Article

Impact of high body mass index on endometrial morphology and function in the peri-implantation period in women with recurrent miscarriage



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Abstract

There is evidence that women with a high body mass index may have a higher risk of miscarriage. It is not known if this is due to an endometrial or embryo defect. The aim of this retrospective study was to examine markers of endometrial function in overweight and obese women with recurrent unexplained miscarriage. A total of 136 women were included in the study and classified according to their body mass index (BMI) into two groups, normal BMI (<25 kg/m^2, n = 70) and high BMI (≥25 kg/m^2, n = 66). Endometrial morphology was examined in all patients. A subgroup of 28 patients was examined for endometrial oestrogen and progesterone receptors in different components of the endometrium, and in a further subgroup of 28 patients, endometrial glandular leukaemia inhibitory factor and leukocyte populations were examined. A modest increase in the BMI (30.4 ± 0.71 kg/m^2) does not have a significant impact on endometrial steroid receptors, leukocyte populations or endometrial morphology. However, there was a significant negative correlation between endometrial glandular leukaemia inhibitory factor concentrations and the BMI (r = –0.4, P = 0.02), warranting further investigation in prospective studies that include patients with higher BMI levels.

Keywords: *BMI, body mass index, endometrium, miscarriage, obesity*

Introduction

Obesity is a significant health problem and is associated with a diverse set of metabolic disorders with reproductive consequences that are complex and not well understood. The adverse effect of obesity on female reproductive function has even been suggested since the time of Hippocrates (Franks, 2006). In pregnancy, it is recognized that obese women are subject to a number of adverse maternal and fetal outcomes (Davies, 2006). There is growing evidence that obesity (BMI ≥30 kg/m^2) is linked with an increased risk of miscarriage, with the risk being as high as 25–37% before the first live born child (Hamilton-Fairley *et al*., 1992). Overweight patients (BMI 25–29.9 kg/m^2) have also been reported to have a higher risk of miscarriage (Hamilton-Fairley *et al*., 1992). This adverse effect of obesity has been suggested in natural and assisted conceptions. As an example, Bellver *et al*. (2006) examined 712 cycles of embryo transfer using donor oocytes. They found that the rate of spontaneous miscarriage increased as the body mass index (BMI) increased; the risk of miscarriage was 38.1% for obese women (BMI ≥ 30 kg/m^2) and 15.5% for overweight women (BMI ≥ 25–29.9 kg/m^2) compared with 13.3% in patients with a normal BMI. Several other studies have shown similar findings (Bussen *et al*., 1999; Wang *et al*., 2002).

Although the evidence for a relationship between increased BMI

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and recurrent miscarriage (defined as three or more consecutive miscarriages) is deficient and more studies are needed, one large retrospective study has suggested that a relationship may exist (Lashen *et al.*, 2004). In this study, data from 1644 obese women were matched with 3288 controls with normal body mass index (BMI). A higher risk of early, late and recurrent miscarriage was found in the obese group. It is possible that the increased risk of miscarriage in obese women is a consequence of poor embryo quality, deranged endometrial function or a combination of both.

The aim of this retrospective study was to determine whether high BMI has an adverse effect on endometrial function by investigating the relationship between increased BMI and endometrial function in a group of women with recurrent miscarriage.

Materials and methods

Subjects

A total of 136 women diagnosed with unexplained recurrent miscarriage and who underwent an LH-timed endometrial biopsy in the mid-luteal phase, were identified from the clinical database of the recurrent miscarriage clinic at the Jessop Wing, Royal Hallamshire Hospital. At the time these patients were seen in the recurrent miscarriage clinic, they were all fertile and ovulatory. The study was approved by the local ethics committee.

