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The therapeutic effects of vitamin D3 administration on the embryo implantation

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ABSTRACT

Various adjuvants have been tested clinically for patients with problems with embryo implantation during *in vitro* fertilization (IVF)-embryo transfer (ET). Vitamin D3, an essential modulator of various physiological processes, has received attention as an important adjuvant for successful pregnancy, as many studies have shown a strong association between vitamin D deficiency and implantation failure and fetal growth restriction. However, vitamin D has been widely utilized in different protocols, resulting in non-reproducible and debatable outcomes. In the present study, we demonstrated that cyclic intrauterine administration of vitamin D3 increased endometrial receptivity and angiogenesis, which could be attributed to increased recruitment of uterus-resident natural killer cells. In particular, cyclic treatment of vitamin D3 promoted stable attachment of the embryo onto endometrial cells *in vitro*, suggesting its merit during the early stage of embryo implantation to support the initial maternal-fetal interactions. Our findings suggest that women with repeated implantation failure may benefit from the use of vitamin D3 as a risk-free adjuvant prior to IVF-ET procedures to improve the uterine environment, and make it favorable for embryo implantation.

1. Introduction

Embryo implantation is a complicated process initiated by the attachment of the blastocyst trophectoderm to the uterine luminal epithelium, which is subsequently followed by more stable adhesion and invasion into the uterine stroma [1,2]. This process is coordinated by the formation of the decidua with an extensive vascular network, which supports the appropriate growth of the implanted embryo [3,4]. Several studies have reported that there is a strong correlation between vitamin D status and successful maintenance of pregnancy [5–7]. Gestational vitamin D levels, which increase during the early stages of pregnancy and continues to increase until delivery, and are crucial for fetal growth [6,8]. However, the detailed physiological functions of vitamin D administration during the process of embryo implantation remain unclear.

Vitamin D3, in an active form of cholecalciferol (vitamin D3; 1,25-dihydroxyvitamin D3) is primarily involved in calcium and phosphorus metabolism, directly relating to the structure and function of the skeletal system [9]. This acts as an essential modulator of the immune system and hormone secretion through its interaction with the vitamin D receptor (VDR) [10]. Vitamin D3–VDR complex activates various transcription factors that regulate hypoxia inducible factor 1α signaling to promote re-endothelialization and angiogenesis, which is critical during embryo implantation and pregnancy [11,12]. VDR-knockout female mice are failed to reproduce displaying severe defects in uterine development and decidualization resulting in impaired embryo implantation [13]. Defects in 25-hydroxyvitamin D-1a-hydroxylase, an enzyme that catalyzes the conversion of 25-hydroxyvitamin D[25(OH)D] to vitamin

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D3, is associated with infertility due to the combined ovarian and uterine defects [14]. Moreover, female rats fed a vitamin D-deficient diet showed reduced overall fertility and litter size [15]. Although many studies have investigated the correlation between vitamin D treatment and female reproduction, the direct therapeutic effects of vitamin D during *in vitro* fertilization (IVF) have not been elucidated. Recent reports have shown a positive correlation of 6 % between the vitamin D levels in the serum and follicular fluid and tendency for successful pregnancy outcome following IVF [16–18]. Moreover, high level of vitamin D was significantly associated with the improved parameters of the controlled ovarian hyperstimulation [16]. However, several other studies have reported that vitamin D deficiency is not a critical factor affecting IVF outcomes [19–21]. In addition, it has been reported that vitamin D negatively affects the quality of embryos, suggesting a lower likelihood of clinical pregnancy in women with sufficient vitamin D levels [17]. Given these contradictory results, further research is needed to directly determine the vitamin D administration protocol for the successful IVF-embryo transfer (ET).

In the present study, we demonstrated that the intrauterine vitamin D3 administration improved the uterine environment and made it favorable for embryo implantation and decidualization. This was the result of improved endometrial receptivity and angiogenesis and increased recruitment of uterus-resident natural killer (NK) cells. In addition, we found that cyclic vitamin D3 administration strengthens the stability of maternal–fetal interactions, resulting in improved rates of early-stage embryo implantation. Based on our findings, we suggest an optimal protocol for the intrauterine administration of vitamin D3 to improve implantation rates.

2. Material and method

2.1. Human samples

Human endometrial tissues were provided by CHA Fertility Center Bundang after diagnostic hysteroscopy. The endometrial tissues used in the experiment were obtained from patients who agreed to use it for research purposes before being collected. This study was allowed by the Institutional Review Board (IRB, approval no. 2020-10-007) of the CHA Bundang Medical Center.

2.2. Animal uses and intrauterine administration of vitamin D3

Nine-week-old C57/B6 strain female mice were purchased from Orientbio (Gyeonggi-do, Korea) and used for *in vitro* evaluation of vitamin D3 treatment. Seven-week-old ICR (CD-1) strain female and ten-week-old male mice were purchased from Orientbio and used for *in vivo* analyses and evaluation of the therapeutic effects of vitamin D3. All animal breeding and experimental procedures were performed by the policies and regulations of the CHA University Institutional Animal Care and Use Committee (IACUC). All reported animal experiments were approved by the CHA University (IACUC 220078). Mice were housed under standard environmental conditions of 12 h light: 12 h dark at a controlled room temperature (20–22 °C and 40–60 % humidity) and fed *ad libitum*. For the experimental use of vitamin D3, it was diluted in 240 μl of 95 % EtOH to the stock concentration (100 μM) and further diluted with saline to adjust to the working concentration (10 nM) prior to use. For the administration of vitamin D3, female mice were randomly selected, and vitamin D3 (D1530, Sigma, Missouri, USA; 10 nM/40 μl) were administered to one side of the mouse uterine cavity. Saline was instilled on the other side of uterine horns for the control using the same procedure as vitamin D3. Both sides of uterine horns were harvested at a designated time for subsequent analyses. In addition, all treatments were conducted at the same stage of estrous cycle to eliminate any variables that could affect the experimental results. Thus, all mice used for the experiments were synchronized in the estrus stage.

2.3. Cell culture

For the uses of primary endometrial cells, harvested human or mouse endometrial tissues were thoroughly minced and enzymatically dissociated with the mixture of collagenase V (C9263-100MG, Sigma, Missouri, USA; 0.4 mg/ml) and dispase II (D4693, Sigma, Missouri, USA; 1.25 IU/ml) for 1 h at 37 °C. Cells were filtered using 100 μm (93100, SPL, Gyeonggi-do, Korea) and 40 μm strainers (93040, SPL, Gyeonggi-do, Korea) to separate the epithelial and stromal fractions. Flow-through containing endometrial stromal cells were collected by centrifugation and cultured with DMEM/F12 media supplemented with 20 % FBS, 1 % L-glutamine (11539876, Gibco, NY, USA), and 1 % penicillin-streptomycin. Human or mouse endometrial tissue-derived primary stromal cells between passage 2 and 5 were used for the study. Human endometrial epithelial cells (Ishikawa cells) were purchased from American Type Culture Collection (ATCC, Virginia, USA), were maintained in DMEM/F12 media (GIB-11320-033, Gibco, NY, USA) supplemented with 10 % fetal bovine serum (FBS; 16000-044, Gibco, NY, USA) and 1 % penicillin-streptomycin (15140-122, Gibco, NY, Virginia, USA). For endothelial cell sprouting assay, human umbilical vein cells (HUVEC; ATCC, USA) were maintained in endometrial cell growth medium 2 (EGM-2; CC-4176, Lonza, Basel, Switzerland) supplemented with 100 ng/ml of bFGF (233-FB-025, R&D system, Minnesota, USA), 50 ng/ml of VEGF-A and C (293-VE-050 and 752-VC-025, R&D system, Minnesota, USA), and 1 nM/ml of S1P (73914, Sigma, Missouri, USA).

