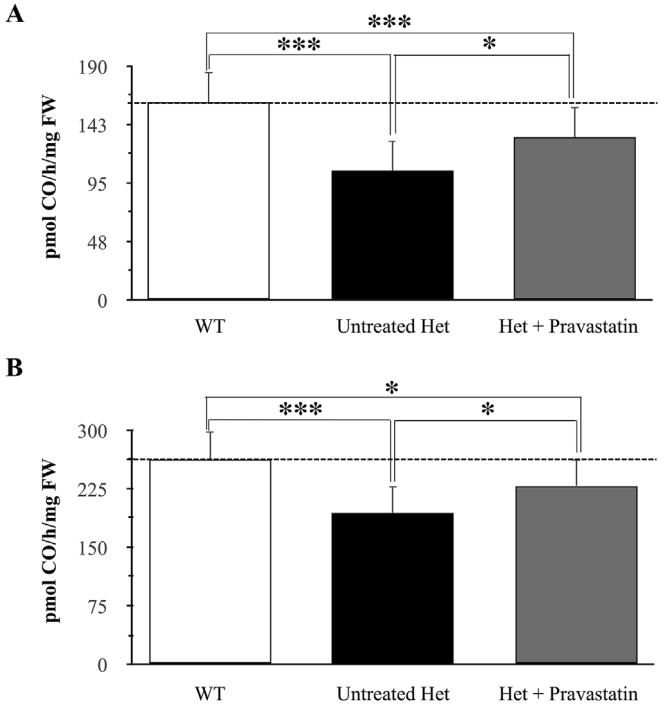

Fig. 3. (A) HO activity in livers were significantly lower (65 ± 18%) in pregnant Untreated Het dams (n = 8) compared with pregnant WT dams (n = 13). After treatment with pravastatin, liver HO activity in pregnant Het dams (Het + Pravastatin, n = 12) significantly increased to 83 ± 7% of that of WT levels. (B) Fetal placental HO activity in the Untreated Het dams (n = 6) was significantly lower (74 ± 7%) compared with that of WT dams (n = 15). After treatment with pravastatin, fetal placental HO activity in pregnant Het dams (Het + Pravastatin, n = 13) significantly increased to 87 ± 14% of that of WT dams.
*P < 0.05; **P < 0.001.

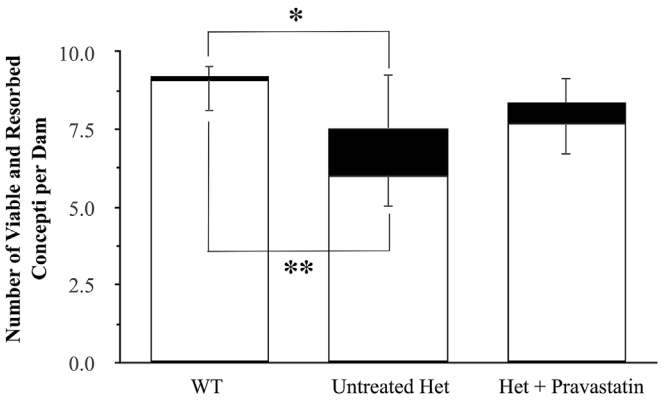

Fig. 4. Untreated Het dams (n = 9) had a significantly lower mean number of viable fetuses (□, 6.0 ± 2.2) compared with WT dams (n = 18, 9.1 ± 1.4) and a significantly higher mean number of resorbed concepti (■, 1.6 ± 1.7 vs 0.1 ± 0.3, respectively). The number of viable fetuses in the Het + Pravastatin (n = 14) group increased to 7.7 ± 2.5 and the mean number of resorbed concepti decreased to 0.6 ± 0.8. Numbers of implantations (viable fetuses *plus* resorbed concepti) for WT, Untreated Het, and Het + Pravastatin pregnancies were 9.2 ± 1.3, 7.6 ± 1.3, and 8.4 ± 2.2, respectively.
*P < 0.05 for the resorbed concepti comparisons; **P < 0.01 for the viable fetuses comparisons and for the number of implantations comparisons between WT and Untreated Het pregnancies.

+ Pravastatin pregnancies, 16 (29.1%) embryos were WT, 37 (67.2%) were Het, and 2 (3.6%) were KO. Although the frequencies of WT and Het fetal placentas in the Het + Pravastatin pregnancies are nearly identical to the Untreated Het pregnancies, it is of note that the HO-1 KO rate increased.

3.2.3. Histology and IHC

Fig. 5A shows a representative resorbed conceptus at E14.5 from an Untreated Het dam with a lack of distinct placental layers and any identifiable fetal tissue. Tissues were also necrotic and admixed with haphazardly-oriented and dilated vascular channels (V) containing vascular thromboses (T) (Fig. 5A, inset).

