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Short Communication

Metalloestrogen cadmium stimulates proliferation of stromal cells derived from the eutopic endometrium of women with endometriosis

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Abstract

Objective: To assess the effect of metalloestrogen cadmium (Cd) on the proliferation of endometrial stromal cells (ESC) isolated from the eutopic endometrium of women with endometriosis

Materials and Methods: ESC were isolated from eutopic endometrial samples from 10 women with endometriosis and 10 women without endometriosis. ESC cultures were established and maintained in RPMI medium. Cultures were treated with Cd at a concentration of 10^{-6} M and after 24 hours and 48 hours, cell number was counted using the Neubauer hemocytometer to calculate the relative cell proliferation. At 48 hours, the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the effect of different concentrations (10^{-8} M to 10^{-3} M) of Cd on ESC cultures. Relative cell proliferation and MTT assay results were analyzed with ANOVA.

Results: At 48 hours, Cd-induced ESC proliferation was higher ($p = 0.02$) in women with endometriosis than in women without endometriosis, which was confirmed by the MTT assay.

Conclusion: The results of this study demonstrate the ability of metalloestrogen Cd to induce the proliferation of stromal cells derived from the eutopic endometrium of women with endometriosis.

Keywords: cadmium; endometrial stromal cells; endometriosis; metalloestrogens; estrogen receptor

Introduction

Cadmium (Cd) is a heavy metal ubiquitously distributed in the biosphere largely due to anthropogenic activity [1]. Numerous adverse health effects in humans secondary to Cd toxicity have been described in the literature [2], which include carcinogenesis [3]. Elevated levels of Cd in blood [4] or urine [5] have been associated with an augmented risk of breast cancer, while long term dietary Cd intake is known to increase the incidence of postmenopausal endometrial cancer [6]. Both breast and endometrial cancer are estrogen-dependent gynecological cancers, which prompted scientists to further investigate the possible estrogenic properties of Cd in order to supplement the knowledge that Cd mediates the malignant transformation of normal breast tissue [7] via several mechanisms [8].

Identification of the ability of Cd to bind and activate the estrogen receptor (ER), has defined a group of xenoestrogens, termed metalloestrogens [9,10], resulting in a paradigm shift with regards to estrogen-dependent diseases. As a consequence, metalloestrogen Cd has been linked with the etiopathology of estrogen-dependent benign gynecological diseases, such as uterine myomas [11] and endometriosis [12].

The estrogenic effects of Cd have mostly been demonstrated in breast cancer cell lines where researchers have established that Cd stimulates proliferation of cells [13] by activating either endogenously or exogenously expressed ERs

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[14]. On the contrary, there is lack of *in vitro* evidence for metalloestrogenic effects of Cd in relation to estrogen-dependent gynecological diseases, including endometriosis. Hitherto published *in vitro* effects of Cd using endometrial cells are confined to few reports, which include that of Tsutsumi et al and the report by Helmestam and coworkers. While Tsutsumi et al describe that Cd causes early induction of decidualization in endometrial stromal cells (ESC) [15], Helmestam and coworkers have published on the Cd-induced expression of mRNA for angiogenic genes in cultured endometrial endothelial cells [16].

Recently, the presence of Cd in ectopic endometrial tissue has been demonstrated [17]. In the absence of any published reports on the effect of Cd on endometrial cells in respect to endometriosis, we faced a dilemma in interpreting the significance the above finding. This study was conducted as a preliminary step to assess the effect/s of metalloestrogen Cd on the proliferation of stromal cells isolated from the eutopic endometrium of women with endometriosis.

Materials and methods

Study participants

Study participants were recruited from women who underwent elective laparoscopy or laparotomy at the Professorial Gynaecology Unit of National Hospital of Sri Lanka, during the period of April 2011 to March 2012. All recruited women were in the reproductive age group and did not receive any hormonal therapy for at least 3 months before the surgery. Indications for surgery in women without endometriosis were investigation of primary or secondary subfertility or ovarian cyst/s on ultrasonography. Ten women with endometriosis and 10 women without endometriosis volunteered for this study.

Preoperatively, participants were given details regarding the nature of the study. The date of the last regular menstrual period was noted to determine the stage of the menstrual cycle in each participant. The presence or absence of endometriosis in each participant was confirmed at the time of laparoscopy or laparotomy. In women with endometriosis, the disease was staged from I to IV according to the revised American Society for Reproductive Medicine (rASRM) classification of endometriosis.

