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ORIGINAL PAPER

Heparin prevents oxidative stress-induced apoptosis in human decidualized endometrial stromal cells

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Abstract
Clinical trials have shown that administering heparin during the luteal phase has beneficial effects on implantation and live birth rates. Heparin exerts direct effects on decidual human endometrial stromal cells (HESCs), which are independent of its anticoagulant effect. However, the accurate effects of heparin on the decidualization process remain unidentified. Here, we demonstrate that HESCs become dramatically resistant to oxidative stress upon decidualization, and we hypothesize a possible direct action of heparin on the decidualization of HESCs, which would lead to improved implantation. To test this hypothesis, we established primary HESC cultures and propagated them, and then we decidualized confluent cultures with 8-bromo-cAMP, with medroxyprogesterone acetate, and with or without heparin. We treated the cells with hydrogen peroxide (H_2O_2) as a source of reactive oxygen species (ROS). Adding heparin to decidualized HESCs induced prolactin secretion. Decidualized HESCs treated with heparin were prevented from undergoing apoptosis induced by oxidative stress. Heparin induced nuclear accumulation of the forkhead transcription factor FOXO1 and expression of its downstream target, the ROS scavenger superoxide dismutase 2. These results demonstrate that heparin-treated decidualized HESCs acquired further resistance to oxidative stress, suggesting that heparin may improve the implantation environment.

Keywords Endometrial decidualization · Heparin · FOXO1 · SOD2 · Oxidative stress

Introduction

Heparin is a mucopolysaccharide that is composed of sulfated glucuronic acids and glucosamine that exists in different lengths of polymeric units and presents different molecular weights. This substance, ubiquitously found in mammalian tissues, is clinically administered to prevent thromboembolic disorders during pregnancy, especially against antiphospholipid antibody syndrome [1, 2]. The anticoagulant properties of heparin have been thought to contribute to the outcome of pregnancy in women with prothrombotic disorders. However, intravascular or intravillous blood clots are rarely found during histological examinations of placentas and decidual samples obtained from patients suffering from miscarriages or complicated pregnancies [3].

Clinical trials have demonstrated that administering heparin during the luteal phase has a beneficial effect on the implantation rate after the embryo transfers as well as on live birth rates in women with repeated implantation failures [4, 5]. Hills et al. suggested that heparin could directly protect human villous trophoblasts against apoptosis in response to a variety of pathological stimuli [6]. In addition, heparin exerts direct effects on the decidualization of human endometrial stromal cells (HESCs), apart from its anticoagulant activity [7]. However, the precise effects of heparin on the endometrial decidualization process remain unclear.

Here, we show that HESCs become resistant to oxidative stress-induced apoptosis upon decidualization [8]. In addition, we previously demonstrated that hCG, one of the earliest and most abundant glycoproteins secreted by embryonic trophoblasts, further promote resistance to oxidative stress [9]. Based on these facts, we investigated whether heparin inhibits apoptosis induced by oxidative stress upon HESC

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decidualization, to clarify the molecular mechanisms underlying this process.

Materials and methods

Tissue collection

We collected human endometrium tissues from patients undergoing hysterectomy at the Saitama Medical University Hospital. This research project was approved by the Institutional Review Board (Approval #16102). We recruited patients with uterine fibroids with regular menstrual cycles who were not on hormonal treatment at the time of the operation. Written informed consent was obtained before tissue collection from all patients.

Isolation of HESCs

We isolated HESCs as mentioned elsewhere [10–13]. Briefly, we cultured the harvested HESCs in a maintenance DMEM/F-12 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% dextran-coated charcoal-treated FBS, supplemented with 2 μg/mL insulin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA), 1×10^{-9} M β-estradiol (Sigma-Aldrich), 1% antibiotic/antimycotic solution (Thermo Fisher Scientific), and 1% L-glutamine solution (Thermo Fisher Scientific).

Decidualization and heparin treatment of HESCs

We stimulated the decidualization of the cultured HESCs with 0.5 mM 8-bromo-cAMP (8-Br-cAMP; Sigma-Aldrich) and 10^{-6} M medroxyprogesterone acetate (MPA; Sigma-Aldrich). Next, we initiated heparin treatment at the time of decidualization medium change using different unfractionated heparin concentrations (Sigma-Aldrich). We performed all experiments before the fourth passage of the cultures.

