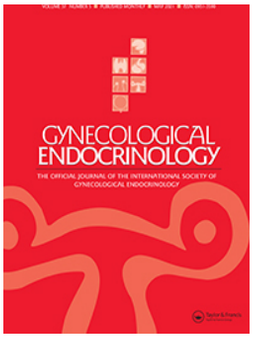

Gynecological Endocrinology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/igye20

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To cite this article: G. Işık, M. Oktem, I. Guler, E. Oktem, C. Ozogul, S. Saribas, A. Erdem & M. Erdem (2021) The impact of granulocyte colony-stimulating factor (G-CSF) on thin endometrium of an animal model with rats, Gynecological Endocrinology, 37:5, 438-445, DOI: 10.1080/09513590.2020.1786508

To link to this article: https://doi.org/10.1080/09513590.2020.1786508

GYNECOLOGICAL ENDOCRINOLOGY
2021, VOL. 37, NO. 5, 438–445
https://doi.org/10.1080/09513590.2020.1786508

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ENDOMETRIUM

The impact of granulocyte colony-stimulating factor (G-CSF) on thin endometrium of an animal model with rats

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ABSTRACT

Purpose: To evaluate whether G-CSF improves the endometrial thickness of thin endometrium by influencing proliferative, angiogenic and apoptotic factors, an experimental rat model was conducted using 24 female adult rats with either thin or healthy endometrium that each was further divided into G-CSF or saline injection groups with six rats.

Materials and methods: After forming of the thin endometrium by uterine injection of 0.2 ml 96% ethyl alcohol to the rats, five days of subcutaneous injections of 40 μg/kg G-CSF or saline were given. Endometrial thickness, immunohistochemically expression of vascular endothelial growth factor receptor-2 (VEGF-R2), proliferative cell nuclear antigen (PCNA) and fibronectin apoptosis with TUNEL method were compared in specimens among four groups of post-model rats.

Results: Endometrial thickness was significantly improved in thin but not in normal endometrium group with GCSF when compared to saline injection. Stromal and glandular epithelial expression of PCNA and pericapillary VEGF-R2 was significantly increased, and apoptosis was significantly decreased with G-CSF. Although fibronectin was also increased with G-CSF in the thin endometrium, the difference was non-significant. In further, G-CSF decreased apoptotic cells and increased expression of PCNA when compared to saline injection in normal endometrium.

Conclusions: G-CSF improves endometrial thickness, proliferation, angiogenesis and DNA fragmentation in thin endometrium.

ARTICLE HISTORY
Received 15 December 2019
Revised 9 May 2020
Accepted 19 June 2020
Published online 2 July 2020

KEYWORDS
Thin endometrium; GCSF; granulocyte colony-stimulating factor; IVF; recurrent implantation failure

Introduction

The thin endometrium is being less than 7 mm of thickness on the day of a trigger of ovulation and unresponsiveness to controlled ovarian hyperstimulation with gonadotropins [1,2]. It is related to decreased pregnancy rates and increased rates of cancelation of IVF cycles with fresh embryo transfer [2]. Currently, no standard effective treatment exists to improve endometrial thickness in these cases [3–5]. Beyond these, G-CSF and autologous stem cells provided better outcomes in some patients with refractory thin endometrium [6]. G-CSF improved endometrial thickness and morphology by immunohistochemistry and western-blot with cytokeratin and vimentin in an experimental animal model [7]. Also, G-CSF instillation between the days of ovulation trigger and embryo transfer was effective for improvement of endometrial thickness with clinical pregnancy rate of 19% [8]. Furthermore, the infusion of G-CSF to the endometrial cavity within 5 min on the day of hCG improved endometrial thickness and implantation rates [9].

Possible mechanisms of G-CSF involved in essential steps of implantation by regulating decidual macrophages, Th2 responses, endometrial cell proliferation. Recently, G-CSF was also used for the recruitment of bone marrow-derived stem cells that is the main step of autologous stem cell therapy of endometrium in cases with Ascherman's syndrome [10].

So, we evaluated the role of subcutaneous injection of G-CSF for improving endometrial thickness and its possible effects on some adhesive, apoptotic and angiogenetic parameters for implantation of an embryo.

Material and methods

We used a total number of 24 adult 200 to 250 g. weighted female rats (Sprague Dawley rat model) from Harlan Laboratory of Holland. All rats were initially recruited into thin ($n = 12$) and normal endometrium ($n = 12$) groups that each was further divided into two groups of treatments with G-CSF or saline injection with 6 rats. The adult rats were synchronized (diestrus) and divided into four groups as control, G-CSF, thin endometrium (TE), TE + G-CSF. Local Ethics Committee for Animal Experiments of the Gazi University approved the study.

For forming thin endometrium, 2 cm lower abdominal skin incision was made under the anesthesia of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) after preparation of skin of the rats with shaving and cleaning with povidone iodine. Then, uterine horns were identified behind the bladder. Two clamps were placed on them at a level as higher as possible and then 0.1 ml of ethyl alcohol 96% was injected in 5 min twice to these horns bilaterally with 2 min interval. Then, these clamps were opened and the layers of the abdomen were closed with sutures.