2.4. RNA extraction and quantitative real-time PCR analysis

Harvested uterine tissues were homogenized in 500 μl of Labozol reagent (CMRZ001, Cosmo, Seoul, Korea), and 1 μg of RNA was converted into complementary DNA using SuperScript™ IV (18091050, Invitrogen, Massachusetts, USA). Amplification was performed in a CFX Connect real-time PCR detection system (Bio-Rad, California, USA) using SYBR Green (RT500M, Enzynomics, Daejeon, Korea). A standard curve was generated for each primer by plotting the log value of the starting quantity of the template against the cycle threshold value obtained during the amplification of each dilution displaying an R^2 value greater than 0.98 and an amplification efficiency of around 100 %. All reactions were conducted in triplicate, and the relative mRNA expression level was normalized to housekeeping gene *Actb* mRNA expression. Primer sequence pairs used for these analyses are shown in Supplementary Table 1.

2.5. Immunostaining and microscopy

Harvested uteruses were fixed in 4 % paraformaldehyde and dehydrated in gradient ethanol series (70–100 %). After immersing with histoclear and paraffin wax, tissues were embedded. 5 μm of trimmed sections were undergone deparaffinization prior to the start of staining, followed by rehydration. Endogenous hydrogen peroxide activity was inactivated by 3 % H_2O_2. For antigen retrieval, sections were incubated in the sodium citrate buffer (pH 6.0) boiled at 95 °C for 30 min. After the blocking procedure with 5 % BSA in PBS, sections were incubated with primary antibodies to integrin β3 (#13166, Cell signaling, Massachusetts, USA; 1:1000), full-length OPN (ab8448, Abcam, Cambridge, UK; 1:1000), and cleaved-OPN (702184, Invitrogen, Massachusetts, USA; 1:100) for immunohistochemistry, and CD31 (ab28364, Abcam, Cambridge, UK; 1:100) for immunofluorescence at 4 °C overnight. Following 1 day, sections were incubated with HRP-conjugated mouse or rabbit secondary antibodies (BIORAD, 170-6516 or 170-6515, California, USA; 1:3000) for 1 h. For immunohistochemistry, DAB substrate solution (Vector Laboratory, VE296420) was used to detected signals after counterstaining with Hematoxylin (HEMH-49/20, BioGNOST, Zagreb, Croatia). DAB-stained sites were measured by IHC profiler open-source plug-in within Image J (National Institutes of Health, Bethesda, MD). For immunofluorescence analyses, sections were stained with DAPI (62248,

Thermo Fisher, Massachusetts, USA; 1:1000) for 10 min after applying secondary antibody (A11011, Invitrogen, Massachusetts, USA; 1:200) for 1 h. Images were captured using Mica microhub and Thunder imager and analyzed by LAS X software (Leica Microsystems, Germany). Intensity of CD31-positive staining was also measured by Image J. Three images of each group were quantified, and quantified values from three independent experiments are presented in graphs.

2.6. Endothelial cell sprouting assay

The device for perform endothelial cell sprouting assay was fabricated with Polydimethylsiloxane (PDMS; Dow Corning, OMNI.0524). PDMS with curing agent (10:1 ratio) was poured into a patterned silicon wafer mold, and hardened at 80 °C. The molded devices were bonded to the cover glass using oxygen plasma and incubated in a dry oven for 3 days before use. Human primary endometrial stromal cells (hESC; 4×10^6/ml) were mixed with 2.5 mg/ml of fibrinogen (F8630, Sigma, Missouri, USA) solution consisted with 0.5 U/ml of serine protease inhibitor aprotinin (T7326, Sigma, Missouri, USA). After thorough mixing, 0.5 U/ml of thrombin (T7326, Sigma, Missouri, USA) were finally added and mixtures were loaded rapidly into the stromal channel. Angiogenesis channel was only filled with fibrinogen solution and thrombin without cells, media channel was filled with EGM-2 medium and then devices were incubated at 37 °C for 24 h. Following 1 day, medium was sucked up and HUVECs (6×10^6/ml) were loaded in the media channel. The devices were vertically placed in the incubator for 40 min to induce the attachment of HUVECs towards the angiogenesis channels. EGM-2 medium sometimes was applied to vitamin D3 according to experimental design. In the devices, cells were cultured and observed up to 13 days.

2.7. Flow cytometry

For flow cytometry analysis, uteruses treated vitamin D3 by intrauterine injection according to experimental design were enzymatically dissociated in the same protocol of 2.3. cell culture section. Collected endometrial primary cells (excluded tissue debris and epithelial fraction) were incubated with antibodies against CD3-APC (100235, Biolegend, California, USA), NK1.1-PE (108707, Biolegend, California, USA), CD49a-FITC (130-125-102, Miltenyi, NRW, Germany), for 30 min at RT. The change of uterine NK proportion in endometrial primary cells was examined by using a Beckman Coulter CytoFlex and analyzed by CytExpert software (Beckman Coulter Inc).

2.8. Mouse embryo collection and embryo attachment assessment

For mouse embryo collection, female mice were applied to 10 IU of PMSG (Prospec, Ness-Ziona, Israel) by intraperitoneal injection and followed by 10 IU of hCG (Sigma, Missouri, USA), after 46–48 h hyperstimulated female mice were mated with fertile male mice for night. In next morning, mice with vaginal plug were sacrificed for oviduct harvest. Fertilized embryos were released by tearing swollen ampulla and washed them into a hyaluronidase/M2 media (Sigma, Missouri, USA). Embryos were cultured during 5 days in KSOM (MR 121-D, Millipore, Massachusetts, USA) under 5 % CO_2 and 37 °C condition. Growing blastocysts were transferred onto Ishikawa cells which were treated saline (for control) or vitamin D3. In the case of the 12 days group, passaging was repeated three times every 4 days of vitamin D3 treatment to mimic the intrauterine three-time cyclic treatment *in vivo*. Stability of embryo attachment was observed and scored at each time point (19, 24, 42 and 48 h after transfer) by applying a standardized scoring protocol [22,23].

2.9. Fertility assessment

Female mice were randomly selected and divided into three groups (1 day, 2 days, and 12 days; three-time cyclic treatments every 4 day). Selected mice were infused with vitamin D3 in one side of uterine horns and the other with saline. Next morning, mice were placed in the same strain of the male cage. The vaginal plug was checked in the next morning and defined as pregnancy day 1. At pregnancy day 1, only mice with the vaginal plug were separated from the male, and uteri were sacrificed after 17 days for further analyses. For the evaluation of vitamin D3 effects on fertility the number of implantation sites were recorded and all fetus and placenta were weighed.

2.10. Statistical analysis

Comparison groups were analyzed with unpaired student t-test for parametric distributions. For multiple comparisons, the ordinary one-way ANOVA analysis with Dunnett's multiple comparison test. For all cases, a p-value that was <0.05 was considered statistically significant ($p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***) and $p < 0.0001$(****)).

3. Results

3.1. Intrauterine administration of vitamin D3 improves endometrial receptivity in mice

To evaluate the effect of vitamin D3 intrauterine administration on the endometrial receptivity, vitamin D3 was administered for 6 h, 1 day, 2 days, 3 days, 4 days, and 12 days (three-time cyclic treatments every estrous cycle, which is a 4 day-cycle. Vitamin D3 (10 nM/40 μl) was administered into one side of uterine horns and saline was instilled into the other side of horns for the control since 0.004 μl of 95 % ethanol was included in 40 μl of vitamin D3 solution (Fig. 1A). ICR(CD-1) female mouse uteri from each group were dissected and subjected to qRT-PCR analyses to evaluate the changes in the expression of endometrial receptivity-related factors. Vitamin D3 administration was found to increase the mRNA expression of *Lif* (leukemia inhibitory factor), *Itgb3* (integrin β3), and *Spp1* (Osteopontin; OPN); however, significant differences were noted at specific time points when compared with the control groups. In particular, *Lif* expression was elevated in the groups that were administered vitamin D3 for 1–4 days, whereas *Itgb3* and *Spp1* expressions in the endometrium were significantly increased in the groups in which vitamin D3 was administered for 1, 2, and 12 days compared with their corresponding saline-treated controls (Fig. 1B–D). This might be due to the involvement of LIF rather than integrin β3 and OPN during the earlier stages of embryo attachment to the maternal epithelium [24]. Moreover, integrin β3, full-length OPN, and cleaved-OPN, the active form of OPN, were significantly highly expressed at protein level both in the luminal and glandular epithelium of the endometrium in the group that was administered vitamin D3 for three-times cyclic treatment for 12 days (three-time cyclic treatments every 4 day) compared with non-treated group (Fig. 1E–G, Supplementary Fig. 1A–C).

