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Endometrial glycogen metabolism during early pregnancy in mice

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Abstract

Glucose is critical during early pregnancy. The uterus can store glucose as glycogen but uterine glycogen metabolism is poorly understood. This study analyzed glycogen storage and localization of glycogen metabolizing enzymes from proestrus until implantation in the murine uterus. Quantification of diastase-labile periodic acid–Schiff (PAS) staining showed glycogen in the glandular epithelium decreased 71.4% at 1.5 days postcoitum (DPC) and 62.13% at DPC 3.5 compared to proestrus. In the luminal epithelium, glycogen was the highest at proestrus, decreased 46.2% at DPC 1.5 and 63.2% at DPC 3.5. Immunostaining showed that before implantation, glycogen metabolizing enzymes were primarily localized to the glandular and luminal epithelium. Stromal glycogen was low from proestrus to DPC 3.5. However, at the DPC 5.5 implantation sites, stromal glycogen levels increased sevenfold. Similarly, artificial decidualization resulted in a fivefold increase in glycogen levels. In both models, decidualization increased expression of glycogen synthase as determine by immunohistochemistry and western blot. In conclusion, glycogen levels decreased in the uterine epithelium before implantation, indicating that it could be used to support preimplantation embryos. Decidualization resulted in a dramatic increase in stromal glycogen levels, suggesting it may have an important, but yet undefined, role in pregnancy.

KEYWORDS

decidualization, glucose, glucose-6-phosphatase, glycogen phosphorylase, glycogen synthase, hexokinase

1 | INTRODUCTION

Pregnancy loss is quite common in humans, with most losses occurring very early in pregnancy (Annual Capri Workshop Group, 2020; Zinaman et al., 1996). Before implantation, embryos are dependent on nutrients secreted into the uterine lumen. Of the nutrients in uterine secretions, glucose is one of the most important. Glucose uptake by embryos is low from fertilization until the eight-cell stage, and before compaction too much glucose is toxic. Around the morula stage glucose uptake starts to increase and is dramatically higher by the blastocyst stage (Dan-Goor et al., 1998; Leese & Barton, 1984). In human embryos produced via in vitro fertilization or intracytoplasmic sperm injection, glucose consumption was higher in the embryos that resulted in a live birth (Gardner et al., 2011). Matching the increased glucose needs of the blastocyst, the glucose concentrations are higher in fluid from the uterus than those in fluid from the oviduct (Gardner et al., 1996; Hugentobler et al., 2010).

At the implantation site (IS), the stromal fibroblasts undergo a morphological and physiological change into decidual cells. Decidualization results in increased glucose flux through the pentose phosphate pathway, and blocking this pathway impairs the decidual response in mice and human endometrial stromal cells (Frolova et al., 2011; Tsai et al., 2013). After decidualization, glucose uptake increases due to a shift to Warburg metabolism (Zuo et al., 2015). Hence, the glucose needs of both the embryo and uterus change in a spatiotemporal manner during early pregnancy.

The uterus lacks the enzymes to make glucose de novo; therefore, all glucose used by the endometrium or secreted into the uterine lumen must come from maternal circulation (Yánez et al., 2003; Zimmer & Magnuson, 1990). The facilitative glucose transporters (GLUTs, gene family *Slc2a*) and sodium–glucose-linked transporter 1 (gene symbol *Slc5a1*) are both expressed in the endometrium (Frolova & Moley, 2011; Zhang et al., 2021). Thus, the uterus may take up glucose from maternal circulation as needed; however, the endometrium can also transiently store glucose as the macromolecule glycogen.

After glucose enters a cell, it is phosphorylated by hexokinase (HK) to produce glucose-6-phosphate. Glucose-6-phosphate can be metabolized by many different pathways. To be stored as glycogen, the glucose-residue is isomerized to glucose-1-phosphate and then transferred to UTP, yielding UDP-glucose. From there, glycogen synthase (GYS) transfers the glucose to a pre-existing glycogen molecule. Glucose-1-phosphate is liberated from glycogen by the enzyme glycogen phosphorylase (PYG). Glucose-1-phosphate is isomerized back to glucose-6-phosphate, which is trapped in the cell. To be secreted, the glucose moiety must be dephosphorylated by glucose-6-phosphatase (G6PC).

In humans, endometrial glycogen concentrations peak during the luteal phase and are correlated with fertility (Maeyama et al., 1977). In rats, uterine glycogen concentrations are high on Day 1 of pregnancy and then decrease over preimplantation. Glycogen concentrations began to increase after implantation (Greenstreet & Fotherby, 1973). However, it is unclear which tissues store the glycogen or where the glycogen metabolizing enzymes are expressed. Mice are important biomedical research models; yet uterine glycogen metabolism has never been characterized in this species.

Our objectives were to 1) characterize glycogen stores in the murine uterus from proestrus through implantation in the glandular epithelium, luminal epithelium, and stroma; 2) localize key glycogen metabolizing enzymes during the same period; and 3) determine if decidualization is sufficient to drive glycogen accumulation in the endometrial stroma independently of pregnancy.

2 | RESULTS

2.1 | Endometrial glycogen levels during early pregnancy

Uteri were collected from mice at proestrus and at days postcoitum (DPC) 1.5, DPC 3.5, and DPC 5.5 and stained with periodic acid–Schiff (PAS), with or without diastase (PASD) pretreatment to localize glycogen. PAS and PASD staining indicated the presence of glycogen in the epithelium at proestrus and in the decidua after implantation (Figure 1a). Quantification of the diastase-labile staining showed that in the glandular epithelium, glycogen content was highest at proestrus, decreased 71.4% at DPC 1.5 ($p < 0.01$), and decreased 62.13% at DPC 3.5 ($p < 0.01$). By DPC 5.5, the glycogen content in the glandular epithelium at the interimplantation site (IIS) increased and was similar to proestrus (Figure 1b). Similar results were found in the luminal epithelium, where glycogen content was highest at proestrus, 46.2% lower at DPC 1.5 ($p = 0.061$), and 63.2% lower at DPC 3.5 ($p < 0.05$). At DPC 5.5-IIS, the glycogen content of the luminal epithelium was 32% lower than that of proestrus, but this was not significant ($p = 0.37$; Figure 1c).