Using a consent form, written consent was obtained from all the participants. The protocol mentioned above is in concordance with the guidelines provided by the Human Tissue Act of 2004 in United Kingdom [18] and the study was approved by the Ethical Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka.

Collection of eutopic endometrial tissue samples

Eutopic endometrial tissue samples were obtained from participants using the MegGyn endometrial sampler (Ref No: 022720, MedGyn Products, Inc., USA). Tissue sampling was always performed before methylene blue dye insufflation, when the latter was performed to ascertain the tubal patency.

The collected tissue fragments were immediately transferred into 1.5 mL sterile micro centrifuge tubes containing 1 mL of Hank's balanced saline solution (HBSS, HyClone Laboratories, Inc., USA) and within 30 minutes of collection, tissue samples were transported from the operating theatre to the cell culture laboratory in HBSS on ice.

ESC

ESC were isolated and cultured as per procedures adopted from Mathews et al [19] and Finas et al [20], with appropriate modifications and optimization.

The tissue fragments were placed in Petri dishes (TPP, Switzerland) and covered with 0.3% collagenase type I solution (Sigma, USA). Tissue fragments were then minced into smaller pieces and incubated in 0.3% collagenase for 120 minutes at 37°C with intermittent agitation. Collagenase digestion was terminated by adding an equal volume (10 mL) of fresh HBSS.

Following collagenase digestion, the solution was passed through a 40 μm cell strainer (BD Falcon, BD Biosciences, USA) and the filtrate was collected into two 15 mL centrifuge tubes (Biologix, USA) and centrifuged at 200 g relative centrifugal force (rcf), for 15 minutes. The resultant cell pellets were resuspended in 5 mL of RPMI 1640 cell culture medium (RPMI 1640; Sigma, USA) supplemented with 10% v/v fetal bovine serum (FBS; Sigma, USA) and 1% v/v antibiotic-antimycotic solution (Gibco, Invitrogen corporation, UK) containing 10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in 0.85% saline. Each cell pellet was dispersed and a homogenous cell suspension was prepared using a 1 mL disposable plastic transfer pipette (Biologix, USA). Aliquots of the cell suspension were used to estimate the cell number using a Neubauer hemocytometer (area 0.0025 mm^2, depth 0.1 mm, Thoma, USA) and cell viability with trypan blue dye (Sigma, USA) exclusion. Once cell number and viability were determined, the ESC were seeded into a 25 cm^2 cell culture flask with vented cap (Nunc, USA) at a density of 2.5×10^5 cells per flask in 5 mL of RPMI medium supplemented with 10% v/v FBS and 1% v/v antibiotic-antimycotic solution. Cell culture flasks were then placed in the carbon dioxide incubator (Mini Galaxi A, RS Biotech, UK) at 37°C with 5% CO_2 and 95% air/humidity.

For the purpose of propagation, until the third subculture (or passage), the cells were grown in either 25 cm^2 or 75 cm^2 cell culture flasks (Greiner Bio One, Germany). After the third subculture, for the relative cell proliferation experiments, cells were seeded into 24-well cell culture cluster plates (Corning, USA) at a density of 2×10^4 cells per well. For the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, 5×10^3 cells per well were seeded into a 96-well cell culture plate (TPP, Switzerland).

Preparation of Cd solutions

Cadmium (II) in the form of cadmium chloride (Sigma, USA; cat no: 202908) a kind donation by the Atomic Energy Authority of Sri Lanka, was used to prepare 2×10^{-2} M of cadmium solution. This was then serially diluted using RPMI medium without FBS to obtain a series of solutions ranging from 2×10^{-3} M to 2×10^{-7} M.

-For the relative cell proliferation experiments, cells grown in 24-well cell culture plates (Corning, USA) at a density of 2×10^{4} cells per well were treated with Cd solution in the following manner. Medium was first removed in each well and replaced with 475 μL of RPMI medium with 10% v/v heat inactivated FCS (Sigma, USA) and 1% v/v antibiotic-antimycotic solution. Then, 25 μL from a 2×10^{-5} M Cd solution was applied into each well, which gave a final Cd concentration of 10^{-6} M in a 500 μL volume.

During the MTT assay, 10 μL each of different Cd concentrations were applied to each well, giving a final volume of 200 μL of RPMI medium supplemented with heat inactivated FBS. This gave concentrations of 10^{-3} M to 10^{-8} M of cadmium in each well.

