# Evidence that the establishment of pregnancy requires activation of lipoxygenase and phospholipase-$A_2$

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*Summary.* The present work investigates the possibility that lipoxygenase products are involved in the biochemical mechanisms of blastocyst implantation by utilizing nordihydroguaiaretic acid (NDGA) and caffeic acid (CA), inhibitors of lipoxygenase enzymes, and quinacrine (QU), an inhibitor of phospholipase-$A_2$. It has been shown previously that inhibition of cyclooxygenase results in blockade of implantation. The inhibitors were dissolved in a standard medium and 5 µl of the solutions were micro-injected into the uterine horns of day-4 pregnant mice. The contralateral horns acted as controls and received only vehicle. A sham-operated group provided normal controls. In 14 NDGA-treated mice, the control horns contained 40 implantations while the treated horns contained only 6 small implantations and 8 resorbing sites. These control horns were comparable to the sham controls. In 14 CA-treated mice, treated horns contained 17 small implantations plus 4 resorptions, whereas the control horns contained 26 small implantations and 4 resorptions. Twelve QU-treated mice exhibited 7 small implantations and 4 resorptions in the treated horns, plus 24 small sites and no resorptions in the control horns. Fourteen sham-operated mice had 95 implantation sites and no resorptions in their 28 horns. The results provide evidence for the involvement of the lipoxygenase enzymes and phospholipase-$A_2$ in the initial implantation process and in the subsequent development of early pregnancy.
*Key words.* Implantation; blastocyst; decidual reaction; lipoxygenase; prostaglandins; leukotrienes.

For some considerable time now it has been known that a signalling system must exist which carries information from the preimplantation blastocyst to the uterine endometrium[1,2]. These signals could be involved in the timing of implantation, the preparation of the nidation site and the prolongation of corpus luteum function. It seems, moreover, that implantation of a blastocyst into a uterine endometrium involves a type of inflammatory response with increased capillary permeability[3], release of endometrial histamine[4], and production of metabolites of the arachidonic acid cascade. Prostaglandins $E_2$ and $F_{2a}$, metabolites in the cyclooxygenase family of the cascade, have already been demonstrated to be necessary for the initiation of blastocyst implantation in hamsters[5,6], mice[7,8] and rats[9].

Biosynthesis of arachidonate metabolites have been even more specifically localized with respect to the implantation process in a number of species. Blastocysts from rabbits[10–12], pigs[13], ewes[14] and cows[15] have been shown to metabolize arachidonic acid to prostaglandins, and prostaglandin-$E_2$ has been demonstrated immunohistochemically in preimplantation mouse embryos[16]. Furthermore, evidence of prostaglandin (PG) synthesis has also been demonstrated in the uterine tissue, for example, in rats[17], rabbits[11,12] and pigs[18], and this PG synthesis is known to be under the control of steroid hormones from the ovary[19–23]. In addition, PG binding to the endometrium has been observed in, for example, pigs[24] and rats[25,26]. The production of prostaglandins in both blastocysts and endometrium could be part of a step-wise stimulatory response necessary for the decidual cell reaction.

Recently, further studies on the involvement of the lipoxygenase pathway of arachidonate metabolism in the implantation process have been reported[27,28]. It seems that rabbit blastocysts are able to utilize this pathway for the production of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE)[11]. This latter research also demonstrated an active lipoxygenase pathway in the rabbit uterus at the time of implantation.

The present work was designed to confirm the reports on the importance of arachidonic acid metabolites for the implantation process in vivo in mice by using specific inhibitors of the lipoxygenase enzymes and phospholipase-$A_2$[29].

*Materials and methods.* Random bred NMRI mice were maintained under controlled lighting to provide a 10-h night centered on midnight, and received a standard diet and fresh water ad libitum. Female mice weighing 25–35 g were individually mated with proven fertile males and successful mating was confirmed by the presence of a vaginal plug between 08.00 and 09.00 h, this being considered day 1 of pregnancy.

