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Immunohistochemistry of Leukemia Inhibitory Factor and Integrin αVβ3 in Mouse Endometrium Following Kisspeptin-54 Ovulation Trigger

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Abstract

Kisspeptin (KP) is a group of hypothalamic neuropeptides encoded by KISS-1 gene. KP-54, a 54-amino-acid peptide, helps regulate the hypothalamic-pituitary-ovarian axis and plays a potential role in implantation. C57BL/6 J female mice were super-ovulated via intraperitoneal injection of 5 International Units (IU) pregnant mare serum gonadotrophin (day 1). Forty-eight hours later, mice (5/group) were injected with phosphate-buffered saline (PBS) (group A), 5 IU human chorionic gonadotrophin (hCG) (group B), or 3 nmol KP-54 (group C). On day 7, mice were euthanized and uteri excised to create paraformaldehyde-fixed paraffin-embedded sections that were immunostained for the implantation markers: leukemia inhibitory factor (LIF) and integrin αVβ3 (ITG αVβ3). Slides were scored for intensity of staining in endometrial glandular epithelium (GE) and stromal cells (SCs) via histoscore (H-score). Data were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney *U* test for pairwise comparisons. LIF expression was significantly higher in GE and SCs of mice triggered with KP-54 compared to placebo ($P = .009$ for both), but only higher than hCG trigger group in SCs ($P = .009$). Meanwhile, ITG αVβ3 expression was significantly higher in SCs of mice triggered with KP-54 compared to placebo ($P = .028$). In conclusion, using KP-54 as an ovulation trigger resulted in higher expression of the implantation markers LIF and ITG αVβ3 in mice endometrium compared to hCG or placebo. This suggests a potential role for KP-54 trigger in improving embryo implantation in clinical IVF. However, further studies are needed to correlate these results with clinical implantation rates and pregnancy outcomes.

Keywords Kisspeptin · Immunohistochemistry · Implantation · LIF · Integrin αVβ3

Introduction

Embryo implantation is a complex process through which an embryo adheres to the luminal surface of the decidual endometrium [1] and comprises three main steps: apposition, adhesion, and penetration [2]. Implantation is regulated by a number of maternal and embryonic factors [3]. However, in human females, abnormalities in uterine receptivity accounts for a large percentage of preclinical pregnancy losses [4]. Thus, understanding the underlying molecular mechanisms involved in uterine receptivity may prove a target to improve embryo implantation. Some such molecules that have been studied as markers for successful implantation include kisspeptin (KP), leukemia inhibitory factor (LIF), and integrin αVβ3 (ITG αVβ3).

KP is a neuropeptide hormone in the arginine phenylalanine amide (RFamide) family (encoded by the KISS1 gene) that acts by stimulating the kisspeptin receptor (KISS1R) [5] and has effects in luteinizing hormone (LH) secretion [6] and preparation of the endometrium for implantation. KP is encoded on the KISS1 gene that translates into 145 amino acid long pro-peptide which is cleaved to give the main active peptide KP-54 [7]. This peptide is further cleaved to smaller peptides KP-10, KP-13 and KP-14 [7]. In human females, KP-54 has been proposed as an ovulation trigger in IVF

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cycles due to its more physiologic stimulation of gonadotropin secretion in comparison to human chorionic gonadotropin (hCG), thus decreasing the risk of ovarian hyperstimulation syndrome (OHSS) [6]. In mice, the 52 amide variant exists, however, KP-54 has been established as a potential ovulation trigger [8, 9] and is commercially available. Additionally, KISS1 and KISS1R genes were found to be expressed in human endometrial epithelial cells [10]. Furthermore, plasma KP levels on the hCG trigger day were positively correlated with the likelihood of implantation after ICSI in patients with unexplained infertility [11, 12]. Similarly, a functional KP/KISS1R system was detected in the mouse endometrial tissues on the day of embryo implantation [13]. Additionally, dynamic elevations of KISS-1 in quantitative PCR occurred in mouse during early pregnancy as well as in artificially induced decidualization [7]. In the same line, KISS-1 inhibition using small interfering RNA (siRNA) blocked the progression of stromal cell decidualization [7]. Furthermore, a study examining heterozygous hormone-rescued KISS-1 $^{+/-}$ mice demonstrated a failure of embryo implantation in KISS-1 $^{+/-}$ mice but not when KISS $^{+/-}$ generated embryos were transferred to wild-type mice, suggesting a uterine factor rather than an embryonic defect [14].

