

**BRUCE EFFECT: IMPLANTATION, LUMINAL AREA, E-CADHERIN**

NOVEL-MALE INDUCED PREGNANCY FAILURE IN MICE: EFFECTS ON  
IMPLANTATION, LUMINAL AREA AND E-CADHERIN

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**TITLE:** Novel-male induced pregnancy failure in mice: effects on implantation, luminal area, and e-cadherin

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## **ABSTRACT**

Adhesion of the blastocyst to the uterine wall is a highly sensitive phenomenon referred to as implantation. Novel-males are capable of disrupting the success of this process (the Bruce effect). A leading hypothesis invokes the transfer of estradiol from the male to the female via urine. This estradiol has direct effect on the uterus which may include morphology and molecular dynamics. Estradiol has been related to closure of the uterus around the blastocyst during implantation, which may assist in bringing the blastocyst close to the uterine wall for strong adhesion. E-cadherin, a cellular adhesion molecule, is found on both blastocyst and uterine surfaces and has been suggested to be involved in their interaction during implantation. Estradiol has been observed to reduce e-cadherin expression in hormonally sensitive tissues like the mammary glands, ovaries and uteri. Here, male-induced disruption of implantation was examined across days 2-8 of gestation. Luminal area was quantified in isolated and male-exposed females as a measure of extent of luminal closure. This area was larger in male-exposed animals. E-cadherin was found to have reduced expression on luminal epithelial cells. I suggest that the reduction in e-cadherin may lead to weaker attachment of the blastocyst to the uterine wall as well as reduced adhesion between opposing uterine walls leading to the “opening” of the uterus observed in male exposed animals. Together, these data may in part explain the blastocyst implantation failure observed in male-exposed animals during the Bruce effect.

## **ACKNOWLEDGMENTS**

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## **DECLARATION OF ACADEMIC ACHIEVEMENT**

All of the research included in this research project was organized and conducted by Nazanin Rajabi under the supervision of Dr. D deCatanzaro. Undergraduate students Marta Sadkowski and Paige Burgess assisted in uteri collection and cell counting procedures, respectively.

## **INTRODUCTION**

### **The Bruce Effect**

Hilda Bruce (1959, 1960) observed a phenomenon by which newly inseminated females lost their pregnancy when exposed to novel males. At first, it was suggested that the Bruce effect is mediated through the males' odor, since pregnancy loss could be prevented with the disruption of the female's olfaction (Parkes and Bruce, 1962). Further support for this theory was the fact that only novel males, and more successfully males of a different strain from those of the female, were able to disrupt pregnancy (Parkes and Bruce, 1962). This theory, referred to as the olfactory memory hypothesis, suggests that the reason why early pregnancy is not disrupted by sire males (i.e. the mating partner of the female) is that he is recognized by the female from his urinary chemosignals (reviewed in Brennan 2004). There is evidence that male urinary chemosignals may be sensed by the female's vomeronasal organ (VNO) and then transformed into a neural signal which has been hypothesized to influence prolactin dynamics and thereby disrupt pregnancy (reviewed in Brennan 2004).

An early study (Marchlewska-Koj, 1981) reported that early pregnancy could be blocked simply by exposing the nasal area of inseminated females to peptides fractioned out of the urine of male mice. More recently, major histocompatibility complex (MHC) peptides found in the urine of males have been suggested to be involved. When these peptides are transferred from the urine of novel-strain males to that of males of the same strain as the female, the ability to disrupt pregnancy is significantly enhanced (Leinders-Zufall et al., 2004). Such experiments were unsuccessful when these peptides were administered to the female in a non-urine medium however, suggesting that independently, they were not responsible for the disruption of early pregnancy.

Since urine alone from the conspecific male has been shown to be capable of inducing the Bruce effect (Parkes and Bruce, 1962), and castrated males cannot induce the Bruce effect (Bruce, 1965; Vella and deCatanzaro, 2001), it is likely that some androgen-dependent constituent of the urine may be in play. Increasingly, evidence indicates that transfer of estrogens via male urine to female reproductive organs may in part be mediating the Bruce effect. Our laboratory has shown that intranasally administered estradiol (a potent estrogen), in doses as low as 0.014 µg per day on days 2-4 of gestation, significantly reduced proportion of births in mice (deCatanzaro, Baptista and Vella., 2001; deCatanzaro, Beaton, Khan and Vella, 2006). Urinary estradiol in male mice rises during the exposure to inseminated females (deCatanzaro et al., 2006). Polydipsia (excessive thirst) and polyuria (frequent urination) are also experienced by males in proximity to inseminated females (deCatanzaro, Khan, Berger and Lewis, 2009). Castration of males leads to gradual reduction in urinary testosterone (a precursor to estradiol) which coincides with a loss of capacity to block pregnancy (Vella and deCatanzaro, 2001). Daily administration of estradiol to these males makes them able to disrupt pregnancy once again (deCatanzaro, Smith and Muir, 1995; Thorpe and deCatanzaro, 2012). Replacement of androgens in castrates restores this capacity as well (Rajendren and Dominic, 1988), while androgen implants in ovariectomized females give them the ability to disrupt pregnancy in other females (Rajendren and Dominic, 1988). These studies suggest that estradiol from the male can have deleterious effects on pregnancy in the female.

Administration of estradiol antibodies to male-exposed females led to pregnancy rates which were comparable to isolated controls (deCatanzaro, Muir, O'Brien and Williams, 1995). Strengthening this hypothesis is direct evidence of the transfer of estradiol from male to female conspecifics through urine. Radioactively labeled estradiol administered to males was traced in

male urine and was specifically in abundance in the reproductive tract of cohabiting females (Guzzo, Jheon, Imtiaz and deCatanzaro 2012). This estradiol at the uterus has important implications on the success and failure of implantation, the adhesion of the fertilized ova (blastocysts) to the uterine wall. While estrogen is required for preparation of the uterus for implantation, above optimal levels, it will induce a rapid end of the receptive period for implantation (Ma, Song, Das, Paria, and Dey, 2003).

### **Hormonal Control of Implantation**

Following fertilization in the fallopian tube, the ovum undergoes multiple cell divisions to mature into a blastocyst (Wang and Dey 2006). The trophoblast cells, which line the outer surface of the mature blastocyst, make contact with the wall of the female uterus in a process referred to as implantation. A brief state of uterine receptivity to the blastocyst is determined by the coordinated actions of estrogen and progesterone on the evening of the 3<sup>rd</sup> day of gestation in the mouse (reviewed in Dey, Lim, Sanjoy, Das, Reese, Paria, Daikoku and Wang, 2004). Implantation can only occur within this “window of receptivity”, after which the uterus becomes refractory to blastocyst attachment (reviewed in Kimber and Spanswick, 2000). Thus, implantation is a highly sensitive process dependent on coordinated dynamics of steroid hormones and their associated actions.