Prolactin (PRL) measurement

We measured PRL concentrations using an electrogenerated chemiluminescence immunoassay (ECLIA) method with an ECLusys Prolactin III Reagent (Roche Diagnostics, Basel, Switzerland) and a Cobas 6000 analyzer (Roche Diagnostics). We then harvested the HESCs on the 3rd day of decidualization to carry out the PRL measurements.

Cell apoptosis detection assay

We induced apoptosis in the cells using hydrogen peroxide (H_2O_2; Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 100 mM in decidualized HESCs either treated or not treated with different concentrations of heparin. We used Cell Death Detection ELISA Plus (Sigma-Aldrich) as per the manufacturer's instructions to perform the apoptosis assays. The results estimate the relative apoptosis level in H_2O_2-treated cells compared to the same level in untreated control cells.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

We extracted total RNA samples from HESCs using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription for synthesis of cDNA from the extracted total RNA was performed using BioScript reverse transcriptase (Bioline, London, UK). We then analyzed the expression of *PRL, FOXO1*, and *SOD2* mRNAs using qRT-PCR. Each qRT-PCR was set up using PowerUp SYBR Green PCR Master Mix (Thermo Fisher Scientific), and we used the PikoReal 96 Real-Time PCR System (Thermo Fisher Scientific) for expression detection. Table 1 shows the primer sequences for each gene. We calculated the mRNA expression levels in relation to the *GAPDH* level using the $2^{-\Delta\Delta c_t}$ method [14].

Immunofluorescent staining

We cultured HESCs on coverslips in six-well culture plates with a maintenance medium. At 80% confluency, we incubated the HESCs with or without a decidualization medium for 3 days. The cells were then fixed with a 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Kyoto, Japan) and stained with a primary antibody (FOXO1, Cell Signaling Technology; SOD2, Abcam, Cambridge, UK; surviving, Abcam). The secondary antibody used was an Alexa Fluor 488 conjugated antibody (Thermo Fisher Scientific). Nuclear staining was performed using 4′,6-diamidino-2-phenylindole- (DAPI-)-containing mounting media (Vector Laboratories, Burlingame, CA, USA). We used a fluorescent microscope to visualize the results (Axiocam; Carl Zeiss, Oberkochen, Germany).

Table 1 Primer sequences used for real-time PCR

Gene name	Primer sequence
GAPDH	Forward: 5′-CGACCACTTTGTCAAGCTCA-3′
	Reverse: 5′-AGGGGTCTACATGGCAACTG-3′
FOXO1	Forward: 5′-ATTCGGAATGACCTCATGGA-3′
	Reverse: 5′-TTTTAAGTGTAACCTGCTCACTAACC-3′
SOD2	Forward: 5′-CTGGACAAACCTCAGCCCTA-3′
	Reverse: 5′-TTTGTAAGTGTCCCCGTTCC-3′

Statistical analysis

We repeated the PRL measurements and apoptosis assays in three separate experiments ($n=3$). The qRT-PCR analysis was repeated six times ($n=6$). Data were analyzed using a two-tailed Student's t test for comparisons within two groups or Tukey's test for multiple comparisons. All graphs of error ranges are indicated as a mean ± standard error. We considered P values <0.05 as statistically significant.

Results

Effect of heparin on the oxidative resistance of decidualized HESCs

In agreement with our published study [8], decidualization reduced the level of H_2O_2-induced apoptosis in HESCs, as assessed by the nucleosomal DNA fragmentation level (data not shown). We found no significant cell apoptosis differences in the decidualizing HESCs treated with heparin at the lowest concentration (0.5 μg/mL) after H_2O_2 treatment. However, cotreatment with higher concentrations (5 μg/mL) of heparin conferred additional protection against H_2O_2-dependent oxidative stress-induced apoptosis as compared to untreated cells (Fig. 1). Accordingly, we used the effective concentration of heparin, 5 μg/mL, as a treatment in subsequent experiments.