Investigations

All the subjects underwent investigations according to an established clinical protocol (Li, 1998), including: prothrombotic studies and antiphospholipid antibody screen, maternal and paternal karyotyping, thyroid function tests and thyroid antibodies, androgen profile (total serum testosterone, sex hormone binding globulin and free androgen index), day 2 FSH, LH and oestradiol, transvaginal ultrasonography, hysterosalpingography and LH-timed Pipelle endometrial biopsy. Hysteroscopy was only performed if an abnormality was suspected on pelvic ultrasound or hysterosalpingography.

Inclusion criteria

Only patients with unexplained recurrent miscarriage were included. Unexplained recurrent miscarriage was defined as the occurrence of at least three consecutive miscarriages with the absence of the following (Li *et al.*, 2000): parental chromosomal abnormalities, acquired or hereditary thrombophilias, or structural uterine abnormalities.

Body mass index (BMI)

The BMI was determined from the hospital records, where body weight and height are routinely recorded at the first clinic visit, usually within 4 weeks of the endometrial biopsy. Overweight was defined as having a BMI of 25–29.9 kg/m^2, and obesity was defined as having a BMI of 30 kg/m^2 or more (Speroff and Fritz, 2005).

LH-timed endometrial sampling

Daily urine testing for LH was performed from the mid-follicular phase onwards until the LH surge was detected. Seven to 9 days following the LH surge, an endometrial biopsy was obtained with the use of a Pipelle sampler (Prodimed, France). The biopsy was immediately fixed in formalin, left overnight and then automatically embedded in paraffin wax. Sections were cut at 5 mm, hydrated, stained with haematoxylin and eosin and the histology dated according to the criteria described by Noyes *et al.* (1950). Retarded endometrial development was diagnosed if histological dating lagged 2 days or more behind chronological dating (Li and Cooke, 1991). Endometrial samples in this study were previously examined for the study of markers of endometrial function in women with unexplained recurrent pregnancy loss (Tuckerman *et al.*, 2004).

Immunocytochemistry

Immunocytochemistry was performed as previously reported by Tuckerman *et al.* (2004). In 28 cases, immunocytochemistry was performed for oestrogen and progesterone receptors in the various components of the endometrium, namely stromal, epithelial glandular and epithelial luminal. Leukaemia inhibitory factor (LIF) expression and analysis of various endometrial leukocyte populations (CD45, CD56, CD3 and CD4) was carried out in a further subgroup of 28 patients.

Immunocytochemistry was performed on de-waxed, re-hydrated 5 mm tissue sections. Antigen unmasking was carried out in a microwave oven and incubation with primary antibodies was performed overnight at 4°C; all other procedures were performed at room temperature. Sections were then quenched in 0.3% hydrogen peroxide in methanol for 20 min. Antigen unmasking was then performed in an 800 W microwave oven; all antigens except CD4 were unmasked in 10 mmol/l citrate buffer, pH 6.0. The buffer was heated in the microwave oven until boiling. Slides were then added to the buffer and left at high heat for 2 min. The slides were then incubated for 12 min at medium heat and left to cool in buffer for 20 min. CD4 was unmasked by heating slides uncovered in 1 l of 0.1 mol/l EDTA pH 8.0 on maximum heat for 30 min, and then allowing the slides to stand in the heated EDTA for 20 min before washing. All slides were blocked for 1 h in the appropriate blocking buffer, which contained 250 μl avidin/ml. Slides were incubated overnight with primary antibody containing 250 ml of biotin/ml. Mouse IgG was substituted for primary antibody in negative controls for mouse anti-human antibodies. In negative controls for rat and goat anti-human antibodies, primary antibody was omitted. Details of the primary and secondary antibodies have been previously described (Tuckerman *et al.*, 2004), and are shown in **Table 1**. Slides were washed twice in either phosphate-buffered saline or Tris-buffered saline between each incubation. Binding was visualized by incubation with peroxidase substrate DAB (3,3'-diaminobenzidine tetrahydrochloride; Vector Laboratories, UK). Sections for CD4 were incubated with DAB for 12 min, and all other sections were incubated for 8 min. Slides were then washed in distilled water for 5 min and stained with 10% haematoxylin (Vector) as follows: oestrogen receptor and progesterone receptor for 20 min, CD45, CD56, CD3, CD4 and LIF for 10 min. Slides were then differentiated, dehydrated, cleared and mounted in DPX.