3.2. Intrauterine administration of vitamin D3 increases endometrial angiogenesis in mice

We next evaluated the effect of intrauterine vitamin D3 administration on the endometrial angiogenesis, which is essentially required for uterine environment that is favorable to the embryo implantation and pregnancy maintenance [25,26]. Vitamin D3-mediated blood vessel growth was visualized using an angiogenesis-on-a-chip in which human umbilical vein endothelial cells (HUVECs) and human primary endometrial stromal cells were plated in each indicated channel (Fig. 2A). Vitamin D3 was applied for 1, 2, and 12 days (three-time treatments every 4 day) through the media channel, and the rates of vessel sprouting were evaluated (Fig. 2B). The time period of vitamin D3 treatment was selected based on the findings of vitamin D3 effects on endometrial receptivity (Fig. 1). Immunofluorescence staining for CD31

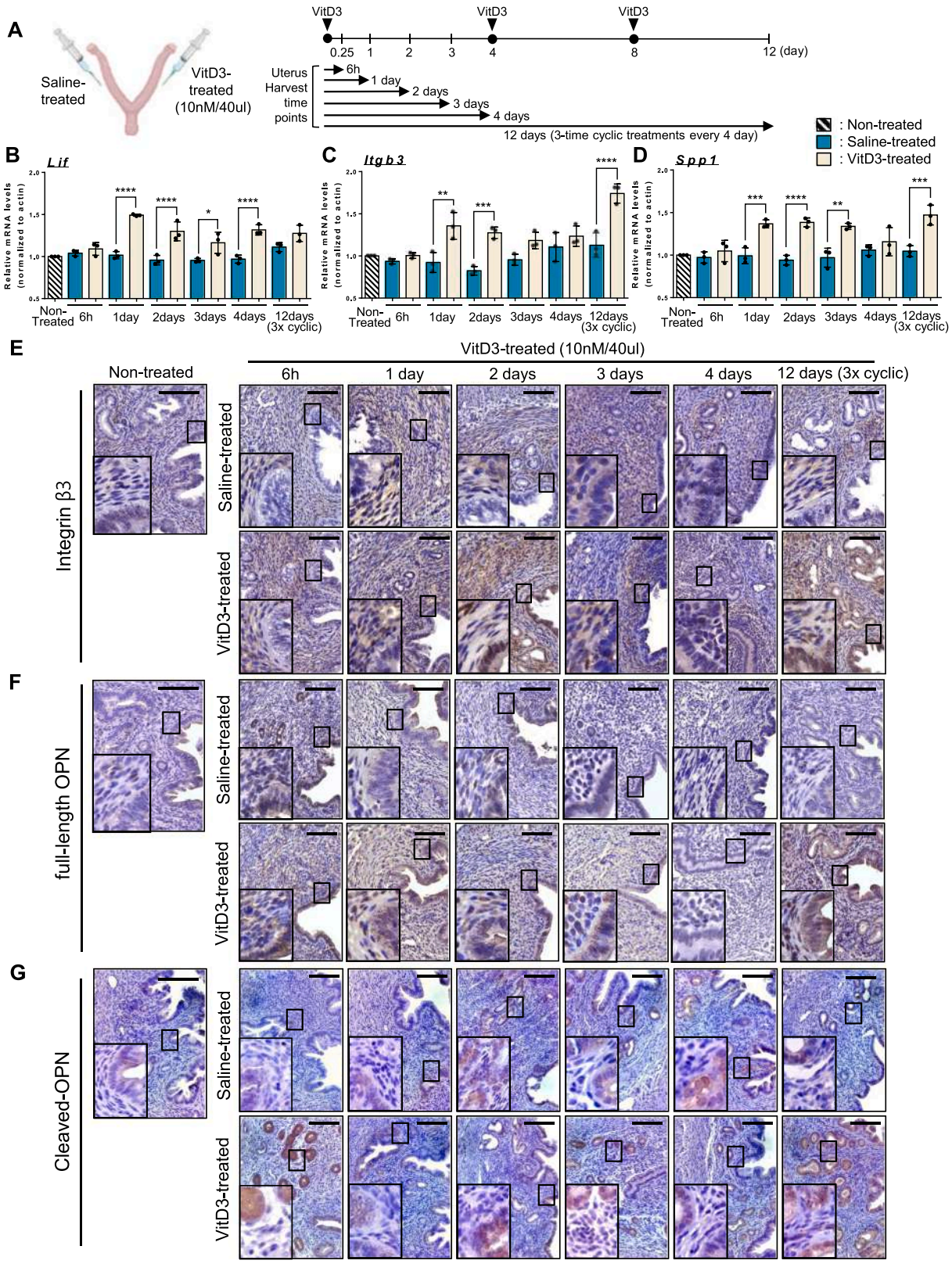

Fig. 1. Intrauterine administration of vitamin D3 improves endometrial receptivity in mice. **(A)** A schematic diagram of vitamin D3 treatment in mice. QRT-PCR analyses of endometrial receptivity-related genes including *Lif* **(B)**, *Itgb3* **(C)**, and *Spp1* **(D)** in vitamin D3-treated (10 nM/40 μl, 6 h, 1 day, 2 days, 3 days, 4 days, and 12 days (3-time cyclic treatments every 4 day)) endometrial tissue compared to non-treated or saline-treated control (n of mice per group = 3, in total 21 mice were used). Data displayed for **(B–D)** are from 3 independent experiments, and represented as mean ± SD and analyzed using the ordinary one-way ANOVA with Tukey's multiple comparisons test including p-values (*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001, NS; not significant). Immunohistochemical analyses of integrin β3 **(E),** full-length OPN **(F)**, and cleaved-OPN **(G)** in vitamin D3-treated mouse endometrial tissues compared to saline-treated group. Scale bar; 100 μm.