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Rats in the study and control groups were followed under standard diet (Korkuteli Food Industry, Turkey) and 12-h of light and dark cycles through 10 days representing 2 estrous cycles. Subsequently, all rats were re-randomized to receive five days of subcutaneous injections of 40 µg/kg G-CSF or saline. After the waiting period of 3 estrous cycles, rats were sacrificed and the uterine horns were resected for histological and immunohistochemical analysis. Microphotograps were taken using a Leica DM 4000 light microscope.

The uterine tissues were fixed in 10% neutral buffered formalin and embedded in paraffin after routine histological procedures were completed. Sections 4 µm thick were then cut from paraffin block and stained with Masson's Trichrome (GBL, Masson Trichrome Staining kit, Cat no:5022, Lot no: A0203) for histological fibrosis evaluation, uterine wall thickness and endometrial thickness measurement. As a result, nuclei, muscles, and collagens were stained as blue-black, red and blue, respectively.

Immunohistochemical analysis

Following deparaffinization, the cross-sections were incubated in citrate buffer (pH: 6.0) and 3% hydrogen peroxide. Ultra V block (LabVision, Fremont, CA, Cat no: TA-125-UB, Lot no: AUB150113AC) was applied for blocking.

Following the blocking stage, tissue sections were incubated fibronectin primary antibody (Abcam, Cat no: ab2413, Lot no: GR248495-1) proliferation cell nuclear antigen (PCNA) primary antibody (Santa Cruz, Cat no: sc-7907, Lot no: K1015); Flk-1 (VEGFR2, VEGF receptor 2) primary antibody (Santa Cruz, Cat no: sc-315, Lot no: H2415) in 1:100 dilutions for overnight at 4 °C. Subsequently, tissue sections were incubated with secondary antibody (Lab Vision, Fremont, CA, Cat no: TS-125-HR, Lot no: SHR150121AA), and then immunoreaction was made visible with streptavidin peroxidase and diaminobenzidin (DAB) (LabVision, Fremont, CA, Cat no: TA-125-HD, Lot no: HD31722) complex. Mayer's hematoxylin was used for background staining. Photomicrographs were taken using a light microscope. At each preparation of Bcl-2 and OPN immunohistochemical stainings, 6 areas were determined randomly at ×400 magnification and the immunological involvement was determined as (%) in the ImageJ program.

Fibronectin and VEGFR-2 immunoreactive cells were calculated as percentages with the ImageJ analysis in six randomly selected areas in six cross-sections from each group. In the program, images obtained with ×100 magnification were used. PCNA immunoreactive cells were counted out of 200 cells in six randomly selected areas in six cross-sections from each group.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method

Apoptosis due to DNA fragmentation was assessed by TUNEL method by using Millipore Apoptag Plus Peroxidase *In Situ* Apoptosis Detection kit (Millipore, Cat no: S7101, Lot No:2693367). Briefly, four µm-thick cross-sections were kept in an incubator with 37 °C for a night, following 57 °C for an hour and 61 °C for 20 min. After deparaffinization, tissues were incubated with 20 µg/ml proteinase K (Millipore, Cat no: 21627) in 37 °C for 25 min. Then, tissue sections were incubated with 3% hydrogen peroxide for the inhibition of endogenous peroxidase activity, in a humid environment for 5 min at room temperature. Equilibration buffer was applied for 5 min at room temperature. After, the slides were incubated in TdT enzyme solution for 1 h

Table 1. Comparison of endometrial and uterine wall thickness among groups.

	Group 1 Thin Endometrium + GCSF		Group 2 Thin Endometrium + Saline		Group 3 Normal Endometrium + GCSF		Grouo 4 - Control G (Normal Endometrium + Saline)		*p* Value[a,b-g]
	Mean ± SD (µm)	M (IQR) (µm)	Mean ± SD (µm)	M (IQR) (µm)	Mean ± SD (µm)	M (IQR) (µm)	Mean ± SD (µm)	M (IQR) (µm)	
Endometrial thickness	239.9 ± 158.4	254.25 (266.98)	151 ± 167	84.27 (150)	283.6 ± 189.1	219.49 (227.78)	282.6 ± 94.9	288.38 (160.86)	$p < .0001^{a}$ $p = .309^{b}$ $p < .0001^{c}$ $p = .102^{d}$ $p < .0001^{e}$ $p = .33^{f}$ $p = .025^{g}$
Uterine wall thickness	551.74 ± 397.51	373.9 (754.19)	290.82 ± 119.18	264.20 (196.53)	1085.58 ± 459.71	1073.47 (644.76)	1262.83 ± 279.69	1230.21 (429)	$p < .0001^{a}$ $p = .076^{b}$ $p < .0001^{c}$ $p < .0001^{d}$ $p < .0001^{e}$ $p < .0001^{f}$ $p = .026^{g}$