On day 4 of pregnancy before 11.00 h, the mice were anesthetized with i.p. sodium pentobarbital. The abdominal hair was removed, the abdomen was opened suprapubically with a small mid-line incision and the overlying fat and bladder were exteriorized to provide a clear view of the bicornuate uterus and cervix. A ligature was placed around the uterocervical junction without incorporating the mesometrial vessels in order to obtain a non-patent lumen without causing necrosis. Thereafter, a hamilton microsyringe was used to inject 5 µl of fluid vehicle into the upper lumen of each uterine horn, the one horn receiving vehicle only and acting as control while the contralateral horn received both vehicle and active substance, the experimental horn. The choice of uterine horn for control and experimental was randomized. The vehicle used was medium 199 with Earles' salts at pH 7.4 without buffer, in which the active enzyme inhibitors were directly dissolved a short time before use.

Three different enzyme inhibitors were used separately for these investigations, nordihydroguaiaretic acid (100 µM NDGA, Sigma Chemical Co., St. Louis, USA) and caffeic acid (10 µM CA, Sigma), specific blockers of lipoxygenase activity, and quinacrine (1 mM QU, Sigma), an inhibitor of phospholipase-$A_2$. Subsequent to the uterine horn instillations of a blocker or just the medium vehicle, the adipose tissue and bladder were returned to the abdomen, this being closed using continuous 6–0 silk sutures both for the abdominal muscle layer and for the skin. With this technique, the active substances came in contact with the blastocyst and endometrium immediately prior to the time of blastocyst activation and of initiation of the decidual response, followed by implantation during the morning of day 5. Post-operatively, these female mice were returned to their respective males where they were maintained until sacrificed by cervical dislocation on day 12. At autopsy, the numbers, positions and sizes of all implantation sites were recorded, as were also the numbers of obviously resorbing implantations (table).

An additional sham-operation group of pregnant mice were anesthetized and treated surgically in an identical manner to those above except that no fluids were injected when the micro-syringe needle was placed in the upper uterine lumen. The implantation figures for the sham-operated controls can be seen in the table and can be compared with both control and experimental horns of the other groups.

Statistical differences between groups were calculated using Student's t-test corrected for small numbers of observations and, where permitted, for paired observations. A probability

Effects of lipoxygenase and phospholipase-$A_2$ inhibitors on implantation and early development

| Treatment | Uterine horns | Implantations Number | Size range (mm) | Number of resorptions | Significance of effect |
|---|---|---|---|---|---|
| NDGA | 14 | 6 | 4–7 | 8 | $< 0.005$ |
| Controls | 14 | 40 | 7–10 | 0 | $> 0.05$* |
| CA | 14 | 17 | 3–10 | 4 | $< 0.05$ |
| Controls | 14 | 26 | 5–10 | 4 | $< 0.05$* |
| QU | 12 | 7 | 3–8 | 4 | $< 0.05$ |
| Controls | 12 | 24 | 3–8 | 0 | $= 0.05$* |
| Sham-operated | 28 | 95 | 7–10 | 0 | – |

* Significant difference between treatment controls and sham-operated controls.

value of 0.05 or less was considered to be indicative of a significant difference.

*Results.* In the 14 female mice treated unilaterally with 5 µl of 100 µM NDGA shown in the table, the control horns contained a total of 40 implantation sites, these being normal in size when compared to the sham-operated controls. No resorptions were seen in the untreated horns of the NDGA treatment mice. The contralateral NDGA-treated horns contained a total of 6 implantation sites and 8 resorbing sites, the implantation sites being considerably smaller in size than under normal conditions. Comparing the control horns with the treated horns, NDGA inhibited normal implantation significantly ($p < 0.05$), and almost completely with the dose used (5 µl of 100 µM NDGA). There was no significant difference between the NDGA control horns and the sham-operated mice.