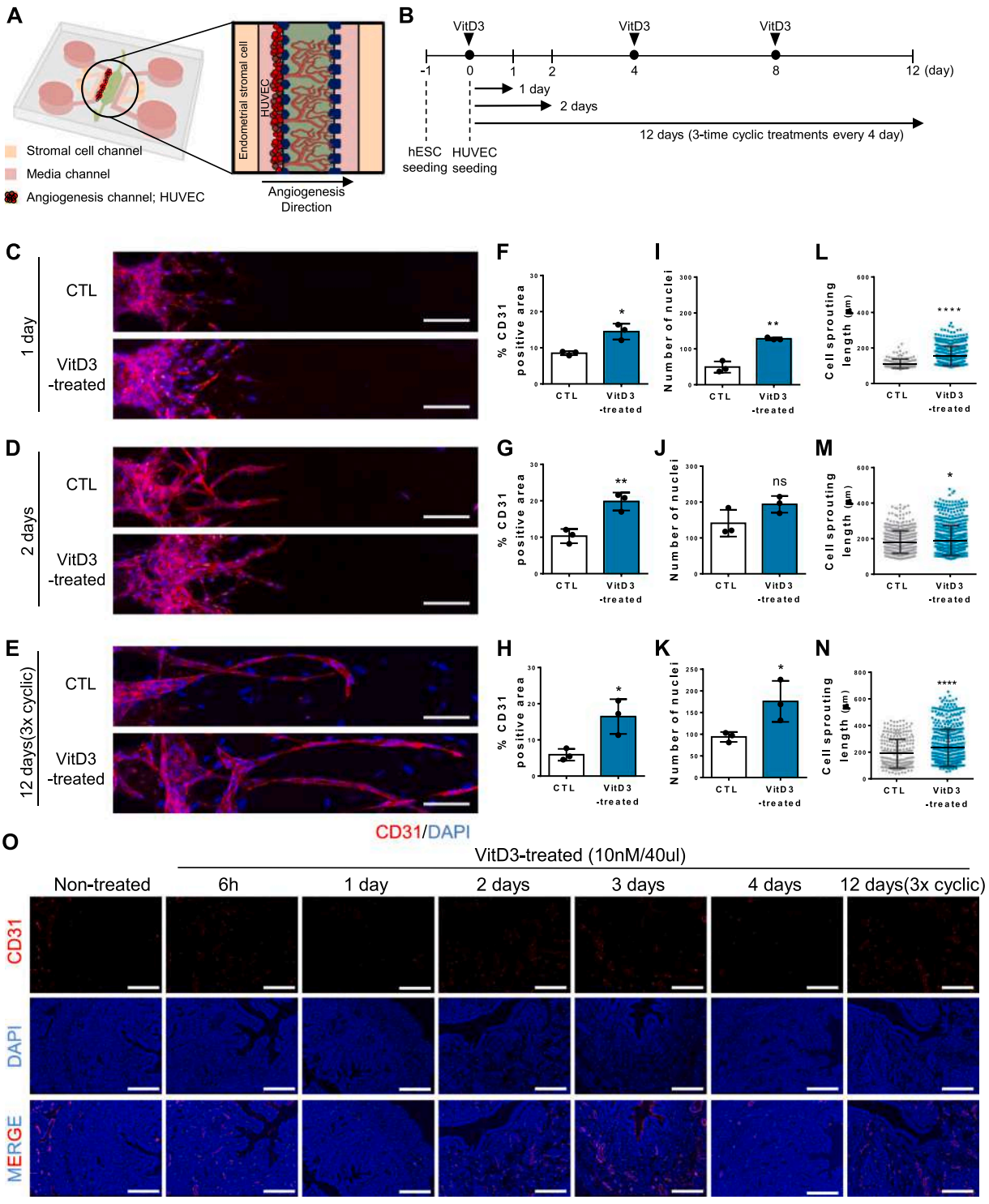

Fig. 2. Intrauterine administration of vitamin D3 increases endometrial angiogenesis in mice. **(A)** An experimental design of a micro-engineered vascular system for the investigation of the effects of vitamin D3 treatment on endometrial angiogenesis. **(B)** Experimental plan of vitamin D3 treatment. Immunofluorescence staining of CD31 (red) and DAPI (blue) in vitamin D3-treated 1 day **(C)**, 2 days **(D)**, and 12 days (3-time cyclic treatments every 4 day) **(E)** in 3D endometrium-on-a-chip. Quantification of CD31-positive area **(F–H)**, the number of nuclei (DAPI) **(I–K)**, and the length of cell sprouting **(L–N).** Data displayed for **(F–N)** are from 3 independent experiments, and represented as mean ± SD, analyzed using the unpaired t test including p-values (*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001, NS; not significant). Scale bar; 100 μm. **(O)** Immunofluorescence analyses of CD31 (red) in vitamin D3-treated (10 nM/40 μl) mouse endometrial tissues compared to non-treated control at indicated time points. Scale bar; 150 μm.

and DAPI demonstrated that blood vessel growth (Fig. 2C–E), which was quantified by measuring the increase in CD31-positive area (Fig. 2F–H), the number of nuclei (Fig. 2I–K) and the endothelial cell sprouting length (Fig. 2L–N), was increased with vitamin D3 administration. These findings were fully supported by increased CD31 protein and elevated *Vegfa* mRNA shown in 12 days(three-time cyclic treatments every 4 day)-treated endometrium compared with control group (Fig. 2O and Supplementary Fig. 2A–B), suggesting an essential role of vitamin D3 in

endometrial angiogenesis during the time of embryo implantation.

3.3. Vitamin D3 administration induces NK cell recruitment in mouse uteri during the early phase of pregnancy

NK cells are recruited where the decidua is formed, and subsequently undergo differentiation and activation expressing CD49a on the surface of NK cells and are stimulated with cytokines, including IL-12 and IL-15 secreted from the decidual endometrial stromal cells [27,28]. Recent studies have reported that vitamin D3 acts as a major regulator of NK cell activities of cytotoxicity, cytokine secretion, and degranulation [29,30]. This led us to investigate whether intrauterine vitamin D3 administration affects NK cell recruitment in the mouse endometrial stroma in pregnant or non-pregnant mice. For the same, vitamin D3 was administered for 1 or 12 days (three-time cyclic treatments every 4 day) into the cavity of one side of the uterus, whereas saline was administered in the other side as the control, and the number of NK cells were evaluated for up to following 10 days (Fig. 3). No difference in the number of NK cells was observed across all time with or without vitamin D3 treatment in the non-pregnant mice (Fig. 3A–D). While in pregnant mice, the number of $CD3^{-}NK1.1^{+}$ cells, denoting the total population of NK cells detected in the uterus of the vitamin D3-treated (1 day) group on gestation day 7–10 was higher (day 7; 1.12-fold, $p = 0.03$, day 10; 1.16-fold, $p = 0.01$) than that in the saline-treated group. In particular, the number of $CD3^{-}NK1.1^{+}CD49a^{+}$ uterine-resident NK cells showed a significant elevation (1.25-fold, $p = 0.05$) until day 10 of pregnancy (Fig. 3A–B and Supplementary Fig. 3B). In the group cyclically treated with vitamin D3 three times every 4 days, although no difference was noted in the number of $CD3^{-}NK1.1^{+}CD49a^{+}$ cells, $CD3^{-}NK1.1^{+}$ cells exhibited a more dynamic variation (day 5; 1.11-fold, $p = 0.03$, day 7; 1.22-fold, $p = 0.009$) on the days 5–7 of pregnancy. Furthermore, a dramatic increase in the number of $CD3^{-}NK1.1^{+}CD49a^{+}$ NK cells (1.37-fold, $p = 0.009$) was observed on day 10 of pregnancy with vitamin D3 treatment (Fig. 3C–D and Supplementary Fig. 3C). These findings suggest that recruitment of NK cells to the uterus is occurred during the early phase of pregnancy, which might be augmented by vitamin D3 administration, which further supports spinal artery remodeling in the decidua for placental formation.

3.4. Vitamin D3 administration increases the rate of embryo implantation

To evaluate the effects of vitamin D3 administration on the rate of embryo attachment, we assessed the stability of embryos attached onto endometrial epithelial cells using an *in vitro* model with or without tapping in the presence or absence of vitamin D3 pre-treatment of endometrial cells (1 day, 2 days, and 12 days; three-time cyclic treatments every 4 day) (Fig. 4A). Assay analyses revealed that there was no significant difference in the stability of attached embryos between the vitamin D3- and saline-pretreated cells at any time points when the embryos were not disturbed during this process (Fig. 4B–D). However, under the condition of disturbance with tapping, the 19 h- and 42 h groups showed higher numbers of strongly attached embryos in the groups with 12 days vitamin D3-treated epithelial cells compared to control (Fig. 4E–G), suggesting cyclic treatments with vitamin D3 may promote stable attachment of embryos onto the maternal epithelium during the early stage of implantation.