Table 1. Details of primary and secondary antibodies (Tuckerman *et al.*, 2004).

Antigen	*Primary antibody*	*Secondary antibody system*
Oestrogen receptors	Monoclonal mouse anti-human ER (M7047; Dako, UK) diluted 1:200 in horse serum/PBS	Universal Elite ABC kit (Vector)
Progesterone receptors	Monoclonal mouse anti-human PR (Novacastra NCL-PGR-312; Vector) diluted 1:100 in horse serum/TBS	Universal Elite ABC kit
CD45	Monoclonal mouse anti-human CD45 (Dako, M0701) diluted 1:50 in horse serum/TBS	Universal Elite ABC kit
CD56	Monoclonal mouse anti-human CD56 (MCA 1814; Serotec, UK) diluted 1:100 in horse serum/TBS	Universal Elite ABC kit
CD3	Monoclonal rat anti-human CD3 (MCA 1477, Serotec) diluted 1:250 in 10% goat serum/TBS 9400 1:100 in 10% goat serum/TBS	Universal Elite ABC kit secondary antibody substituted + Vector BA-
CD4	Monoclonal mouse anti-human CD4 (MCA 1803; Serotec) diluted 1:20 in horse serum/TBS	Universal Elite ABC kit
LIF	Polyclonal goat anti-human LIF (AF-250-NA; R&D Systems, UK) diluted 1:25 in horse serum/PBS	Universal Elite ABC kit secondary antibody substituted + Vector BA-9500 diluted 1:50 in 2% horse serum/PBS

PBS = Phosphate-buffered saline, pH 7.5; TBS = Tris-buffered saline, pH 7.6; LIF = leukaemia inhibitory factor.

Leukocyte populations (CD+ cells)

The number of CD^+ cells was calculated as a percentage of the total stromal cell number in sections stained for CD cell populations (CD45, CD56, CD3 and CD4). The number of positive cells and the total number of stromal cells were counted in 10 high-power (×400) microscope fields by two independent observers.

Immunostaining and H-score

Oestrogen receptors, progesterone receptors and LIF staining intensity in tissue sections was graded, and assigned an H-score (Lessey *et al.*, 1996) calculated according to the following equation: H-score = $\sum pi\ (i + 1)$, where i = staining intensity (1 = weak, 2 = moderate, 3 = strong) and pi = percentage of cells staining at each intensity (0–100%). Each parameter was assessed separately in the stroma, luminal and glandular epithelium by two independent observers.

Data analysis

Patients were classified into two groups based on the BMI; normal (BMI 19–24.9 kg/m^2) and high BMI (BMI ≥ 25 kg/m^2). Results for demographic factors were expressed in the form of the mean ± SEM, median, range, interquartile range, and percentages, as appropriate. The Mann–Whitney U and Student's t-tests were used for comparison between groups, as appropriate. The Pearson correlation coefficient was used to examine the relationship between the BMI and the following: oestrogen/progesterone receptor H-scores, CD45, CD56, CD3 and CD4 positive cells, and leukaemia inhibitory factor H-scores. The chi-squared test was used to compare between proportions. Statistical significance was set at $P < 0.05$ and analysis was performed using SPSS version 13.

Results

Patient demographics

Among the 66 women with a high BMI (BMI ≥ 25 kg/m^2), 43 patients had a BMI of 25–29.9 kg/m^2, 12 had a BMI of 30–34.9 kg/m^2 and 11 patients had a BMI of ≥ 35 kg/m^2 The demographic characteristics of both groups are shown in **Table 2**.

Outcome measures

Endometrial morphology

Among the 136 endometrial biopsies examined, 31 biopsies were found to have retarded histological development by ≥2 days (luteal phase defect). These included 15 endometrial samples from the normal BMI group and 16 from the high BMI group. Two-by-two contingency table analysis showed that there was no difference in the prevalence of luteal phase defect (LPD) between the two groups.