In contrast, the stroma stored little glycogen during the preimplantation period. Glycogen content was low and did not change significantly from proestrus through DPC 3.5. At DPC 5.5, glycogen content was still low in the stroma at the IIS; however, the glycogen level increased sevenfold at the IS compared to the stroma of proestrus or the IIS ($p < 0.0001$; Figure 1d).

2.2 | Glycogen metabolizing enzymes during early pregnancy

The levels of glycogen metabolizing enzymes (HK1, GYS, phosphoglycogen synthase [pGYS], and PYG) in the uterus of mice at proestrus and pregnant mice were analyzed by western blot. Tissue at DPC 5.5 contained both ISs and IISs. There were no significant differences in the levels of the glycogen synthesizing enzymes HK1, GYS, and pGYS (Figure 2a–c). Similarly, no difference in the level of the glycogen catabolizing enzyme PYG was detected during early pregnancy (Figure 2d).

Immunohistochemistry demonstrated that glycogen synthesizing enzymes (HK1 and GYS) were highly expressed in the uterine epithelium. HK1 was localized to the glandular and lumen epithelium and was undetectable in the stroma. Immunostaining in the epithelium was consistent from proestrus to DPC 5.5 IIS (Figure 3 top). GYS was present in the luminal and glandular epithelium. Immunostaining was higher on DPC 1.5 and 3.5 compared to proestrus or DPC 5.5 IIS. Some immunostaining for GYS was observed in the stroma on DPC 3.5 (Figure 3 bottom).

Similar to glycogen synthesizing enzymes, glycogen catabolizing enzymes (PYG and G6PC) were also found primarily in the glandular and luminal epithelium. PYG immunostaining was higher after mating (DPC 1.5–5.5) than at proestrus (Figure 4 top). There was moderate PYG immunostaining in the stroma at DPC 3.5 (Figure 4 top). G6PC was localized to the uterine epithelium and the expression appeared higher from DPC 1.5 to DPC 5.5 when compared to proestrus (Figure 4 bottom).

HK1 expression was undetectable in the decidualized stroma at the DPC 5.5 IS by immunohistochemistry, similar to the stroma at the IIS. In contrast, there was a dramatic increase in immunostaining for