3.5. Intrauterine vitamin D3 administration improves embryo implantation rates in vivo

To further examine the effect of vitamin D3 on the embryo implantation in mice, vitamin D3 was administered directly into the uterine cavity. Based on the findings revealing increased endometrial receptivity and angiogenesis of the endometrium with vitamin D3 treatments (1 day, 2 days or 3-time cyclic treatments for 12 days) (Figs. 1–2), vitamin D3 was administered in a same manner (for 1 day, 2 days or three-times for 12 days) prior to natural mating, and the number of implantation sites, fetal weight, and placental weight were analyzed on the 17 days of pregnancy (Fig. 5A). Interestingly, three-time cyclic treatment group clearly showed a significantly increasing pattern in the number of implantation sites and fetal weight compared to those in the saline-treated group. However, other groups with 1 or 2-day vitD3 treatment showed no significant difference compared to control (Fig. 5B-M). Moreover, the number of embryos detected in the oviduct of vitamin D3-treated uterus was similar to that detected in control group (Supplementary Fig. 4A–C), implying that increased rate of embryo implantation in vitamin D3-administered uterus was mediated by improving the uterine microenvironment not by increasing the number of embryos fertilized.

4. Discussion

Various types of supplements or adjuvants are clinically tried for patients with the problems with embryo implantation during IVF-ET. Vitamin D3, an essential modulator of various physiological processes, has been thought to be an important adjuvant for successful pregnancy since many studies have shown a strong association of vitamin D deficiency with the failure of implantation and fetal growth restriction [6]. However, vitamin D has been widely utilized with different protocols, resulting in non-reproducible and debatable outcomes. Moreover, many of research on vitamin D3 treatment have focused on the oral delivery or intramuscular injection. In the present study, we demonstrated that cyclic intrauterine administration of vitamin D3 increased endometrial receptivity and angiogenesis recruiting more uterine-resident NK cells during the pregnancy. In particular, vitamin D3 administration induced more stable attachment of the embryo onto endometrial cells *in vitro*, suggesting its merit during the early stages of embryo implantation to support the initial maternal-fetal interaction.

Our findings showed that the expression of adhesion molecules (*Itgb3* and *Spp1*) was most significantly increased in the endometrium in the group that was administered vitamin D3 cyclically, with especially higher expression in both luminal and glandular epithelia. The most significant difference in the expression of *Lif*, a secretion factor, was observed in the group treated only once (1–4 days treatments) (Fig. 1). Unlike the adhesion molecules expressed in the uterine epithelium, these results may be related to the unique properties of LIF, which is expressed in the endometrial glands and secreted in the endometrial epithelium during the initial stage of implantation [24]. Moreover, the expression of angiogenesis-related markers was found to be increased following intrauterine treatment of vitamin D3. However, the time points at which the expression of the two angiogenic markers (CD31 and *Vegfa*) increased differed. There was a significant elevation in CD31 expression with 3-time cyclic intrauterine administration of vitamin D3, whereas *Vegfa* expression was increased in the 1–4 days groups subjected to a single intrauterine administration (Fig. 2). This difference can be attributed to the fact that Vegfa stimulates the proliferation of vascular endothelial cells and subsequently increases the expression of CD31, a surface protein on vascular endothelial cells [31,32].

During the pregnancy, the maternal immune system surrounding the endometrium largely changes with respect to the proportion, localization, and the number of immune cells, specifically dendritic cells, T cells, macrophages, and NK cells, which recognize fetal antigens [33]. In particular, vitamin D3 has recently attracted an attention for its potential roles in the management of recurrent implantation failure and repeated miscarriage, which are characterized by a lower number or higher cytotoxicity of NK cells compared to normal women [34,35]. Based on this information, in our study we aimed to investigate whether vitamin D3 intrauterine administration affects the number of NK cells in pregnant mice compared to the saline-treated or non-pregnant mice (Fig. 4). Our findings, revealing vitamin D3 administration directly into the uterine cavity prior to the initiation of gestation induces significantly higher rates of $CD3^{-}NK1.1^{+}CD49a^{+}$ NK cell recruitment, strongly