M: Median; IQR: Interquartile Range.
[a]Kruskall–Wallis test, [b-g]Mann–Whitney *U* Test, [b]Group 4 vs 3, [c]Group 4 vs 2, [d]Group 4 vs 1, [e]Group 3 vs 1, [f]Group 3 vs 2, [g]Group 1 vs 2.

at 37 °C in a humidity chamber. The slides were incubated in the stop/wash buffer for 10 min, then slides were incubated in anti-digoxigenin peroxidase solution at room temperature for 30 min in a humidity chamber. Then, staining with DAB was performed to identify TUNEL-positive cells. Methylene green was used for background stain. TUNEL positive cells were counted out of 200 cells in six randomly selected areas in six cross-sections from each group

Statistical analysis

The software of Statistical Program for Social Sciences (SPSS, version 21.0, IBM, Chicago, IL, USA) and Microsoft Excel 2013 were used for the statistical analysis. The distribution of data was tested by the Shapiro Wilk test and graphically. The data of the variables that were not normally distributed were presented by using the median (M) and Interquartile Range (IQR). Comparison of non-parametric variables was performed by Kruskal–Wallis and Mann–Whitney *U* tests, otherwise, ANOVA and *t*-test were used. The level of statistical significance was accepted as $p < .05$.

Results

Comparison of the thickness of endometrium and uterine wall among 4 groups is shown in Table 1. In this analysis, there was a significant increase in mean endometrial and uterine wall thickness with G-CSF in comparison with saline injection among thin endometrial groups (239 ± 158.4 vs. 151 ± 167, $p = .025$ and 551.74 ± 397.51 vs 290.82 ± 119.1, $p = .026$, respectively).

Masson's Trichrome Staining revealed a similar pattern and density of stain of collagen fibers with G-CSF when compared to saline injection in normal endometrium groups (Figure 1(a,b)). The absence of staining of lamina propria of thin endometriums was also shown due to the nonexistence of collagen fibers in the extracellular matrix in groups 1 and 2. However, some increment on the density of collagen fibers in the tunica adventitia of thin endometrium was shown with G-CSF injection (Figure 1(c,d)).

The results of immunohistochemical analyses and TUNEL method were presented in Table 2. The bounding of fibronectin among groups was shown in Figure 2. The rates of bounding of fibronectin were similar among groups ($p = .071$), although there

