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Original article

TNF-alpha Differentially Regulates Ghrelin Expression in Endometrial Cancer Cell Lines and Primary Cell Cultures

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Abstract
Tumor necrosis factor-alpha (TNF) and Ghrelin exhibit autocrine and paracrine functions and are present in several tissues. Their biological effects have been observed in the endometrium under both normal and pathological conditions. This experiment was set out to determine whether TNF alpha changes ghrelin expression in primary endometrial cell culture and endometrial cancer cell lines. Endometrial tissue was taken after ethical permission from 25–40-year-old women who had gynecological surgery at Jessop Hospital, Sheffield, UK. TNF- alpha was added to cultivated Ishikawa and HEC1B endometrial cancer cell lines at 1,10 ng/ml. similar treatments were conducted to isolate primary endometrial epithelial and stromal cells. TNF-induced Ghrelin expression was evaluated by immunocytochemistry. TNF induced a concentration-dependent increase in immunostaining of ghrelin expression and apoptosis in endometrial cancer cell lines. Similarly, primary endometrial epithelial showed a concentration-dependent increase in the apoptotic appearance and ghrelin expression, whereas stromal cells did not demonstrate any detectable changes. TNF-alpha showed a Concentration-dependent effect on apoptotic appearance and ghrelin-expressing in endometrial cancer cell lines (Ishikawa, Hec1B cells) and epithelial cells but not in stromal cells. This finding points to TNF-alpha's intricate influence on the biology and reproduction of the endometrium. Further in vivo quantitative assessments for endometrial expression of ghrelin peptide, mRNA, and the related molecular aspects for these endometrial effects are needed.
Keywords: *Ghrelin, Primary Endometrium Cultures, Ishikawa, Hec1B, TNF alpha.*

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Introduction

Self-renewing endometrium is composed primarily of epithelial cells (luminal and glandular), stromal cells (supporting cells), endothelial cells, and leukocytes [1,2]. While the luminal epithelial layer first encounters the implanting blastocyst, the glandular epithelium extending into stromal tissue maintains endometrial secretory function and supports pregnancy [3,4]. The human endometrium goes through three cyclical changes that include menstrual phase, proliferative phase, and secretory phase. Principally, hormonal effects combined with other factors such as pro-inflammatory cytokines and ghrelin influence these endometrial sequential phases and are significant to the appropriate embryo implantation into the endometrium [5,6]. Interestingly, the endometrial layer differentiates during the secretory phase under the effect of progesterone while rebuilding when estrogen level is increased throughout the proliferative stage and shed in a menstrual phase when fertilization or implantation does not occur [7].

Ghrelin is an autocrine and paracrine 28 amino acids hormone, which is synthesized and secreted by endocrine cells in the gastrointestinal tract. This peptide hormone plays a significant physiological role, controlling processes such as, food intake, energy homeostasis, and the release of growth hormone. However, digestive tissue is not the only source for ghrelin, but it is present in other tissues including reproductive organs for example, testes, ovaries, breast and endometrium. Growth hormone secretagogue receptors (GHS-R) mediate the action of Ghrelin [8-10]. Several biological processes are influenced by ghrelin such as cellular growth, differentiation, and apoptosis, regulating endometrial physiology. In addition, ghrelin is linked to embryo implantation along with endometrial remodeling processes. However, several endometrial pathologies such as hyperplasia and cancer of the endometrium have been connected to ghrelin action. Importantly, the effect of ghrelin on reproduction either in normal or pathological conditions can be recognized by studying the molecular factors that control the expression of ghrelin in the endometrium [10-12].

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Tumor necrosis factor-alpha (TNF alpha) plays a key role in endometrial immune modulation, tissue remodeling, and inflammation. This pro-inflammatory cytokine has two corresponding receptors (TNFR1 and 2) which mediate and trigger its function. Notably, TNFR1 controls pro-inflammatory and apoptotic (programmed cell death) signaling cascades, resulting in shedding endometrium during the menstrual phase, TNFR2 is expressed by immune and endothelial cells, regulating NF-κB (NF Kappa B) and mitogen-activated protein kinase (MAPK pathways), regulating cellular growth, tissue remodeling and angiogenesis [13-15]. The proper equilibrium between TNFR1 and TNFR2 receptors in the endometrium maintains reproductive health because any abnormal expression results in disorders including recurrent miscarriages and endometrial cancer [13,16,17].
Ishikawa and HEC-1B (endometrial cancer cell lines) are derived from an endometrial carcinoma and have become common models to investigate normal and pathological endometrial function since they have well-characterized response to hormonal and inflammatory stimuli. Their properties of being reproductive and stable under controlled experimental conditions provide an ideal tool for testing the regulation of ghrelin expression in endometrial cells under inflammatory stress conditions [18].
Since the endometrium is prone to inflammatory and hormonal signals, suggesting the role of TNF-alpha in controlling multiple cellular expression responses that are fundamental to the endometrium's functions, including decidualization and implantation. In addition, the endometrial ghrelin expression signifies its functional role. Thus, this experiment is conducted to investigate whether TNF alpha could regulate the expression of ghrelin in endometrial primary cell culture and endometrial cancer cells lines. This may offer novel information about endometrial physiology and pathology and perhaps the discovery of new therapeutic targets and biomarkers for endometrial pathology.