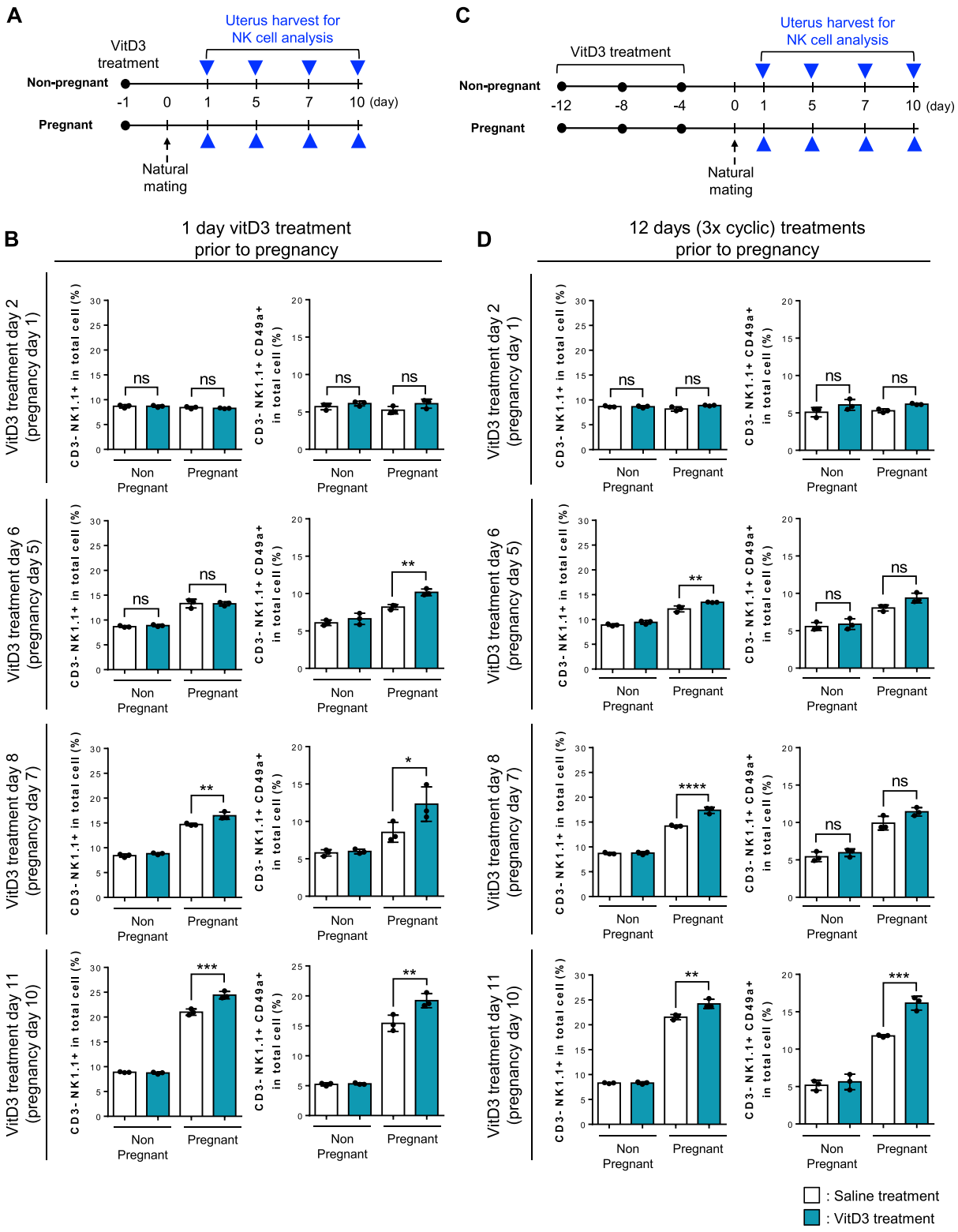

Fig. 3. Vitamin D3 administration induces NK cell recruitment in mouse uteri during the early phase of pregnancy. **(A)** An experimental plan of intrauterine administration of vitamin D3 for 1 day. **(B)** Flow cytometry analyses of CD3, NK1.1, and CD49a in vitamin D3-treated mouse endometrial stromal cells compared with saline-treated group in non-pregnant or pregnant mice (number of mice used in total = 48, 3 mice per group). The proportion of $CD3^{-}NK1.1^{+}$ and $CD3^{-}NK1.1^{+}CD49a^{+}$ cells with vitamin D3 treatment for 1 day prior to natural mating are quantified. **(C)** An experimental plan of intrauterine administration of vitamin D3 for 12 days (3-time cyclic treatment every 4 day). The proportion of $CD3^{-}NK1.1^{+}$ and $CD3^{-}NK1.1^{+}CD49a^{+}$ cells with vitamin D3 treatment for 12 days **(D)** prior to natural mating are quantified. All data are from 3 independent experiments and represented as mean ± SD and analyzed using the ordinary one-way ANOVA with Tukey's multiple comparisons test including p-values (**< 0.01, ****< 0.0001, NS; not significant).

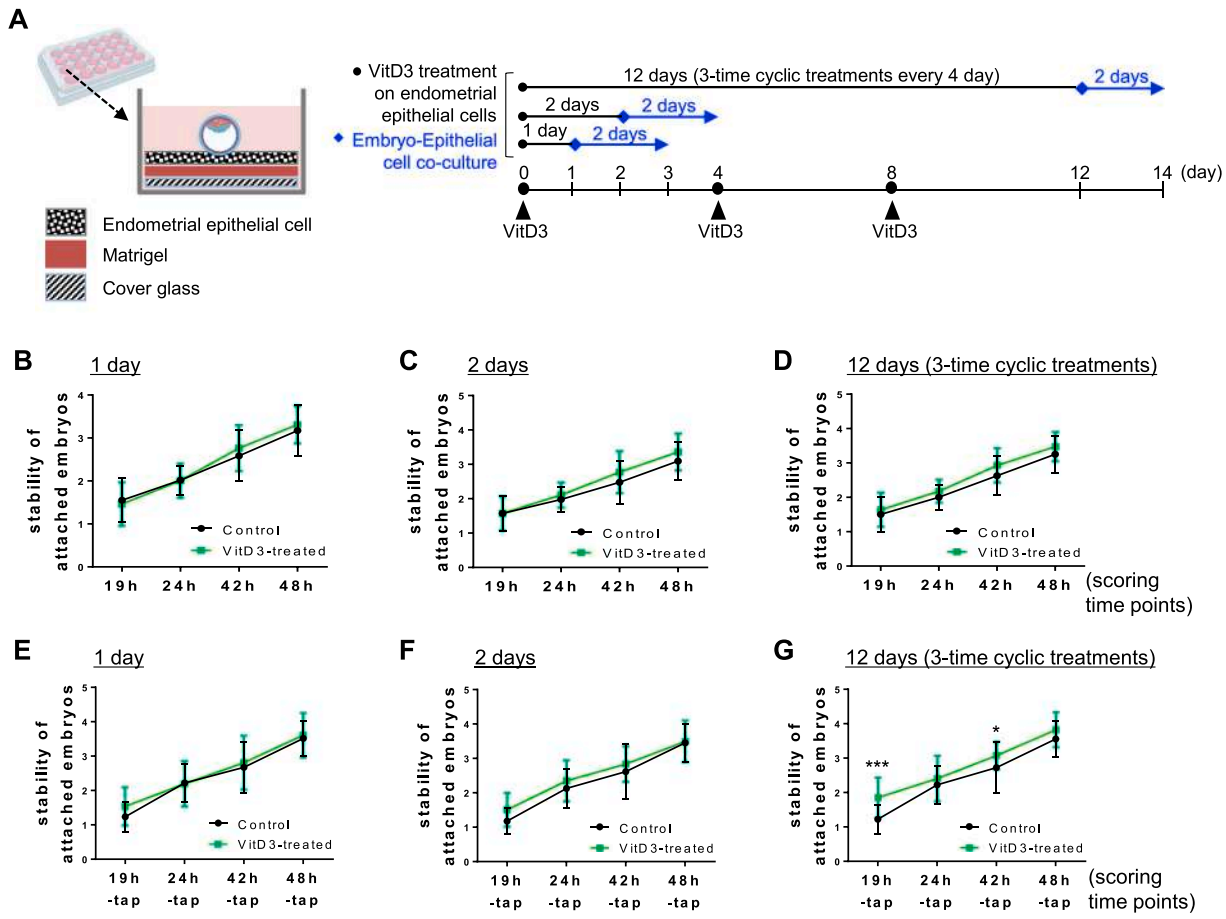

Fig. 4. Vitamin D3 administration increases of the rate of embryo attachment. **(A)** An experimental design of embryo attachment assay (total number of embryos = 146 (from 14 female mice)). Stability of embryos attached on vitamin D3-treated Ishikawa cells (y-axis) was scored at indicated time-points (x-axis) without tapping at 1 day **(B)**, 2 days **(C)**, and 12 days (3-time cyclic treatments every 4 day) **(D)** or with tapping at 1 day **(E)**, 2 days **(F)**, and 12 days **(G)**. **(B-G)** Data are analyzed by unpaired t test including p-values (*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001).

suggest that vitamin D3 intrauterine administration prior to IVF-ET recruits more uterine-resident NK cells which support fetal development by secreting pro-angiogenic factors or various cytokines to regulate their characteristics [36]. In particular, an increase in the proportion of $CD3^{-}NK1.1^{+}CD49a^{+}$ NK cells in the total endometrial stromal cell population were noted on the day 10 of pregnancy. This may correspond to an increase in NK cells that functionally remodel arteries between 8.5 and 12.5 days of pregnancy when spinal artery remodeling actively occurs for placental formation [37,38]. Consistently, elevated CD31 expression, which is indicative of increased vessel formation (Fig. 2A) provides supportive evidence for the increased number of uterine-resident $CD3^{-}NK1.1^{+}CD49a^{+}$ NK cells following vitamin D3 administration. However, further studies are required to investigate the origin of these vitamin D3-induced NK cells, which may be imported from the periphery or the result of proliferation of uterine-resident NK cells. Although the present study was not disease-specific, based on our findings, we suggest that adequate vitamin D treatment may be beneficial for the patients with infertility due to NK cell deficiency, aberrantly high NK cell cytotoxicity, and vitamin D3 deficiency.

Moreover, we evaluated the therapeutic effects of vitamin D3 on embryo implantation both *in vitro* and *in vivo*. Our findings revealed that cyclic administration of vitamin D3 resulted in more stable attachment of embryos on vitamin D3-primed endometrial cells (Fig. 4). Additionally, the number of implantation sites and fetal weight on day 17 of pregnancy were increased in the group subjected to three cycles of vitamin D3 administration (Fig. 5). Collectively, these findings suggest a supportive role for vitamin D3 in maternal–fetal interactions during the early stage of implantation and pregnancy maintenance. However, there might be limitation that could be occurring during two independent intrauterine administration to each uterine horn for comparison. In our study, potential leakage or mix between two independent solutions of intrauterine administered vitamin D3 from one side of horn to the other was prevented as possible by adjusting the appropriate volume of solution and tight monitoring of mice by examining dry tissue placed under the body during the treatment, which was adapted from the previous studies [23,39]. Further investigation with technically more refined experimental strategies and disease-specific studies of vitamin D3 effects on maternal-fetal network with more detailed time-line of embryo implantation stages or fetal and placental growth are needed.