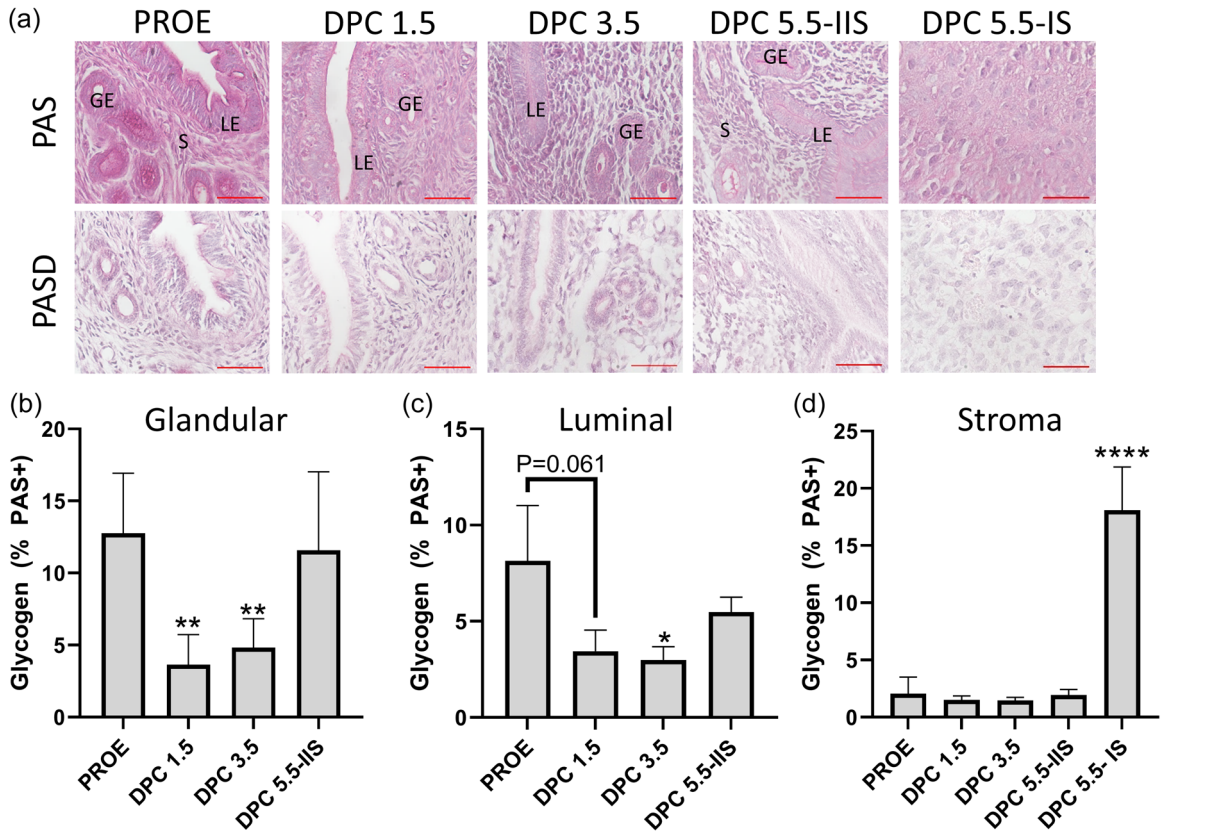

FIGURE 1 Glycogen levels in the murine endometrium during the first 6 days of pregnancy. (a) Representative images from the murine uterus collected at proestrus (PROE), days postcoitum (DPC) 1.5, DPC 3.5, and DPC 5.5. Sections were stained with periodic acid–Schiff (PAS, top). Other slides were pretreated with diastase (PASD) to digest glycogen before PAS staining (bottom). (b–d) Glycogen content of the glandular epithelium (GE; b), luminal epithelium (LE; c), and stroma (S; d) as calculated with ImageJ. Glycogen content was determined by measuring the area occupied by each tissue and the area PAS positive. The percent area PAS positive in PASD slides was subtracted from the area positive in PAS slides to account for nonspecific PAS staining. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ relative to PROE. $n = 6$. Scale bar = 50 μm. IIS, interimplantation site; IS, implantation site.

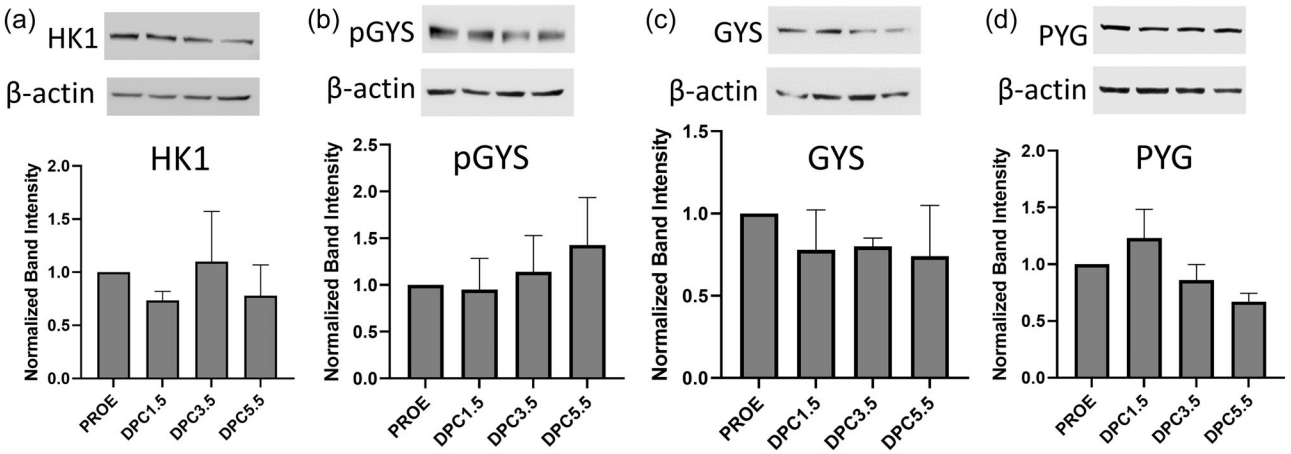

FIGURE 2 Levels of glycogen metabolizing enzymes in uterine homogenates. (a–d) Western blots for hexokinase 1 (HK1, a), phospho-glycogen synthase (pGYS, b), glycogen synthase (GYS, c), and glycogen phosphorylase (PYG, d) in uteri collected from mice at proestrus (PROE) and days postcoitum (DPC) 1.5, 3.5, and 5.5. $n = 4$.

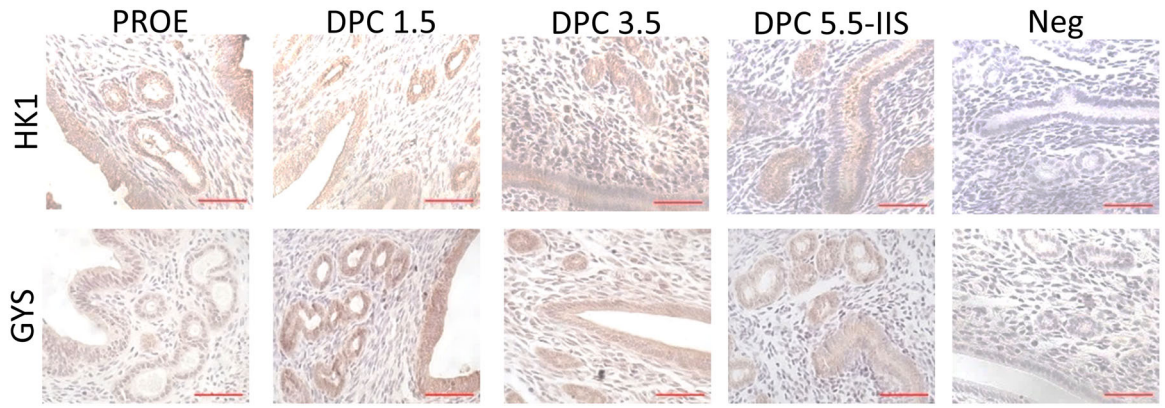

FIGURE 3 Localization of glycogen synthesizing enzymes in the endometrium from proestrus (PROE) until days postcoitum (DPC) 5.5. Immunohistochemistry for hexokinase 1 (HK1) and glycogen synthase (GYS) in uteri collected at PROE, DPC 1.5, DPC 3.5, and DPC 5.5 IIS. $n = 4$. Scale bar = 50 µm. IIS, interimplantation site; Neg, negative control.