Material and Methods

Human subjects

Samples of endometrial tissues were collected from women (25-20 years) who had gynecological procedures at Jessop Hospital in Sheffield, United Kingdom. This experiment was performed following obtaining approval from the ethical committee and acquisition of written consent.

Cell Culture and TNF-alpha Treatment

Ishikawa and HEC1B endometrial cancer cell lines were acquired from Department of Biology, Sheffield Hallam University. Cells were placed in 75-ml culture flasks and cultivated in minimal essential medium (MEM) (Invitrogen, Carlsbad, California), 10% fetal calf serum (FCS), 1% penicillin-streptomycin and 1% glutamine and maintained at 37°C, 5% CO2. Next, cells were re-cultivated and seeded at concentration of $1x10^5$ cells/mL on 8-well chamber slides. Subsequently, when cells reached 70-80% confluence, they washed with fresh medium, detached using trypsin-EDTA, and centrifuged for further purposes. Each chamber slide filled with 400 µl portions of cell suspension and after reaching 60-70% in confluence, they were treated with TNF (Propro Tech) at 1 and 10 ng/ml in triplicate per well. Fresh medium was dispensed in all control wells and incubated for 48 hours. Medium was discarded and cells were BPS washed before fixing them with 3.7% paraformaldehyde, methanol, and acetone. To avoid crystal formation, cells were preserved in sucrose solution at -20°C for until further analysis.

Isolation and treatment of primary endometrial epithelial and stromal cells with TNF-alpha

The obtained samples of endometrial tissue were cut into small pieces, incubated (15 minutes, 37°C) in DMEM (4ml) containing 1.25 µl /ml collagenase, 1% penicillin/streptomycin and 1% glutamine. Next, tissue samples were mixed with Pasteur pipette and incubated for another 20 minutes. The obtained cell suspension was centrifuged (300 rpm) for 5 minutes, the supernatant that contains stromal cells was removed, and the pellet was kept for further use. The pellet of stromal cells was formed by centrifugation (1000 rpm) for 5 minutes of stromal cell suspension. The resulted pellet was resuspended in collagenase (4ml of 1.25 µg/ml) and incubated for 15 minutes at 37 ◦C.
Following two times of mixing and centrifugation, resulted stromal cells supernatant was pooled with the earlier suspension, while the obtained pellet was reconstituted in complete medium to make epithelial cell suspension. The pooled stromal cell suspension was centrifuged at 1000 rpm for 5 minutes) and the supernatant was removed. Then, each cell types pellet was resuspended in complete media (27 ml) and 400 µl portions were located on 8-well chamber slides.
Epithelial cells were incubated for 48 hours at 37°C with 5% CO2 in 50 µl of FCS to enhance adhesion, and TNF (1 and 10 ng/ml) were added to each chamber slide and cells were fixed according to the Ishikawa and HEC1B protocol.

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Immunocytochemistry

Immunocytochemical procedure was conducted by using VECTASTAIN Elite ABC Kit (Vector Labs) to visualize the antibody binding on stromal, epithelial, cancer cell lines cells. After removal of sucrose solution, all cells were washed twice (5 minutes each) with PBS and incubated with 100 µl of the secondary antibody solution which consisted of 10 ml PBS mixed with 45 µl normal serum and 15 µl biotinylated antibody at room temperature for 30 minutes. Next, cells were washed two times for 5 minutes in PBS and treated with VECTASTAIN Elite ABC Reagent (Vector Laboratories) solution (10 ml PBS, 30 µl Reagent A, and 30 µl Reagent B) for 30 minutes at room temperature and followed by washing twice in PBS (5 minutes each).
Antibody binding was visualized using DAB substrate kit (Vector Laboratories). Cells were incubated with solution of DAB substrate for 10–15 minutes. Then washed thoroughly with tap water, followed by counterstaining for 5 minutes using 20% haematoxylin solution. Cells were then washed twice PBS (5 minutes each) and sequentially dehydrated through passing into ascending concentrations of ethanol solution (50%, 70%, 90% and 100%), with each step last for 10 minutes. Then, slides were cleared by passing through three containers containing Histo-clear (five minutes each) and mounted in DPX and left to dry before microscopic examination.