Correlation between BMI and immunocytochemistry

There was a significant negative correlation between glandular epithelial LIF H-scores and BMI ($r = -0.4$, $P = 0.02$) (**Figure 1**); however, there was no significant correlation between the BMI and any of the following: H-scores for progesterone receptors

in the stromal epithelium ($r = 0.2$), glandular epithelium ($r = -0.15$) or luminal epithelium ($r = -0.04$); H-scores for oestrogen stromal epithelial receptors ($r = 0.2$), glandular epithelial receptors ($r = 0.01$) or luminal epithelial receptors ($r = -0.01$); percentage of endometrial stromal CD45-positive cells (total leukocytes) ($r = 0.2$), CD56-positive cells (natural killer cells) ($r = -0.04$), CD3-positive cells (T-cells) ($r = 0.1$) or CD4-positive cells (helper T-cells) ($r = -0.01$).

Comparison of immunocytochemistry results between groups

The percentages of various CD+ cell populations ($n = 28$) and H-scores of endometrial oestrogen and progesterone receptors ($n = 28$) and LIF ($n = 28$) were compared between patients with normal BMI (< 25 kg/m^2) and high BMI (≥ 25 kg/m^2). The results are shown in **Tables 3** and **4**. There was no significant difference in any of the measurements between the two groups.

Table 2. Demographic data of the study groups.

	BMI < 25 (n = *70*)	*BMI ≥ 25* (n = *66*)
Mean age in years (± SEM)	33.0 (± 0.66)	33.2 (± 0.71)
Median no. of previous pregnancies (range)	4.0 (3–13)	4.0 (3–15)
Median no. of previous miscarriages (range)	3.0 (3–13)	3.0 (3–14)
No. with past history of infertility (%)[a]	24 (36.4)	23 (37.1)
No. with primary miscarriage (%)	41 (58.6)	35 (53.0)
No. with secondary miscarriage (%)	29 (41.4)	31 (47.0)
Mean BMI (kg/m^2) (± SEM)	22.2 (± 0.24)[b]	30.4 (± 0.71)[b]

BMI = body mass index; [a]Data missing from four cases; [b]$P < 0.0001$.

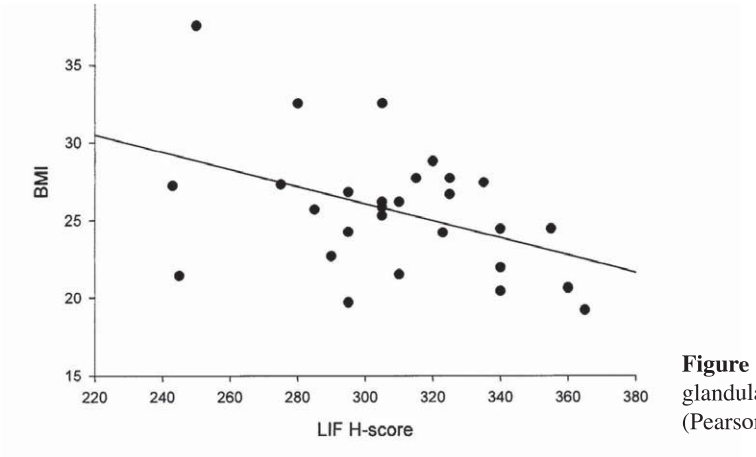


Figure 1. Correlation between the body mass index (BMI) and glandular epithelial leukaemia inhibitory factor (LIF) H-scores (Pearson correlation, $r = -0.4$, $P = 0.02$).

Table 3. Comparison of the various immunocytochemical measurements of oestrogen and progesterone receptor H-scores between the study groups (median and interquartile range).