5. Conclusions

Herein, we report that cyclic intrauterine administration of vitamin D3 increased endometrial receptivity and angiogenesis, induced the recruitment of uterine-resident NK cells, and promoted stable maternal–fetal interactions during the early phase of implantation. Based on our findings, we suggest that women suffering from repeated implantation failure might benefit from the use of vitamin D3 as a risk-free adjuvant during IVF-ET.

Ethics approval and consent to participate

All experimental procedures using animals and animal use protocol have been approved by the Animal Ethics Committee of the CHA

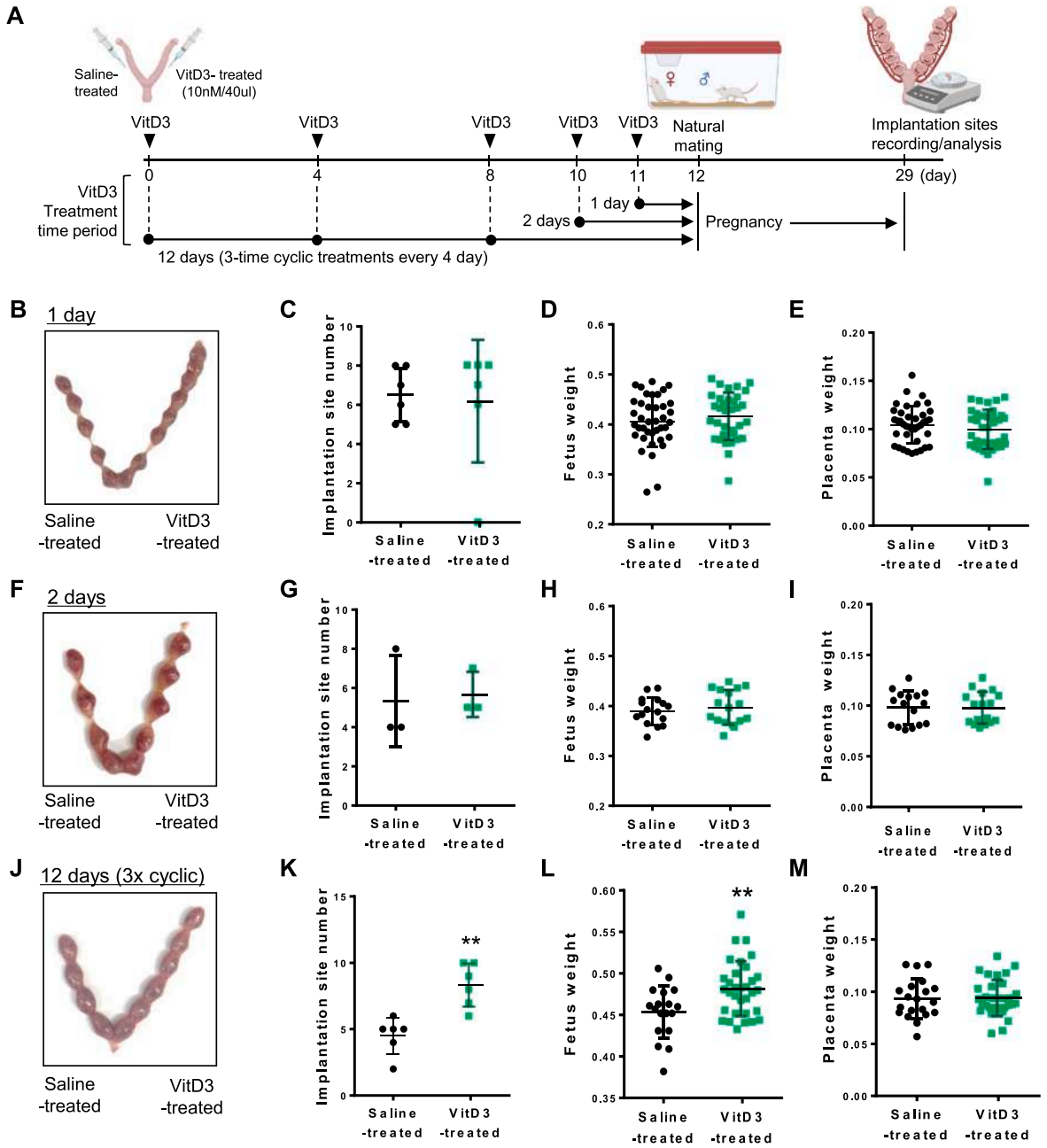

Fig. 5. Intrauterine vitamin D3 administration improves embryo implantation rates *in vivo.* **(A)** An experimental plan of fertility assay of vitamin D3 for 1 day, 2 days, and 12 days (3-time cyclic treatments every 4 day). Representative images of uteri with implantation sites on pregnancy day 17: vitamin D3 administered 1 day **(B–E)**, 2 days **(F–I)**, or 12 days (3-time cyclic treatments every 4 day) **(J–M)** prior to natural mating. Total number of mice used=15. The number of implantation sites (per uterine horn) and fetal/placental weight were measured. Data are analyzed by unpaired t test including p-values (*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001).

University (IACUC, approval no. #200220). Moreover, informed consent for human samples was obtained from each patient and this study was allowed by the Institutional Review Board (IRB approval number, 2020-10-007) of the CHA Bundang Medical Center.

Informed Consent Statement

Not applicable.

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CRediT authorship contribution statement

Youn-Jung Kang: Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Gaeun Lee:** Visualization, Data curation. **Sun-Young Hwang:** Data curation. **Hwa Seon Koo:** Conceptualization. **Jungho Ahn:** Methodology, Formal analysis. **Hwijae Cha:** Formal analysis. **Danbi Lee:** Methodology, Data curation. **Yu-Gyeong Lee:** Writing – original draft, Visualization, Validation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116853.