Results

Effect of Tumour necrosis factor alpha (TNFa) on ghrelin expression in endometrial cancer cell lines.

Using Immunostaining the current experiment investigates whether TNF effects ghrelin expression in endometrial cancer cell lines. Ishikawa and Hec1B cancer cell lines were used as endometrial epithelial cell models and were incubated with different concentrations of TNF- alpha (1, 10, and 100 ng/m) and compared to untreated and negative control group. The Ishikawa cells were examined using phase contrast microscopy for viability after 24 hours of TNF incubation. Ishikawa cells showed a confluent grow, normal and undamaged epithelial shape (Figure 1a). TNF incubation (10 ng/ml) reduced the cell density and increased the apoptotic appearance (Figure 1b). In addition, the apoptotic cells were higher after exposure to 100ng/ml of TNF (Figure 1c). Similarly, Hec1B cells treated with 10 and 100 ng/ml TNF showed a concentration-dependent increase in apoptotic effects (no picture). To decrease apoptosis, Ishikawa and Hec1B cells were stimulated with lower levels of TNF 1, 10 ng/ml.
Immunocytochemical examination showed a concentrations dependent increase in ghrelin expression in both Ishikawa and Hec1B cells (Figure 2). Ghrelin immunostaining in Ishikawa cells was especially strong in cells treated with 10 ng/ml TNF (Figure 2B), and moderately enhanced with cells exposed to 1 ng/ml TNF (Figure 2A). However, the basal ghrelin immunostaining was low in untreated Ishikawa cells (figure 2C). The specificity of immunostaining was approved by incubation negative control cells without primary antibodies (Figure 2D). Similar observations were detected for Hec1B cells (Figure 3), where TNF alpha treated cells exhibit concentration-dependent ghrelin expression. Hec1B cells treated with TNF alpha at 10 ng/ml showed the greatest immunostaining (Figure 3B). Conversely, TNF alpha at concentration of 1 ng/ml resulted in mild intensity of immunostainning in Hec1B cells (Figure 3A), while these cells showed weak ghrelin immunostainning in the absence of TNF alpha treatment (Figure 3C). Negative control slides confirmed the non-specific staining (Figure 3D).

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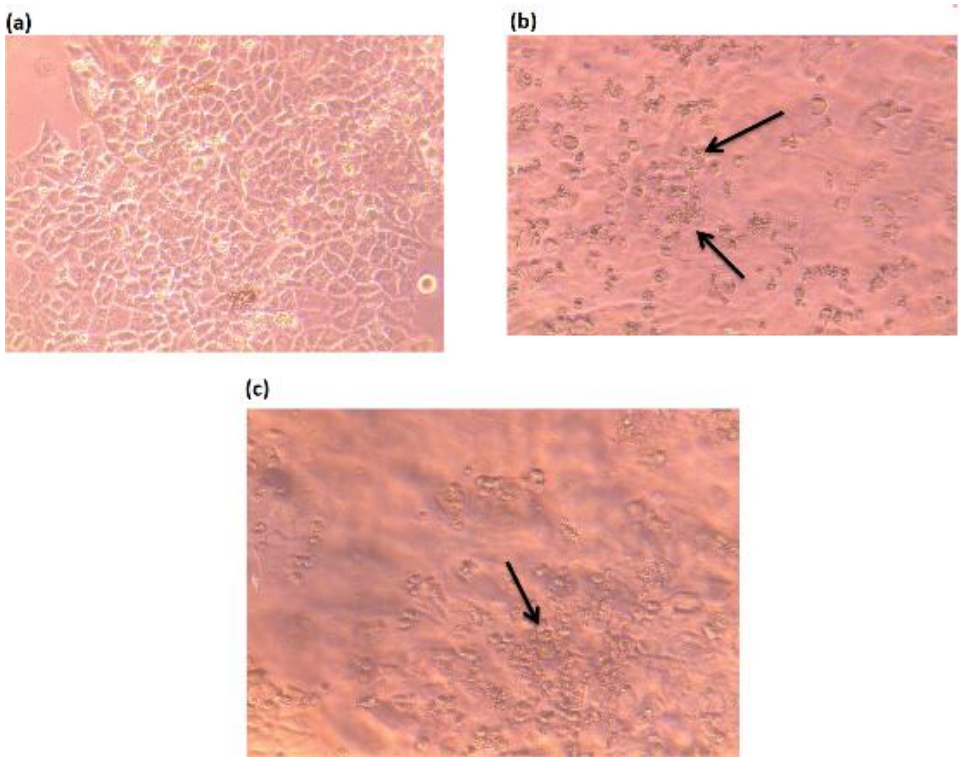