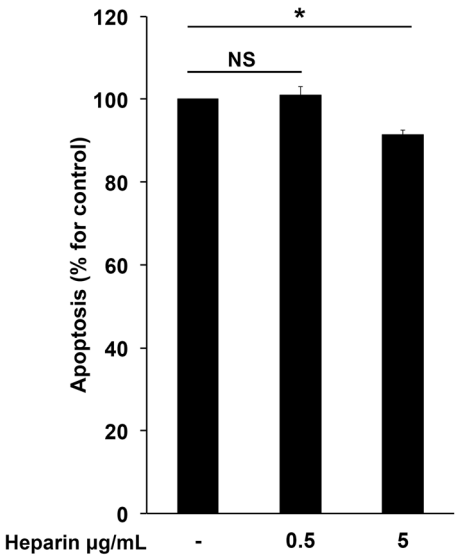

Fig. 1 Decidualized HESCs resistant to H_2O_2-induced apoptosis after heparin treatment. We cultured decidualized HESCs with or without heparin (0.5 or 5 μg/mL) for 3 days and exposed them to H_2O_2 (100 μM) for 4 h. Data represent the percentage of apoptosis in comparison to the same percentage in untreated control cells. Data are represented as mean ± standard error. $^*P<0.05$

Heparin modulates PRL expression on decidualized HESCs

Next, we investigated whether heparin stimulates PRL secretion, a widely used biochemical marker for decidualizing HESCs [11, 15]. We treated primary HESC cultures with or without 8-Br-cAMP and MPA, as well as with heparin, for 6 days. As expected [16], the enhanced expression of PRL secretion required both 8-Br-cAMP and MPA signaling. Heparin significantly induced PRL secretion in cultures treated with decidualized HESCs. In contrast, it had no effect on PRL secretion in undifferentiated HESCs (Fig. 2a).

HESCs are found as spindle-shaped fibroblast-like cells when cultured without treatment. Treatment with 8-Br-cAMP and MPA induced morphological decidual changes (larger and rounder cells than before) to the phenotype of HESCs. Heparin did not affect these morphological changes,

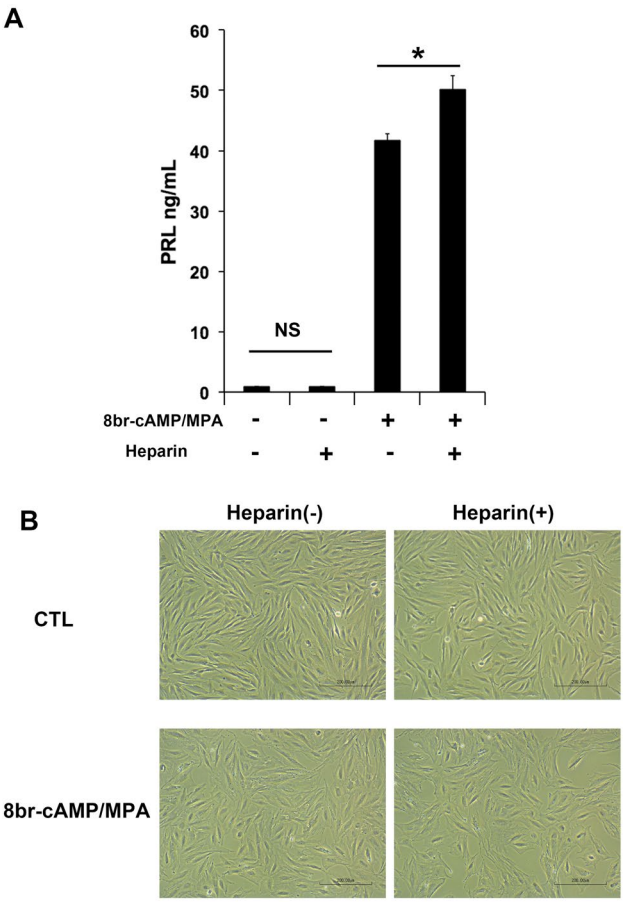

Fig. 2 Effect of heparin on the phenotype of HESCs. **a** PRL production by primary HESCs in culture media. We treated HESCs with or without 8-Br-cAMP/MPA or 5 μg/mL heparin for 3 days. The data represent the mean PRL protein concentration of three different HESC cultures ($n=3$). Data are presented as mean ± standard error. $^*P<0.05$. **b** Morphological changes of decidual HESCs in response to heparin. Scale bar: 200 μm

and the phenotypes were undistinguishable from those of control cells. The addition of heparin to decidualized HESCs also had no discernible effects on the morphological appearance of the decidual cultures (Fig. 2b).