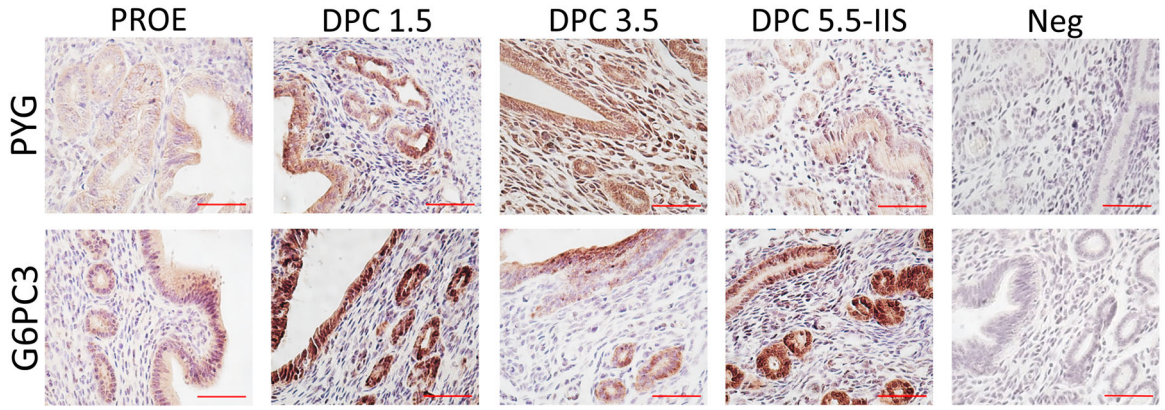

FIGURE 4 Localization of glycogen catabolizing enzymes in the endometrium from proestrus (PROE) until days postcoitum (DPC) 5.5. Immunohistochemistry for glycogen phosphorylase (PYG) and glucose-6-phosphatase (G6PC) in uteri collected at PROE or DPC 1.5, DPC 3.5, and DPC 5.5 IIS. $n = 4$. Scale bar = 50 µm. IIS, interimplantation site; Neg, negative control.

GYS at the DPC 5.5 IS compared to the stroma at the DPC 5.5 IIS, which agrees with the increased glycogen levels at the IS (Figure 5 top). Interestingly, there were modest increases in immunostaining for PYG and G6PC in DPC 5.5 IS stroma compared to DPC 5.5 IIS stroma (Figure 5 bottom).

Western blot were used to further examine the glycogen metabolizing enzymes at DPC 5.5 IIS and IS. The levels of HK1 tended to be lower in the IS than the IIS ($p = 0.097$; Figure 6a). There were no significant difference in pGYS levels between DPC 5.5 IIS and IS (Figure 5b). In agreement with the immunohistochemistry data, GYS levels were 2.4-fold higher at the IS compared to IIS ($p < 0.05$; Figure 6c). The level of PYG was the same at the IISs and ISs (Figure 6d).

2.3 | Endometrial glycogen metabolism after artificial decidualization

Next, we induced decidualization artificially to determine if decidualization, by itself, increased glycogen storage. Mice were ovariectomized, primed with ovarian steroids, and the left uterine horn was stimulated to initiate the decidual reaction. The right uterine horn was unstimulated and served as an internal control. The stimulated uterine horn appeared larger and weighed significantly more than the nonstimulated horn, confirming successful decidualization (Figure 7a). Quantification of PAS and PASD staining showed that the glycogen content was five times higher in the stimulated horn than that in the unstimulated horn (Figure 7b; $p < 0.05$). Similar to the data from the DPC 5.5 IIS and IS, HK1 immunostaining was undetectable in the stroma of the unstimulated horn and stimulated horn. GYS immunostaining was absent in the stroma of the unstimulated horn and was markedly increased in the stimulated horn (Figure 8 top). In addition, immunostaining for both PYG and G6PC appeared to slightly increase in the stimulated horn compared to the stroma of the unstimulated horn (Figure 8 bottom). Western blots revealed that HK1 tended to be lower in the stimulated horn relative to the unstimulated horn ($p = 0.064$). pGYS showed no significant difference between the unstimulated and stimulated horn (Figure 9b). The level of GYS was fivefold higher in the stimulated

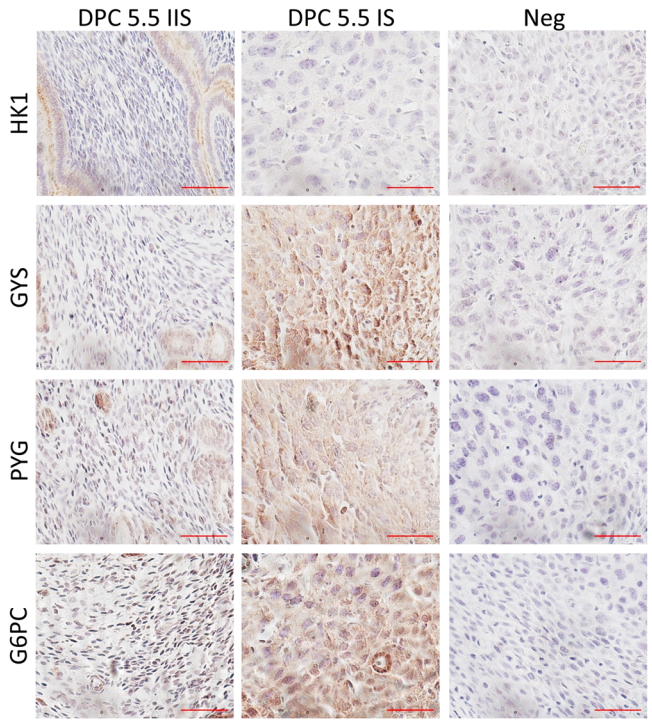

FIGURE 5 Immunostaining for glycogen metabolizing enzymes at the implantation site (IS) and the interimplantation site (IIS) on DPC 5.5. Immunohistochemistry for hexokinase 1 (HK1), glycogen synthase (GYS), glycogen phosphorylase (PYG), and glucose-6-phosphatase (G6PC) in uteri at DPC 5.5. $n = 4$. Scale bar = 50 μm. DPC, days postcoitum; Neg, negative control.

horn compared to unstimulated horn ($p < 0.01$; Figure 9c). Furthermore, no difference in PYG levels was detected between the unstimulated horn and stimulated horn (Figure 9d).