In the 14 mice treated unilaterally with 5 µl of 10 µM caffeic acid shown in the table, the treated horns contained 17 implantation sites which were smaller in size than the sham-operated control sites, plus 4 resorptions. This represents a significant ($p < 0.05$) reduction in the implantation rate compared with sham-operated controls but not so compared with the contralateral controls. The contralateral control horns of the CA treated mice had a total of only 26 implantation sites of reduced size plus 4 resorptions. These control horns exhibited a significant reduction in implantation rate ($p < 0.05$) with reduced size of the sites compared to the sham-operated group.

In the 12 mice treated unilaterally with 5 µl of 1 mM quinacrine as shown also in the table, the treated horns contained a total of 7 implantation sites, which were smaller in size than the sham-operated control sites, and 4 resorbing sites. The contralateral control horns contained 24 small implantations and no resorptions. The implantation rate was significantly ($p < 0.05$) reduced after QU treatment, not only compared with the sham-operated controls, but also with the contralateral controls. Moreover, there was an apparent inhibition in the contralateral control horns compared with the sham controls ($p = 0.05$), although no resorbing sites were present.

*Discussion.* The primary finding of the present study is that specific inhibitors of the lipoxygenase enzymes[30] of the mouse blastocysts and/or endometrium inhibit successful implantation and subsequent development of pregnancy. An additional important finding is that a blocker of phospholipase-$A_2$ activation and arachidonic acid release also inhibits successful blastocyst implantation and subsequent pregnancy development. Hence, this study both confirms and extends the results reported previously[27,28].

In the CA and the QU treated mice, it was observed that the contralateral control horns of the treated mice exhibited a partial inhibition of implantation and subsequent pregnancy when compared to the sham-operated control mice. Such was not observed with the NDGA treatment. The most probable explanation for this observation is that CA and QU must have diffused to the contralateral uterine horn to produce the effect. It is very doubtful that the effect seen is mediated more centrally via the hypophysis or ovaries since the observations remain unilateral not only with NDGA but also with CA and QU.

The fact that QU, an inhibitor of arachidonic acid release, decreases the rate of implantation and subsequent development is not so surprising. With this inhibitor the production of prostaglandins should also be decreased and they have previously been shown to be involved in implantation[5–9] in rodents. As described in the introduction, it seems that both blastocysts and endometria of a number of species can metabolize arachidonic acid. Consequently, one cannot know from the present results whether the inhibitions of implantation seen were due to a biochemical blockade in the blastocysts, the endometria or both.

The presence of a resorption in any of the groups is considered by the authors to represent a successful implantation followed directly by a severe growth inhibition. With this interpretation in mind and when one counts all resorption sites as normal implantation sites, there remain significant differences between treated and control groups. This indicates that inhibitors of lipoxygenase and phospholipase-$A_2$ detrimentally affect both the initial implantation and the development of pregnancy thereafter.

It remains to investigate which specific mechanism is affected by inhibition of the leukotriene pathway enzymes.

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## Regulation of mouse trophoblast giant cell nucleus development in hatched mouse blastocysts by cyclic cytidine 3′,5′-monophosphate (cCMP)

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*Summary.* The dibutyryl analog of cCMP enlarged the nuclei of trophoblast gian cells and promoted blastocyst development. The result suggests that cCMP has a trophic effect on embryonic development, specifically by altering the size of the trophoblast cell nucleus but does not enhance trophoblast cell proliferation processes.
*Key words.* cCMP; cyclic nucleotides; blastocyst; trophoblast; mouse.

Mouse blastocysts cultured in tissue culture media will subsequently hatch out of the zona pellucida and attach to the substratum [1–4]. The attachment of the hatched blastocysts appear to require certain tissue culture conditions such as the presence of a collagen substratum [5], a serum source such as human cord serum [6], essential amino acids [7, 8] and glucose [9, 10]. The inner cell mass (ICM) differentiates into the ectoderm and endoderm layers after culturing the blastocysts for 96 h [11]. The stages of embryonic development have been reported [6, 12, 13]. Recently, a less well-known cyclic nucleotide, cCMP, found endogenously in cells [14] has been implicated in cell proliferation [15, 16], activation of protein kinases [17], initiation of hemoglobin synthesis [18] and is found at high intracellular levels in rapidly-growing hepatoma cells [19]. It has been shown that the mouse embryo is responsive to cCMP and that cCMP promotes the attachment process of the hatched blastocyst [20]. The purpose of the present study was to further define the action of cCMP in the trophoblast giant cells and determine if cCMP is involved in the regulation of cell proliferation in the implanted embryo.