LIF is a member of the interleukin-6 (IL-6) cytokine family and has been suggested to play a significant role in the implantation of mouse and human embryos [15]. In fertile women, a moderate to high level of LIF expression was observed during the proliferative and secretory phases of the menstrual cycle, while low levels of LIF expression were seen in women with implantation failure [16]. In mice, uterine gland-derived LIF initiates embryo-uterine communication, which leads to embryo attachment and decidualization of endometrial stromal cells [17, 18]. Previous studies have shown that estradiol is essential for endometrial LIF expression [19], and treatment with exogenous estradiol upregulates uterine LIF either in wild type or ovariectomized mice [20, 21]. Estradiol treatment has also been shown to upregulate expression of KISS-1 mRNA in ovariectomized mice [7]. In KISS-1$^{-/-}$ mice, estrogen treatment did not stimulate LIF expression and only exogenous LIF treatment in hormone-primed KISS-1 $^{-/-}$ female mice partially rescued implantation [14]. Together, these results suggest that estradiol acts upstream to both KISS-1 and LIF, and KISS-1 is a necessary regulator of LIF expression and thus, integral to embryo implantation.

Another important implantation marker is ITG αVβ3 which is a heterodimeric glycoprotein that has been reported to be essential for implantation in mice [22]. ITG αVβ3 is expressed on the apical surface of human luminal endometrial epithelial cells [23], and its expression is synchronous with fully developed pinopodes [24]. Moreover, blocking ITG αVβ3 using intrauterine injection of monoclonal neutralizing antibodies was effective in significantly reducing the number of implantation sites in mice compared to control [22]. Additionally, a temporal expression pattern similar to that of LIF has also been reported for ITG αVβ3, strongly suggesting a role in the initial stages of blastocyst adhesion [25].

The current study aimed to examine the novel role of KP-54 as an ovulation trigger specifically as it relates to its potential role as a mediator in embryo implantation using a mouse model. To accomplish this, we compared LIF and ITG αVβ3 expression in the endometrium of superovulated female mice using KP-54, hCG, or placebo as ovulation triggers. We hypothesized that KP-54 trigger will be associated with upregulated endometrial implantation markers, specifically LIF and co expressed ITG αVβ3.

Materials and Methods

Setting and Ethics Approval

The study was conducted in the British Columbia Children's Hospital Research Institute (BCCHR) in conjunction with the University of British Columbia (UBC), in Vancouver, BC, Canada. The study was approved by the UBC Research Ethics Board (#A16-0295). All experiments were conducted in accordance with the regulations of the UBC Animal Care Committee and the Canadian Council on Animal Care.

Kisspeptin-54 Dose Calculation

Given that KP-52 is not commercially available and a previous study has established KP-54 as an effective ovulation trigger in mice [9], the KP-54 dose was calculated based on a previous human study. In a phase 2, randomized clinical trial, four doses of KP-54 were used: 3.2, 6.4, 9.6 and 12.8 nmol/kg body weight, with the latter having the highest yield of oocytes [26]. Study dose was then calculated by multiplying the maximal response human dose (12.8 nmol/kg) by a simplified dose conversion for human to animal (12.3), resulting in 157.44 nmol/ kg [27]. Study dose was further adjusted per mouse based on an average body weight of 20 g to be 3.14 nmol/mouse which was then rounded to 3 nmol/mouse.