During the first step of the implantation reaction, apposition occurs in which the trophoblast of the blastocyst gains proximity to the uterine wall. The lumen of the uterus is usually filled with fluid, but in response to progesterone (Mayer, Nilsson and Reinus, 1967), epithelial cells lining the uterus remove fluid through pinocytotic activity (Bansode, Chauhan, Makker and Singh, 1998). This area is reduced, in return, as the walls of the uterus “close” around the implanting blastocyst (Mayer et al., 1967). Uterine closure has repeatedly been

suggested to mediate blastocyst apposition by bringing it closer to the attachment site (Salleh, Baines, Naftalin and Milligan, 2005; Finn and Martin 1969; Mayer et al., 1969). Simultaneously, progesterone primes the lining of the uterine cavity by promoting cell proliferation and vascularization in a process known as decidualization (Huet-Hudson and Dey, 1990). The luminal epithelium is considered the site of hormonally controlled receptivity for implantation since without its preparation by progesterone, implantation cannot occur and this can only be mediated with manual damage to the surface epithelium (Cowell, 1969). Estrogen has been observed to facilitate transport of the pre-implantation embryo from the oviduct to the uterus (Roblero and Garavagno, 1979) however its role in implantation is further evident at the site of blastocyst attachment.

Pinopodes, visualized in a wide variety of species including mice, rats and humans, are uterine apical protrusions which increase uterine surface area and are specified markers of uterine receptivity and pinocytotic activity (*e.g.* Psychoyos and Mandon, 1971; Ferenczy, Richart, Agate, Purkerson and Dempsey, 1972). Under normal conditions, pinopodes appear just prior to implantation, a phenomenon that can be mimicked by through administration of physiologically typical doses of estradiol to progesterone-primed uteri in rats (Martel, Monier, Roche and Psychoyos, 1991). Once attachment is successful, the endometrium can be penetrated by the blastocyst and the trophoblast cells can embed themselves in the stromal cells located below the surface epithelium of the uterus (Wang and Dey, 2006). Optimal levels of estrogens on progesterone primed uteri are responsible for increasing chances of successful implantation through morphological changes (*i.e.* pinopodes, luminal closure) as well as by promoting expression of cell adhesion molecules involved in blastocyst attachment during implantation (Guliver and Hurst, 2012).

## **E-Cadherin**

Epithelial-cadherin (e-cadherin) is part of a larger family of calcium-dependent adhesion molecules. It is associated with intercellular junctions between epithelial cells, cell sorting, inhibiting apoptosis and implantation (reviewed in Rowlands, Symonds, Farookhi and Blaschuk, 2000). E-cadherin's dynamic expression between uterine cells throughout all the stages of implantation has been studied in a range of species. Forced expression of e-cadherin in non-receptive human endometrial epithelial cells *in vitro* causes a significantly enhanced receptivity for embryo attachment (Rahnama, Thompson, Steiner, Shafiei, Lobie and Mitchell 2009). In the rat, a ubiquitous probe for several classes of cadherins was used to follow the migration of these molecules from basal to apical membranes of luminal epithelial cells in preparation for implantation (Hyland, Shaw, Png, and Murphy, 1998). In the mouse, e-cadherin has been observed on both opposing surfaces that are in contact during the implantation reaction - on trophoblast cells of the blastocyst as well as on the luminal epithelial cells throughout the uterus (Kadokawa et al., 1989). It is expressed on the mouse uterus with the initiation of implantation (Paria, Zhao, Das, Dey and Yoshinaga, 1999). The co-localization of this molecule both temporally and spatially with uterine-blastocyst interaction is evidence for its involvement during the process of implantation.

Adhesion molecules have been observed to be regulated by several steroid hormones that are present during implantation. Hormonal regulation of e-cadherin is most often studied in mammary carcinoma cells that are estrogen receptor  $\alpha$  positive as are epithelial cells of the uterus. In these cells, e-cadherin is down-regulated by administration of estrogen, and reversal of this effect can be achieved through administration of anti-estrogen drugs (Osterreich et al., 2003). Since estrogens are non-polar steroidal hormones, they can easily pass through the cell

membrane to bind with their cytoplasmic receptors, at which point they may enter the nucleus to regulate gene transcription (reviewed in Heldring et al., 2007). The antagonistic effect of estrogen on e-cadherin is explained by the observation that following estrogen administration, there is suppression of e-cadherin's promoter activity which leads to decreased transcription of this adhesion molecule (Park, Cheung, Wong and Leung 2008). As well, several genes that naturally inhibit e-cadherin, are transcriptionally activated further reducing e-cadherin expression (Park, Cheung, Wong and Leung 2008).

E-cadherin's role in the uterus has been studied, with virtually indistinguishable results from those seen in other extra-uterine cells. In general, estrogens are observed to down-regulate e-cadherin in the uterus while progesterone opposes this action (Guo, Han, Tian, Zhang, Jiang, Liu and Yue 2010; Potter, Gaza and Morris, 1996; Jha, Titus, Saxena, Kumar and Laloraya, 2006). Down-regulation of e-cadherin follows administration of estradiol to a progesterone primed uterus (Jha et al., 2006). As well, administration of a progesterone receptor antagonist which would block the action of progesterone is found to lead to a further reduction in e-cadherin (Jha et al., 2006). This finding suggests that progesterone promotes e-cadherin expression in the uterus. The actions of estrogen on e-cadherin expression may be more complicated, however. In the mouse, e-cadherin is degraded in estradiol-injected ovariectomized mice (Potter et al., 1996), but in older mice, while a single injection of estradiol increases e-cadherin expression, repeated injections lower it on the ovarian surface epithelium (Guliver and Hurst, 2012). The definite relationship between estrogen and e-cadherin in the uterus makes this adhesion molecule a suitable candidate in the study of implantation failure.

A recent study looked at the effect of an estrogen-like molecule, bisphenol-A, on various uterine dynamics during implantation failure (Berger, Foster and deCatanzaro, 2010). An

increase in luminal area representing extent of luminal closure was observed on the 6<sup>th</sup> day of gestation in response to administration of bisphenol-A (Berger et al., 2010). Here we wanted to investigate whether this change in luminal area is reproducible in animals under the influence of the Bruce effect. This prediction was based on evidence that the Bruce effect is mediated through estrogenic impact on implantation like that which is seen with bisphenol-A exposure.

The aim of the current study was to add to the understanding of novel-male induced implantation failure (the Bruce effect) by studying a possible underlying mechanism based on prior knowledge of these estrogen-related changes in the uterus. We extended the observation of the Bruce effect over the first 8 days of gestation to explore dynamics across these days. Once implantation failure with novel-male exposure was confirmed, measures were taken of uterine luminal area, and the involvement of estrogen-regulated e-cadherin on luminal epithelial cells in both implantation success and maintenance of luminal closure was explored.

## **MATERIALS AND METHODS**

### **Animals**

Female subjects and inseminating males were CF-1 strain mice of stock originally obtained from Charles River Breeding Farms of Canada (St. Constant, Quebec). CF-1 female mice were aged 3-5 months and inseminating males were aged 2-6 months. Novel (stimulus) male mice were aged 5-10 months and were of heterogeneous strain (HS), derived from interbreeding C57-B6, Swiss Webster, CF-1 and DBA-3 strains originally obtained from Charles River Breeding Farms (St. Constant, Quebec). All animals were housed in standard polypropylene cages (28x16x11cm height) unless otherwise stated, with continuous access to water and food (8640 Teklad Certified Rodent Chow, Harlan/Teklad, Madison, WI, USA).