3 | DISCUSSION

The early embryo prefers pyruvate and lactate as energy substrates but has switched to glucose by the blastocyst stage (Gardner & Leese, 1990; Leese & Barton, 1984). Too much glucose during cleavage development is toxic to the embryo (Cagnone et al., 2012; Pantaleon et al., 2010). As a result, preimplantation embryos require optimal glucose concentrations to survive. Given the near-ubiquitous expression of GLUTs in the endometrium and their facilitated diffusion mechanism of action (Frolova & Moley, 2011), GLUTs themselves are unlikely to adequately regulate glucose secretion into the uterine lumen.

In other species, endometrial glycogen content peaks during estrus and then declines during the luteal phase or pregnancy (Dean et al., 2014; Demers et al., 1972; Sandoval et al., 2021). This has led to the theory that glycogen acts as an energy reservoir for preimplantation embryos (Dean, 2019). In support of that, we show that glycogen mobilized during the preimplantation period is coming from the uterine epithelium, the cells that secrete histotroph. We also showed that the epithelium expresses G6PC, which is necessary for the secretion of glucose liberated from glycogen. G6PC has also been localized to the uterine epithelium of cyclic cows (Sandoval

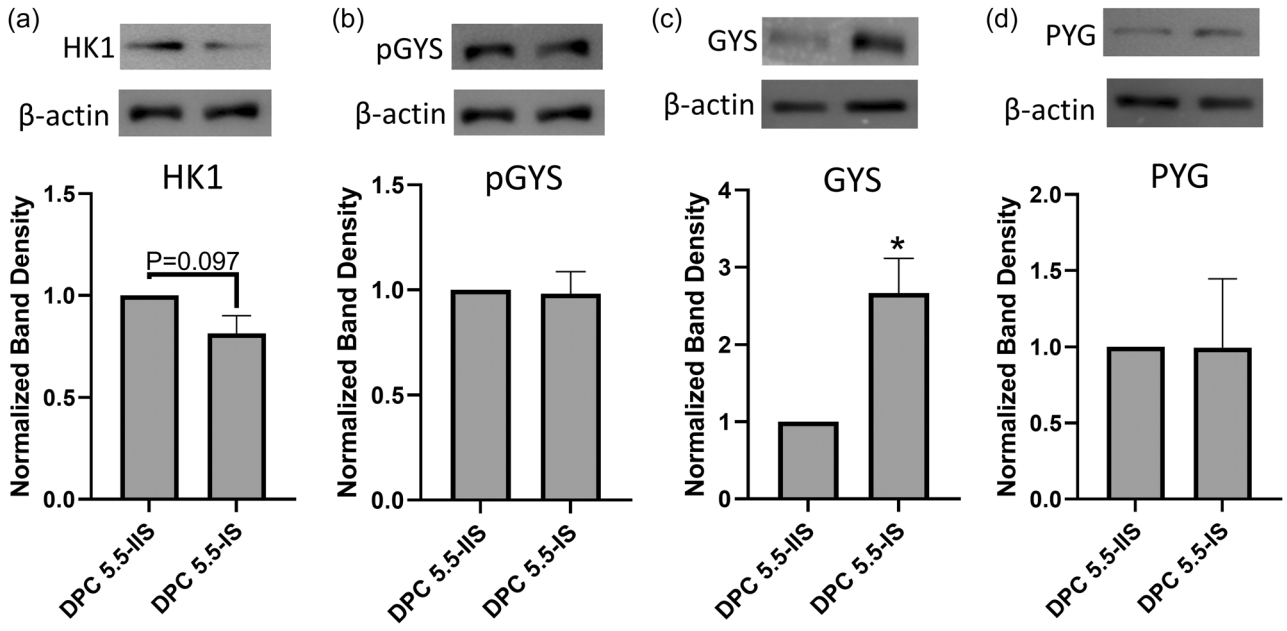

FIGURE 6 Western blots comparing levels of glycogen metabolizing enzymes in the uterus at the interimplantation site (IIS) and implantation site (IS) on DPC 5.5. Levels of hexokinase 1 (HK1, a), phospho-glycogen synthase (pGYS, b), glycogen synthase (GYS, c), and glycogen phosphorylase (PYG, d). *$p < 0.05$ relative to DPC 5.5-IIS. $n = 4$–5. DPC, days postcoitum.

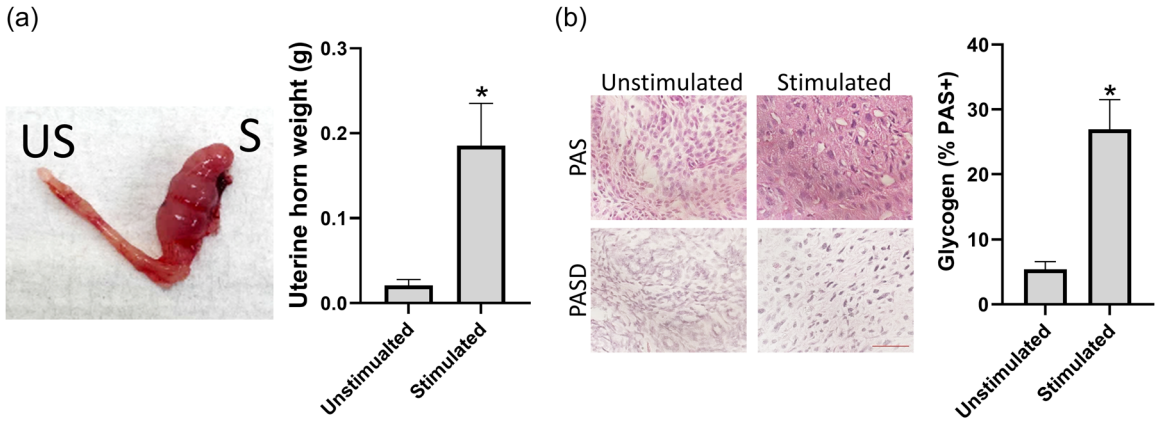

FIGURE 7 Uterine size and glycogen content after induction of artificial decidualization. (a) Representative image showing the stimulated (S) and unstimulated (US) uterine horns, and corresponding uterine horn weight. (b) PAS and PASD staining showed an increase of glycogen detected in the stimulated uterine horn compared to the unstimulated uterine horn. *$p < 0.05$. $n = 4$. Scale bar = 50 μm. PAS, periodic acid–Schiff; PASD, PAS with or without diastase.