Animals and Treatments

C57Bl/6 J female mice aged 8–14 weeks were housed and bred in the animal care facility at the BCCHR under controlled conditions including 12-h photo schedule, controlled temperatures (21–23 °C), standard ventilation with 20 air exchanges per day allowing low air movement throughout

the day, and optimum humidity maintained by HVAC (60–70%). Mice had free access to non-irradiated pelleted food (Envigo Teklad 2918) and chlorinated reverse osmosis water. On day 1, all mice received intraperitoneal (IP) injection of 5 IU pregnant mare serum gonadotrophin (PMSG) (Cat#:367222, EMD Millipore Corp.) to stimulate superovulation [28, 29]. Bedding from male cages was then transferred to female cages as an added stimulus to induce estrus. On day 3, mice were divided into 3 ovulation trigger groups ($n = 5$/group) as follows: (A) placebo control (1 × phosphate-buffered saline (PBS) Cat#:14–190-144, Gibco), (B) hCG (5 IU, Cat#: 230734, EMD Millipore Corp.), (C) KP-54 (3 nmol, Cat#: SCP0186, Sigma-Aldrich). All treatments were administered by IP injection using a 27-gauge hypodermic needle with a volume of 0.1 mL. To simulate day 4 of pregnancy [30], on experiment day 7, mice were euthanized using isoflurane inhalational anesthesia followed by cervical dislocation. Briefly, the animals were moved to an anaesthetic induction chamber. Then, oxygen flow was turned on at 1–2 l/min flow rate for couple of minutes. After that, the isoflurane vaporizer was turned on to its maximum setting at 5% with observation of animals' activity until the breathing became very shallow. While under deep anesthesia, animals were rapidly taken out from the anesthetic chamber and cervical dislocation was performed.

Sample Collection and Preparation

Immediately following euthanasia, mice were dissected, uteri were cut, then washed with 1 × PBS, and fixed with 4% formaldehyde. Uteri were then sent to the Histology Department at the BCCHR for further processing and paraffin embedding. Formalin-fixed, paraffin-embedded (FFPE) blocks of mice uteri were sectioned into 4-µm slices, with two samples arising from each uterus. The slides were subsequently stained using previously published protocols [31–33]. Briefly, after de-paraffinization using histological grade xylenes, slides were rehydrated using gradually decreasing concentrations of ethyl alcohol then tap water. Antigen retrieval was completed with a preheated antigen retrieval reagent in a steamer (Preheat Dako Retrieval Solution modified citrate buffer pH 9, Cat#: S2367). Endogenous peroxidase activity was blocked using Dako Dual Endogenous Enzyme block for autostainer (Cat#: S2003) and additional blocking was completed using 5% bovine serum albumin solution (Amresco Albumin, Bovine, Cat#: 9048–46-8). Slides were then incubated with either anti-LIF antibody (Cat#: ab135629, Abcam) at 1:200 concentration or anti-ITG αVβ3 (Cat#: SC-7312, Santa Cruz Biotechnology Inc.) at 1:50 concentration at 4 °C overnight. Negative control slides were prepared by omitting the primary antibody step. To detect primary antibody, slides were incubated for 30 min with secondary antibodies (EnVision + Dual Link System-HRP, Dako, Cat# K4061). The sections were then exposed to a chromogen reaction for 10 min (Liquid DAB Chromogen System, Dako, Cat# K3468) and counterstained with Harris hematoxylin for 1 min (Sigma-Aldrich Cat# HHS-80). Finally, the slides were re-dehydrated through gradually increasing concentrations of ethyl alcohol then xylene and mounted using mounting medium xylene (Fisher Scientific, Cat# 245–691).

Study Outcomes

The primary outcome measure for this study was the histoscore (H-score) calculated for each individual slide and the comparison of mean rank H-scores for both LIF and ITG αVβ3 between the 3 study groups at the level of endometrial glandular epithelium (GE) and stromal cells (SCs). For secondary outcomes, we compared H-scores for both LIF and ITG αVβ3 in GE compared to SCs within the respective study groups. The gross appearance of uteri as well as observations of immunoreactivity were also examined and discussed narratively.