Based on the assessment criteria in Supplementary Table 1, no significant morphologic differences between placentas from surviving concepti of WT, Untreated Het, and Het + Pravastatin dams were found (Fig. 5B, C, and D, respectively). Thicknesses of the SP layers were similar between pregnant WT (349 ± 123 μm, n = 9, Fig. 5B), Untreated Het (455 ± 95 μm, n = 7, Fig. 5C), and Het + Pravastatin (499 ± 93 μm, n = 10, Fig. 5D) placentas. However, compared with WT placentas (Supplementary Fig. S1A), some Untreated Het placentas had focal losses of cellular architecture and compaction of vascular spaces (indicative of reduced blood flow) in the labyrinth (Supplementary Fig. S1B). Although we did not observe these characteristics in Het + Pravastatin placentas, some had areas with moderate dilation and congestion of the vascular spaces in the labyrinth (Supplementary Fig. S1C) and focal dilation and engorgement of venous sinuses in the junctional zone (Supplemental Fig. S1F) compared with the junctional zones of WT and Untreated Het placentas (Supplementary Figs. S1D and E, respectively). In addition, these changes were not accompanied by any signs of inflammation.

CD31 IHC revealed that compared to WT placentas (Fig. 6A), there was a qualitative reduction in vascular density within the labyrinth layers in the placentas of viable concepti from Untreated Het (Fig. 6B, arrowheads) as well as Het + Pravastatin placentas (Fig. 6C, arrowheads).

4. Discussion

Daily treatment with pravastatin for a minimum of 2 weeks was required to significantly increase HO enzyme activity for the liver and 3 weeks for the spleen and uterus. In the uterus, HO activity increased 143% and 161% following 5 and 10 mg/kg/day of pravastatin, respectively. This observed duration of treatment and doses were similar to our previous study using the more potent cholesterol-lowering statins, atorvastatin and rosuvastatin [21], where we found increases in heart HO activity of 133% and 163%, respectively.

In contrast, in a study using pregnant mice overexpressing sFlt-1, Fox et al. [36] did not observe any increase in HO-1 expression in the aorta after 10-days of 5 mg of pravastatin/kg/day. Although the dose was the same as our lower dose, their short duration of treatment might have accounted for the apparent absence of increases in HO-1 expression in the aorta. For example, when we investigated the role of rosuvastatin on the progression of AAAs, we found that 35 days of daily IP injections was required to significantly induce aortic HO activity and suppress AAA progression [22]. In this study, pravastatin was administered orally in the drinking water and thus its effective dose may be affected by incomplete intestinal absorption and also subject to the "first-pass" effect [37] as well as increases in renal and hepatic clearance during pregnancy [38]. These factors could explain some of the variabilities in our degree of HO-1 induction, histologic changes, and fetal survival in pregnant Het + Pravastatin dams. Although we did not directly measure plasma pravastatin levels or placental uptake of pravastatin, we did observe increases in HO-1 expression in the liver and spleen of non-pregnant mice similar to our previous statin studies [21,22].

Although rare, hepatotoxicity resulting from statin use has been reported [39] and could contribute to any upregulation of liver HO-1 expression as a stress response [40]. Therefore, we measured liver enzymes after 3 weeks of treatment with the 5 and 10 mg/kg/day doses. No significant differences in liver enzymes were found between all