Figure (1) Representative Phase contrast images of Ishikawa cells after 24 hours incubation with TNF alpha. (a) Confluent cells. (b) Ishikawa cells treated with 10 ng/ml TNFa and. (c) increased numbers of apoptotic cells following treatment with 100 ng/ml of TNFa (indicated by arrows).

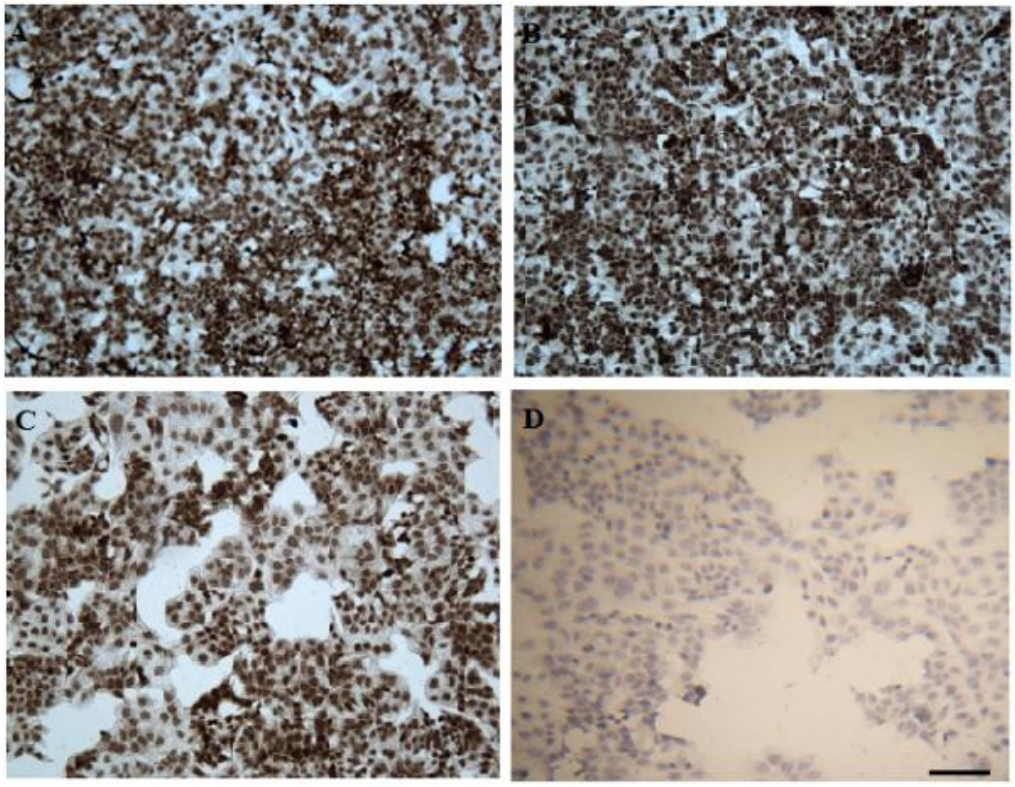

Figure (2) ghrelin Immunoexpression in Ishikawa cells after treatment with 1 and 10 ng/ml TNF alpha. A, the pattern of ghrelin expression in Ishikawa cells stimulated with 1 ng/ml TNF alpha. B Ghrelin expression in Ishikawa cells after treatment with 10 ng/ml TNF alpha. C, ghrelin immunoexpression without treatment with TNF alpha. D, Negative control slide.