H-Score Calculation

The slides were examined under a light microscope (Leica DM4000B, Leica Microsystems Wetzlar GmbH) using × 100 magnification. Slides were scored according to the intensity and abundance of the expression signal in GE and SCs using the H-score method. The immunoreactivity of LIF and ITG αVβ3 was calculated using an intensity strength (I) score of 1, 2, or 3 correlating to weak, moderate, or strong, respectively. A proportion score ($P = 0$–100%) was determined for each slide and an H-score was calculated with the following formula: $\text{H-score} = \Sigma P\,(I + 1)/100$. The final H-score for each slide ranged from 0 to 4 [34, 35].

Statistical Analysis

Statistical analysis was performed using the Kruskal–Wallis test for the primary outcome followed by the Mann–Whitney *U* test for pairwise comparisons and Wilcoxon test for the secondary outcomes. A *p*-value of less than 0.05 was considered significant. Analysis was completed through IBM SPSS Statistics for Windows (version 24.0. Armonk, NY: IBM Corp. 2016).

Results

Mean rank H-scores for implantation markers LIF and ITG αVβ3 tended to be higher among mice receiving KP-54 ovulation trigger (Table 1), although not all relationships were

Table 1 Mean rank H-score of implantation markers leukemia inhibitory factor (LIF) and integrin (ITG) αVβ3 in mouse uteri among different ovulation trigger groups

	LIF (GE)	LIF (SCs)	ITG αVβ3 (GE)	ITG αVβ3 (SCs)
PBS	4.8	4.4	6.0	3.4
hCG	7.2	6.6	7.4	10.0
KP-54	12.0	13.0	10.6	10.6
P value	**0.034**	**0.007**	0.248	**0.018**

Data is reported using mean rank for 5 animals per group and compared utilizing Kruskal–Wallis Testing. Significant *P* values are shown in bold font

GE, endometrial glandular epithelium; *hCG*, human chorionic gonadotropin; *ITG αVβ3*, integrin αVβ3; *KP-54*, kisspeptin-54; *LIF*, leukemia inhibitory factor; *PBS*, phosphate-buffered saline; *SCs*, endometrial stromal cells

statistically significant. In samples examining LIF expression, LIF expression was significantly higher in both GE and SCs in the KP-54 ovulation trigger group when compared to both hCG ovulation trigger and control, $P=0.034$ and $P=0.007$ respectively. In samples evaluating ITG αVβ3, ITG αVβ3 expression was significantly higher in SCs when KP-54 ovulation trigger was used ($P=0.018$), although there were no significant differences in GE ITG αVβ3 expression among groups ($P=0.248$).

On microscopic examination, immunoreactivity to either LIF or ITG αVβ3 was detected in GE and SC samples (Fig. 1). However, the abundance and intensity of the signal were much greater in the GE collectively without ovulation trigger subcategorization compared to those in the SCs for both LIF (median [interquartile range (IQR)], 2.43[2.4–2.8] vs. 1.35[0.9–2.15], $P=0.001$) or ITG αVβ3 (2.43[2.08–2.6] vs. 1.43[0.9–2.15], $P=0.002$). H-score median and IQR for both tissue types were stratified by the ovulation trigger and displayed in Fig. 2. When pairwise comparisons were performed among the three ovulation trigger groups (Fig. 2), the median H-score in samples examining LIF in GE was significantly higher in the KP-54 group compared to the PBS group ($P=0.009$), but there were no statistically significant differences between the KP-54 group and the hCG group. When the same comparisons were performed in SCs, KP-54 ovulation trigger displayed a significantly higher LIF expression when compared to both hCG and PBS ($P=0.009$). Additionally, ITG αVβ3 was significantly higher in the KP-54 group compared to PBS ($P=0.03$) and hCG ($P=0.009$) in the SCs only, with no difference among ovulation trigger groups in the GE (Fig. 2).