Rooms were maintained on a reversed 14h light: 10h darkness cycle at 21°C. This research was approved by the McMaster University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

### **Bruce Effect Protocol**

Sexually naive female mice were randomly paired with inseminating males. Following pairing, female hindquarters were inspected three times daily during the dark phase of the light cycle for the presence of a vaginal sperm plug. The day of sperm plug detection was designated as gestational day 0. Around the start of the dark phase of the next light cycle (gestational day 1), each female was randomly assigned to one of the experimental conditions and isolated in the lower chamber of a double-decker exposure apparatus (30x21x27cm height) previously described (deCatanzaro, Zacharias and Muir, 1996). Separated from the female below by a wire mesh grid, two stimulus males were each placed in one half of the upper chamber divided by an opaque plastic barrier. The opaque barrier prevented aggressive behaviour between stimulus males. In this way, the males' excretions could fall through to the female below but the males were not able to re-inseminate the female. Control females were housed in similar exposure cages with the above compartment left empty. All females were housed in exposure cages until immediately prior to sacrifice on one of days 2 through 8 of gestation.

### **Sample Collection**

Assignment to the gestational day on which the uterus would be collected was performed such that there was a random set of conditions on each day of uterine collection (i.e. on a certain day, there were control and experimental animals in any of their 2-8<sup>th</sup> day of gestation). Females were isolated and anaesthetized using isoflurane administered through a gas mask placed over the nasal area of each subject. Cardiac puncture was used to collect approximately 1 mL of blood

per mouse. The blood was allowed to clot overnight at 4°C and was centrifuged on the following day at 8000 rpm for about 12 minutes. The supernatant was collected and stored at -20°C until analysis could be completed. Terminal urine was collected from each mouse at cervical dislocation under anaesthesia. This urine was stored at -20°C for further analysis. Uteri from each animal were excised through a single abdominal incision and the total number of implantation sites in each uterine horn was counted by an investigator without knowledge of the experimental condition of the animal. Implantation sites were defined as spherical protuberances in either uterine horn (Berger, Shaw and deCatanzaro 2008).

### **Uterine Histomorphology**

Uteri excised from females were trimmed of mesentery and fixed in 10% neutral buffered formalin for at least 48h at 4°C. Several 5 mm pieces were taken from randomly chosen areas along the uterine horns of each individual regardless of the location of implantation sites. Uterine sections were embedded in paraffin and 5 µm thick sections were cut and mounted on glass slides. A random subset of these samples were deparaffinized in xylene, rehydrated in descending grades of ethanol and rinsed in phosphate buffered saline (PBS). One slide from each subject was stained with hematoxylin and eosin, dehydrated in graded ethanol solutions, cleared in xylene and mounted with Permount for bright-field microscopy. A single trained investigator blind to condition measured the area in a minimum of 3 sections for each subject using 2x field images in Image Pro Plus (v. 4.5 Silver Spring, MD). Luminal area for each individual was calculated based on the average of 3 measurements.

### **Immunohistochemical Staining**

Due to time constraints, a subset of undamaged samples was used for immunohistochemical staining. Slides were deparaffinized in xylene, rehydrated in descending

grades of ethanol and rinsed in phosphate buffered saline (PBS). To decrease nonspecific binding, these were incubated with normal goat serum in PBS for 1 hour in a covered humidified tray. This was followed by overnight refrigerated (4°C) incubation with e-cadherin (H-108 from Santa Cruz, Santa Cruz, CA, USA) polyclonal rabbit antibodies at a determined optimal dilution of 1:100. PBS was used as a negative control on each slide. On the following day, sections were incubated with biotinylated secondary antibody for 2 h in a covered humidified tray and incubated with avidin-biotin peroxidase complex for 2 h separated and followed by a PBS wash. A DAB solution of 50mg DAB dissolved in 200 mL PBS with 2 drops of H<sub>2</sub>O<sub>2</sub> was used to conduct the DAB reaction for 2 minutes. The reaction was terminated with distilled water and Carazzi's hematoxylin was used to counterstain. The sections were then dehydrated through graded ethanol solutions, cleared in xylene and mounted with Permount in preparation for bright-field microscopy. At a 10x field, images were digitally acquired and a blind investigator assessed presence or absence of staining on the apical border of luminal epithelial cells using an image analysis program (Image Pro Plus 4.5, Silver Spring, MD). The percent of positively stained cells was used to indicate the proportion of cells expressing the e-cadherin molecule.

### **Statistical Analysis**

Statistical analyses were completed using SPSS statistical software. Statistical significance was designated at the conventional level of  $p < 0.05$ . For each measure, factorial analysis of variance was used to examine effects of gestational day and exposure condition. Multiple pairwise comparisons were examined using Duncan's method. Number of implantation sites counted was also related to gestational day through a quadratic regression with associated  $R^2$  value tested with an  $F$ -test.

## RESULTS

### Implantation of the Blastocyst

The number of subjects in either isolated-control and male-exposed conditions are shown in Table 1. The average numbers of implantation sites in each condition from days 2-8 are shown in Table 2. Prior to day 4, no implantation sites were counted in either condition however the average numbers of implantation sites were found to increase in both isolated and male-exposed animals from days from days 4-8 (Figure1). The increase in implantation sites followed a significant quadratic trend,  $R^2= 0.438$ ,  $F(2,164)= 63.78$ ,  $p<0.001$ . Uteri excised on day 4 of gestation had observable implantation sites but their variable size and appearance made them difficult to count with confidence and so these counts were excluded from further statistical analyses. Factorial analysis of variance looking at days 5-8 showed a significant effect of day,  $F(3,98) = 4.155$ ,  $p= 0.008$ . Male-exposed females also had an overall lower number of implantation sites compared to isolated controls,  $F(1,98) = 6.47$ ,  $p =0.012$ . The interaction did not reach significance. Multiple comparisons showed that there were significantly fewer implantation sites in uteri of male-exposed animals excised on days 6 and 8 of gestation.

### Uterine Luminal Area

A random subset of uteri from the original set of subjects was stained for measurement of luminal area (see Table 3). Typical uterine slices from the 5<sup>th</sup> day of gestation in each exposure condition are shown in Figure 2. Uteri from day 8 of gestation were not measured due to tearing artifacts in samples however average luminal area measured on the remaining days of gestation

	<b>Day of Gestation</b>						
	2	3	4	5	6	7	8
<b>Isolated Control</b>	10	10	14	17	15	9	12
<b>Male-Exposed</b>	7	12	13	15	14	9	10

Table 1: The number isolated control and male-exposed animals. Gestational day refers to day in which uterus was excised.

	<b>Day of Gestation</b>						
	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>
<b>Isolated Control</b>	0±0	0±0	3±1.20	8.65±1.57	13.93±0.50	12.44±1.63	12.33±1.20
<b>Male-Exposed</b>	0±0	0±0	1.69±1.03	6.60±1.66	9.07±1.90	13.11±0.68	7±2.37

Table 2: The mean±S.E number of implantation sites. upon uterine excision on gestational days 2-8 in isolated control and male-exposed animals.