Determination of relative cell proliferation

Relative cell proliferation was assessed using a method described by Sampey et al [21] with necessary modifications. After 24 hours and 48 hours of metal treatment, the monolayers in 24-well cell culture plates were first washed three times with 1 mL of HBSS. Then the cells were subjected to trypsinization with 500 μL of 0.05% trypsin in 0.02% of EDTA (Sigma, USA) for 15 minutes at 37°C. Once all the cells were detached, trypsinization was terminated with an equal volume (500 μL) of RPMI medium without FBS and a cell suspension (1 mL) was obtained by careful mixing of the contents in each well with a 1000 μL pipette tip (Nichiryo, Japan) attached to a micropipette (Nichiryo, Japan).

An aliquot of the above cell suspension was used to count the number of viable cells. After adding 1% w/v Trypan blue, the cells were loaded into the hemocytometer and the number of viable cells was determined for 1 mL of cell suspension. The relative cell proliferation was calculated by dividing the number of viable cells in each treatment well by that of the untreated well (control).

MTT assay

The effect on overall cell activity was determined by performing the MTT assay [22] on cell monolayers following 48 hours of treatment with Cd. MTT solution (20 μL) was added to the 200 μL medium in each well of the 96-well plate, and the plate was incubated at 37°C for 4 hours. After removing the medium and adding 100 μL of 0.04 N HCl in isopropanol per well, the plate was shaken using the mini orbital shaker for 30 minutes and the absorbance read at 620 nm using a microplate reader (EL_x 800 universal microplate reader, Biotek instruments, USA). Results were expressed as a percentage of the control value.

Results

Characteristics of the study participants

In both groups, the eutopic endometrial tissue samples were obtained during the proliferative phase of the menstrual cycle from Day 3 to Day 7. While the majority of women with endometriosis ($n = 5$) belonged to the Stage III in the rASRM classification, four women belonged to the Stage IV and one participant was in Stage II. None of the women who participated in the study were current smokers.

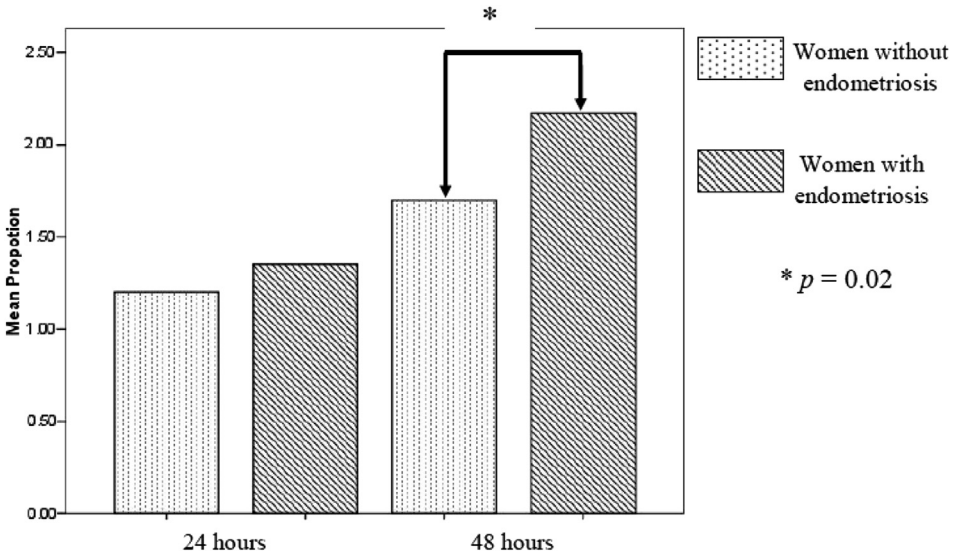

Fig. 1. Relative proliferation of endometrial stromal cells from women without endometriosis and women with endometriosis after 24 hours and 48 hours of treatment of 10^{-6} M Cd.