Gross examination of mice uteri showed observable differences in the size and vascularity. The KP-54 ovulation trigger group mice had larger and more vascular uteri than the hCG and PBS groups in a descending manner. Representative gross section images are displayed in Fig. 3.

Discussion

This study is the first to provide biological evidence of increased expression of both LIF and ITG αVβ3 in mouse uteri after exogenous KP-54 is utilized as an ovulation trigger compared with standard hCG or placebo. Thus, providing a tangible biological mechanism for exogenous KP-54 aiding in embryo implantation through upregulating implantation markers such as LIF and ITG αVβ3.

Our study also observed differential expression of LIF and ITG αVβ3 in mouse uteri among those triggered with KP-54. All samples of SCs demonstrated statistically significant rises in expression while only select samples of GE demonstrated such statistical significance. Specifically in GE, only the LIF mean rank H-score in the KP-54 triggered group compared to both hCG trigger as well as placebo and LIF median H-score in the KP-54 triggered group compared to placebo was significantly increased. Blastocyst implantation requires a receptive luminal epithelium, then GE for initial adhesion followed by SCs for successful penetration [36]. However, in mice, the window for receptivity of the epithelium is extremely brief, lasting at most from days 3.5 to 4.5 of pregnancy [36]. Additionally, LIF expression is likely biphasic, with evidence showing its presence by day 1, decreasing on day 2, then increasing again on day 4 around the time of the embryo attachment reaction [37–39]. While some authors support LIF expression solely in uterine epithelial cells [37], there is data to support stromal production of LIF [39, 40]. Specifically, a mouse model demonstrated through in situ hybridization the second peak at day 4 of LIF expression to be localized to the SCs around the blastocyst and concurrent expression of LIF receptor (LIF-R) in luminal epithelium [39]. In this model, LIF produced by the SCs acts in a paracrine fashion on the LIF-R in the luminal epithelium at the time of embryo attachment [39]. A delayed implantation model further supports the importance of LIF stromal production [40]. In this model, 3 ng of estrogen was insufficient to induce LIF in GE, however, was sufficient to induce LIF production in SCs and subsequent implantation [40]. By day 5.5 of pregnancy, the epithelium undergoes apoptosis, which allows the blastocyst to interact with the SCs that are decidualizing [36]. These decidual cells are necessary for pregnancy and appropriate placental formation [36]. It is possible that within our study protocol the slight variations in hours could have resulted in missing the statistically significant increase of implantation markers in GE or that examining LIF-R expression in GE instead of LIF may have yielded a different result. Furthermore, GE may only require a certain level of implantation marker expression to establish implantation given the brevity of its interaction with the blastocyst and successful implantation may depend on SC implantation markers to a greater degree.