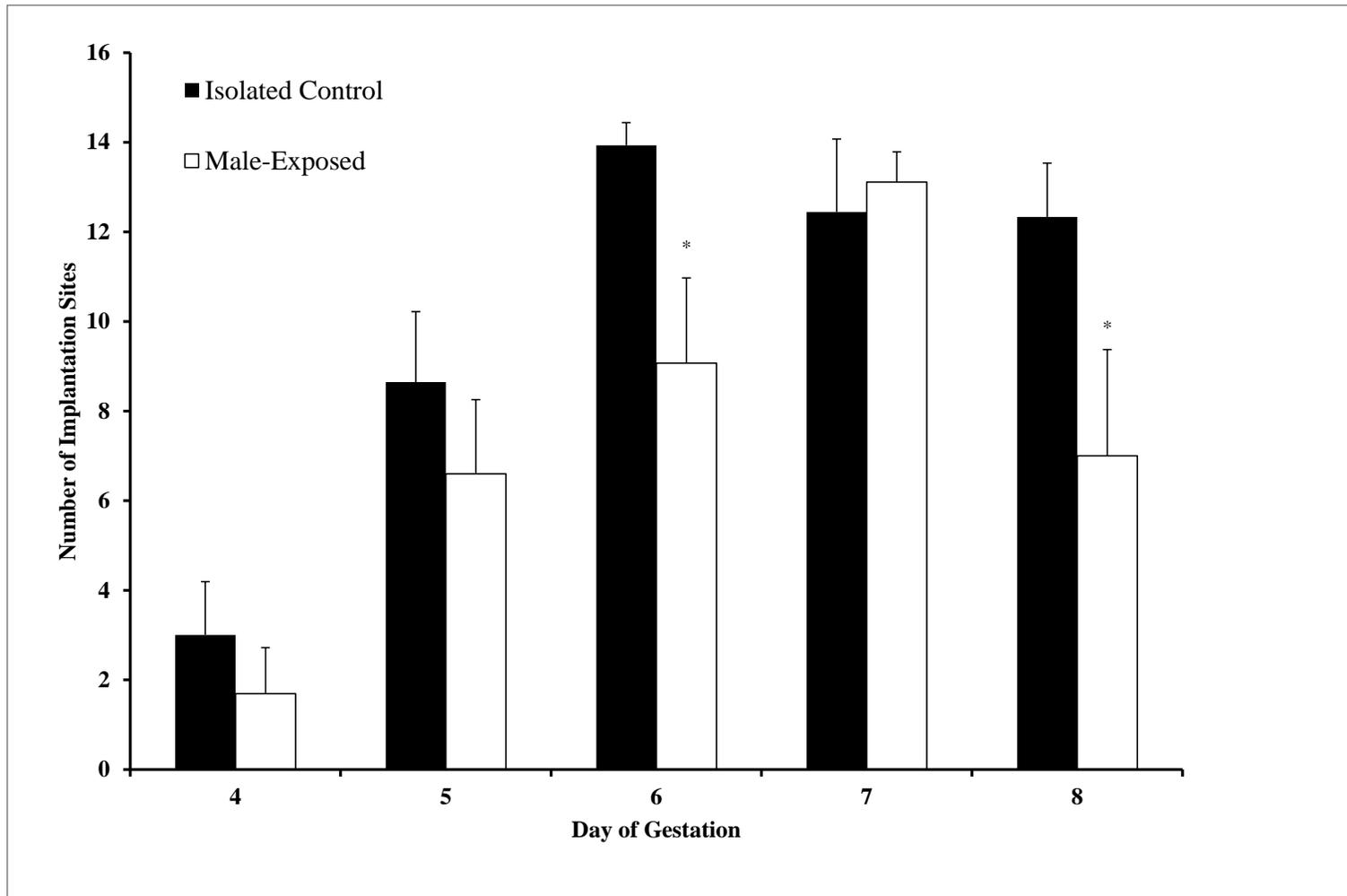


Figure 1: The mean±S.E. number of implantation sites on gestational days 4-8 in isolated control and male-exposed animals. There were fewer implantation sites in male-exposed compared to isolated control uteri. The number of implantation sites follows a quadratic trend regardless of exposure condition. \* Denotes significant differences from control condition on the same day of gestation.

	<b>Day of Gestation</b>					
	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
<b>Isolated Control</b>	7	6	8	3	5	5
<b>Male-Exposed</b>	7	6	5	7	5	4

Table 3: The number of isolated control and male-exposed animals from which uteri were excised for luminal area measurements on days 2-7 of gestation.

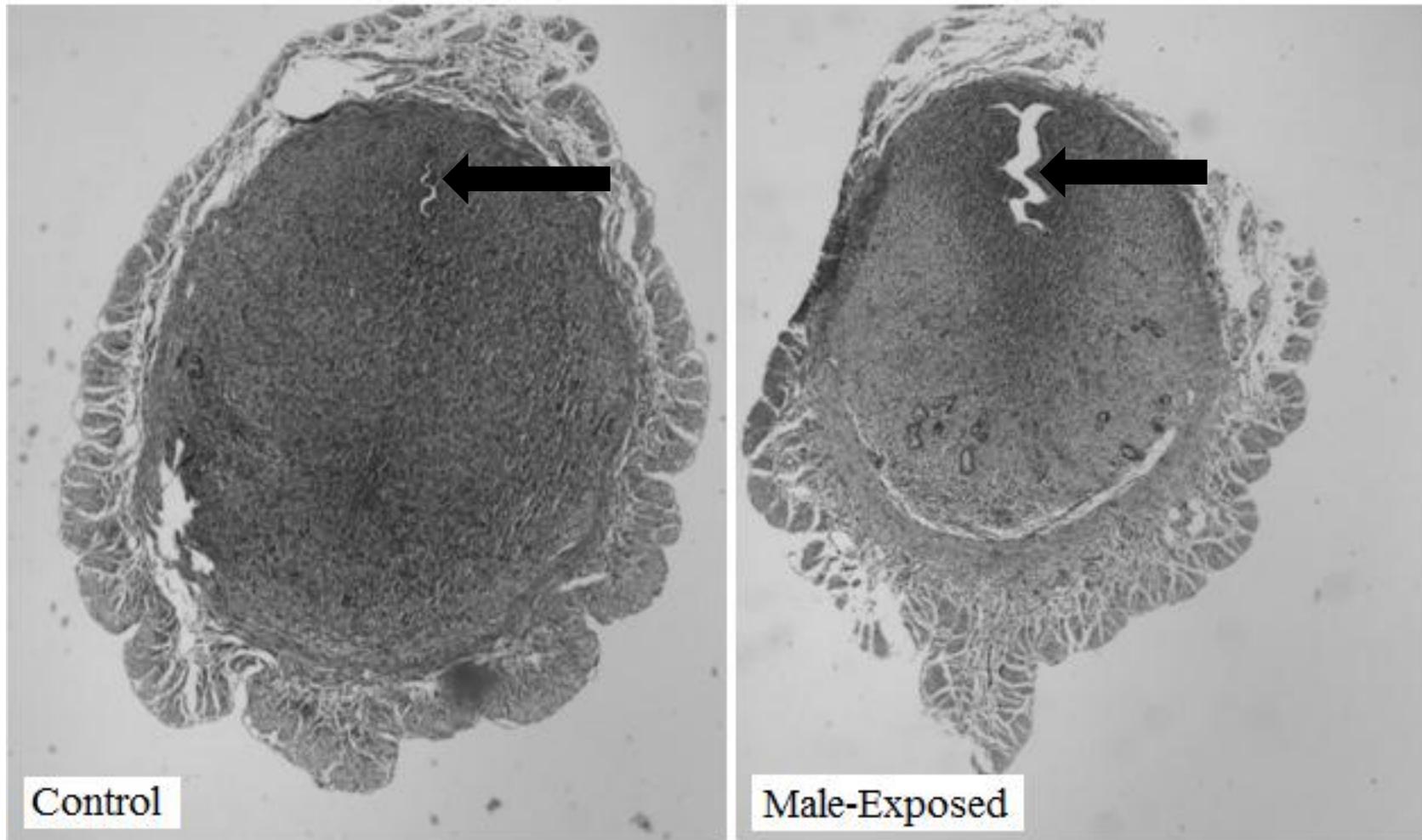


Figure 2: 2x magnification of representative Hemotoxylin and Eosin stained uterine sections from control and male-exposed uteri on day 5 of gestation. Arrows point to lumen of each uterus.