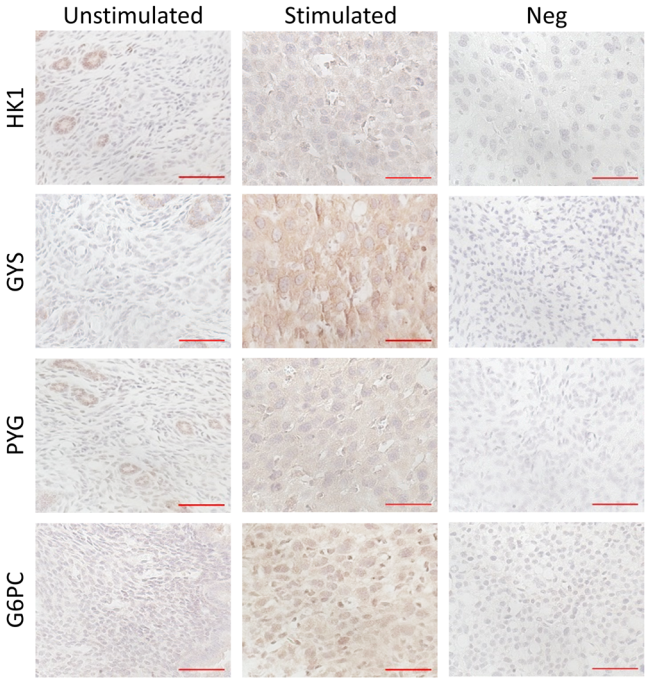

FIGURE 8 Localization of glycogen metabolizing enzymes in the decidualized and undecidualized uterine horn in an artificial decidualization model. Immunohistochemistry for hexokinase 1 (HK1), glycogen synthase (GYS), glycogen phosphorylase (PYG), and glucose-6-phosphatase (G6PC) in hormonally primed mice. One horn was simulated to decidualize. The unstimulated horn served as a nondecidualized control. $n = 4$. Scale bar = 50 μm.

et al., 2021). Global knockout of G6PC leads to a 50% decrease in litter size in mice, suggesting that G6PC is important for pregnancy (Jun et al., 2012), though systemic effects of G6PC knockout cannot be ruled out.

Before implantation, all four enzymes detected by immunohistochemistry (HK1, GYS, PYG, and G6PC) were primarily localized in the glandular and luminal epithelium, which is consistent with the significant change of glycogen content in the epithelium instead of the stroma. The expression of GYS, PYG, and G6PC in the uterine epithelium appeared to increase during the preimplantation period (DPC 1.5 and 3.5). These results agree with a study in mink that found uterine expression of *Gys*, *Pyg*, and *G6pc* messenger RNA increased after progesterone treatment with estradiol priming (Bowman & Rose, 2016). Western blots detected significant differences in GYS between the IS and IIS but no differences in HK1, PYG, or G6PC expression. However, western blots cannot differentiate between enzymes in the uterine epithelium, stroma, and myometrium. The trend for lower HK1 levels in the decidua is probably due to high expression in the uterine epithelium, which contributes a smaller part of the endometrium after decidualization. The concurrent expression of glycogen synthesizing and catabolizing enzymes in the uterine epithelium suggest that synthesis and catabolism are occurring concurrently within the epithelium. This may facilitate the continued transport of glucose from maternal blood to the uterine lumen even as glycogen levels are decreasing.

We also observed a substantial increase of glycogen in decidualized stromal cells at the IS and after artificial decidualization. In agreement, electron microscope studies detected glycogen deposits in the decidual cells in hamsters (Blankenship et al., 1990). In humans, endometrial glycogen concentration peaks in the luteal phase, presumably due to glycogen in the decidua, though glycogen has also been observed in the epithelium during the luteal phase (Gordon, 1975; Jones et al., 2015). Endometrial glycogen deficiency during the luteal phase is correlated to infertility in humans (Maeyama et al., 1977). Collectively, these results suggest that glycogen synthesis may be an inherent feature of decidualization and might be critical for maintaining a successful pregnancy.

The increase in GYS expression at the IS and in the artificially decidualized endometrium agrees with the dramatic increase of glycogen in the same tissues. The purpose of this glycogen reserve is currently unclear. Decidualization is a glucose-intensive process, requiring glucose metabolism via the pentose-phosphate pathway