Receptor	*H-score*	
	BMI < 25 (n = *11*)	*BMI ≥ 25* (n = *17*)
Stromal progesterone receptors	275.0 (70.0)	295.0 (63.0)
Glandular progesterone receptors	250.0 (155.0)	215.0 (140.0)
Luminal progesterone receptors	220.0 (192.0)	210.0 (158.0)
Stromal oestrogen receptors	113.0 (130.0)	125.0 (130.0)
Glandular oestrogen receptors	140.0 (160.0)	110.0 (148.0)
Luminal oestrogen receptors	110.0 (100.0)	135.0 (145.0)

There were no statistically significant differences between the two study groups.

Table 4. CD populations (median and interquartile range), expressed as a percentage of total stromal cell count in 10 high-power fields, and endometrial glandular leukaemia inhibitory factor (LIF) H-scores (median and interquartile range) in the study groups.

	BMI < 25 (n = *12*)	*BMI ≥ 25* (n = *16*)
LIF H-score	331 (56)	305 (38)
CD45 (% of total stromal cells)	23.6 (6.30)	21.0 (9.40)
CD56 (% of total stromal cells)	8.2 (6.90)	8.3 (5.0)
CD3 (% of total stromal cells)	4.4 (3.60)	3.9 (2.34)
CD4 (% of total stromal cells)	2.6 (1.8)	2.5 (1.2)

BMI = body mass index. There were no statistically significant differences between the two study groups.

Discussion

Whilst there is significant evidence that increased BMI is associated with a higher risk of spontaneous miscarriage, both after spontaneous and assisted conception, the exact mechanism of this adverse effect remains unclear. The question of whether the problem lies in the embryo or the endometrium remains a matter of controversy in the literature, as highlighted by a recent review article (Bellver *et al.*, 2006). The present study was concerned with the endometrial factor only.

There are several issues which need to be taken into account when analysing the results of this study. Firstly, the results of this study are based on a group with a modest increase in BMI (**Table 2**) and the number of patients with a BMI of ≥35 kg/m^2 was limited (11 subjects, 17% of subjects of the high BMI group); thus, it was not possible to perform a subgroup analysis of endometrial markers in this specific group of patients, which may have shown a more pronounced alteration in endometrial function compared with those with a lower BMI. Secondly, this was a retrospective study of archived endometrial samples that had already been studied for the various endometrial markers, thus limiting the number of markers that could be examined. It is therefore important to address this question further in an adequately powered prospective study.

Maternal age is also a very important confounding variable in any study on miscarriage; in this study, there was no difference in the mean age between the two groups, and therefore it was not an issue for concern, Nevertheless, it should be stressed that further studies should always account for this variable, and where a more diverse population is included, a study design in which participants are stratified by age should be employed.

Finally, it is important to note that endometrial expression of the markers examined in this study is dependant on the phase of the menstrual cycle, and therefore all the biopsies were taken in the mid-luteal phase and precisely timed after the LH surge, thus eliminating the possibility that the phase of the menstrual cycle acted as a confounding factor.

Leukaemia inhibitory factor (LIF)

In this retrospective study, no obvious impact for increased BMI on endometrial function was found in patients with recurrent unexplained miscarriage. However, a significant

negative correlation was found between endometrial glandular LIF concentrations and increasing BMI. It is apparent from **Figure 1** that patients with particularly high BMI levels had the most influence on the slope of the regression curve. This may indicate that changes in endometrial function, and particularly changes in LIF concentration, may only be evident in patients with more severe degrees of obesity. Again, this finding needs to be verified in future studies.

LIF is an important member of the IL-6 family of cytokines that has been shown to have an important role in the central inhibition of food intake and decrease in body fat mass (Beretta *et al*., 2002), mediated through the gp130 signal transducing receptor (Jansson *et al*., 2006).

In animals, endometrial LIF has been shown to be essential for implantation (Stewart *et al*., 1992). This may also be true for humans, since LIF concentrations peak around the time of implantation, and patients with unexplained infertility have been shown to have lower endometrial LIF concentrations (Laird *et al*., 1997; Lass *et al*., 2001; Aghajanova, 2004; Kralickova *et al*., 2006). This is the first study to report on the relationship between endometrial glandular LIF concentrations and BMI, particularly in patients with recurrent miscarriage.