Female Swiss-Webster mice were superovulated with an intraperitoneal injection (i.p.) of 5 IU pregnant mares serum gonadotropin (PMSG) followed by 5 IU i.p. of human chorionic gonadotropin (hCG) 48 h later. The animals were mated and the presence of a vaginal plug the following morning indicated successful copulation. Embryos at the blastocyst stage were flushed out of the uteri 90 h after hCG administration, and were placed into Falcon petri dishes (Falcon No. 3037) containing either 1 ml culture medium as control or 10 µM dibutyryl cCMP (dbcCMP; Sigma Chemical Co., Cat No. D-7392). The dibutyryl analog of cCMP was used in this study, instead of cCMP or cytosine because the dibutyryl group facilitates penetration into the cell [21] and is more resistant to phosphodiesterase degradation [22]. The cultures were incubated at 37 °C in a moist 5% $CO_2$ in air mixture.

The culture medium consisted of Ham's F-10 (with L-glutamine, 5.6 mM D-glucose; GIBCO, Grand Island, NY) supplemented with 2.1 g/l sodium bicarbonate, 245.2 mg/l calcium lactate, 75 mg/l penicillin G and 75 mg/l streptomycin [20, 23, 24]. In addition, the medium contained 4 mg/ml human serum albumin fraction V (HSA-V; Sigma, St. Louis, MO). The pH and osmolarity were adjusted to 7.2 and 290 mOsm respectively and the medium was filter-sterilized and equilibrated overnight in the incubator prior to use the following day. The blastocysts were examined under a Nikon Diaphot inverted microscope equipped with a microscope incubator on days 1, 3 and 5 after the start of incubation (day 0). Photomicrographs were taken at the appropriate stages of development for each embryo. On day 3 of culture, a portion of the implanted embryos in the treatment and control groups were fixed with methanol and stained with Giemsa and photomicrographs were taken of the preparations. Measurements of the photomicrographs of the trophoblast cells of implanted embryos (at the egg cylinder stage) were made using the Zeiss Videoplan computerized image analyzer equipped with statistical software. The area and perimeter of the trophoblast cell nuclei were measured. Measurements of individual trophoblast cells and ICM cells were not carried out because the borders between each cell were not clear and appeared to be fused into syncytiums. The experimental results were expressed as mean ± standard error of the mean (SEM). Categorical data were analyzed using the chi-square statistic. The area and perimeter measurements were analyzed using the Student's t-test statistics. A $p < 0.05$ value was considered significant.

After 3 days of culture (table 1) the mean area of the nucleus was significantly ($p < 0.05$) larger in the trophoblasts cells exposed to dbcCMP (1478.4 ± 519.9 µm²) when compared with the control (1110.9 ± 363.0 µm²). The mean perimeter of the nucleus which is a reflection of the nuclear area was also larger in the dbcCMP treatment group. There were no differences in the number of trophoblast giant cells associated with the inner cell mass in both treatment and control groups.

Table 1. Morphometric analysis of trophoblast giant cell nucleus development in the presence of cyclic cytidine 3′,5′-monophosphate (day 3)

| Treatment | No. of cells examined | Mean area of cell nucleus (µm² ± SEM) | Mean perimeter of nucleus (µm ± SEM) |
|---|---|---|---|
| Control | 74 | 1110.9 ± 363.0 | 138.1 ± 23.2 |
| 10 µM dbcCMP | 49 | 1478.4 ± 519.9[a] | 157.6 ± 27.1[a] |

[a] Significantly different from control ($p < 0.05$).