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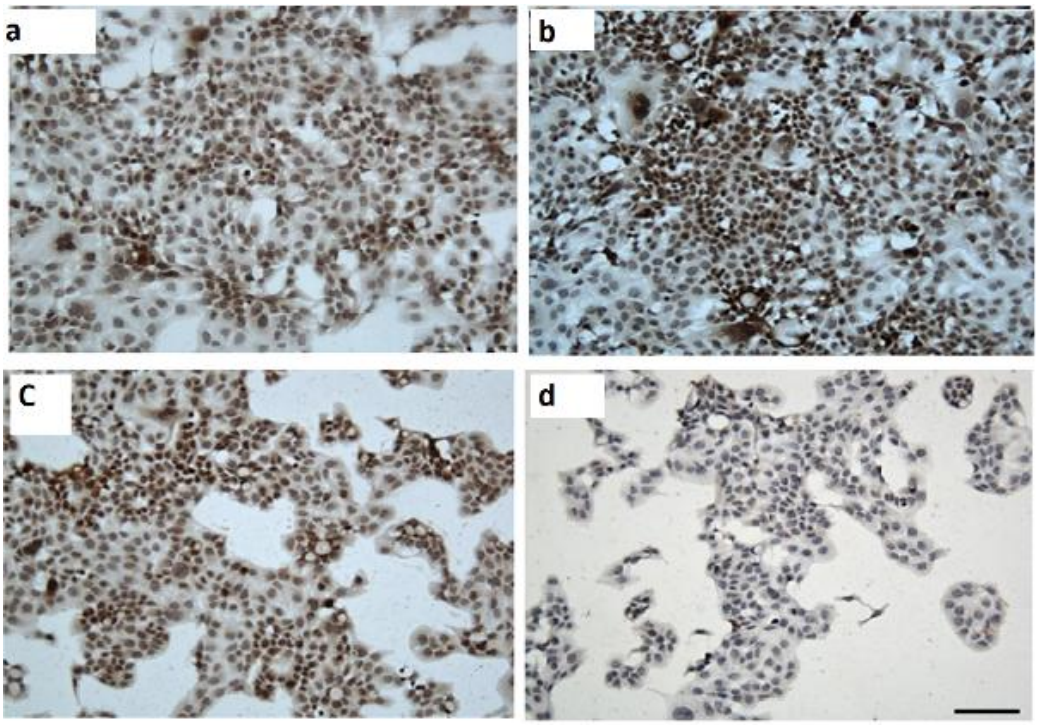

Figure (3) the pattern of Immunostaining for ghrelin expression in Hec1B cells following stimulation with 1, 10 ng/ml of TNF alpha. (a) Ghrelin immunostainning in Hec1B cells following treatment with TNF alpha (1ng/ml). (b) Ghrelin expression after stimulation with 10 ng/ml TNF alpha. (c) Ghrelin expression in absence of TNF alpha. (d) Negative control slide.

Effect of TNFa on Ghrelin Expression in Primary Endometrial Cell Cultures

Primary epithelial cells demonstrated a concentration dependent increase in the intensity of ghrelin immunostaining after beings stimulated with 1 and 0 ng/ml of TNF alpha. Interestingly, the strongest staining was detected in the cultured epithelial cells following 10 ng/ml TNF alpha treatment (Figure 4A). However, at the lower concertation of TNF alpha (1ng/ml) there was a slight increase in the ghrelin immunostainning (Figure 4B), while cells untreated with TNF alpha showed weak expression pattern (Figure 4C). The specificity of the staining procedure was confirmed in the negative control slides (Figure 4D). In compression with epithelial cells, there was not distinguished effect on ghrelin immunostaining in cultivated stromal cells after treatment with 1 and 10 ng/ml of TNF alpha (Figure 5A, B). Moreover, the expression of ghrelin in the untreated stromal cells was similar to that in cells treated with TNF alpha (Figure 5C).