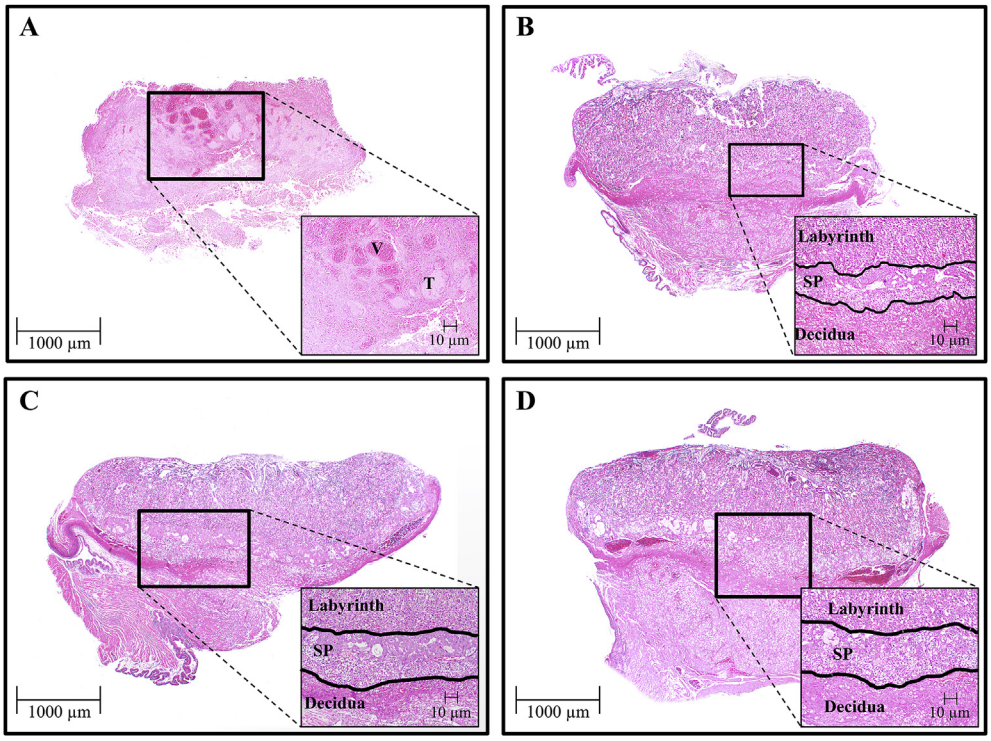

Fig. 5. (A) A representative H&E-stained section of a resorbed Untreated Het conceptus shows a lack of distinct placental layers and/or identifiable fetal tissue. Residual tissue was necrotic and admixed with haphazardly-oriented and dilated vessels (inset, V). Vessels frequently contain intravascular fibrin thrombi (inset, T). Representative H&E-stained sections of surviving placentas associated with viable concepti from (B) WT, (C) Untreated Het and (D) Het + Pravastatin dams did not differ based on histopathologic grading criteria. The spongiotrophoblast (SP) thicknesses (insets) were similar between all groups.

groups, and we concluded that there were no hepatotoxic effects of pravastatin and our observed increases in HO activity was due to the induction of HO-1 by pravastatin.

Because a growing embryo and fetus is dependent on maternal and fetal cholesterol for normal development [41], administration of statins during pregnancy has not been advocated. However, the hydrophilic properties of pravastatin and reduced transfer of pravastatin from the mother to the fetus [42] have been shown to minimize fetal exposure to the drug. Additionally, pravastatin is less potent than simvastatin or atorvastatin in lowering cholesterol [43]. In our study, we did not observe a reduction in cholesterol levels following 3 weeks of treatment with the 5 or 10 mg/kg/day of pravastatin in non-pregnant WT mice. Similarly, in the above-mentioned study by Fox et al. [36], no lowering of maternal total cholesterol after 11 days of oral pravastatin (5 mg/kg/day) was observed. Therefore, it appears that our observed induction of HO activity was likely independent of the cholesterol-lowering property of pravastatin. Translation of these doses from mice to humans is limited by species differences in body surface area, hepatic intake, and other factors [44]. Although we did observe a statistically significant 2-mg/dl decrease in LDL after 3 weeks of treatment with the 10 mg/kg/day dose, this difference was below the detection limit of the LDL assay (Automated LDL Cholesterol Flex®) and therefore should be interpreted with circumspection. Nonetheless, it should be possible to identify a dose of pravastatin that selectively induces HO-1 expression without lowering maternal cholesterol levels for human studies. In a pilot randomized study evaluating the use of pravastatin for preventing preeclampsia, 10 mg is given daily to women, but found to reduce cholesterol levels [10].

We have previously reported that during pregnancy, HO-1 expression (mRNA and protein) in WT and Het placentas are similar until E12.5 to E14.5, when levels in Het placentas diverge and remain low until delivery [30]. We found that the number of viable fetuses in untreated Het dams was significantly lower than that of WT controls and similar to our [30] and other [13] previous reports. We hypothesized that the maintenance of HO-1 expression during this critical gestational window might improve placental development and conceptus survival in Het pregnancies. Therefore, we chose to initiate pravastatin treatment 7 days prior to breeding so that by E14.5, pregnant Het dams would receive 3 weeks of 10 mg of pravastatin/kg/day. We found that liver and placental HO activity increased to levels near those of WT pregnant dams confirming that pravastatin treatment can induce HO-1 expression in the placenta.

Moreover, after treatment with pravastatin, fetal survival increased and the number of resorbed concepti decreased compared with Untreated Het pregnancies and approached numbers of WT controls. In addition, the total number of implantations (viable fetuses *plus* resorbed concepti) per pregnancy was also similar (8.4 ± 2.2 and 9.2 ± 1.3 for Het + Pravastatin and WT, respectively) and higher than that of Untreated Het dams (7.6 ± 1.3). Resorbed concepti could not be genotyped since there is no discernible maternal or fetal interface visible to allow accurate separation. Furthermore, the RR for concepti resorption significantly increased in Untreated Het pregnancies but decreased 6-