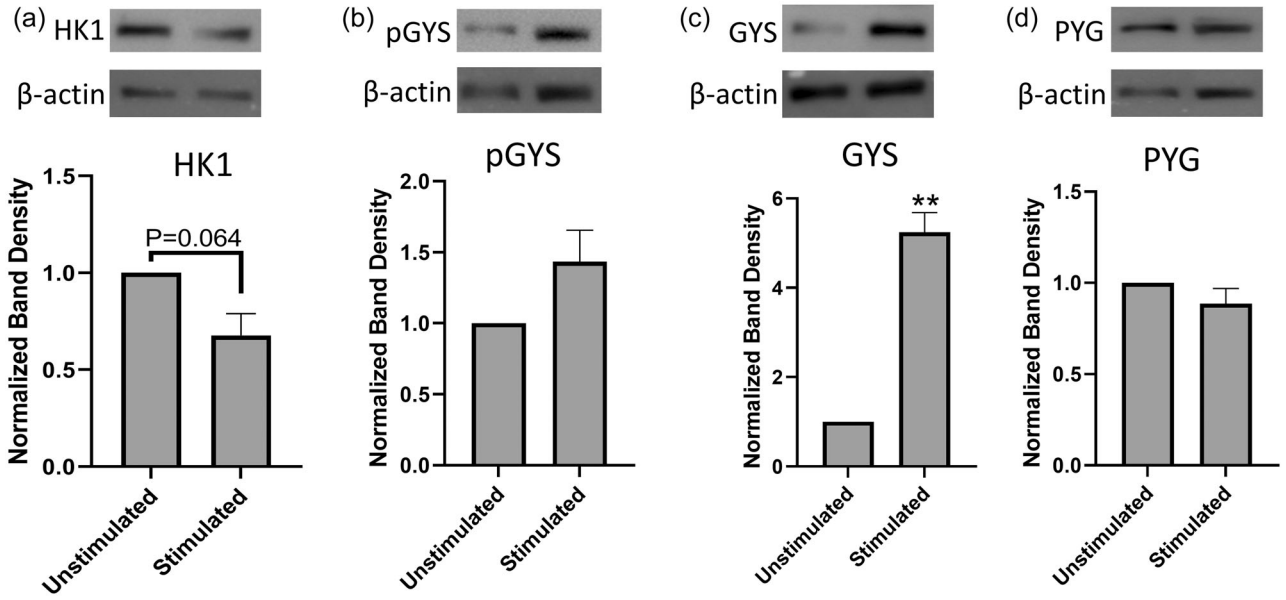

FIGURE 9 Levels of glycogen metabolizing enzymes in uterine horns stimulated to decidualize or left unstimulated. (a–d) Western blots for hexokinase 1 (HK1, a), phospho-glycogen synthase (pGYS, b), glycogen synthase (GYS, c), and glycogen phosphorylase (PYG, d) in uterine horns stimulated to decidualization with corn oil or unstimulated after hormonal priming. $^{**}p < 0.01$ relative to unstimulated horn. $n = 5$.

(Frolova & O'Neill, & Moley, 2011; Tsai et al., 2013). After decidualization, the decidua switches to Warburg metabolism, metabolizing a large amount of glucose via glycolysis (Zuo et al., 2015). Yet we consistently found high levels of glycogen after decidualization. It is possible that glycogen in the decidua is used to regulate the supply of glucose to decidual cells themselves, preventing negative effects of hyperglycemia, or to supply glucose to the developing embryo (Favaro et al., 2013; Zuo et al., 2015). More work is needed to elucidate the role of glycogen in the decidua and to determine if it is required for a successful pregnancy.

In conclusion, we show that the glycogen content of the glandular and luminal epithelium decreased during early pregnancy. This decrease was accompanied with increased expression of PYG and G6PC, suggesting glycogen is broken down into glucose and possibly secreted into the uterine lumen. The increased levels of glycogen in the decidual cells correlated with increased expression of GYS and stable expression of PYG and G6PC, suggesting concurrent synthesis and breakdown of glycogen in the decidua. Currently, the role of glycogen in the decidua is unclear, but it could be utilized by the uterus or secreted to supply nutrient for the invading embryo.

4 | MATERIALS AND METHODS

4.1 | Animals

CD-1 mice were purchased from Charles River Laboratories and maintained at the University of Illinois animal facility. Mice were kept on 12L:12D light cycle and were fed a standard chow diet. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #190624). To obtain uteri at proestrus, vaginal lavages were examined daily to monitor the estrous cycle in mice. After at least two normal estrous cycles, mice were killed at proestrus and uterine horns were collected. To collect uteri after mating, female mice were housed with males of proven fertility and examined every morning for the presence of vaginal plug. Observance of a vaginal plug was designated as DPC 0.5. The female mice were killed at DPC 1.5, 3.5, or 5.5 accordingly. Uterine horns were collected and fixed in 4% paraformaldehyde (PFA; Fisher Scientific; ICN15014601) or snap frozen in liquid nitrogen.

To induce artificial decidualization of the uterus, CD-1 female mice (Charles River Laboratories) were ovariectomized via two mid-dorsal incisions and given 2 weeks to heal and to eliminate circulating steroid hormones. Next 100 ng estradiol in 0.1 ml corn oil was given subcutaneously every 24 h for 3 consecutive days. Then, after 2 days of rest, 10 ng estradiol (Sigma-Aldrich; E2758) and 1 mg progesterone (Sigma-Aldrich; P8783) in 0.1 ml corn oil were injected subcutaneously daily for 3 consecutive days. Decidualization was initiated 4 h after the last injection. Corn oil (15 µl) was injected into the lumen of the left uterine horn through a flank incision. The right uterine horn was left unstimulated and served as an internal control. The mice were treated with 1 mg progesterone in 0.1 ml corn oil daily for 4 days and were euthanized 96 h postinducing decidualization. Both uterine horns were collected, weighed, and fixed in 4% PFA or snap frozen in liquid nitrogen.

4.2 | PAS staining

Tissues were sectioned at 5 µm. Two slides were used for PAS and PASD staining separately. Slides were deparaffined in xylene

(Avantor; 8668-16) for 10 min and rehydrated with graded concentrations of ethanol. Slides for PAS staining were incubated in PBS, and slides for PASD staining were immersed in PBS with 0.5% diastase (Sigma-Aldrich; 09962) at 37°C for 60 min. After incubation, slides were incubated in 0.5% periodic acid solution (Fisher Scientific; AC453171000) for 5 min at room temperature and washed three times with distilled water. Sections were immersed in Schiff's reagent (Sigma-Aldrich; 3952016) for 15 min at room temperature, followed by 5 min wash in lukewarm running tap water. Then, slides were counterstained with hematoxylin (Fisher Scientific; 22-050-206) and dipped in ammonium hydroxide buffer (Thermo Fisher; A669S) for 20 s. Slides were dehydrated in an ethanol series and incubated in xylene overnight. Slides were mounted with Permount mounting media (Fisher Scientific; SP15100). Images were captured using Zeiss Axioskop with 305 Axoicam color camera.