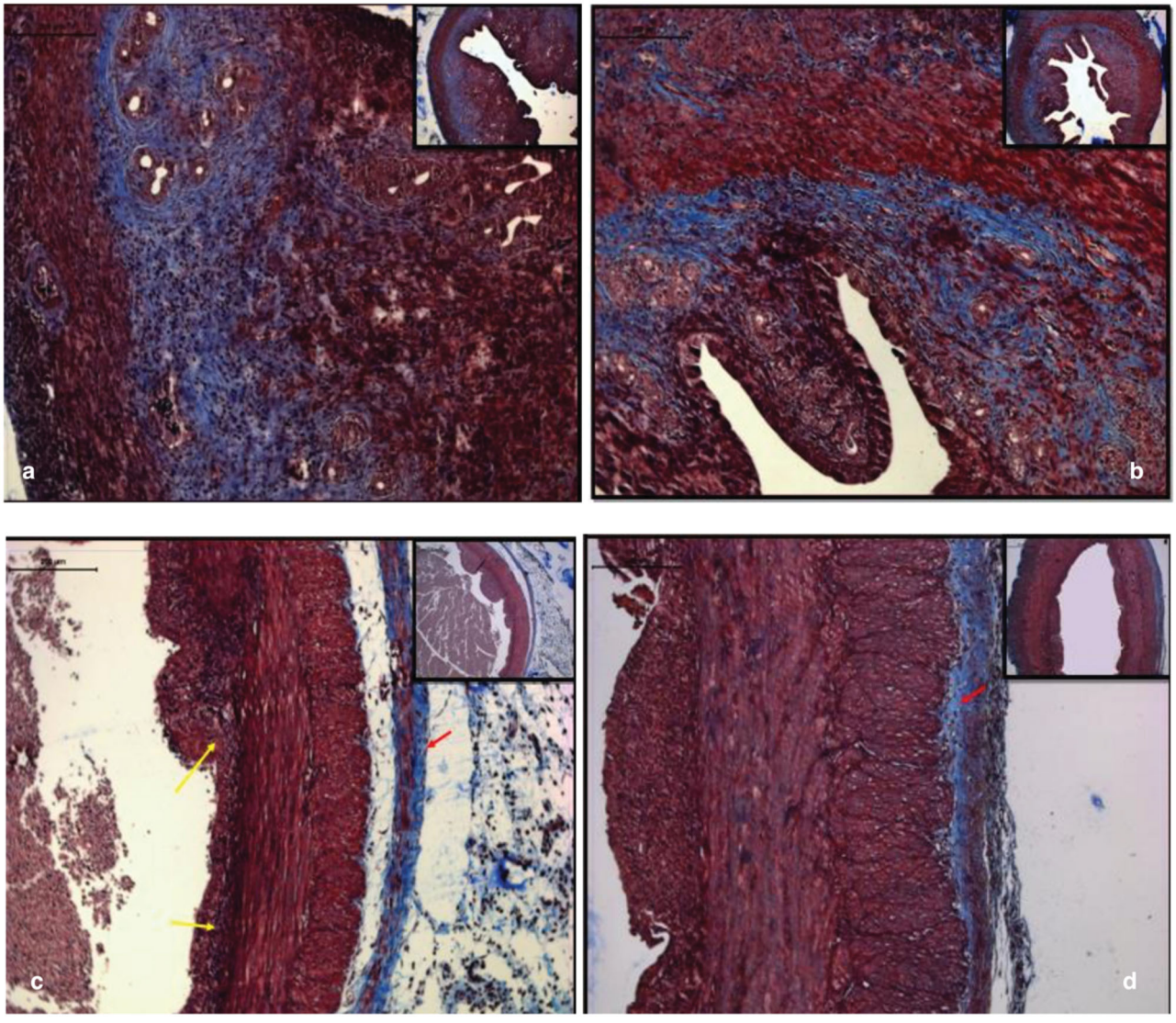

Figure 1. Masson's trichrome staining in normal and thin endometrial groups. Blue color indicates the distribution of collagen fibers in the lamina propria of normal endometrium with saline (a) and GCSF injection (b). Yellow arrows indicate absence of such staining in the thin endometriums and red arrows show staining only in tunica adventitia of thin endometriums with saline (c) and GCSF injection (d).

Table 2. Comparison of Immunohistochemical analyses among grouops.

	Group 1 Thin Endometrium + GCSF	Group 2 Thin Endometrium + Saline	Group 3 Normal Endometrium + GCSF	Gropu 4 Normal Endometrium + Saline	*p* Value[a,b-g]
Fibronectin, %	0.79	0.71	0.98	0.96	$p = .071^{a}$
VEGFR2, %	3.11	1.84	3.33	5.93	$p = .0011^{a}$
					$p = .143^{b}$
					$p = .005^{c}$
					$p = .172^{d}$
					$p = .770^{e}$
					$p = .083^{f}$
					$p = .037^{g}$
PCNA, n (Mean ± SD)	10.56 ± 4.25	2.31 ± 2.01	21.31 ± 5.33	16.25 ± 6.21	$p < .001^{a}$
					$p = .001^{b}$
					$p < .001^{c}$
					$p < .001^{d}$
					$p < .001^{e}$
					$p < .001^{f}$
					$p < .001^{g}$
TUNEL, n (Mean ± SD)	16.97 ± 4.42	28.50 ± 7.92	8.39 ± 3,26	15.06 ± 5.86	$p < .001^{a}$
					$p = .001^{b}$
					$p < .001^{c}$
					$p < .195^{d}$
					$p < .001^{e}$
					$p < .001^{f}$
					$p < .001^{g}$

[a]Anova or Kruskall–Wallis test, [b-g]*t*- test or Mann–Whitney *U*-Test, [b]Group 4 vs 3, [c]Group 4 vs 2, [d]Group 4 vs 1, [e]Group 3 vs 1, [f]Group 3 vs 2, [g]Group 1 vs 2.