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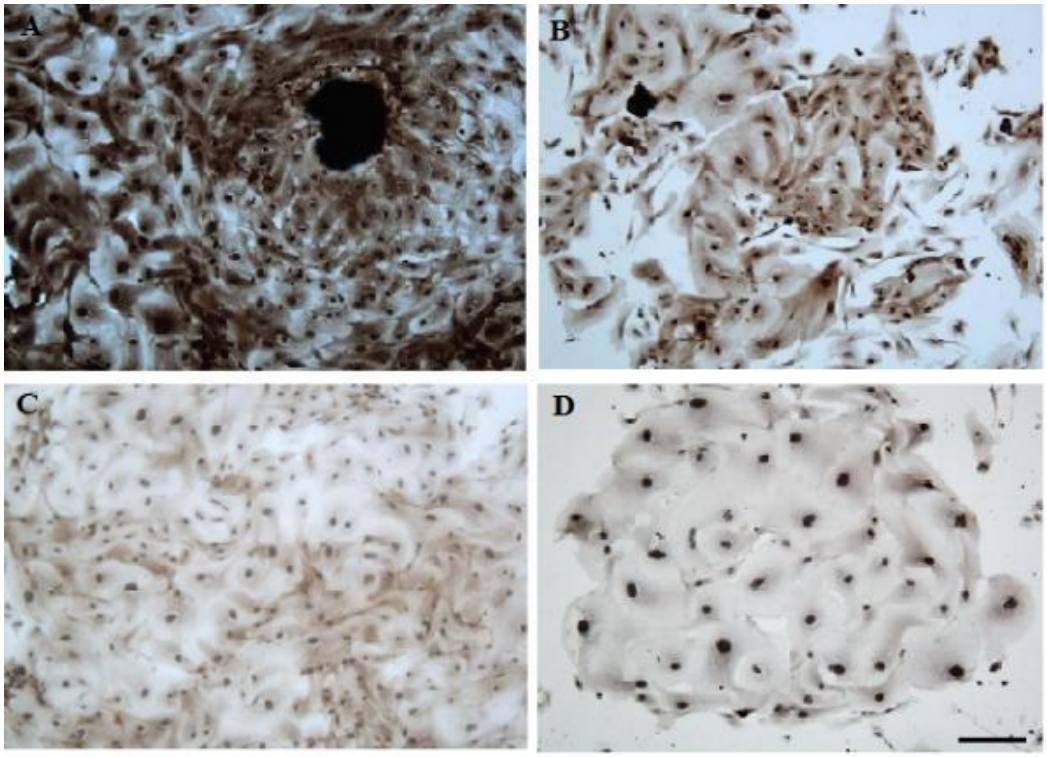

Figure (4) Patterns of ghrelin immunostaining in endometrial epithelial cells following TNF alpha (1 and 10 ng/ml) treatment. A, the immunostainning of ghrelin after treatment with 10 ng/ml TNF alpha. B, Ghrelin expression in cultured endometrial epithelial cells after treatment with 1 ng/ml TNF alpha. C, Ghrelin expression in cultured endometrial epithelial cells without treatment with TNF alpha. D, negative control slide. The staining intensity for Expression of ghrelin was increased after treatment with 10 ng/ml TNF alpha.

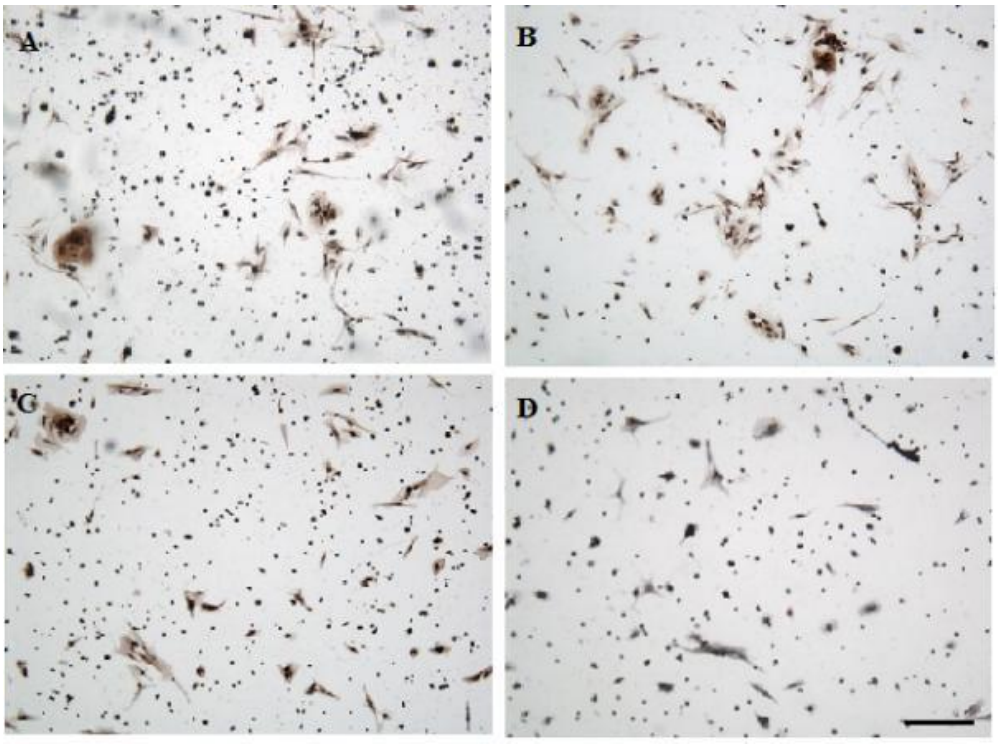

Figure (5) Immunostaining of ghrelin in cultured endometrial stromal cells after treatment of TNF alpha (1 and 10 ng/ml). A, Ghrelin expression after treatment with 10 ng/ml TNF alpha. B, Ghrelin expression in endometrial stromal cells after treatment with 1 ng/ml TNF alpha. C, Ghrelin expression in endometrial stromal cells without treatment with TNF alpha. D, negative control slide. No significant differences was observed in the ghrelin expression after treatment with 1 and 10 ng/ml TNF alpha.