Heparin induced the expression levels of SOD2 in decidualized HESCs

To explore the underlying mechanism of oxidative resistance, we focused on the forkhead transcription factor FOXO1, an important cAMP-dependent transcription factor in decidualizing HESCs [8, 10, 12]. FOXO1 is also implicated in antioxidative-resistance responses because it regulates the expression of superoxide dismutase 2 (SOD2) [8], also known as manganese superoxide dismutase (MnSOD). As expected, FOXO1 mRNA expression levels were extremely upregulated upon HESC decidualization by 8-Br-cAMP and MPA. However, contrary to our expectation, heparin did not alter the FOXO1 mRNA expression on nondecidualized and decidualized HESCs. In contrast, the SOD2 mRNA expression levels rose upon HESC decidualization by 8-Br-cAMP and MPA, and heparin further stimulated SOD2 mRNA expression on the decidualized HESCs (Fig. 3).

Heparin regulates FOXO1 protein subcellular localization and increases the protein levels of SOD2

In response to the activation of the PI3K/AKT signaling pathway or other kinases, phosphorylates FOXOs on conserved residues, triggering their export from the nucleus and binding to 14-3-3 chaperone proteins in the cytosol and, hence, loss of transcriptional activity [17]. We speculated that the reciprocal effects of heparin on FOXO1 and SOD2 mRNA expression on decidualized HESCs could reflect changes in the subcellular distribution of the FOXO1 protein. Confocal microscopy demonstrated that the FOXO1 protein in 8-Br-cAMP- and MPA-treated cells was localized in both the cytoplasm and the nuclei, in comparison with untreated decidualized cells that had the protein mostly in. We observed the nuclear accumulation of FOXO1 protein immunoreactivity in decidualized HESCs treated with heparin (Fig. 4). Consistent with our qRT-PCR analysis, the amount of SOD2 protein increased upon HESC decidualization by 8-Br-cAMP and MPA. Heparin further enhanced the production of SOD2 protein on decidualized HESCs, as seen by the increased immunoreactivity (Fig. 5).

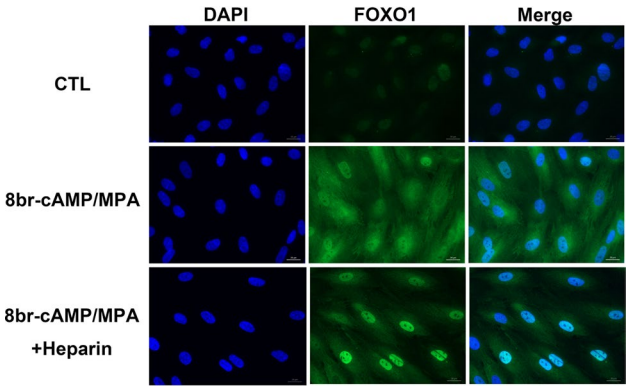

Fig. 4 Heparin induces the cytoplasmic localization of FOXO1 in decidualized HESCs. We treated primary HESC cultures with 8-Br-cAMP/MPA for 3 days with or without heparin (5 μg/mL). Confocal micrographs showing FOXO1 stained with Alexa Fluor 488 (green) and nuclei stained with DAPI (blue). Scale bar: 20 μm