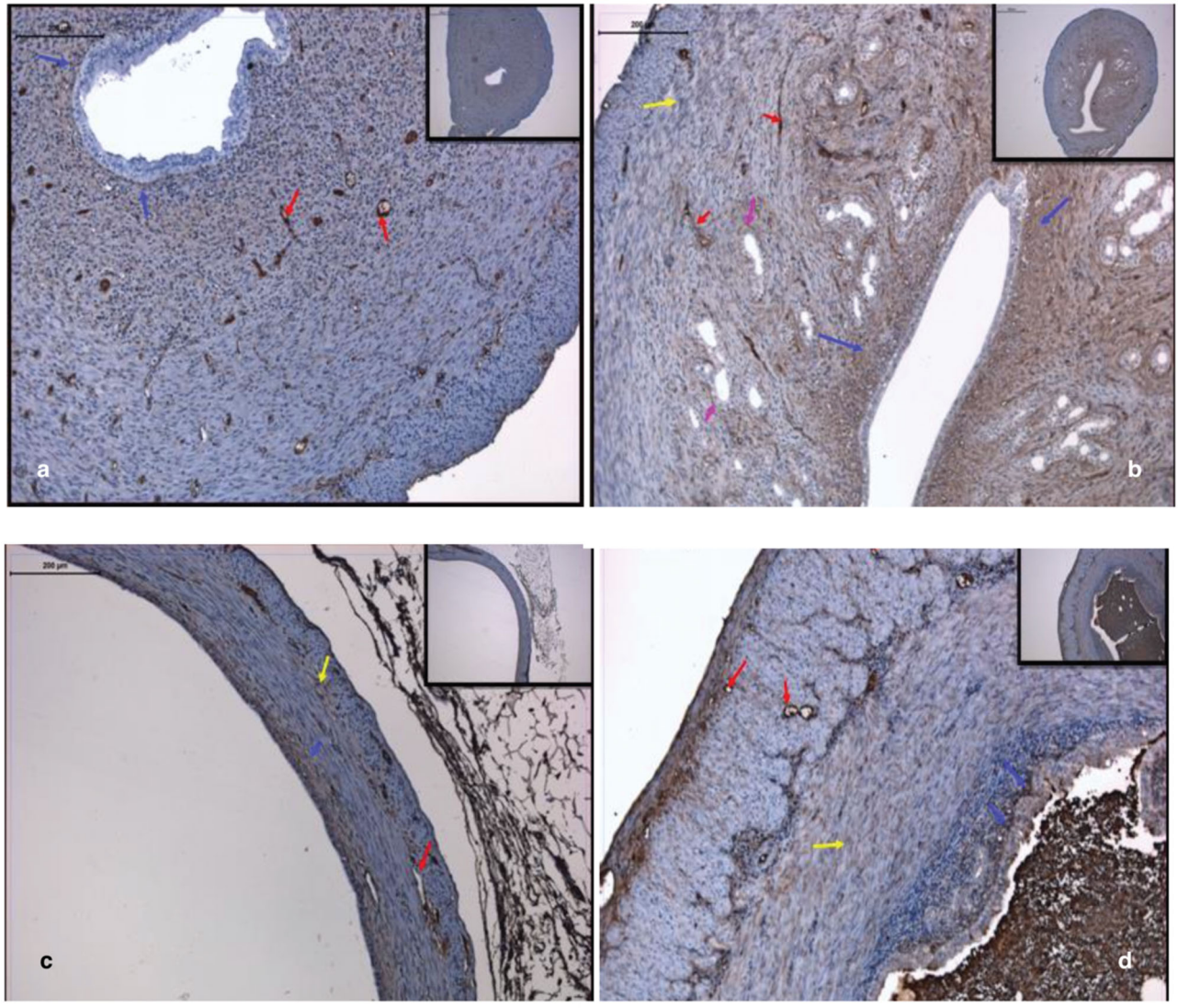

Figure 2. Immunoreactivity of fibronectin among groups. Bounding of fibronectin in subepithelial matrix, perivascular matrix, basal membrane of endometrial glands and bands of muscle cell was indicated with blue, red, pink and yellow arrows, respectively in groups of normal endometrium with saline (a) and GCSF injection (b), and thin endometriums with saline (c) and GCSF injection (d). There was a stronger bounding of fibronectin with GCSF than saline in thin endometrium groups.