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Discussion

Ghrelin and TNF-alpha, along with their associated receptors are expressed and showed multifaceted effects on physiological functions of several tissues including endometrium [8] [9]. This trial was conducted to investigate the potential effects of TNF-alpha on ghrelin expression in endometrial cancer cell lines and primary endometrial cells. The results of this experiment reveal that TNF alpha's induced dose dependent apoptotic effect in endometrial cancer cell lines (Ishikawa and Hec1B). A higher level of TNF (10,100 ng/ml) resulted in increased number of apoptotic cells in both cell lines and the most prominent effect was seen following incubation with 100 ng/ml of TNF alpha. This finding agreed with other data indicating that TNF alpha was an inducer for programmed cell death in cancer cell lines including endometrium [19]. TNF alpha's apoptotic activity has been observed across various other cancer cell lines including those from breast and colon cancers emphasizing TNF's crucial effects on cellular survival regulations throughout different tissues [20,21].

TNF alpha promotes programmed cell death through TNFR1 activation, which attract death adaptor proteins TRADD and FADD to trigger caspase-mediated apoptosis [22] Additionally, TNF alpha causes cell death through mitochondrial damage by reducing respiratory enzyme activity and generating more reactive oxygen species [23]. However, previous data have also demonstrated that TNF alpha is able to trigger NF-κB pathway, which in turn result in enhancing the cellular proliferation ability and inhibits apoptosis. This finding highlights a TNF alpha context-dependent effect that based on concentration and cell type [24,25]. Moreover, the differential TNF alpha induced cell death response detected in endometrial cancer cell lines might be related to the differential expression of TNFR1 and TNFR2. It has shown that TNFR1 is mainly related to activation of apoptotic processes, while another data suggested that TNFR2 is more likely activates the signals for survival via activation of NF Kappa B (NF-κB) and Mitogen-activated protein (MAP) Kinase pathways [26]. Our results regarding apoptotic activity induced by high TNF alpha concentrations could be attributed to TNFR1 activation, magnifying apoptotic messages and overriding TNFR2 signals for survival.

This experiment also highlights endometrial cell-type specific response induced by TNF alpha. The patterns of immunostaining showed higher ghrelin expression in epithelial cells treated with TNF alpha (particularly at the 10 ng/ml), but not in stromal cells treated at the same concentration. The varied TNF- response might linked to the particular molecular environments in these cells. A previous data indicate that TNF alpha induces apoptosis via activation of several interrelated pathways such as JNK, which involved in the release of Smac/DIABLO after cleavage of Bid. This disturbs the intracellular TRAF2-cIAP1 complex, stimulates caspase 8 activation, and promote cellular death. In TNF-alpha-sensitive cells, the lack of JNK inhibition that mediated by NF-κB- result in prolonged JNK activation and apoptosis. [27-31].

Stimulatingly, ghrelin anti-apoptotic action was detected in several tissues. Previous research has demonstrated that ghrelin suppresses TNF-α-induced apoptosis in intestinal epithelial cells and hepatocytes via regulating the apoptosis related proteins. Our results suggests that endometrial epithelial cells, known for their role in the secretory phase may produce ghrelin as adaptive mechanism to mitigate TNF alpha induced inflammatory stress. This protective mechanism could contribute to reproductive functions such as receptivity, implantation, decidualization, and menstruation. However, the absence of ghrelin expression in stromal cells may indicate that these cells are less responsive to TNF alpha and might highlight their other critical roles in immune modulation, tissue remodeling rather than ghrelin production [31] [32]

Compared with other cells types, endometrial epithelial cells exhibit a pronounced sensitivity to TNF alpha which peaked during the late secretory phase and menstrual bleeding, stimulating multiple molecular cascades, such as NF-κB, MEK [27]. The sensitivity of endometrial epithelial cells to TNF alpha is supported by expression of both TNFRI and TNFRII, in contrast to stromal cells, which demonstrate undetectable expression [33-35]. In this experiment, the obtained increased endometrial epithelial expression of ghrelin following treatment with TNF alpha may suggest the important regulatory role of TNF alpha on ghrelin expression in endometrium and this in line with other results that showed TNF alpha dose dependent effects on ghrelin expression [36]. In addition, our results indicate that TNF-alpha induces autocrine effects in endometrial epithelial cells, stimulating ghrelin expression and reveal the complex interactions between tumor necrosis factor-alpha (TNF-α) and ghrelin in endometrial function.