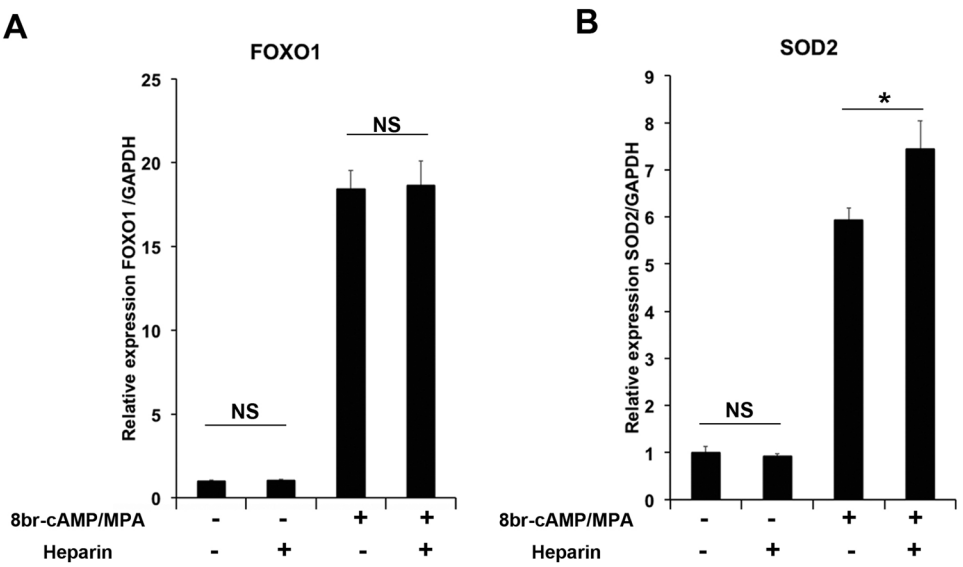

Fig. 3 Heparin stimulated the expression of SOD2 mRNA but not of FOXO1 mRNA in decidualized HESCs. Relative mRNA expression levels of FOXO1 (**a**) and SOD2 (**b**). We treated primary HESCs with or without 8-Br-cAMP/MPA or heparin (5 μg/mL), as indicated, for 3 days. Data are represented as mean ± standard error. $^*P < 0.05$. *NS* not significant

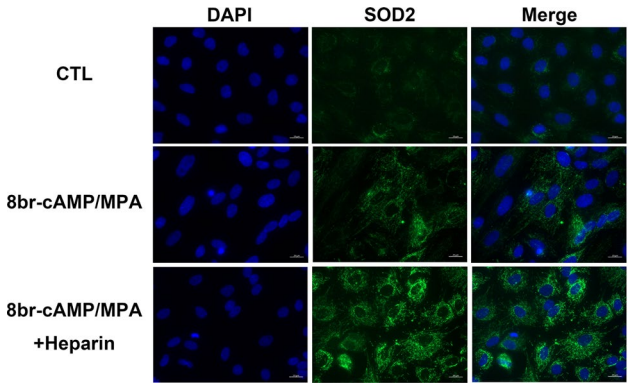

Fig. 5 Heparin had no effect on the subcellular localization of SOD2 on decidualized HESCs. We treated HESC cultures with 8-Br-cAMP/MPA for 3 days with or without heparin (5 μg/mL). Confocal micrographs showing SOD2 stained with Alexa Fluor 488 (green) and nuclei stained with DAPI (blue). Scale bar: 20 μm

Discussion

Since the initiation of in vitro fertilization-embryo transfer (IVF-ET) [18], improvements in the protocols of ovarian stimulation, preparation of follicles, embryo culture conditions, and others have given considerable hope to couples with infertility. However, the live birth rate of IVF-ET is not satisfactory. However, the rate-limiting factor appears to be the implantation rate, which results from a complex process that depends on many variables [19]. Successful implantation depends on three interdependent processes: embryo development, placenta formation, and decidualization of the endometrium. In particular, decidualization involves the transformation of the endometrial stromal cells into specialized secretory cells, a process that is further characterized by an influx of specialized immune cells and vascular remodeling [20]. The decidual process is indispensable for the formation of a functional fetal–maternal interface as it controls trophoblast invasion and tissue homeostasis, and it confers resistance to environmental stress signals, protecting against oxidative stress [8]. Impaired decidualization is associated with an increase in miscarriage rates in an exponential fashion [21].

Although the use of heparin in IVF-ET procedures lacks both molecular and clinical evidence to support it, practitioners use it empirically to improve the implantation rates [22]. Clinical trials have demonstrated that administering heparin during the luteal phase has beneficial effects on the implantation and live birth rates in women with repeated implantation failures [4, 5]. The mechanism of this positive effect of heparin on implantation is unclear, but some hypotheses have been proposed, and we wanted to investigate whether heparin has direct effects on the decidualization process.

In this study, we demonstrated that heparin increases PRL secretion in decidualized HESCs without changing the cell morphology, which is widely accepted as a highly sensitive and specific decidual maker and is known to play an important role in human endometrial differentiation and implantation [23, 24], in agreement with another report [25]. The expression of PRL in the endometrium has been shown to be impaired in patients with recurrent pregnancy loss, both in vivo and in primary cultures subjected to a decidualizing stimulus [26]. Additionally, a lack of expression of endometrial PRL during the implantation window has been proven in some patients suffering from unexplained infertility and repeated miscarriages [27]. These observations suggest that heparin may improve the uterine environment for implantation by inducing PRL secretion upon decidualization.