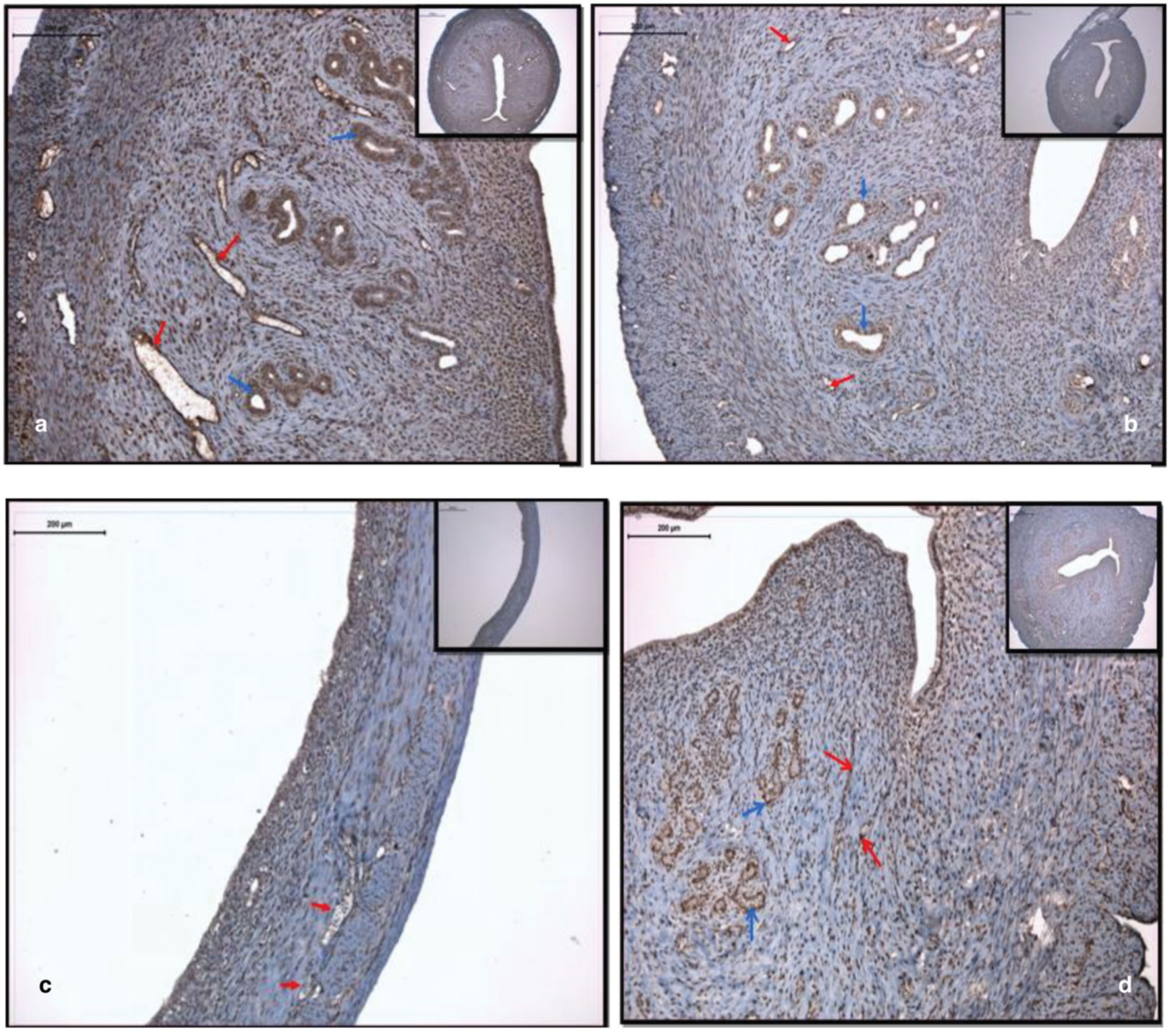

Figure 3. Comparison of VEGF receptor 2 stain among groups. Red and blue arrows shows immunoreactivity of VEGF receptor 2 of vascular basal membrane and endometrial glandular epithelium cells, respectively. (b) is GCSF injection in normal endometrium. Thin endometrium (c) shows significantly lighter bounding of VEGF receptor 2 than normal endometrium (a), and this bounding was significantly improved with GCSF in thin endometrium (d).

was a non-significant improvement with G-CSF in groups with a thin endometrium (0.79% vs 0.71%).

VEGF Receptor 2 immunoreactivity was significantly lower in thin than normal endometrium (1.84% vs 5.93%, $p = .005$) and bounding of VEGF receptor 2 was significantly improved with G-CSF when compared to saline injection in thin endometrium (3.11 vs 1.84, $p = .037$) (Figure 3).

PCNA immunoreactivity, an indicator of cell proliferation, was found to be significantly different among groups ($p < .001$), significantly lower in thin than normal endometrium (16.2 ± 6.21 vs 2.31 ± 2.01, $p < .001$), and also significantly improved with G-CSF injection in thin endometrium (10.56 ± 4.25 vs 2.31 ± 2.01, $p < .001$) (Figure 4).

The number of TUNEL positive cells was significantly higher in thin than normal endometrium (28.50 ± 7.92 vs 15.06 ± 5.86, $p < .001$). Injection of G-CSF significantly lowers TUNEL positivity in thin endometrium (16.97 ± 4.42 vs 28.50 ± 7.92, $p < .001$) as well as in normal endometrium when compared to saline ($8.39 \pm 3{,}26$ vs 15.06 ± 5.86, $p = .001$) (Figure 5).

Discussion

In this study, we evaluated the impact of subcutaneous administration of G-CSF on endometrial thickness, TUNEL positivity for apoptosis and immunoreactivity of fibronectin, VEGF receptor 2 and PCNA for being possible factors of implantation in thin endometriums of an experimental rat model. At first, thin endometrium showed significantly lower levels of fibronectin, cell proliferation, and angiogenesis and increased apoptosis when compared to normal endometrium. In further, our findings revealed that G-CSF induced cell proliferation in endometrial cells of luminal epithelium and stroma by increased levels of staining of PCNA for the regeneration of thin endometrium. This was also supported with decreased levels of apoptosis by the TUNEL method in the areas of thin endometrium where PCNA positive cell groups were prominent after G-CSF injection. VEGF receptor 2, an angiogenic factor of tissue regeneration was also improved in thin endometrium with G-CSF injection. However, increased levels of fibronectin expression did not reach statistical