are shown in Table 4. Across both conditions, average luminal area decreased until day 5 of gestation. It remained constant until day 7 in controls and increased slightly in male-exposed animals. Pre-implantation days 2-4 had larger areas than did post-implantation days 5-7. Average luminal area was greater on all days except gestational day 4 in male-exposed as compared to isolated animals. Average luminal area measurements on post-implantation days 5-7 are shown in Figure 3. Average luminal area significantly differed across the two groups on these days,  $F(1,28) = 5.386$ ,  $p=0.03$ . Multiple comparisons on these days showed that luminal area in uteri from male exposed animals was significantly higher than in isolated animals on day 7 of gestation.

### **Apical Luminal E-Cadherin**

A subset of uteri collected from the original set of animals, which include but are not limited to those used to observe luminal area, were used to stain for luminal e-cadherin (see Table 5). Figure 4 shows typical stained uterine slices from the 4<sup>th</sup> day of gestation in each exposure condition. The proportion of apical luminal epithelial cells with positive staining for e-cadherin was not dependent on day of gestation at which counting was completed (see Table 6). The proportions of luminal epithelial cells that stained positively for e-cadherin on post-implantation days 4-7 are shown on Figure 5. The average proportion of e-cadherin positive cells was lower in uteri from male-exposed compared to isolated control females across all measured days, with analysis of variance approaching significance,  $F(1, 52) = 3.191$ ,  $p = 0.082$ . This effect reached significance in a separate analysis conducted on only the post-implantation period (gestational days 4-7),  $F(1, 23) = 5.657$ ,  $p= 0.026$ . Multiple comparisons indicated a significantly lower proportion of e-cadherin positive cells in gestational day 7 uteri of male-exposed animals.

	<b>Day of Gestation</b>					
	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
<b>Isolated Control</b>	0.11± 0.04	0.09±0.02	0.06±0.01	0.02±0.01	0.03±0.02	0.02±0.01
<b>Male-Exposed</b>	0.12±0.04	0.13±0.04	0.06±0.01	0.04±0.01	0.05±0.01	0.05±0.01

Table 4: Mean±S.E.luminal area (mm<sup>2</sup>) in uteri from isolated control and male-exposed animals on days 2-7 of gestation.

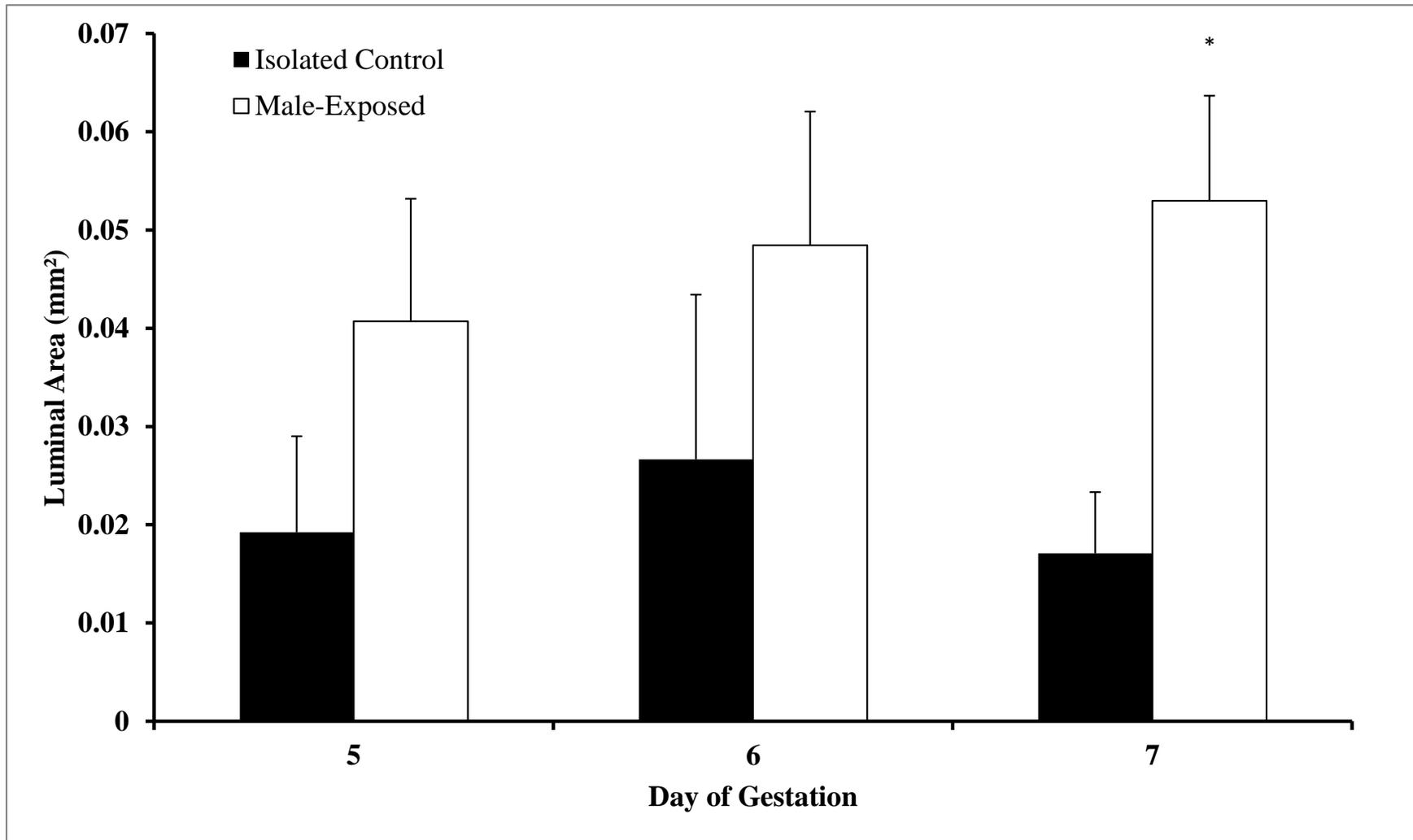


Figure 3: Mean±S.E. uterine luminal area on days 5-7 of gestation of control and male-exposed inseminated females. Luminal area in male-exposed animals is greater than that of isolated controls. \*Denotes significant difference with average luminal area in control animals of the same gestational day.

	<b>Day of Gestation</b>					
	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
<b>Isolated Control</b>	6	5	5	3	4	2
<b>Male-Exposed</b>	5	4	4	5	5	4

Table 5: The number of isolated control and male-exposed animals from which uteri were excised days 2-7 of gestation for immunohistochemical preparation to visualize e-cadherin.