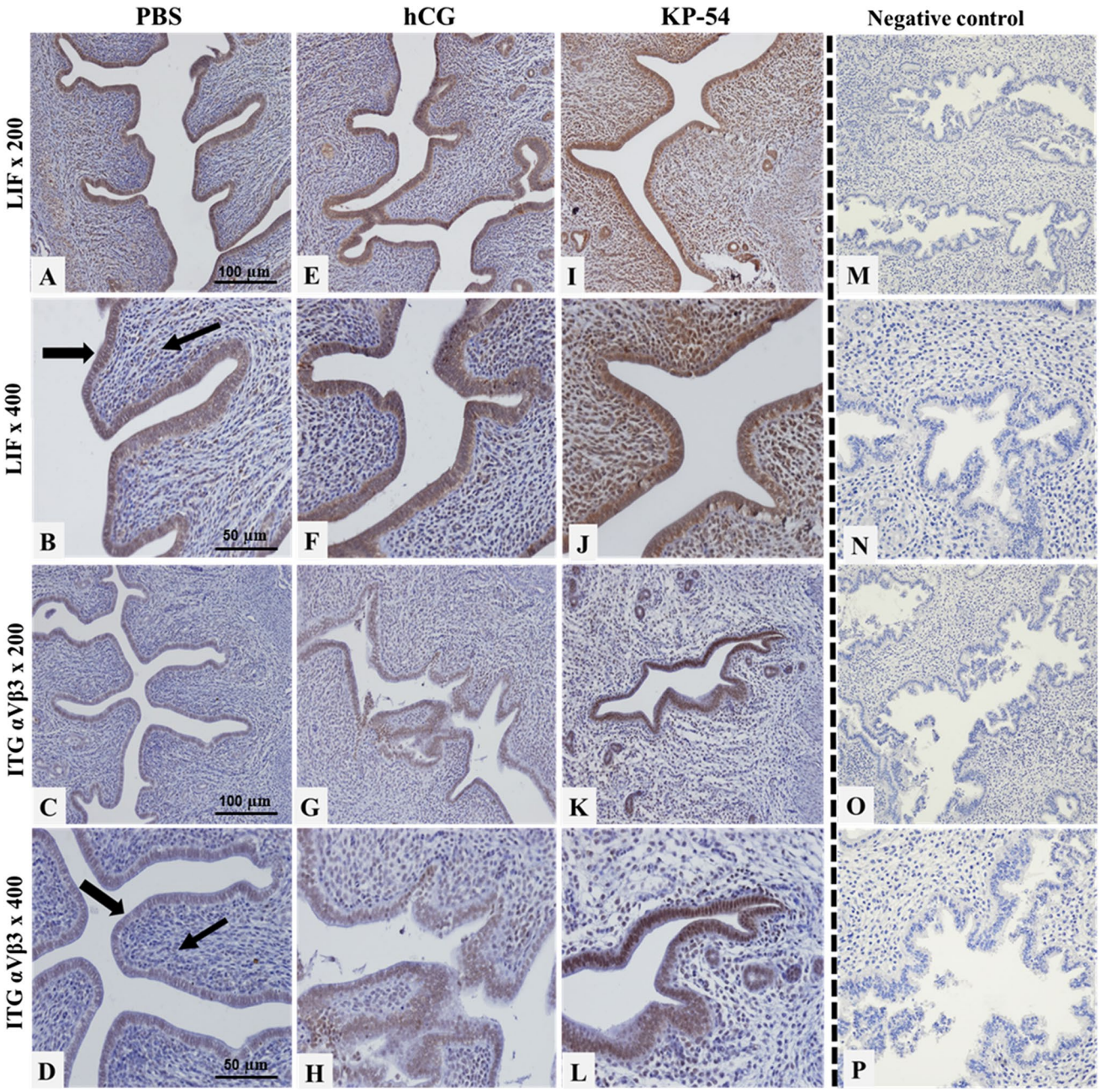

Fig. 1 Immunohistochemical staining of leukemia inhibitory factor (LIF) and integrin (ITG) αVβ3 in representative sections of mouse uteri among different ovulation trigger groups. **A**–**D** Phosphate-buffered solution (PBS) placebo ovulation trigger. **E**–**H** Human chorionic gonadotropin (hCG) ovulation trigger. **I**–**L** Kisspeptin-54 (KP-54) ovulation trigger. **A**, **B**, **E**, **F**, **I**, and **J** are stained with anti-leukemia inhibitory factor (LIF) antibodies and **C**, **D**, **G**, **H**, **K**, and **L** are stained with anti-integrin (ITG) αVβ3 antibodies. **M**–**P** act as a negative control. **B** and **D** demonstrate endometrial glandular epithelium (bold arrow) and endometrial stroma (thin arrow)

While this study has a number of strengths, it also has several limitations. First and foremost, the study objectives involved utilizing surrogate markers of implantation, including LIF and ITG αVβ3 expression in GE and SC mouse endometrial tissue. Additionally, secondary outcomes from observing gross pathology of uteri were primarily descriptive and observed changes could have been from more than the surrogate implantation markers studied. Moreover, our examination was limited to effects on mouse uteri and did not examine the potential effect of KP-54 on the mouse blastocyst. A previous in vitro study demonstrated that KP increased mouse blastocyst adhesion to collagens