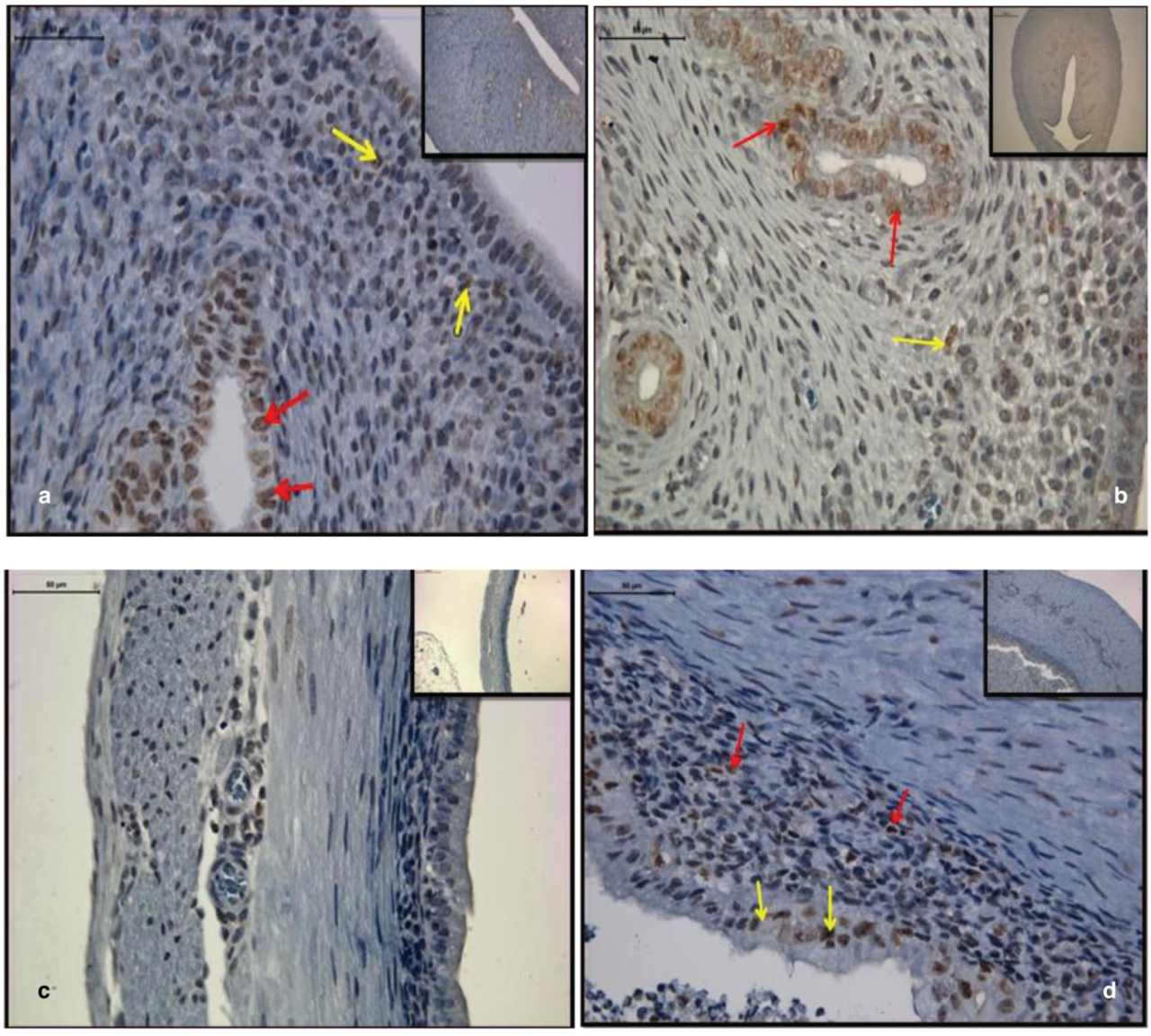

Figure 4. Comparison of PCNA stain among groups. Red and yellow arrows show PCNA stain in endometrial glandular epithelium and endometrial stromal cells, respectively. There was no PCNA stain in thin endometrium + saline injection (c) and GCSF lead to PCNA positive cells in thin endometrium (d). (a) normal endometrium + saline and (b) normal endometrium + GCSF.

significance with G-CSF in thin endometrium. With respect to its effects on normal endometrium, fibronectin and VEFGR were not but apoptosis and cell proliferation were significantly improved with G-CSF.

The acting mechanisms of G-CSF on normal and thin endometrium is not well known. Monocytes, fibroblasts, endothelial and mesothelial cells in many tissues and decidual cells produce G-CSF that stimulates differentiation of precursor cells and inhibits apoptosis [11,12]. So, the production of G-CSF may be insufficient along with the depletion of stem cells and other growth factors in thin endometrium [13]. In a prospective observational study, it was found that thin endometrium was associated with poor angiogenesis and decreased VEGF expression as well as epithelial proliferation [14]. Consistently, thin endometrium showed decreased angiogenic factors and cell proliferation that were significantly increased after G-CSF injection in our study. Pala et al also demonstrated protective effects of G-CSF on endometrium as lesser endometrial gland degeneration and stromal fibrosis was seen with G-CSF application when compared to saline on impaired endometrium with oxidative stress-induced damage by diabetes in a rat model [15].

The clinical effects of G-CSF got attention after the first report by Gleicher et al. regarding its effectivity for improving endometrial thickness of thin endometrium that is unresponsive to standard treatments in 2011 [4]. Subsequent studies showed no relationship between such improvement on endometrial thickness and clinical pregnancy rates [8,16,17]. On the contrary, it was found that the application of G-CSF significantly increased implantation and clinical pregnancy rates [18]. In a recent guideline reported that although G-CSF usage for thin endometrium in IVF-ET cycles was significantly associated with increased pregnancy rates (RR: 1.678, 95% CI: 1,108-2540) in 4 observational studies, one randomized trial did not show a significant difference from this point (OR: 0.990, 95% CI: 0,545 – 1800) [19].