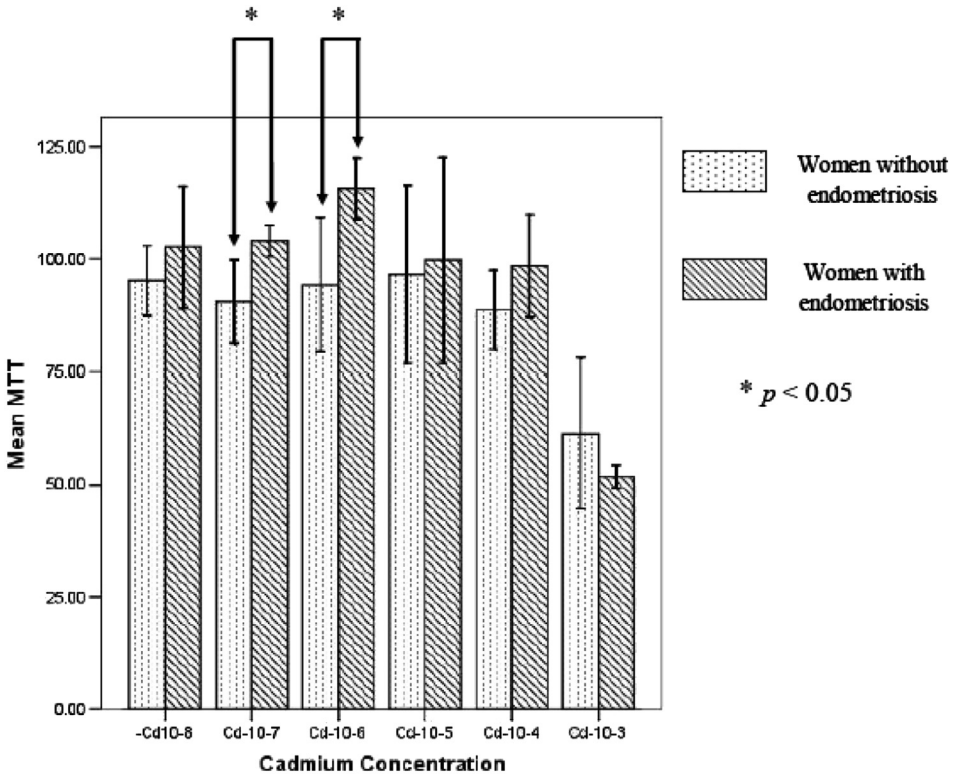

Fig. 2. Results of MTT assay following 48 hours of incubation with different concentrations of Cd (10^{-8} M to 10^{-6} M).

Relative cell proliferation

Relative proliferation of ESC in women with endometriosis increased in response to treatment with 10^{-6} M Cd. This increase reached statistically significant levels ($p = 0.02$; two way ANOVA) at 48 hours of incubation when compared to the relative proliferation of ESC in women without endometriosis. (Fig. 1)

MTT assay

When treated with Cd concentrations of 10^{-7} M and 10^{-6} M, the MTT results were significantly higher ($p < 0.05$) in women with endometriosis, when compared with the same values from women without endometriosis (Fig. 2). Similar mean MTT results were observed in both groups in respect to other Cd concentrations that were tested. There was no clear dose response relationship in either group in respect to any of the Cd concentrations.

Discussion

In this study, the effects of Cd on cultured ESC were investigated, where a comparison was made between the cultures from women with and without endometriosis. Cell proliferation in response to Cd treatment was assessed by means of relative cell proliferation experiments and the MTT assay. In the relative cell proliferation experiments, significant differences were noted in cultures that originated from women with endometriosis following 48 hours of Cd treatment. When the MTT assay was performed using ESC incubated with different concentrations of Cd for 48 hours, there were differences in MTT results only in the endometriosis group in respect to 10^{-7} M and 10^{-6} M of Cd, with no clear dose–response relationship.

In the present study, the method of direct counting of viable cells using a hemocytometer was adopted to calculate the relative proliferation after assessing stromal cell viability by Trypan blue dye exclusion. These experiments were based on a protocol described by Sampey et al who tested the effects of phytoestrogen genistein on cultured ESC [21]. Direct counting of stromal cells has been employed by Gazvani et al and Klemmt et al as a measure of stromal cell proliferation [23,24]. The latter research group used a Neubauer hemocytometer for counting viable stromal cells following trypan blue exclusion.

However, direct counting of cells to derive a measure of cell proliferation is considered by some as a subjective method [25]. Thus, different concentrations of Cd were used in the MTT assay to confirm the stimulation of stromal cell proliferation. The MTT assay is considered as a good indicator of viable and glycolytically active cells [22]. It is noteworthy that many authors, including Chang and coworkers who probed the effect of arsenic on primary ESC cultures [26], have used the MTT assay to assess the proliferation of stromal cells. Helmestam and colleagues opted to use the WST-1 assay to gauge

the proliferation of endometrial endothelial cells in response to cadmium chloride [16]. The WST-1 assay is an improved version of the MTT assay where a tetrazolium dye is used to detect cell viability [25].