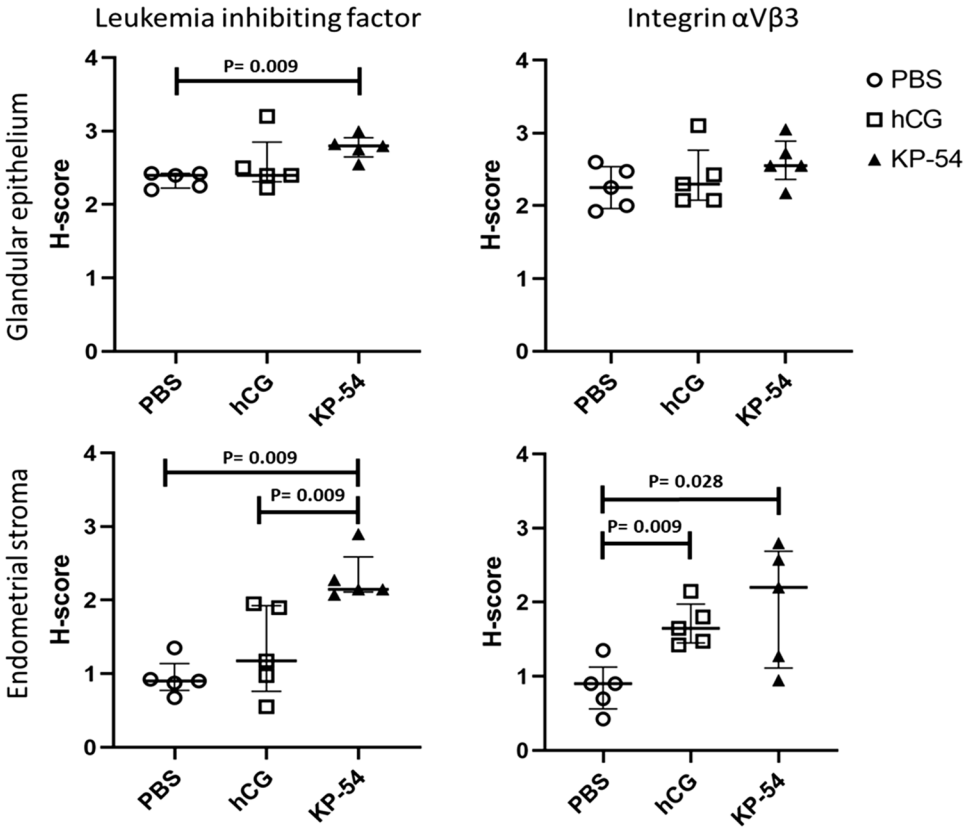

Fig. 2 Graphical median H-scores of leukemia inhibitory factor (LIF) and integrin (ITG) αVβ3 immunoreactivity in mouse uteri glandular epithelium and stromal cells among different ovulation trigger groups. Immunostaining was scored separately in endometrial glandular epithelium (upper row) and endometrial stromal cells (bottom row). For all panels, vertical lines represent median and interquartile ranges, while pairwise comparisons are represented by horizontal lines. hCG, human chorionic gonadotropin; KP-54, kisspeptin-54; PBS, phosphate-buffered saline

[41], potentially mediated through the downregulation of MMP-2 and MMP-9 activity via the ERK1/2 and protein kinase C signaling pathways [41–43]. Further studies are required to establish a correlation between these results and effects on mouse blastocysts, implantation rates, as well as pregnancy outcomes. The study is also limited by the relatively small sample size of five animals per group. Given that this was an early study in establishing biological mechanisms, we felt it prudent to be conservative with the number of animals utilized. Additionally, we attempted to overcome the limited sample size by employing non-parametric statistical analysis to increase the specificity of our results. We also attempted to mitigate bias by blinding the investigator completing H-scoring to group allocations.