The studies of G-CSF on normal endometrium in patients with a history of unexplained recurrent miscarriage or implantation failure revealed improved pregnancy outcomes and rates,

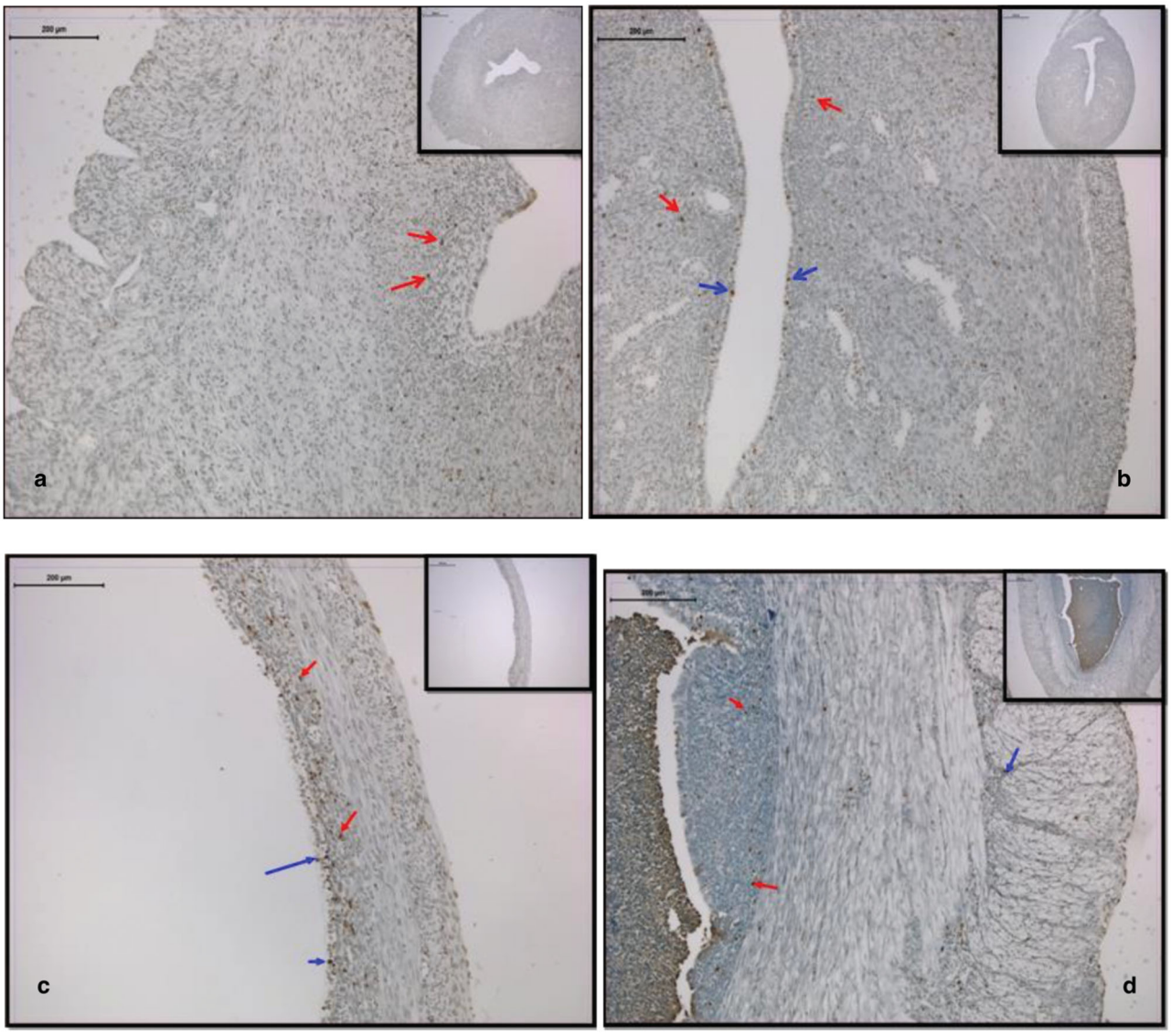

Figure 5. Comparison of TUNEL positivity among groups. Red and blue arrows show TUNEL positivity in endometrial glandular epithelium and endometrial stromal cells, respectively. GCSF injection significantly decreases the TUNEL positivity in groups of normal and thin endometrium when compared to saline.

respectively [12,20]. In our study, GCSF significantly improved apoptosis and cell proliferation in normal endometrium that may be involved as an acting mechanism of G-CSF in patients with recurrent miscarriage or recurrent implantation failure with undetermined causes. In an *ex-vivo* study model on endometrial samples, G-CSF has some significant impact on tissue remodeling by cell migration and local angiogenesis and sufficient amount of its production is required for acting on T cells to express genes for vascular and cell growth and some immune mechanisms of adequate endometrial changes for implantation [21].

In conclusion, experimental thin endometrium was associated with increased apoptosis and decreased angiogenesis, cell proliferation, and fibronectin. Subcutaneous use of G-CSF was effective to improve the thickness, angiogenesis, cell proliferation and apoptosis of thin endometrium.

Acknowledgements

We thank Gazi University to support this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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