	<b>Day of Gestation</b>					
	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
<b>Isolated Control</b>	30.12±7.88	41.62±14.41	42.14±6.58	34.09±16.36	57.07±8.73	51.72±11.02
<b>Male-Exposed</b>	26.59±7.01	46.60±14.48	26.76±10.87	34.62±12.11	21.68±5.45	33.98±5.81

Table 6: Mean±S.E. proportion of positively stained luminal epithelial cells. in uteri from isolated control and male-exposed animals on days 2-7 of gestation.

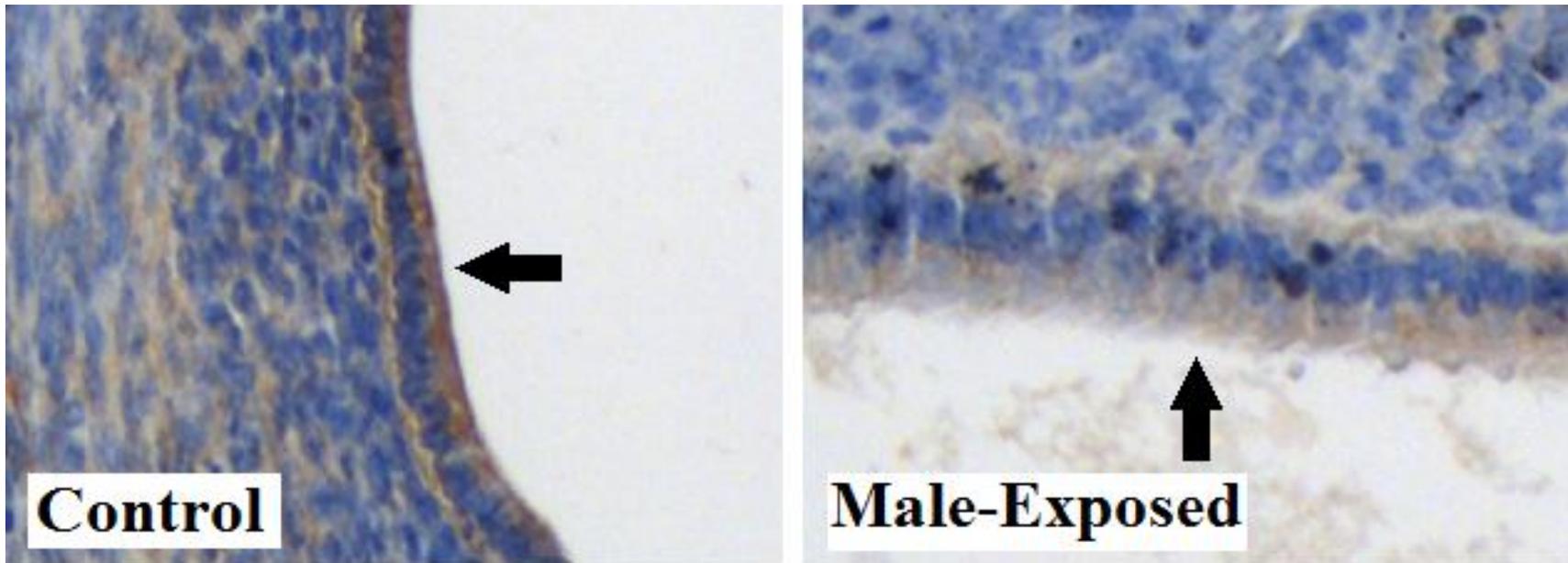


Figure 4: 10x magnification of representative control and male-exposed uteri on day 4 of gestation. Arrows point to apical border of luminal epithelial cells. Cells with brown staining are positive for E-cadherin. Note intensity of stain is not directly related to concentration of antigen.

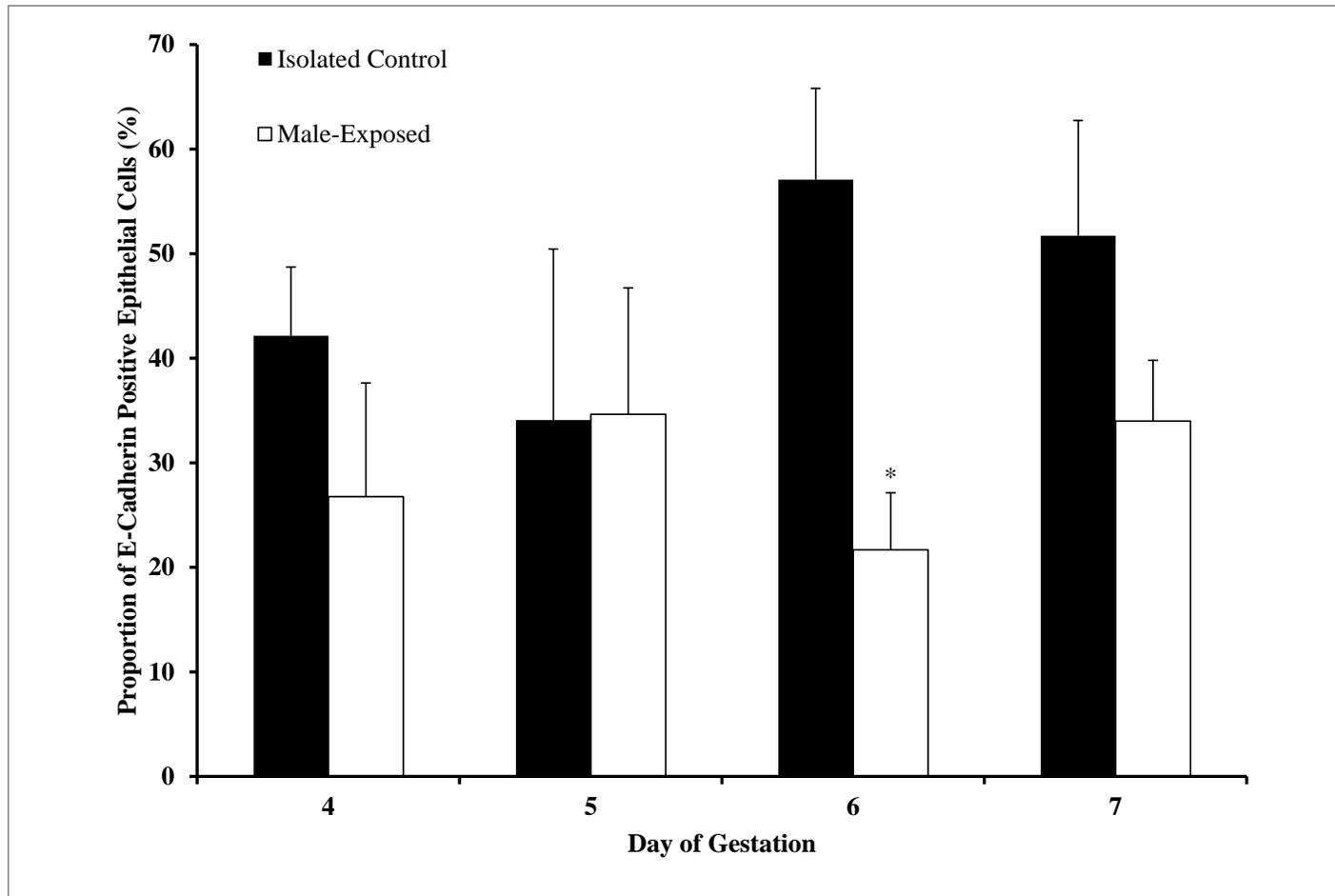


Figure 5: Mean±S.E. proportion of e-cadherin positive epithelial cells in uteri from control and male-exposed animals on gestational days 4-7. E-cadherin expression was diminished in male exposed animals on days 4-7. \*Denotes significant difference in average proportion of e-cadherin positive cells between male exposed and control animals of the same gestational day.