In recent times, new evidence suggests a potential connection between ghrelin and TNF-alpha in recurrent miscarriage development (RM). Endometrial ghrelin expression increases in women who experience miscarriage that could indicate its contribution to this medical condition [37]. TNF-alpha has been also involved in RM as researchers found significant upregulation of this proinflammatory cytokines in decidual tissues from women suffering from RM. Over expression of ghrelin in women with failed pregnancy outcome may suggest a ghrelin compensatory response to counteract inflammatory processes and unsuccessful implantation induced elevated levels of TNF alpha [38,39]. These evidences could suggest ghrelin as a valuable diagnostic and therapeutic biomarker or target to optimize pregnancy outcome.

Conclusion

In conclusion, this study showed that TNF alpha induced concentration-dependent effects on apoptosis and ghrelin expression in endometrial cancer cell lines and endometrial epithelial but not stromal primary cell cultures. These results contribute to the current evidences on the complex interplay between pro-inflammatory cytokines such as TNF alpha and ghrelin, highlighting their role in reproductive physiology. Our findings underscore ghrelin as potential therapeutic targets for infertility and endometrial pathology. Further in vivo studies are needed to understand the molecular mechanisms behind TNF alpha effects on ghrelin expression and endometrial function.

Conflict of interest. Nil

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المستخلص

عامل النخر الورمي ألفا والغريلين لديهما وظائف ذاتية الافراز وموضعية التأثير (نظير صماوي)، ويوجد كل منهما في عدة انسجة، وقد تم رصد تأثيرهم البيولوجي في الحالات الطبيعية والمرضية لبطانة الرحم. الهدف: تم اجراء هذه التجربة لتحديد إذا ما كان عامل النخر الورمي الفا له تأثير على تعبير القريلين في خطوط الخلايا الأولية وخطوط الخلايا السرطانية لبطانة الرحم. المواد والطرق: تم اخذ عينات انسجة بطانة الرحم بعد الموافقة الأخلاقية من نساء تتراوح أعمارهم من 25 الي 40 عاما واللائي خضعن لجراحة نسائية في Jessop Hospital, Sheffield, UK . تم إضافة عامل النخر الورمي الفا لخطوط الخلايا السرطانية لبطانة الرحم (Ishikawa, Hec1B cells) بتركيز 1 و 10 نانوجرام \ مل . نفس المعاملة السابقة تم اجراؤها على الخلايا الطلائية والسدوية الأولية المعزولة من عينات بطانة الرحم وتم تقييم التغييرات المستحدثة بإضافة عامل النخر الورمي الفا على تعبير القريلين باستخدام كيمياء المناعة الخلوية. النتائج: أدت المعاملة بعامل النخر الورمي الفا الي زيادة معتمدة علي التركيز في صفات الموت الخلوي وكذلك في التعبير عن القريلين في خطوط خلايا سرطان بطانة الرحم . (Ishikawa, Hec1B cells) وبالمثل؛ أظهرت الخلايا الطلائية الأولية لبطانة الرحم زيادة معتمدة على التركيز في مظاهر الموت الخلوي وتعبير القريلين. بينما لم تظهر الخلايا السدوية لبطانة الرحم أي تغيير ملحوظ. الاستنتاج: عامل النخر الورمي الفا لديه تأثير يعتمد علي التركيز في ظهور الخلايا المبرمجة للموت و أيضا في تعبير القريلين في خطوط خلا سرطان بطانة الرحم (Ishikawa, Hec1B cells) والخلايا الطلائية ولكن ليس في الخلايا اللحمية لبطانة الرحم. تشير هذه النتائج الي التأثير البيولوجي المعقد لعامل النخر الفا على وظيفة بطانة الرحم والتكاثر. هناك حاجة الي دراسات اخري تجري في الجسم الحي لتقييم تأثير عامل النخر الفا على تعبير القريلين وكذلك فهم الالية الجزيئية المرتبطة بهذا التأثير.