Although the results of LIF expression are significantly related to BMI ($r = –0.4$, $P = 0.02$), there were no differences in the expression of endometrial oestrogen or progesterone receptors between the studied groups. The results therefore suggest that the impact of BMI on endometrial expression of LIF is not mediated through oestrogen or progesterone receptor pathways. Alternative pathways are therefore likely to be involved, including androgen receptors, leptin, or other pro-inflammatory cytokines such as interleukins (IL-1β and IL-γ), tumour necrosis factor-α, insulin-like growth factor (IGF)-I and II and transforming growth factors (TGF-β and TGFα) or human chorionic gonadotrophin (Sawai *et al*., 1997; Bulletti *et al*., 2005; Kimber, 2005). Furthermore, it is not known if this beneficial role for LIF on implantation is mediated through genomic or non-genomic mechanisms. It is therefore postulated that LIF may act as the missing link between the body fat compartment and the endometrium in this group of patients, and this hypothesis needs to be tested in future larger studies.

Endometrial morphology

For some time, luteal phase defect (LFD) has been considered as a possible contributing factor to recurrent miscarriage (Li and Cooke, 1991; Tuckerman *et al*., 2004). Li *et al*. (2000) reported a significantly higher prevalence of LPD in patients with recurrent miscarriage (27%) compared with normal controls (11%). In this study, no evidence for a significant difference in the prevalence of LPD between patients with a normal BMI (21.4%) and those with a high BMI (24.2%) was found.

There has been much controversy about the role of LPD, mainly due to the large incidence of inter- and intra-observer variability when interpreting the results (Gibson *et al*., 1991). However, in the present study, all the endometrial samples were precisely timed based on the LH surge and examined by experienced histopathologists, thus minimizing the risk of error.

Endometrial steroid receptors

The endometrium is responsive to the effects of the steroid hormones, namely oestradiol, progesterone and androgens, via receptors present in the tissue (Giudice, 2006). The metabolism of these steroid hormones is known to be affected by obesity, mainly as a result of the associated hyperinsulinaemia and decreased concentrations of sex hormone-binding globulin (Pasquali and Gambineri, 2006). Previous studies have shown that the concentration of endometrial oestrogen receptors may increase and progesterone receptor concentrations decrease in obese patients (Moran *et al*., 1996). This study, however, does not support these previous findings.

Uterine leukocyte populations

Obesity may represent a form of chronic inflammation. As body mass increases, a relative state of hypoxia occurs within the clusters of adipocytes, evoking an inflammatory response and resulting in the release of various substances including, cytokines, chemokines and acute-phase proteins (Trayhurn and Wood, 2004; Tsai *et al*., 2006). There is also evidence that obesity is associated with a state of peripheral reactive leukocytosis (Herishanu *et al*., 2006). A number of recent studies have suggested that uterine natural killer cells (uNK cells), which are the predominant leukocytes present in the endometrium, are increased in the endometrium of patients with recurrent miscarriage (Quenby and Farquharson, 2006; Rai and Regan, 2006). The results of this study, however, showed that women with a high BMI do not have a significantly altered profile for uNK cells or for any of the other measured leukocyte subpopulations, compared with women with normal BMI.

In conclusion, this study has shown that a modest increase in BMI is not associated with any clear effect on endometrial morphology, steroid receptors or leukocyte populations; however, a significant negative correlation was demonstrated between endometrial glandular LIF and BMI. A well-powered prospective study is needed to further confirm these results, especially in patients belonging to the higher BMI classes. If future studies confirm the findings that LIF is indeed a key factor in pregnancy failure in obese patients, then it would make a strong case for recommending weight loss to obese patients with recurrent pregnancy loss prior to contemplating further pregnancies.

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