Implantation and subsequent placenta formation are profound inflammatory processes that are characterized by an influx of immune cells, extensive remodeling, and vascular changes [20]. Reactive oxygen species (ROS) are invariably generated during inflammatory processes and are even more pronounced at the fetal–maternal interface because of the profound fluctuations in oxygen tension associated with the onset of placental perfusion in the first trimester of pregnancy [28]. In the luck of effective against mechanisms, ROS cause indiscriminate damage to proteins and nucleic acids. Thus, impaired decidualization inevitably predisposes the fetal–maternal interface to oxidative damage. In addition, compelling evidence has suggested that oxidative stress underpins a spectrum of pregnancy disorders, ranging from miscarriages to fetal growth restriction and preeclampsia [29].

FOXO1, a member of the mammalian FOXO subfamily of forkhead transcription factors, is an important regulator of the decidual process in HESCs. FOXOs are downstream targets of the phosphatidylinositol-3-kinase pathway, and phosphorylation of FOXOs by Akt and other kinases results in their nuclear exclusion and loss of their transcriptional activity [30, 31]. FOXO proteins are critical mediators of cell fate because of their ability to regulate either proapoptotic genes [32–36] or genes involved in differentiation, cell cycle arrest [37], oxidative defenses [30, 38, 39], and DNA repair [31, 40]. We have demonstrated that decidualized HESCs acquire heightened defenses against oxidative stress, which is partly explained by the FOXO1-dependent induction of SOD2 [8]. Herein, we demonstrated that heparin enhances resistance to oxidative stress-induced apoptosis. Moreover, it stimulates SOD2 expression in parallel with the accumulation of FOXO1 in the nuclei of decidualized HESCs. In addition, heparin induces apoptotic inhibitor surviving protein expression in decidualized HESCs. Therefore, the possible beneficial effects of heparin on implantation probably consist in modulating trophoblast invasion [41] and endometrial differentiation.

Low-molecular-weight heparins (LMWHs) are derived heparin by enzymatic or chemical depolymerization and have largely replaced UFH for preventing obstetrical complications in women with thrombophilic disorders because of its simpler usage, more predictable anticoagulant activity and safety profile [42]. We employed only UFH as heparin, but not LMWHs fir the present study. However, both UFH and LMWHs improve the pregnancy outcome in women suffering from thrombophilia and pregnancy complications [1, 43]. Furthermore, various studies demonstrated that UFH has similar direct effect as LMWHs for decidualized HESCs in vitro [7, 25, 44]. Hence, additional studies are need to evaluate LMWHs in prevention of apoptosis induced by oxidative stress.

In addition to its anticoagulant activity, heparin has biological properties that may be critical for the prevention of tissue injuries at the fetal–maternal interface. For instance, it suppresses natural killer cell cytotoxicity [45, 46], prevents leukocyte adhesion/influx [47–49], and antagonizes interferon-γ (IFN-γ) signaling [50]. On top of these anti-inflammatory and immunomodulatory effects, an increasing number of cytokines and growth factors, including the hepatocyte growth factors, epidermal growth factors (EGFs), heparin-binding epidermal growth factors (HB-EGFs), and fibroblast growth factors (FGFs), bind glycosaminoglycans of the heparin and heparin-sulfate family, thereby regulating their bioactivities [51, 52]. Further studies should be performed to clarify the association between heparin and anti-inflammatory or immunomodulatory effects on the decidualization process.

Conclusion

In conclusion, to the best of our knowledge, this is the first study showing that heparin confers resistance to oxidative stress-induced apoptosis in decidualizing HESCs. Our results suggest that the mechanism accounting for this resistance to oxidative stress-induced apoptosis involves an increase in SOD2 expression through the accumulation of FOXO1 in nuclei to enhance the free-radical-scavenging potential in decidualized HESCs. Moreover, we demonstrated that heparin increases PRL secretion in decidualized HESCs. All in all, our results suggest that heparin may improve the uterine environment for successful implantation by preventing apoptosis due to oxidative stress and by modulating the decidual process.

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Compliance with ethical standards

Conflict of interest The authors declare having no conflicts of interest.

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