## **DISCUSSION**

Inseminated females were either isolated or exposed to novel males, and their uteri were measured on days 2-8 of gestation for differences in number of implantation sites, luminal area, and expression of e-cadherin on apical membranes of epithelial cells. There was a general increase in number of sites over the initial days that levelled out in controls by the 6<sup>th</sup> day of gestation, and there were fewer implantation sites in male-exposed animals as compared to isolated controls. Uterine luminal area was greater in females exposed to males. In all subjects, this area decreased until implantation day; however while in controls it remained constant following this day, an increase was seen in male-exposed animals. The proportion of epithelial cells expressing e-cadherin was greater in control compared to male-exposed animals, however there was no day-dependent pattern observed for this measure. The combination of these findings points to a plausible way in which novel males can block pregnancy in the Bruce effect.

### **Implantation of Blastocysts**

The Bruce effect can be characterized by the number of implantation sites observed in the uterus of male-exposed as compared to isolated females. Implantation sites are bumps along the uterine horn (i.e. points at which a blastocyst has attached and are visible to the naked eye) (Berger et al., 2008). Here I found fewer implantation sites in uteri of male-exposed females considered collectively across all days of post-implantation measurement. This result replicated the Bruce effect indicating that the number of implantation sites in a newly inseminated female is affected by the presence of a non-sire male (reviewed in deCatanzaro, 2011). Although there is no direct statistical comparison, the Bruce effect in the current experiment was arguably weaker than that previously seen this lab when females were allowed to bear their litters (e.g.

deCatanzaro et al.,1996; Beaton and deCatanzaro 2005), but closer to that seen when implantation sites were also counted (Thorpe and deCatanzaro 2012). Typical sample sizes required for demonstration of the effect are over 15 animals per condition, which would have been difficult to achieve across all the days of gestation studied here. Insofar as littering animals results in greater differences between male-exposed and isolated animals, some post-implantation disruptions to pregnancy could be involved in the Bruce effect. One novelty of the current study is the examination of implantation failure across the first 8 days of gestation, which includes both pre- and post-implantation phases. In most studies of the Bruce effect, implantation was only observed on the 6th day of gestation. Here the range of days in which implantation sites were counted was expanded to study peri-implantation dynamics in the uterus.

I found that the number of implantation sites visible to the naked eye increased in a quadratic manner in both male-exposed and isolated control conditions. Prior to the 4<sup>th</sup> day of gestation, no implantation sites were observed. After this day, the average number of these sites increased until gestational day 6, following which they remained significantly equal in controls and dropped until gestational day 8 in male-exposed animals. Only implantation site counts from days 4-8 were sufficient to study statistically. Gestational day 4 implantation sites were found to be small and presence or absence was difficult to discern with confidence. That early in gestation, it is possible that implantation has yet to occur in some animals that were inseminated later on the initial day. By the 6<sup>th</sup> day of gestation, it seems that all implantation sites are visible to the naked eye, confirming that this day is the first day on which this measure should be used. Significantly fewer implantations observed on the 8<sup>th</sup> day of gestation in male-exposed animals indicate the need to look at later days of gestation when studying the Bruce effect, as post-implantational effects may be at play. The replication and extension of the Bruce effect with this

measure gave the confidence needed to observe subsets of uteri in these animals for changes in luminal area and e-cadherin expression.

### **Uterine Luminal Area**

We found that the lumen of the uterus was most open on average across the days before any implantation sites were counted (days 2 and 3) in both control and male-exposed animals. Where the day of insemination is labelled as day 0 of gestation, implantation in the mouse is believed to occur on the evening of gestational day 3 (Ma et al., 2003), and days 2 and 3 are prior to the secure attachment of the blastocyst to the uterine wall. Luminal area is dynamic throughout the gestational days surrounding implantation in the rodent (Parr, 1983). Measurement of the luminal area was a method of quantitatively comparing extent of luminal closure. Luminal closure is described as the decrease in the lumen of the uterus caused in part by increased ability of epithelial cells to remove fluid from the lumen (pinocytotic activity) (Bansode et al, 1998). Such closure occurs immediately prior to embryo implantation and so has been suggested to assist in the apposition phase of implantation by decreasing the space available for the blastocyst within the walls of the uterus (Parr, 1983). In this way luminal closure is thought to help the blastocyst gain proximity with the site of implantation on the uterine wall (Salleh et al., 2005; Finn and Martin, 1969; Mayer et al., 1967). Consistent with this theory, prior to implantation, the lumen is still open and by the 4<sup>th</sup> day of gestation on which implantation likely occurred in most animals, the area of the lumen has decreased significantly to help strong attachment of the blastocyst. On the days following implantation (5-6) the luminal area remained consistently smaller than before. The closed form of the uterus in which no lumen is visible is that which has long been associated as the typical appearance of the pregnant uterus (Martin,

Finn and Carter, 1970; reviewed in Enders, 2010). On these days, implantation has occurred and the uterus remains closed around the growing blastocyst.

The uterine luminal area was measured in both isolated and novel-male exposed females. The area was found to be significantly greater in male-exposed animals on all but day 4, where it appeared to be equivalent in the two conditions. On the days after implantation, there was a trend in which the luminal areas in uteri of isolated females remained fairly stable, whereas in male exposed animals they become larger on each consecutive day of measurement. By day 7 of gestation, the average luminal area of male-exposed females was significantly greater than that of the control animals. The sensitive dynamics of estrogen and progesterone during the peri-implantation period make them likely participants in this observed transformation of the uterus (Parr, 1983). Closure can be achieved artificially with progesterone treatment alone or with the treatment of estrogen after progesterone priming in mice and rats (Martin et al., 1970, Parr 1983). However, slight excess of estrogen above optimal levels had been shown to be detrimental to implantation perhaps by inhibiting the closing process as is suggested here. In a previous study observing luminal closure in bisphenol-A treated animals, it was found that luminal area was greater in animals on day 6 of gestation when they had been exposed to the chemical on days 2-4 of gestation (Berger et al., 2010). As an estrogen-like molecule, bisphenol-A is able to mimic the Bruce effect and reduce proportion of implantation sites (Berger, Hancock and deCatanzaro, 2007). This lab has shown that estradiol transfer from male to females is the key to the male ability to block pregnancy as described in the Bruce effect (*e.g.* deCatanzaro et al., 2006, Guzzo et al., 2012, Vella and deCatanzaro, 2001). Some possible mechanisms by which estrogens exert actions like their influence on the rate of blastocyst transport, blastocyst mortality and slowed growth and decreased functionality have been explored (Ortiz, Villalon and

Croxatto, 1979; Roblero and Garavagno, 1979; Safro, O'Neill and Saunders, 1990). However, the effects that estrogens have on luminal closure present a novel approach to the relationship between estrogens and implantation failure.