References

[1] J.D. Aplin, P.T. Ruane, Embryo-epithelium interactions during implantation at a glance, J. Cell Sci. 130 (2017) 15–22.
[2] K. Diedrich, B.C. Fauser, P. Devroey, G. Griesinger, G. Evian Annual Reproduction Workshop, The role of the endometrium and embryo in human implantation, Hum. Reprod. Update 13 (2007) 365–377.
[3] S.K. Dey, H. Lim, S.K. Das, J. Reese, B.C. Paria, T. Daikoku, et al., Molecular cues to implantation, Endocr. Rev. 25 (2004) 341–373.
[4] A.M. Kelleher, J. Milano-Foster, S.K. Behura, T.E. Spencer, Uterine glands coordinate on-time embryo implantation and impact endometrial decidualization for pregnancy success, Nat. Commun. 9 (2018) 2435.
[5] H. Du, G.S. Daftary, S.I. Lalwani, H.S. Taylor, Direct regulation of HOXA10 by 1,25-(OH)2D3 in human myelomonocytic cells and human endometrial stromal cells, Mol. Endocrinol. 19 (2005) 2222–2233.
[6] K. Jakubiec-Wisniewska, H. Huras, M. Kolak, Effect of vitamin D supplementation on the fetal growth rate in pregnancy complicated by fetal growth restriction, Children 9 (2022).
[7] D. Li, Y. Xu, K. Wang, Z. Yang, H. Li, S. Lei, et al., Maternal Vit D supplementation in AMA mice and the role of Vit D/VDR signaling in the offspring's cognition, Am. J. Transl. Res. 13 (2021) 12650–12661.
[8] K. von Websky, A.A. Hasan, C. Reichetzeder, O. Tsuprykov, B. Hocher, Impact of vitamin D on pregnancy-related disorders and on offspring outcome, J. Steroid Biochem. Mol. Biol. 180 (2018) 51–64.
[9] P. Skowronska, E. Pastuszek, W. Kuczynski, M. Jaszczol, P. Kuc, G. Jakiel, et al., The role of vitamin D in reproductive dysfunction in women – a systematic review, Ann. Agric. Environ. Med. 23 (2016) 671–676.
[10] H. Hou, J.Y. Zhang, D. Chen, F. Deng, A.N. Morse, X. Qiu, et al., Altered decidual and placental catabolism of vitamin D may contribute to the aetiology of spontaneous miscarriage, Placenta 92 (2020) 1–8.
[11] X. Ge, L. Wang, M. Li, N. Xu, F. Yu, F. Yang, et al., Vitamin D/VDR signaling inhibits LPS-induced IFNgamma and IL-1beta in Oral epithelia by regulating hypoxia-inducible factor-1alpha signaling pathway, Cell Commun. Signal. 17 (2019) 18.
[12] M.S. Wong, M.S. Leisegang, C. Kruse, J. Vogel, C. Schurmann, N. Dehne, et al., Vitamin D promotes vascular regeneration, Circulation 130 (2014) 976–986.
[13] T. Yoshizawa, Y. Handa, Y. Uematsu, S. Takeda, K. Sekine, Y. Yoshihara, et al., Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning, Nat. Genet. 16 (1997) 391–396.
[14] D.K. Panda, D. Miao, M.L. Tremblay, J. Sirois, R. Farookhi, G.N. Hendy, et al., Targeted ablation of the 25-hydroxyvitamin D 1alpha-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction, Proc. Natl. Acad. Sci. USA 98 (2001) 7498–7503.
[15] H. Safari, M. Hajian, M.H. Nasr-Esfahani, M. Forouzanfar, J.R. Drevet, Vitamin D and calcium, together and separately, play roles in female reproductive performance, Sci. Rep. 12 (2022) 10470.
[16] S. Ozkan, S. Jindal, K. Greenseid, J. Shu, G. Zeitlian, C. Hickmon, et al., Replete vitamin D stores predict reproductive success following in vitro fertilization, Fertil. Steril. 94 (2010) 1314–1319.
[17] K. Garbedian, M. Boggild, J. Moody, K.E. Liu, Effect of vitamin D status on clinical pregnancy rates following in vitro fertilization, CMAJ Open 1 (2013) E77–E82.
[18] N.P. Polyzos, E. Anckaert, L. Guzman, J. Schiettecatte, L. Van Landuyt, M. Camus, et al., Vitamin D deficiency and pregnancy rates in women undergoing single embryo, blastocyst stage, transfer (SET) for IVF/ICSI, Hum. Reprod. 29 (2014) 2032–2040.
[19] A. Aleyasin, M.A. Hosseini, A. Mahdavi, L. Safdarian, P. Fallahi, M.R. Mohajeri, et al., Predictive value of the level of vitamin D in follicular fluid on the outcome of assisted reproductive technology, Eur. J. Obstet. Gynecol. Reprod. Biol. 159 (2011) 132–137.
[20] J.M. Franasiak, T.A. Molinaro, E.K. Dubell, K.L. Scott, A.R. Ruiz, E.J. Forman, et al., Vitamin D levels do not affect IVF outcomes following the transfer of euploid blastocysts, Am. J. Obstet. Gynecol. 212 (2015) (315 e1–6).
[21] R.D. Firouzabadi, E. Rahmani, M. Rahsepar, M.M. Firouzabadi, Value of follicular fluid vitamin D in predicting the pregnancy rate in an IVF program, Arch. Gynecol. Obstet. 289 (2014) 201–206.
[22] Y.J. Kang, K. Forbes, J. Carver, J.D. Aplin, The role of the osteopontin-integrin alphavbeta3 interaction at implantation: functional analysis using three different in vitro models, Hum. Reprod. 29 (2014) 739–749.
[23] H.S. Koo, M.J. Yoon, S.H. Hong, J. Ahn, H. Cha, D. Lee, et al., CXCL12 enhances pregnancy outcome via improvement of endometrial receptivity in mice, Sci. Rep. 11 (2021) 7397.
[24] H. Achache, A. Revel, Endometrial receptivity markers, the journey to successful embryo implantation, Hum. Reprod. Update 12 (2006) 731–746.
[25] E.V. Schulz, L. Cruze, W. Wei, J. Gehris, C.L. Wagner, Maternal vitamin D sufficiency and reduced placental gene expression in angiogenic biomarkers related to comorbidities of pregnancy, J. Steroid Biochem. Mol. Biol. 173 (2017) 273–279.
[26] M. Grundmann, M. Haidar, S. Placzko, R. Niendorf, N. Darashchonak, C.A. Hubel, et al., Vitamin D improves the angiogenic properties of endothelial progenitor cells, Am. J. Physiol. Cell Physiol. 303 (2012) C954–C962.
[27] F. Cyprian, E. Lefkou, K. Varoudi, G. Girardi, Immunomodulatory effects of vitamin D in pregnancy and beyond, Front. Immunol. 10 (2019) 2739.
[28] T. Hydes, A. Noll, G. Salinas-Riester, M. Abuhilal, T. Armstrong, Z. Hamady, et al., IL-12 and IL-15 induce the expression of CXCR6 and CD49a on peripheral natural killer cells, Immun. Inflamm. Dis. 6 (2018) 34–46.
[29] K. Ota, S. Dambaeva, M.W. Kim, A.R. Han, A. Fukui, A. Gilman-Sachs, et al., 1,25-Dihydroxy-vitamin D3 regulates NK-cell cytotoxicity, cytokine secretion, and degranulation in women with recurrent pregnancy losses, Eur. J. Immunol. 45 (2015) 3188–3199.
[30] S. Oh, S. Chun, S. Hwang, J. Kim, Y. Cho, J. Lee, et al., Vitamin D and exercise are major determinants of natural killer cell activity, which is age- and gender-specific, Front. Immunol. 12 (2021) 594356.
[31] M.B. Nourse, D.E. Halpin, M. Scatena, D.J. Mortisen, N.L. Tulloch, K.D. Hauch, et al., VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 80–89.
[32] D. Guo, Q. Wang, C. Li, Y. Wang, X. Chen, VEGF stimulated the angiogenesis by promoting the mitochondrial functions, Oncotarget 8 (2017) 77020–77027.
[33] F. Yang, Q. Zheng, L. Jin, Dynamic function and composition changes of immune cells during normal and pathological pregnancy at the maternal-fetal interface, Front. Immunol. 10 (2019) 2317.
[34] G. Babayeva, Y.E. Purut, B. Giray, P. Oltulu, R. Alakus, M.C. Colakoglu, Endometrial CD56+ natural killer cells in women with recurrent implantation failure: an immunohistochemical study, Turk. J. Obstet. Gynecol. 17 (2020) 236–239.
[35] K. Sfakianoudis, A. Rapani, S. Grigoriadis, A. Pantou, E. Maziotis, G. Kokkini, et al., The role of uterine natural killer cells on recurrent miscarriage and recurrent implantation failure: from pathophysiology to treatment, Biomedicines 9 (2021).
[36] B. Fu, Y. Zhou, X. Ni, X. Tong, X. Xu, Z. Dong, et al., Natural killer cells promote fetal development through the secretion of growth-promoting factors, Immunity 47 (2017) 1100–1113, e6.
[37] J. Zhang, Z. Chen, G.N. Smith, B.A. Croy, Natural killer cell-triggered vascular transformation: maternal care before birth? Cell. Mol. Immunol. 8 (2011) 1–11.
[38] J. Kieckbusch, L.M. Gaynor, F. Colucci, Assessment of maternal vascular remodeling during pregnancy in the mouse uterus, J. Vis. Exp. (2015) e53534.
[39] I. Pavlidis, O.B. Spiller, G. Sammut Demarco, H. MacPherson, S.E.M. Howie, J. E. Norman, et al., Cervical epithelial damage promotes Ureaplasma parvum ascending infection, intrauterine inflammation and preterm birth induction in mice, Nat. Commun. 11 (2020) 199.