Images were analyzed with ImageJ (https://imagej.nih.gov/ij/) as previously described (Sandoval et al., 2021). Using hue, saturation, and brightness a threshold was set to define PAS positive pixels. The same threshold was used for all images. In each image, the area positive for PAS and total area occupied by each tissue (the glandular epithelium, luminal epithelium, and stroma) was measured. The percent area positive for PAS in each tissue was calculated in PAS and PASD images. To account for non-glycogen staining in the PAS images, the percent area positive for PAS in PASD images was subtracted from the percent area stained in the corresponding PAS images. The results are expressed as percent area PAS positive (%PAS+).

4.3 | Immunohistochemistry

Tissues were sectioned at 5 µm, added to slides, deparaffinized, and rehydrated. Slides were boiled in sodium citrate buffer (Fisher Scientific; S271-3) and then cooled to room temperature. Then, the slides were incubated in 3% hydrogen peroxide (Fisher Scientific; H325-500) for 15 min. Nonspecific blocking was inhibited with block containing 10% goat serum (Vector Laboratory; S-1000-20) and 5% bovine serum albumin (BSA; Fisher Scientific; BP9706100) in Tris-buffered saline (TBS) for 1 h at room temperature. After the serum block, previously validated primary antibodies (Table 1) were diluted in the block, added to tissue sections, and incubated at 4°C overnight (Sandoval et al., 2021). All incubations were performed in hydrated chamber. The next day, slides were washed in TBS with tween (TBS-T) three times and incubated with secondary antibody (Vector Laboratories; BA-5000-1.5) diluted in the block for 30 min at room temperature. Then, slides were washed three times and incubated with avidin–biotin complex reagent (Vector Laboratory; SP-2001) for 30 min at room temperature. After three washes in TBS-T, 3, 3′-diaminobenzidine (Vector Laboratory; SK-4100) was applied. Slides were counterstained with hematoxylin for 2 min. Then, the tissues were dehydrated, mounted, and imaged with a Zeiss Axioskop with 305 Axoicam color camera. Negative controls were treated as described above, except that the primary antibody was replaced with an isotype control (anti-green fluorescent protein) antibody.

4.4 | Western blots

When comparing ISs and IISs, the uterus was removed and ISs and IISs were separated. The IS was then cut longitudinally and the embryo was carefully separated from the uterus under a dissecting scope. In all circumstances, the uterine segments contained myometrium and endometrium. Tissues were then snap frozen at after processing.

Uterine tissues were homogenized in radioimmunoprecipitation assay buffer supplemented with phosphatase and protease inhibitors

TABLE 1 Primary antibodies and summary of conditions used for western blot (WB) and immunohistochemistry (IHC)

Antigen	Catalog No.	Technique	Dilution	Block
Hexokinase 1	2024 Cell signaling	WB	1:500	BSA
		IHC	1:20	Goat serum
Glycogen synthase	3886 Cell signaling	WB	1:500	BSA
		IHC	1:40	Goat serum
Phospho-glycogen synthase	47043 Cell signaling	WB	1:500	Milk
Glycogen phosphorylase	Ab231963 Abcam	WB	1:500	BSA
	A9392 ABclonal	IHC	1:100	Goat serum
Glucose-6-phosphatase	PA5-70653 Invitrogen	IHC	1:50	Goat serum
GFP	2956 Cell signaling	IHC	variable	Goat serum
β-actin	A2066 Sigma-Aldrich	WB	1:1000	Milk

Note: Block for WB consisted of 5% powdered milk or 5% BSA in TBS-T. Block for IHC was 3% BSA and 10% goat serum in TBS.

Abbreviations: BSA, bovine serum albumin; GFP, green fluorescent protein; TBS, Tris-buffered saline; TBS-T, TBS with tween.

(Sigma-Aldrich; P0044-1ML and Thermo Fisher; A32953, respectively), and the protein concentration was determined by BCA assay. Proteins (25 μg) were separated in a 10% SDS-PAGE gel using constant voltage. Then, proteins were transferred onto polyvinylidene difluoride membranes and were blocked for 1 h with either 5% BSA in TBS-T or 5% nonfat dry milk in TBS-T depending on the primary antibody. The membranes were incubated in primary antibody (Table 1) overnight at 4°C. The next day, the membranes were washed three times with TBS-T and incubated in block in TBS-T containing anti-rabbit secondary antibody (Cell Signaling; 7074S) for 30 min. SuperSignal West Pick PLUS chemiluminescent substrate (Thermo Scientific; 34577) was used for developing signals and images were obtained using an ImageQuant LAS 4000 (GE Healthcare)

4.5 | Statistical analysis

Statistical calculations were performed using GraphPad Prism version 8.3.1. Data collected during early pregnancy was analyzed by a One-way analysis of variance followed by a Dunnett's analysis. Western blot of DPC 5.5 IIS and DPC 5.5 IS was analyzed by a paired *t*-test. For the artificial decidualization experiments, glycogen content, uterine weight, and western blot were analyzed using a paired *t*-test. Results are presented as mean ± SEM and differences were considered statistically significant when $p < 0.05$.

AUTHOR CONTRIBUTIONS

Matthew Dean designed the experiments. Matthew Dean and Kassandra Sandoval monitored the mice and collected uteri. Ziting Chen carried out the analysis. Matthew Dean and Ziting Chen wrote the manuscript. All authors approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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