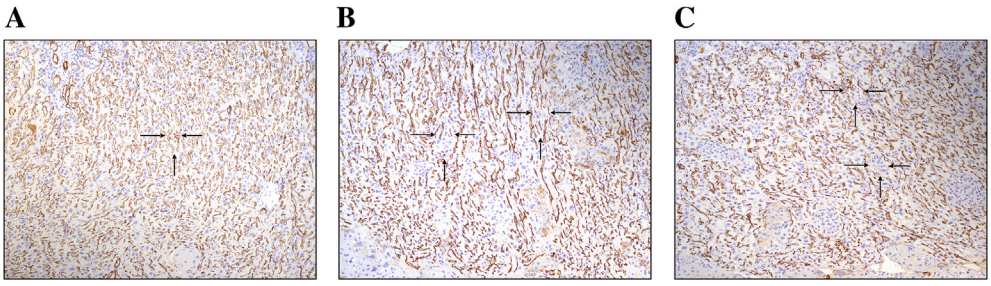

Fig. 6. Immunohistochemical staining with CD31 was used to highlight the vascular endothelium (immunoreactivity = brown). Compared with that of (A) WT placentas, vascular densities within the labyrinth layer of surviving (B) Untreated Het and (C) Het + Pravastatin placentas were reduced (see arrows). All figures are oriented with the spongiotrophoblast (SP) layer at the bottom and luminal surface at the top.

fold after treatment with pravastatin. Because an under-expression of HO-1 has been reported to adversely affect ovulation [45] and/or uterine receptivity [46], our findings suggest that HO-1 is also important for the maintenance of fetal survival by affecting placental function.

Histological examination of the placentas did not reveal significant differences between those from WT, Untreated Het, and Het + Pravastatin pregnancies. However, this was expected as the Het placentas were taken from those containing viable fetuses, and thus less likely impacted by a partial deficiency of HO-1. However, in some placentas, there were changes in the labyrinth that were suggestive of reduced blood flow. In Het + Pravastatin placentas, these changes were not apparent, but contained areas with moderate dilation and congestion of vascular spaces in the labyrinth layer as well as focal dilation and congestion of venous sinuses in the junctional zone. These findings are suggestive of increased blood flow to these areas and could be due to an increase in vasodilation due to increased CO production mediated by increased placental HO activity. It has previously been reported that the administration of CO can significantly improve the viability of concepti in HO-1-deficient dams [13]. Furthermore, it has been shown that CO formation is significantly lower in women with preeclampsia [47], and that cigarette smoking during pregnancy is associated with a lower risk of preeclampsia [48]. Sacrificing dams at earlier timepoints, e.g., at E10.5 or earlier, may have afforded us an opportunity to observe some concepti in the midst of resorption and perhaps reveal more detailed ongoing pathological changes within the placenta.

In addition, CD31 IHC revealed reduced vascular densities in the labyrinth layers of placentas from both untreated and treated Het dams compared with those from WT dams. Reduced vascular density has been shown to reflect abnormal vessel formation and penetration [34]. We have previously reported that HO-1 is crucial for normal angiogenesis in the developing placenta, and disrupted in HO-1 deficiency [28]. To this end, we performed preliminary PCR arrays and found that certain angiogenic factors (e.g., Vegfb, Plg, Cc12, and Figf) in the decidua of Het placentas returned to WT levels after treatment with pravastatin (data not shown). This suggests that the improved fetal survival in Het + Pravastatin pregnancies may also be related to improved, albeit not normal, placental angiogenesis as well as to vasorelaxation and increased blood flow. However, more detailed work is needed to validate these preliminary findings.

It is well-known that HO-1 is upstream to sFlt-1 and can down-regulate sFlt-1 [17], an anti-angiogenic protein that has been implicated in the pathogenesis of preeclampsia [7–9,49]. In fact, we have previously shown that plasma sFlt-1 levels are increased in pregnant HO-1-deficient mice compared with pregnant WT mice [30]. In addition, it has been shown in mice overexpressing sFlt-1 that sFlt-1 secretion is affected early by pravastatin (within 10–11 days) [5,36,50] before any increases in HO-1 expression would occur. Our current findings suggest that the value of pravastatin lies in its dual ability to not only decrease sFlt-1 secretion and improve vascular function; but also induce HO-1 expression in women at risk for preeclampsia.

In summary, we demonstrated that pravastatin induces maternal liver and fetal placental HO activity and can prevent concepti resorption related to low HO-1 expression. We speculate that an increased production of CO induced by HO-1 expression and consequent vessel relaxation may play a role in the improved fetal survival.

Disclosure statement

None of the authors have any financial conflicts of interest to disclose.

Statement of financial support

This work was supported in part by the Mary L Johnson Research Fund, the Christopher Hess Research Fund, the Prematurity Research Fund, and the March of Dimes Prematurity Research Center at Stanford University and the Stanford Child Health Research Institute.

Acknowledgements

We like to thank Dr. Christina M. Konecny and our summer high school trainees Veronica K. Zehnder and Zoe Onderdonk for their help in these studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2018.11.001.

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