In future studies, exogenous KP-54 administration may take the form of an ovulation trigger, as in this study, or an intrauterine infusion on the day of or prior to ovulation trigger, where the ovulation trigger may or may not include KP-54. The latter would assess the effect of locally administered KP-54 on LIF and ITG αVβ3 expression as well as the clinical outcome of embryo implantation. Future studies could also investigate whether differing levels of implantation markers in the GE and SCs has any form of temporality, as suggested by the sequence involved in implantation, and

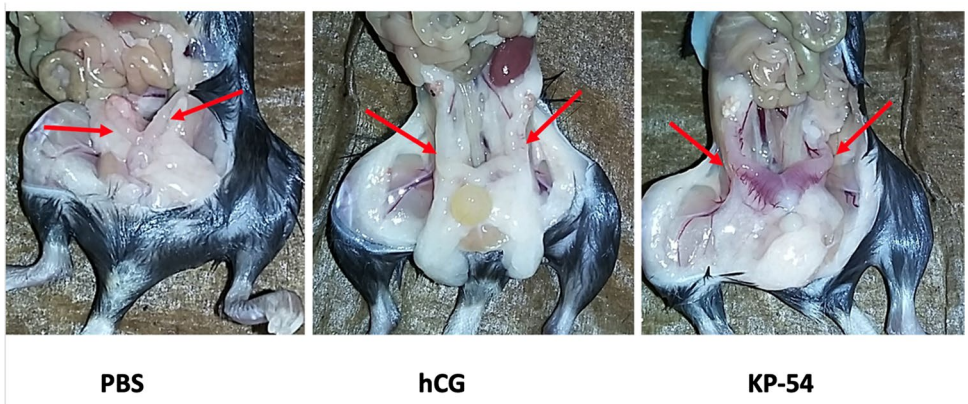

Fig. 3 Gross examination of mouse uteri among different ovulation trigger groups at sample collection. Red arrows demonstrating either horn of the mouse uterus. hCG, human chorionic gonadotropin; KP-54, kisspeptin-54; PBS, phosphate-buffered saline

whether a threshold level of implantation markers is GE is sufficient given its short-lived role in implantation. Lastly, future studies could examine the effect of exogenous KP-54 on the blastocyst and whether early exposure to exogenous KP-54 could improve embryo implantation.

Cumulatively, our study confirmed the role of KP-54 in the biological regulation of embryo implantation and specified a potential biological mechanism through the increased expression of LIF and ITG αVβ3 in mouse uteri. We postulated that this increased expression, coupled with the increased vascularity observed in mice receiving KP-54 ovulation trigger, might lead to improved embryo implantation. As such, KP-54 as an ovulation trigger might represent a novel method to increase implantation rates when used in IVF. Finally, our study added to the paradigm for further research on utilizing exogenous KP-54 through various routes to improve embryo implantation rates.

Author Contribution A.S. Ait-Allah and M.A. Bedaiwy were involved in idea conceptualization. A.O. Abdelkareem, A.S. Ait-Allah, S.M. Rasheed, Y.A. Helmy, and M.A. Bedaiwy were involved in study design. A.O. Abdelkareem was involved in animal experiments, immunohistochemistry, and data analysis. M.S. Iews was involved in animal experiments. A.O. Abdelkareem and R. Habte were involved in manuscript writing. R. Habte, F.F. Abdelhafez, and M.A. Bedaiwy were involved in manuscript critical revision and submission.

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Data Availability The data that support the findings of this study are available from the corresponding author.

The research ethics board at the University of British Columbia approved laboratory animal care and use (#A16-0295).

Declarations

Conflict of Interest Dr. Bedaiwy has received grants from the Canadian Institutes of Health Research & Ferring Pharmaceuticals. He is also on the advisory boards of AbbVie and Baxter. All other authors have no competing interests to declare.

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