Once attached to the endometrium, the blastocyst in a hormonally normal uterus is tightly surrounded by uterine tissue (Mayer et al., 1967). Luminal closure may be a way in which the uterus physically holds the blastocyst in place. It is possible that without it, a weakly attached blastocyst may have a greater risk of detachment. In male-exposed animals, larger average luminal area may be the result of excess estrogen transferred from the male (see also Guzzo et al., 2012; Thorpe and deCatanzaro, 2012). Opening of the lumen could explain the lower average number of implantation sites in these animals. Transfer of the blastocyst has also been shown to be accelerated with the presence of excess estrogen (Ortiz et al., 1979). The premature entry of the blastocyst to the uterine chamber in combination with the diminished luminal closing could mean that some blastocysts are unable to be implanted altogether. Another plausible explanation for the difference in luminal area in the two conditions could be that this measurement is the result of failed implantation. It is likely that if a blastocyst is unsuccessful in implanting, the uterus will begin its transformation back to its pre-implantation form (Ma et al., 2003) where the lumen is wide open. However, this notion does not explain why on the 7<sup>th</sup> day of gestation, luminal area was significantly greater in male-exposed animals, yet the number of implantation sites counted was not reduced in this condition compared to controls.

### **Apical Luminal E-Cadherin**

One molecule that may be involved in the relationship of the luminal area to the Bruce effect is e-cadherin. This cellular adhesion molecule is found throughout the uterine epithelial cells (Aplin, 1997) and can migrate through the various layers of the epithelium in response to

hormonal manipulations (Hyland et al., 1998; Jha et al., 2006). Here, we documented the proportion of cells which expressed e-cadherin on their apical membranes. This membrane of the epithelial layer is the site of blastocyst adhesion during implantation (Hyland et al., 1998). We found no difference in the expression of e-cadherin across the first 8 days of gestation. In the rat uterus, cadherins have been documented to migrate from basal to apical membranes of epithelial cells in preparation for implantation (Hyland et al., 1998). In the current experiment, this would have been characterized by a daily increase in e-cadherin expression on the apical surface of epithelial cells. This effect was not captured here for reasons which may include small sample size, differences in rat and mouse implantation dynamics as well as the possibility that another cadherin (e.g. p-cadherin) dominated in the migration observed in that previous study. It is also likely that the expression of e-cadherin is influenced by too many factors which would make this method of studying its expression not specific enough to observe day dependent changes.

We did find that the proportion of cells expressing e-cadherin was diminished in male-exposed as compared to isolated animals. This is the first demonstration of e-cadherin dynamics in the Bruce effect. The Bruce effect is believed to be mediated through estrogen transfer from novel males to females (reviewed in deCatanzaro, 2011). Radioactively-tagged estradiol can be traced as it transfers from novel males to the uteri of inseminated females (Guzzo et al., 2012). Since injection and nasal administration of estradiol doses as low as 0.014 µg per day on days 2-4 of gestation can significantly reduce proportion of births (deCatanzaro et al., 2001; deCatanzaro et al., 2006), the Bruce effect is likely to be mediated through greater than optimal levels of this hormone in the female. The detrimental effect of exogenous estrogens, whether through artificial administration or natural transfer from males as observed in the Bruce effect, has been studied repeatedly. E-cadherin is a hormonally-regulated molecule in the uterus (Guo

et al., 2010; Jha et al, 2006; Potter et al., 1996). Its expression has been shown to be upregulated by progesterone in the mouse uterus (MacCalman, Farookhi and Blaschuk, 1994). As well, on a progesterone-primed uterus, administration of estradiol is followed by downregulation of e-cadherin expression (Jha et al., 2006). It is likely that during the Bruce effect, estrogen is responsible for the decrease in e-cadherin on the surface epithelium of the uterus. This decrease in e-cadherin is detrimental for blastocyst implantation in two major ways: 1) decreased adhesion between blastocyst and endometrium at the appositional and attachment phases of implantation, and 2) decreased adhesion between uterine walls during uterine closure.

#### *1) Adhesion between blastocyst and uterus*

E-cadherin is a prime adhesion molecule involved in blastocyst adhesion with the endometrium and so its down-regulation may diminish the chances of successful attachment. E-cadherin is found on both the blastocyst and the endometrial epithelial cells and as such has been suggested to be directly involved in the attachment of these two surfaces (Kadokawa, Fuketa, Nose, Takeichi and Nakatsuji, 1989). In humans, forced expression of e-cadherin in non-receptive endometrial epithelial cells *in vitro* leads to enhanced receptivity of the endometrium for the blastocyst (Rahnama et al., 2009). Steroid hormones are responsible for enhancement of receptivity on the endometrium luminal epithelium through promoting appearance of a variety of adhesion molecules including e-cadherin (reviewed in Aplin, 1997). Implantation is not successful in e-cadherin-null mice due to problems with formation of the trophoblast on the blastocyst, which is usually the outer layer of cells that makes contact with the endometrium during implantation (Riethmacher, Brinkmann, and Birchmeier, 1995). It is likely that there are changes in the expression of e-cadherin on the trophoblast cells of the blastocyst which may influence the overall adhesiveness between the uterine and blastocyst surfaces. Further

exploration into the dynamics of this molecule on the blastocyst could shed some light onto an additional way in which implantation may be failing. It is likely that e-cadherin expression is reduced on both adhesive surfaces during the Bruce effect. Reduction in adhesiveness at the uterine epithelium is a clear way in which proportion of successful implantation may be reduced in male-exposed animals.

## *2) Adhesion between uterine walls*

The expression of e-cadherin across the entire luminal epithelial surface suggests that it could be involved in maintaining luminal closure once it has occurred (Hyland et al., 1998). Although the process of the closure involves pumping of the fluid from the lumen into the epithelial cells through pinocytosis (Bansode et al., 1998), once the lumen is free of liquid, the two walls of the uterus must form a strong adhesion with each other. This idea emerged from a study (Hyland et al., 1998) where cadherins were noticed migrating to the apical side of the epithelial cells, the sites at which cohesion between cells of opposite sides of the uterus would occur. It is likely that the estrogen-induced downregulation of e-cadherin on the uterine lining observed during the Bruce effect leads to weaker attachment of the opposing uterine walls followed by opening of the lumen. This is consistent with the increased luminal area observed in male-exposed animals. The result of this may be that the blastocysts which have implanted or are weakly attached due to the diminished e-cadherin expression may then have a smaller chance of survival without the tight hold of the uterus, thus causing the failure of implantation in the Bruce effect.

## **CONCLUSIONS**

It has been shown here that the presence of males induces a decrease in expression of e-cadherin on luminal epithelial cells of inseminated females. This can both decrease adhesion between the blastocyst and uterine wall as well as decrease adhesion between opposing uterine walls which explains the increase in luminal area (luminal opening) in male-exposed animals. The opening of the lumen of the uterus can be deleterious for newly implanted blastocysts, documented as the fewer implantation sites in male-exposed animals described in the Bruce effect. The current study has provided a plausible mechanism by which novel males can terminate pregnancy in the well-established Bruce effect.

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