Published literature on the effects of cadmium on ESC is confined to two studies conducted by Tsutsumi et al and Kawano and colleagues. While the former group investigated the effect of Cd on the decidualization of ESC following Cd treatment, the latter evaluated the metallothionein levels due to Cd exposure. Neither group report on the proliferation of ESC subsequent to administration of Cd. According to the results of Tsutsumi et al, the most effective dose of Cd was 1 μmol/L [15], while Kawano et al noted the highest metallothionein expression in ESC at a concentration of 10 μM of cadmium [27]. In the present study, a Cd concentration of 10^{-6} M was able to enhance the proliferation of ESC from women with endometriosis after 48 hours, which is similar to the dose of Cd described by Tsutsumi and coworkers. However Tsutsumi et al incubated the ESC for 12 days, to elicit the effects of Cd at the said concentration. Induction of metallothionein is an indicator of toxicity of Cd on cells that occurs at higher doses of the metal [28]. Therefore, a Cd concentration greater than 10^{-5} M appears to be toxic to cultured ESC as observed in the present study where the cell viability decreased in doses above 10^{-5} M (Fig. 2).

The growth stimulatory effects of metals on estrogen sensitive cells are largely confined to the experiments conducted on breast cancer cell lines such as MCF-7, TD47, and SKBR3. Both Garcia-Morales et al and Choea et al report on the significant proliferative effects of Cd on MCF-7 cells at a dose of 10^{-6} M following a 24 hour incubation [13, 29]. The latter group was able to elicit a dose-dependent response of MCF-7 cell growth due to Cd treatment, where the EC_{50} was calculated as 1.08×10^{-7} M. After incubating for 24 hours at a Cd concentration of 50–500 nM, Yu et al were able to demonstrate the proliferation of SKBR3 breast cancer cells [30]. In the present study, a significant proliferation of ESC was observed in the endometriosis group after the administration of 10^{-6} M cadmium for 48 hours. Although the concentration of Cd is comparable to previous experiments conducted on MCF-7 cells, the incubation time differs with a longer time period being required to observe the growth stimulatory effects of Cd of ESC. The MCF-7 cell line is derived from malignant breast cancer tissue with a faster growth rate that has a shorter doubling time [31], while in comparison, the ESC are noted to have slow growth rates [19]. It is probable that the mitogenic effects of Cd attenuate the innate growth potential of the estrogen responsive cells *in vitro*. Nonetheless, negative results in respect to the stimulation of MCF-7 cell proliferation following Cd treatment at a dose of 10^{-7} M have been reported [32].

A dose-dependent response for Cd could not be observed in this study, contrary to findings of previous researchers on MCF-7 cell lines. Neither Tsutsumi et al nor Kawano et al have reported any dose-dependent effects of Cd in their studies involving cultured ESC, raising the possibility of Cd-induced responses on cultured endometrial cells being devoid of a dose-dependent relationship. This inference is further strengthened considering the results of Helmestam and coworkers who reported on the lack of a dose-dependent response after exposing endometrial endothelial cells to different concentrations of cadmium chloride, where the expression of VEGF-A was measured [16].

Taken together, the findings of this study indicate the ability of Cd to induce proliferation of ESC, which was prominently observed in women with endometriosis. Cadmium probably induced these effects in ESC from women with endometriosis by acting through the ER. The lack of similar effects in women without endometriosis is perhaps partly attributable to the differences in the ER levels in the eutopic endometria of the two groups, where higher numbers of ERs are expressed in women with endometriosis [33,34]. However, confirmation of the above requires the demonstration that an antiestrogen such as fulvestrant will abrogate the observed changes secondary to Cd treatment in ESC from women with endometriosis. In addition, increased Cd deposition, initially in ESC of women with endometriosis, is another possibility that has to be entertained in interpreting the findings of this study. Ideally, the levels of Cd in the ESC monolayers should have been measured before Cd treatment in the present study. Lack of such data, a limitation of the present study, was due to the fact that the detection limits of the available instruments were insufficient to measure minute amounts of Cd that may have been present in the cell culture samples.

In conclusion, the results of this study demonstrate the ability of Cd to induce the proliferation of stromal cells derived from the eutopic endometrium of women with endometriosis. As women are susceptible to ill effects of Cd due to widespread environmental pollution, further research is recommended to shed more light on the role of Cd on estrogen-dependent gynecological diseases, especially endometriosis.

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